Studies towards the synthesis and mode of action of ~~epi-3,6-dithio-2,5-diketopiperazines~~

EPIDITHIO-3, 6-DIKETO-2, 5-DIPERAZINES.

A thesis presented by

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In partial fulfilment of the requirements
for the award of the degree of

Doctor of Philosophy of the University of London

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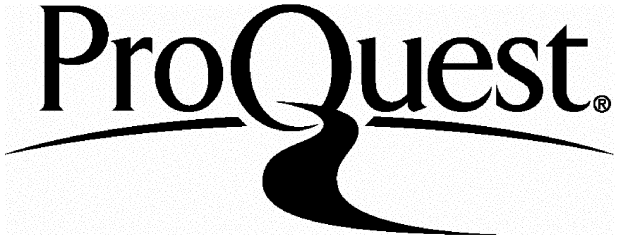
July 2004



Abstract

The present thesis is concerned with two differing but equally important aspects of the chemistry of the epi-3,6-dithio-2,5-diketopiperazine family of natural products. The first of these is related to studies involving new and existing synthetic methodologies for the construction of the disulfide bridge whilst the second is concerned with probing a possible mode of action for these compounds both through in *vitro* and *in vivo* experiments. The thesis is divided into three sections.

The first chapter provides a detailed introductory review and is organised into four sections. The first of these highlights both the structural diversity and wide range of biological activities found in this class whilst a second section outlines the biosynthetic studies which have been performed. The synthetic strategies adopted by various groups are then described and in a final section the spectral and physical properties which provide some insight into the unusual nature of the disulfide bridge are then examined.

The results of our own studies are then presented in the second chapter which opens with the synthesis of 2,5-diketopiperazines and then leads on to an examination of the scope and limitations of the classical methodologies which have been developed to date for introduction of the disulfide bridge from these cyclic dipeptides. Although a number of simple epi-3,6-dithio-2,5-diketopiperazines were prepared, the tolerance of selectively protected derivatives proved problematic. Efforts to develop a xanthate based elimination-readdition strategy from either *N*-hydroxy- or *C*-hydroxy-2,5-diketopiperazines were also studied but no evidence for formation of the desired carbon sulfur bond was obtained. In similar vein, obtention of an unsaturated *N*-oxide derivative for further elaboration was unsuccessful. Efforts were then made to probe the idea that a thiyl radical produced from the opened form of the disulfide would be sufficiently reactive to abstract the hydrogen atom of a tetrahydrofuran based DNA model. Two polarity reversal catalysed based reactions were examined and it was demonstrated that whilst thiyl radicals derived from a *cis*-3,6-dithiol-2,5-diketopiperazine were not sufficiently reactive in the key reaction, they could function as catalysts for hydrosilylation of an enol lactone. Finally the reactivity of some epi-3,6-dithio-2,5-diketopiperazines was investigated with plasmid DNA under anaerobic conditions.

The third chapter provides a formal description of the experimental results and procedures.

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Abbreviations

Ac	Acetyl
Ad	Adamantan-1-yl
AIBN	2,2'-Azobis(2-methylpropionitrile)
Bn	Benzyl
Boc	*Tert* -butoxycarbonyl
Bp	Boiling point
br	Broad
Bu	Butyl
°C	Degrees Celsius
cat	Catalyst
Cbz	Carbobenzyloxy
CC	Covalently closed
CI	Chemical ionisation
CSA	Camphorsulfonic acid
d	Doublet
DCC	*N,N'*-Dicyclohexylcarbodiimide
DIBAL-H	Diisobutylaluminium hydride
DMAP	*N,N*-Dimethyl-4-aminopyridine
DME	Dimethoxyethane
DMF	*N,N*-Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DNTB	5,5'-dithiobis-(2-nitrobenzoic acid)
% ee	% Enantiomeric excess
EDCI	1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride
EDTA	Ethylenediamine tetraacetic acid
EI	Electron impact
Enz	Enzyme

ESP	Electrospray
Et	Ethyl
EtOAc	Ethyl acetate
FAB	Fast atom bombardment
FT	Fourier transform
g	Gram(s)
G	Glutathione
Gly	Glycine
h	Hours(s)
HOBt	1-Hydroxybenzotriazole
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
IR	Infrared spectroscopy
J	Coupling constants
L	Linear
LDA	Lithium diisopropylamide
lit	Literature
LRMS	Low resolution mass spectrometry
m	Meta
M	$Mol.l^{-1}$
m	Multiplet
*m*CPBA	*Meta*-chloroperbenzoic acid
Me	Methyl
MEM	β-Methoxyethoxymethyl
mL	Millilitre(s) l
mmol	Millimole(s)
mol	Mole(s)
MOM	Methoxymethyl
Mp	Melting point
NBS	*N*-Bromosuccinimide
NMR	Nuclear magnetic resonance
o	Ortho

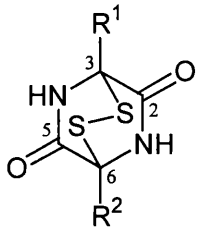
OC	Open circular
p	Para
P	Phosphate
Ph	phenyl
PMB	*Para*-methoxybenzyl
ppm	Part per million
Pr	Propyl
q	Quartet
rac	Racemic
RNA	Ribonucleic acid
rt	Room temperature
s	Singlet
sec	Secondary
SET	Single electron transfer
t	Tertiary
t	Triplet
TBDMS	*Tert* -butyldimethylsilyl
TBDPS	*Tert* -butyldiphenylsilyl
TBHN	*Trans*-di-*tert*-butylhyponitrite
t-Bu	*Tert*-butyl
Tf	Trifluoromethanesulfonyl
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TRITONB	Benzyltrimethylammonium hydroxide
UV	Ultraviolet spectroscopy

Chapter 1

Introduction

1.1 Preface

The present thesis focuses on the synthesis and possible mode of action of an unusual product family, the epi-3,6-dithio-2,5-diketopiperazines. The basic structural unit of epi-3,6-dithio-2,5-diketopiperazines is characterised by a cyclic dipeptide skeleton, substituted at the 3 and 6 positions by a disulfide bridge (Figure 1). Although cyclic dipeptides are recognised as one of the most common peptide classes found in nature, the chemistry of these sulfur bridged derivatives has not been significantly reviewed since 1975.[1]



R^1, R^2 = amino acid side chains

Figure 1

This very special core is present in a wide range of natural products, which show a variety of potent and interesting biological properties, and it has been demonstrated that the disulfide linkage is absolutely crucial for biological activity.

When these molecules were first isolated, interest was concentrated on their biosyntheses, and their syntheses. More recently, as the disulfide bridge is the unusual feature, the nature of the sulfur-sulfur bond has been investigated by both physical and chemical methods.

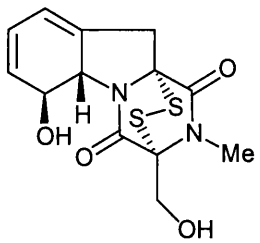
The present introduction will therefore concentrate on reviewing those biologically active natural products which contain the epi-3,6-dithio-2,5-diketopiperazine core, along with the studies that have been carried out on both their biosynthesis and synthesis. In particular, approaches taken to understand the particular nature and function of the disulfide bridge will be highlighted.

1.2 The isolation, structure and biological activity of epi-3,6-dithio-2,5-diketopiprazines

In the following section, attention will focus on the wide variety of structural types which can be generated from the simplest cyclic dipeptides through incorporation of the sulfur bridge and further oxidative transformations of the pendant amino acid chains. The resultant compounds exhibit a very wide range of potent biological activities[2] and it is tempting to speculate that the disulfide bridge core may be responsible for a common mode of action and the surrounding architecture for molecular recognition.

1.2.1 Natural products containing one epi-3,6-dithio-2,5-diketopiperazine unit

Of all the natural products which contain the epi-3,6-dithio-2,5-diketopiperazine core, gliotoxin **1** (Figure 2) must take pride of place since it has emerged as of significant interest to a considerable number of scientists. Because of its wide range of biological properties, the synthesis of this oxygenated metabolite and the understanding of its mode of action both constitute a formidable range of challenges.

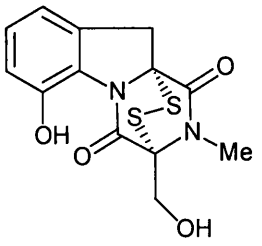


Gliotoxin **1**

Figure 2

Gliotoxin **1** is produced by miscellaneous species of pathogenic yeasts, moulds and fungi. It was first isolated in 1936, from the fungus *Gliocladium* by Weindling and Emerson[3] and, at a later stage, from the fungi *Aspergillus fumigatus* mut. *Helvola* Yuill[4] and the strains of *Trichoderma viride*.[5] Another fungus found to produce gliotoxin is *Penicillium obscurum* Biourge[6] subsequently corrected to *Penicillium*

terlikowski Zaleski by Johnson *et al.*[7] This fungus also produced the aromatised form of gliotoxin **1**, dehydrogliotoxin **2**, an antibacterial metabolite (Figure 3). More recently, gliotoxin **1** was found in *Gliocladium deliquescens*[8] and in *Gliocladium flavofuscum.*[9]



Dehydrogliotoxin **2**

Figure 3

Crowfoot and Rogers-Law reported the first analysis of gliotoxin **1**, in 1944[10] and proposed $C_{13}H_{14}O_4N_2S_2.1/2H_2O$, as the empirical formula. Later, on the basis of chemical experiments carried out on dethioglitoxin, Johnson *et al.* proposed the first structure, a pentacyclic ring system, shown in Figure 4. [11]

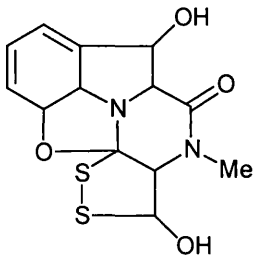


Figure 4

Five years later, further experimental observations allowed Johnson in a collaboration with Woodward to elucidate the structure of gliotoxin **1**.[12] However, no absolute configuration was assigned by these authors. Finally, an X-ray analysis, carried out by Fridrichsons and Mathieson, revealed the *S* configuration of the alcohol on the 1,3-cyclohexadiene moiety and the *R,R* configuration of the two disulfide bridgehead carbons[13] (Figure 2).

Gliotoxin **1** exhibits a surprisingly large variety of biological properties.[14] Firstly, it was recognised as an antibacterial and antiviral agent, but it is also a toxic metabolite. It can inactivate a number of fundamental enzymes and suppress the immune system cells. Apoptosis and DNA damage can also be induced by this compound.

Soon after the discovery of gliotoxin **1**, its antimicrobial properties, such as antibacterial, antifungal and antiviral, proved of great interest to the scientific community. Initially, gliotoxin **1** was demonstrated to be a powerful bacteriostatic agent[15] and to inhibit growth of bacteria *in vitro*.[16] In addition, an activity towards the bacteria *Bacillus subtilis* was observed[17] and gliotoxin **1** also showed antimalarial activity.[18] The inhibition, irreversibly and specifically, of the process of viral RNA replication in infected cells,[19] and reverse transcriptase (RNA-directed DNA polymerase) activity[20] are also a demonstration of the antiviral properties[21] of gliotoxin **1**. However, when used in high concentrations, gliotoxin **1** becomes a toxic metabolite. Moreover, the genotoxicity of gliotoxin **1** has very often been associated with allergic diseases because of its production by moulds.[22]

One of the other properties of gliotoxin **1** is its inhibition of enzymes. Initially, it was shown to inactivate the activity of horse liver alcohol dehydrogenase.[23] But recently, Waring *et al.* also reported the inhibition of rabbit muscle creatine kinase by gliotoxin **1**.[24]

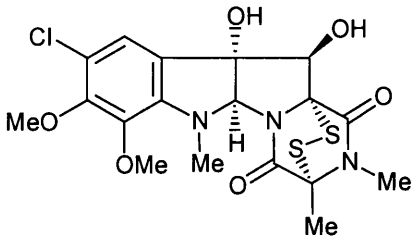
Furthermore, gliotoxin **1** shows potent immunosuppressive properties, by inhibiting the activation and proliferation of a large number of cells, both *in vitro* and *in vivo*.[25] Thus, gliotoxin **1** inhibits the nuclear factor-kappa B, which is a crucial protein for the activation and proliferation of T and B cells.[26] Nuclear factor-kappa B, as a regulator of the transcription of inflammatory cytokines, plays an important role in inflammatory bowel disease. Consequently, gliotoxin **1** not only inactivates the activity of nuclear factor-kappa B, but also suppresses intestinal inflammation (dextran sodium sulfate-induced colitis) in rats.[27] Two mechanisms for the anticolitis of gliotoxin **1** have been proposed, namely that this metabolite

suppresses or blocks the inflammatory cytokin, based on the inhibition of nuclear factor-kappa B. It is also considered that gliotoxin **1** may cause apoptosis of the inflammatory cells.[25b-c]

Apoptosis is defined as a programmed cell death, which is detected by morphological changes and by a specific DNA fragmentation. Gliotoxin **1** causes apoptosis in a variety of cells; for example, T cells, thymocytes, lymphocytes and macrophages,[13c] as witnessed by the typical DNA fragmentation.[28] The mechanism of induction of apoptosis by gliotoxin **1** is, however, still under investigation.[29]

Amongst those natural products containing the epi-3,6-dithio-2,5-diketopiperazine core, the sporidesmins constitute one very important family. Their common feature is not only the epi-3,6-dithio-2,5-diketopiperazine unit but a tricyclic ring system based on tryptophan.

Sporidesmin A **3** (Figure 5) was first isolated and purified in 1959, by Synge and White,[30] from the spores of the fungus *Phytomyces Chartarum* (formerly named *Sporidesmium bakeri*). The structure was determined by X-ray analysis,[31] and the absolute configuration at the disulfide bridgehead carbons was shown to be *R*,*R*.[13] This agreed with the circular dichroism curves of sporidesmins, obtained by Taylor *et al.*[32]



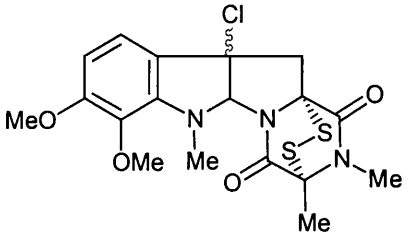
Sporidesmin A **3**

Figure 5

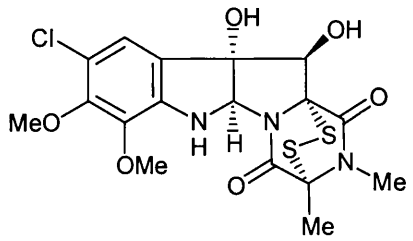
From 1958, this mycotoxin was of great interest to the scientific community as it was found to be responsible for poisoning animals, especially sheep, causing facial eczema.[33,30] In New Zealand, the cost of this problem has been estimated, at between 10–63 million NZ$ annually. Administering zinc salts has been shown to

prevent facial eczema in lactating dairy cows, and this may be due to the formation of a complex between zinc and the reduced form of sporidesmin A **3**.[34] Hendersen *et al.* have recently identified these types of complexes using electrospray ionisation mass spectrometry techniques.[35]

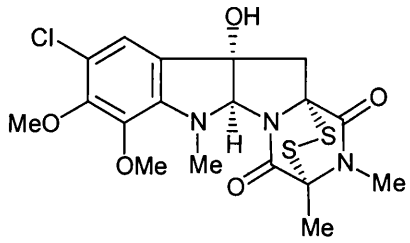
Other metabolites have also been isolated by Taylor *et al.*, from the culture extracts of the same fungus; namely, sporidesmin H **4**, containing a 3-chloroindoline, and sporidesmin J **5** (Figure 6), N^6-desmethylsporidesmin, the most polar one, produced by the fungus in *ca.* 1% yield.[36] Sporidesmin B **6** (Figure 6) has been shown to be a sporidesmin A derivative.[37]



Sporidesmin H **4**



Sporidesmin J **5**

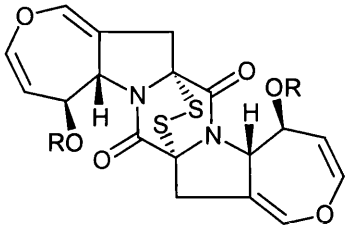


Sporidesmin B **6**

Figure 6

Chronologically, the aranotin family was the second class of epi-3,6-dithio-2,5-diketopiperazines to be discovered. Aranotin **7** and acetylaratonin **8** (Figure 7) were first isolated and fully characterised by Nagarajan, Neuss *et al.*, from the fungus

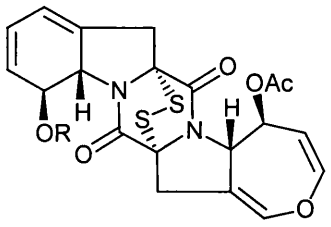
Arachniotus aureus[38] and possess two dihydrooxepin rings. At the same time, other studies showed that acetylaranotin exhibits the same configuration as sporidesmin.[39] Acetylaranotin **8**, also named LL–S88α, was isolated from another fungus: *Aspergillus terreus*.[40] Acetylaranotin **8** inhibits RNA viruses in tissue culture and in animals, as well as inhibiting viral RNA replication.[41]



R = H Aranotin **7**
R = Ac Acetylaranotin **8**

Figure 7

Two other members of the aranotin family, apoaranotin **9** and acetylapoaranotin **10**, which are produced by the fungus *Arachniotus aureus*, have been isolated by Neuss, Nagarajan *et al.* (Figure 8).[42] Both of these compounds contain a dihydrooxepin ring and a cyclohexadiene moiety.



R = H Apoaranotin **9**
R = Ac Acetylapoaranotin **10**

Figure 8

More recently, SCH 64874 (Figure 9), which has been isolated from an unidentified fungus by Hedge *et al.*, has been shown to be diester of an eight carbons aliphatic acid of aranotin **7** and shows antitumoural properties.[43]

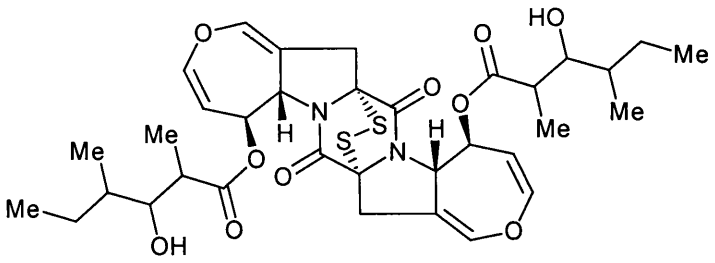
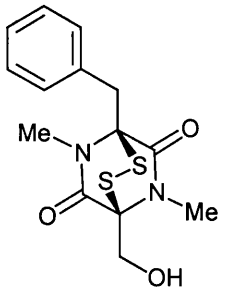


Figure 9

Hyalodendrin **11**, which has the simplest structure based around *cyclo*-phenylalanine-serine (Figure 10), was isolated, by Strunz *et al.*, from culture filtrates of *Hyalodendron* species and showed antifungal properties.[44] However, the configuration of the disulfide bridgehead carbons has been proven to be *S,S*.[45] Moreover, hyalodendrin is a bacterial growth inhibitor and has antimicrobial properties against fungi causing plant and tree diseases, such as *Ceratocystis*, which is responsible for Dutch elm disease.[46]

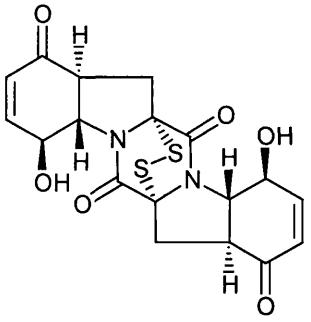


Hyalodendrin **11**

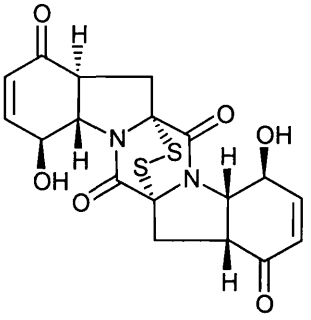
Figure 10

Epicorazine A **12** and B **13** (Figure 11) were isolated in 1976 by Baute et *al.*, from the cultures of one stump of the fungus *Epicoccum nigrum*. Both these compounds are antibiotic metabolites and show activity against methicillin-resistant *Staphylococcus aureus*.[47] By comparison of the circular dichroism spectra with known epi-3,6-dithio-2,5-diketopiperazines, the chirality of the asymmetric centres at the disulfide bridgehead was demonstrated to be *R*. Their structures are derived from oxygenated indole moieties and X-ray studies showed that the two molecules differed from each other solely in the stereochemistry of one ring junction.[48] More recently, Dornberger *et al.* isolated epicorazine C **14** (Figure 11) from the same

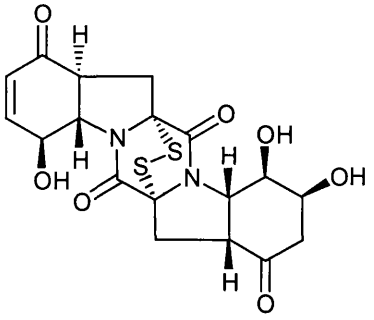
fungus and this mycotoxin is an antimicrobial metabolite exhibiting activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* as well as *Candida albicans*.[49]



Epicorazine A **12**



Epicorazine B **13**

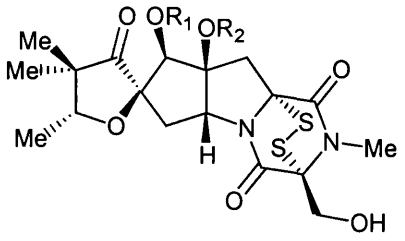


Epicorazine C **14**

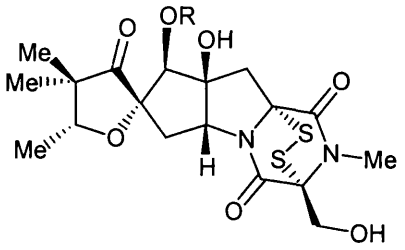
Figure 11

Sirodesmins A **15** and G **18** (Figure 12) are two antibiotics produced by *Sirodesmium diversum*. They were first reported in 1977 by Hesp, Cameron *et al.*[50] and are epimers characterised by a tetrahydrofuranone ring spiro-fused to a cyclopentylpyrrolidine moiety. The configuration of the disulfide bridgehead carbons has been established as *R,R*. In the same year, sirodesmin G **18** was reported as sirodesmin PL by Barbier *et al.*, with deacetylsirodesmin PL **19** from the fungus *Phoma lingam* Tode, a parasite of cruciferea (Figure 12).[51] Later, Harada *et al.* isolated three new metabolites from the fungus *Microsphaeropsis* sp:

TAN-1496 B **15**, A **16** and D **17** (Figure 12).[52] TAN-1496 B is identical to sirodesmin A **15** and TAN-1496 D is the deacetyl sirodesmin A. TAN-1496 A **16** is an antibiotic and an inhibitor against mammalian DNA topoisomerase I.[52]



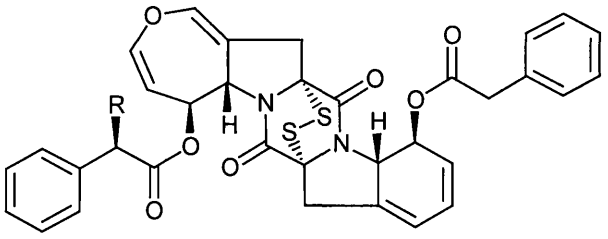
R_1 = Ac R_2 = H Sirodesmin A or TAN-1496 B **15**
R_1 = Ac R_2 = Ac TAN-1496 A **16**
R_1 = H R_2 = H TAN-1496 D **17**



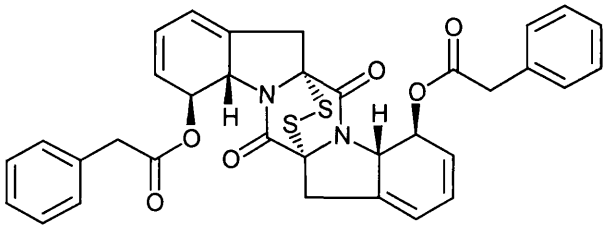
R = Ac Sirodesmin G and PL **18**
R = H Deacetylsirodesmin PL **19**

Figure 12

Emethallicin A **20** (Figure 13) was isolated from the mycelial chloroform extract of *Emericella heterothallica* (mating type A)[53] and one year later, Kawai *et al.* reported two new structures, emethallicin F **21** and E **22**, produced by the same mycelial extract (Figure 13).[54]



R = OH Emathallicin A **20**
R = H Emathallicin F **21**

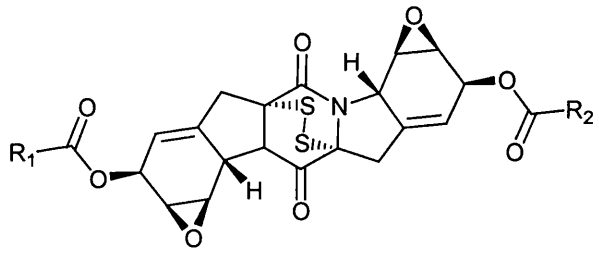


Emathallicin E **22**

Figure 13

Emethallicin A **20** and F **21** have structures related to apoaranotin **9**, whereas emethallicin E **22** contains two cyclohexadiene moieties. Furthermore, these three metabolites show potent inhibitory activity on histamine release.[53,54]

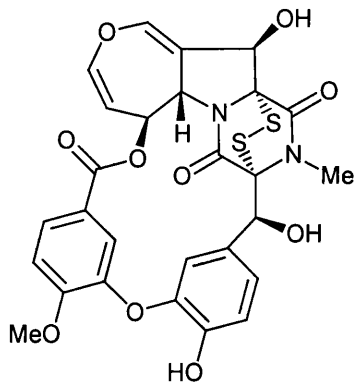
In 1978, the scabrosin esters **23**, **24**, **25** and **26** were isolated from the lichen *Xanthoparmelia scabrosa* Hale by Elix *et al.* (Figure 14).[55] Their structures, characterised by their oxirane rings, were finally elucidated in 1999, by the same group, along with the isolation of a new ester **27** (figure 14). The scabrosin esters exhibit potent cytotoxicity.[56] In 1998, ambewelamides A **28** and B **29**, which have a similar structure, were isolated from the lichen *Usnea* species by Andersen *et al.* (Figure 14).[57] The extracts of this lichen show potent *in vitro* cytotoxicity.



R_1 =	CH_3	R_2 = CH_3	Scrabosin ester	**23**
R_1 =	CH_3	R_2 = C_3H_7	Scrabosin ester	**24**
R_1 =	C_3H_7	R_2 = C_3H_7	Scrabosin ester	**25**
R_1 =	CH_3	R_2 = C_5H_{11}	Scrabosin ester	**26**
R_1 =	C_3H_7	R_2 = C_5H_{11}	Scrabosin ester	**27**
R_1 =	$-(CH_2)_2CH_3$	R_2 = $-(CH_2)_2CH_3$	Ambewelamide A	**28**
R_1 =	$-(CH_2)_4CH_3$	R_2 = $-(CH_2)_4CH_3$	Ambewelamide B	**29**

Figure 14

More unusually, emestrin **30** (Figure 15) is a macrocycle containing an embedded epi-3,6-dithio-2,5-diketopiperazine unit. It was isolated, by Kawai *et al.*, from the mycelium of *Emericella striata*, a fungus isolated from cumin (the seeds of *Cuminum cymium* L.) collected in Nepal.[58] The structure has been elucidated, proving that the configuration at the two disulfide bridgehead carbons was *R,R*. Emestrin **30** was also isolated from the fungus, *E. foveolata*, along with related epi-3,6-polythio-2,5-diketopiperazines.[59] Emestrin **30** exhibits strong antifungal properties.[60]

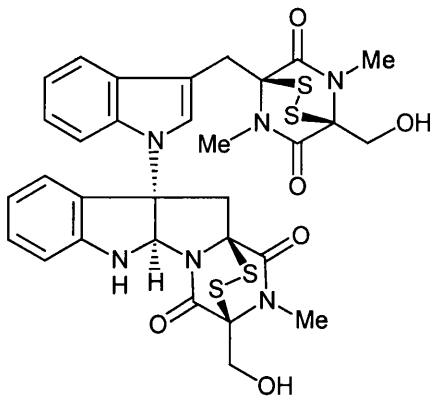


Emestrin **30**

Figure 15

1.2.2 "Dimeric" natural products containing one or two epi-3,6-dithio-2,5-diketopiperazine units

Chaetomin **31** (Figure 16), an antibiotic metabolite, was isolated by Taylor *et al.* from the fungus *Chaetomium cochliodes.*[61] Renamed chetomin, it is a toxic metabolite of *Chaetomium cochliodes* and *Chaetomium globosum.*[62] The structure of chetomin has been elucidated by Taylor *et al.*[63] and confirmed, later, by ^{15}N and ^{13}C nuclear magnetic resonance.[64] It specifically inhibits the synthesis of viral RNA.[65]

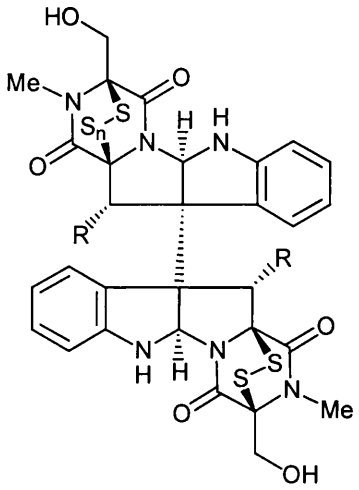


Chetomin **31**

Figure 16

Chaetocin[66] **32** and 11α,11'α-dihydroxychaetocin[67] **33** (Figure 17) were isolated in 1970 and 1972 respectively, from the fungus *Chaetomium minutum* by Hauser *et*

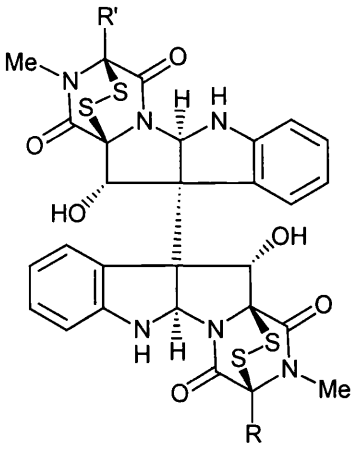
al. The X-ray study shows that chaetocin contains the same unit as does chemotin and is a dimeric structure. Also, the configuration at the disulfide bridgehead carbons has been found to be *S,S*, which is opposite to that in the sporidesmins **3-6**. In 1988, Chaetocin B **34** (Figure 17) was isolated by Saito *et al.* from *Chaetomium thielavioideum* and possesses one disulfide bridge, one trisulfide bridge and the same skeleton as chaetocin **32**.[68] These molecules possess both antibacterial and antimitotic properties.



n = 1 R = H Chaetocin **32**
n = 1 R = OH Dihydrochaetocin **33**
n = 2 R = H Chaetocin B **34**

Figure 17

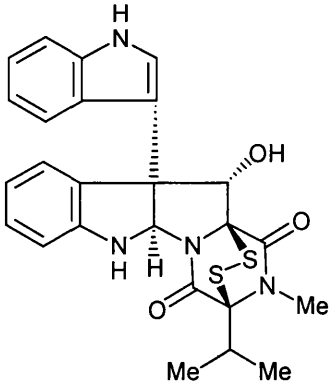
A similar pattern is observed with verticillins. Verticillin A **35** (Figure 18) is an antibiotic agent, first isolated from *Coltricia cinnamomea* (*Polystictus cinnamomeus*) by Minato *et al.*[69] Verticillins A **35** and B **36** were also isolated from the *Verticillium* species, which is a fungus of *Coltricia cinnamomea.*[70] Verticillin A **35** and B **36** both exhibit antibiotic properties.[70] In 1995, two new antitumoural products, Sch 52901 **37** and Sch 52900 **38**, were isolated from the fungal *Gliocladium* species by Chu *et al.*[71] Four years later, Gloer *et al.* isolated verticillins D **39**, E **40** and F **41** from the fermentation cultures of sclerotial mycoparasite *Gliocladium canenulatum.*[72]



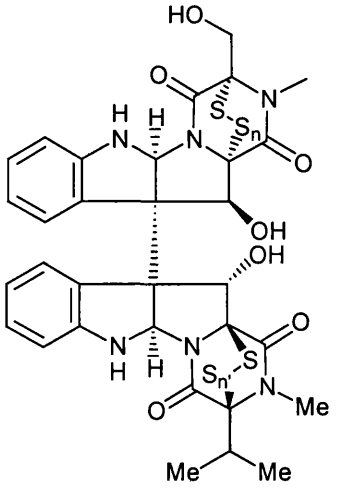
R = CH_3	R' = CH_3	Verticillin A **35**
R = CH_3	R'= CH_2OH	Verticillin B **36**
R = CH_3	R' = CH_2CH_3	Sch 52901 **37**
R = CH_3	R' = $CH(OH)CH_3$	Sch 52900 **38**
R = $CH(OH)CH_3$	R' = $CH(OH)CH_3$	Verticillin D **39**
R = $COCH_3$	R' = $COCH_3$	Verticillin E **40**
R = $COCH_3$	R' = $CH(OH)CH_3$	Verticillin F **41**

Figure 18

Leptosin D **42** and C **43** are natural products containing one or two epi-3,6-dithio-2,5-diketopiperazine molecules respectively (Figure 19). These compounds clearly have a similar structure to the chaetocins and verticillins and have been isolated from the fungal *Leptosphaeria* species of a marine alga *Sargassum tortile*, by Numata *et al.*[73]



Leptosin D **42**



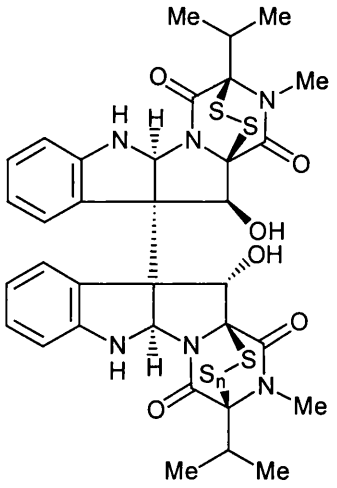
n = 1 n' = 1 Leptosin C **43**
n = 3 n' = 1 Leptosin A **44**
n = 2 n' = 1 Leptosin B **45**
n = 1 n' = 2 Leptosin G_2 **46**
n = 1 n' = 3 Leptosin H **47**

Figure 19

Leptosin A **44** and Leptosin B **45** have also been isolated from the same fungus and possess one tetrasulfide bridge and one trisulfide bridge respectively, on one of the

2,5-diketopiperazine rings (Figure 19).[73] However Leptosin G2 **46** and H **47** (Figure 19), possess these trisulfide and tetrasulfide bridges on the other 2,5-diketopiperazine ring.[74] All of these metabolites exhibit potent cytotoxicity and leptosin C also shows antitumoural properties.

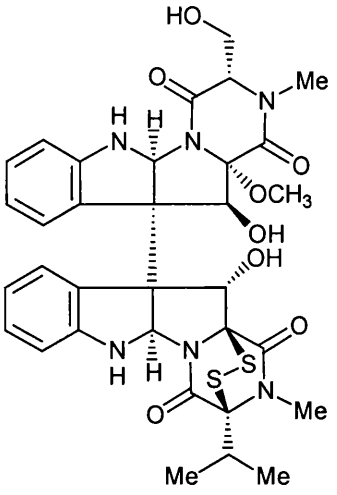
In 1995, Numata reported three new members of the leptosin family, leptosin K **48**, leptosin K_1 **49** and leptosin K_2 **50**, which were isolated from the same fungal *Leptosphaeria* species (Figure 20).[75] All three compounds are potent cytotoxic metabolites. Leptosin K **48** and C **43** both contain the same dimeric structure, but differ in the absolute configuration of the epi-3,6-dithio-2,5-diketopiperazine rings. Indeed, the two epi-3,6-dithio-2,5-diketopiperazine rings have the opposite configuration. Leptosin K_1 **49** and leptosin K_2 **50** possess one trisulfide and one tetrasulfide bridge respectively.



n = 1 Leptosin K **48**
n = 2 Leptosin K_1 **49**
n = 3 Leptosin K_2 **50**

Figure 20

Most recently, leptosin M_1 **51**, a new member of the leptosin family was isolated by the same researchers, from the fungal *Leptosphaeria* species (Figure 21).[76] It contains a single epi-3,6-dithio-2,5-diketopiperazine unit with the *S,S* absolute configuration and shows cytotoxic properties.



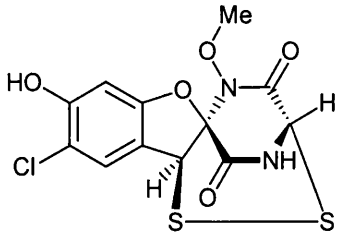
Leptosin M_1 **51**

Figure 21

1.2.3 Natural products with an alternative disulfide bridge linkage

Although our attention has been focused on natural products containing the most common epi-3,6-dithio-2,5-diketopiperazine moiety, other natural products of interest contain a disulfide bridge in which a carbon sulfur bond is formed from one of the amino acid substituents.

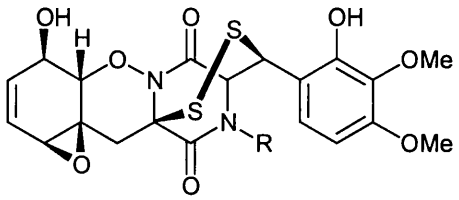
Aspirochlorine is one of these natural products. It was isolated from the fungus *Aspergillus flavus* and is an antifungal antibiotic.[77] In 1982, Sakata *et al.* believed that the molecule possessed a 1,3-disulfide linkage[78] but a revision of the structure of aspirochlorine **52** (Figure 22), published five years later by the same group,[79] showed that the linkage was between one carbon of the 2,5-diketopiperazine skeleton and a different carbon of the molecule. Aspirochlorine **52** is an antiviral and antitumoural metabolite that alters DNA and RNA[80] and also inhibits fungal protein synthesis.[81]



Aspirochlorine **52**

Figure 22

Gliovirin[82] **53** and *N*-methylated gliovirin, FA-2097,[83] **54** have been isolated from the fungus *Gliocaldium virens* (Figure 23). These molecules also possess the same unusual disulfide bridge as does aspirochlorine **52**. Moreover, gliovirin **53** is an antibiotic against *Phytium ultimum*.[82]



R = H Gliovirin **53**
R = Me FA-2097 **54**

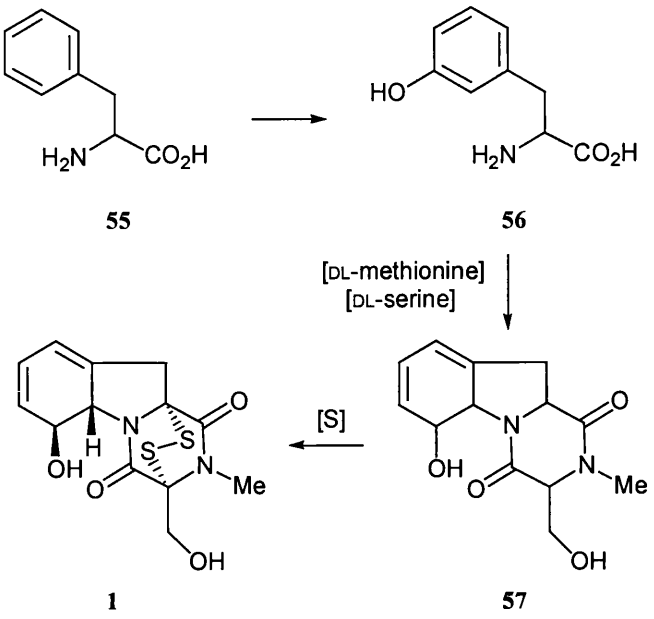
Figure 23

1.3 Biosynthetic studies

1.3.1 Gliotoxin

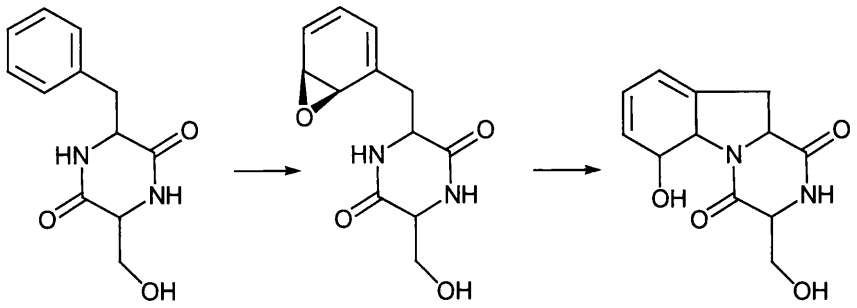
Of all epi-3,6-dithio-2,5-diketopiperazines, gliotoxin **1** has been the most extensively studied from a biosynthetic standpoint. Suhadolnik *et al.* were the first to propose a biosynthesis based on radiolabelling experiments.[84] They showed that *Trichoderma viride* (cultures of *Gliocladium deliquescens*) incorporated DL-phenylalanine **55**, DL-methionine, DL-serine and glycine into gliotoxin **1**, which is then secreted into the medium (Scheme 1). DL-*m*-Tyrosine **56** was also shown to be incorporated into gliotoxin. As a result DL-phenylalanine **55** was indicated as the precursor to the indole moiety and DL-*m*-tyrosine **56** an intermediate between DL-phenylalanine **55** and gliotoxin **1**. DL-Serine was a second precursor of gliotoxin **1**

whilst DL-methionine was incorporated as the methyl donor for dethiogliotoxin **57**. In a final step dethiogliotoxin **57** was oxidised to gliotoxin **1** (Scheme 1).



Scheme 1

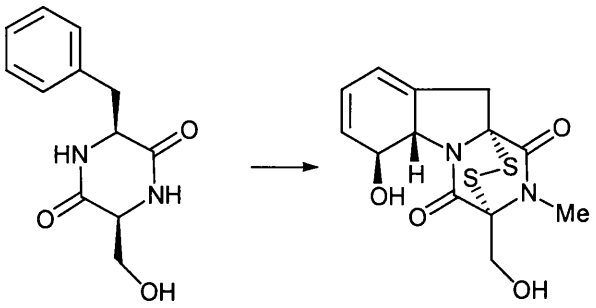
However, both Bu'Lock *et al.*[85] and Kirby *et al.*[86] contested that DL- *m*-tyrosine **56** was an intermediate in the biosynthesis of gliotoxin **1** and demonstrated that DL-*m*-tyrosine **56** was not incorporated into gliotoxin **1** by *Trichoderma viride*. However, their radiolabelling studies showed that DL-phenylalanine **55** and DL-serine were incorporated into gliotoxin **1** by *Trichoderma viride*, and that, consequently, DL-phenylalanine **55** was a direct precursor of gliotoxin **1**. They postulated the formation of 2,5-diketopiperazine **58** and the 2,3-epoxide **59**, which would undergo a nucleophilic ring opening by the amide nitrogen of the 2,5-diketopiperazine ring **59** to produce the gliotoxin skeleton **57** (Scheme 2).



58 **59** **57**

Scheme 2

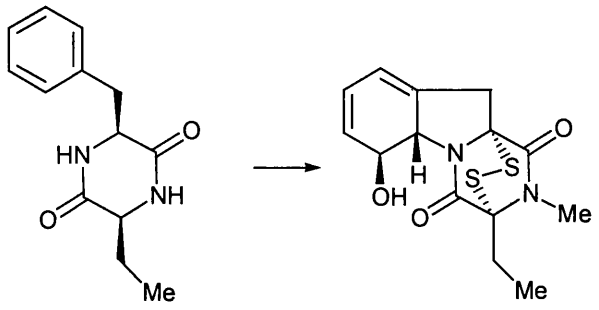
Bu'Lock *et al.* also reported the incorporation of a mixture of *cyclo*-L-phenylalanine-L-serine and *cyclo*-L-phenylalanine-D-serine into gliotoxin **1** in *Trichoderma viride*.[87] Moreover, Kirby *et al.* fed four stereoisomers of *cyclo*-phenylalanine-serine to *Trichoderma viride* and demonstrated that only *cyclo*-L-phenylalanine-L-serine **58a** was incorporated efficiently into gliotoxin **1** (Scheme 3).[88] They both concluded that *cyclo*-L-phenylalanine-L-serine **58a** was a natural metabolite of *Trichoderma viride* and was either a normal biosynthetic intermediate or was reversibly converted into an intermediate on the biosynthetic pathway between phenylalanine **55** and gliotoxin **1**.



58a **1**

Scheme 3

Concurrently, Kirby *et al.* observed the conversion of *cyclo*-L-alanine-L-phenylalanine **60** to an unnatural metabolite, 3a-deoxygliotoxin **61** in *Trichoderma viride* with the same efficiency as the corresponding "natural" process (Scheme 4).[89] This experiment also demonstrated that metabolite analogues could be obtained from biosynthesis of unnaturally designed precursors.

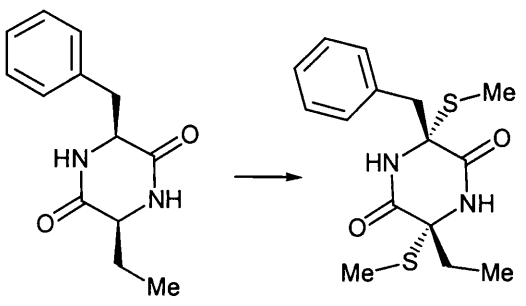


60 **61**

Scheme 4

It would therefore appear that, from the intact 2,5-diketopiperazine unit **58a**, three major steps are required for the formation of gliotoxin **1**. These are *N*-methylation, oxidative cyclisation and the introduction of the disulfide bridge. The *N*-methyl group has been demonstrated to be derived mainly from DL-methionine and cyclisation could occur after the formation of the 2,5-diketopiperazine ring **58a**. However, to date, there is no evidence to suggest the origin of the sulfur, nor how it is introduced.

The order of these steps was of great interest to Kirby *et al.* and they showed that the 2,5-diketopiperazine **60** unsubstituted on nitrogen, was transformed by the fungus *Gliocladium deliquescens*, to the dithioether-2,5-diketopiperazine **62** (Scheme 5).[90] These authors also demonstrated that the introduction of sulfur occured after formation of the 2,5-diketopiperazine ring **60** and before *N*-methylation and oxidative cyclisation.

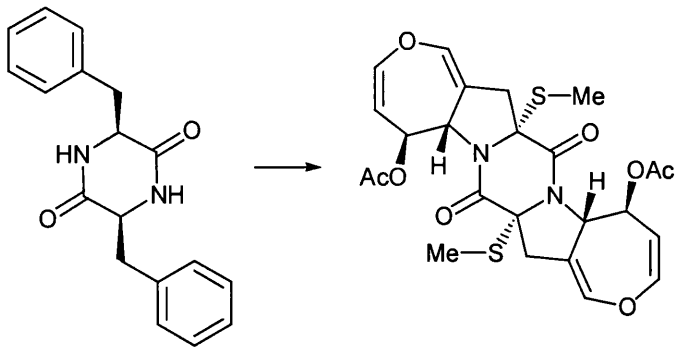


60 **62**

Scheme 5

1.3.2 Aranotins

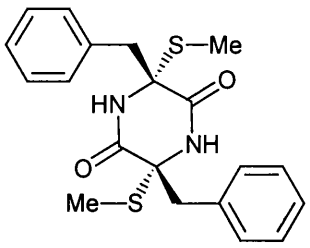
The biosynthesis of aranotins has also been studied due to the fact that the basic structure is related to that of gliotoxin **1**. Kirby *et al.* found that *cyclo*-L-phenylalanine-L-phenylalanine **63** was incorporated intact into *bis*-dethio-bis-(methylthio)acetylaranotin **64** in *Aspergillus terreus*, indicating the importance of natural 2,5-diketopiperazines in biosynthetic pathways (Scheme 6).[91]



63 **64**

Scheme 6

They also isolated from the same fungus a similar metabolite, which was a *bis*-thio-ether **65** derivative of *cyclo*-L-phenylalanine-L-phenylalanine **63** (Figure 24), proving again, that sulfur was incorporated after the formation of the 2,5-diketopiperazine ring.

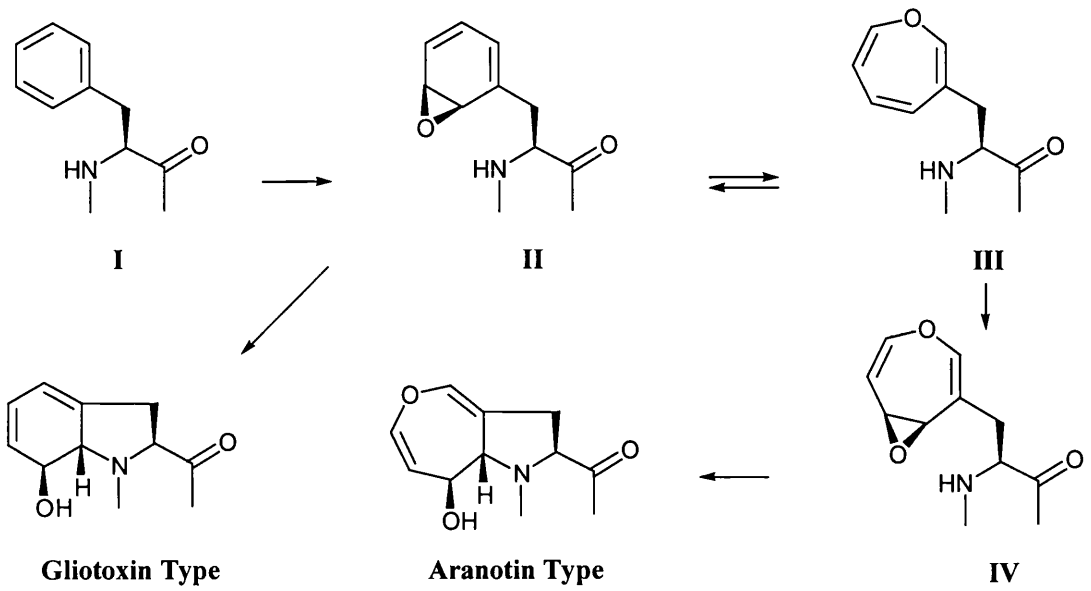


65

Figure 24

Oxepin ring systems in aranotins are believed to be formed *via* a benzene oxide intermediate **II**, which can undergo disrotatory electrocyclic ring opening to give the isomeric oxepin **III** (Scheme 7). Further oxidation of the oxepin ring **III**,

followed by a nucleophilic attack by the amide group of the 2,5-diketopiperazine **IV** on the epoxide could then produce the observed oxepin ring of aranotins (Scheme 7). Alternatively direct nucleophilic attack by the amide group of 2,5-diketopiperazine **II** on the benzene oxide can also lead to the gliotoxin type of skeleton (Scheme 7). To date, however, there is no direct evidence for these mechanisms.



Scheme 7

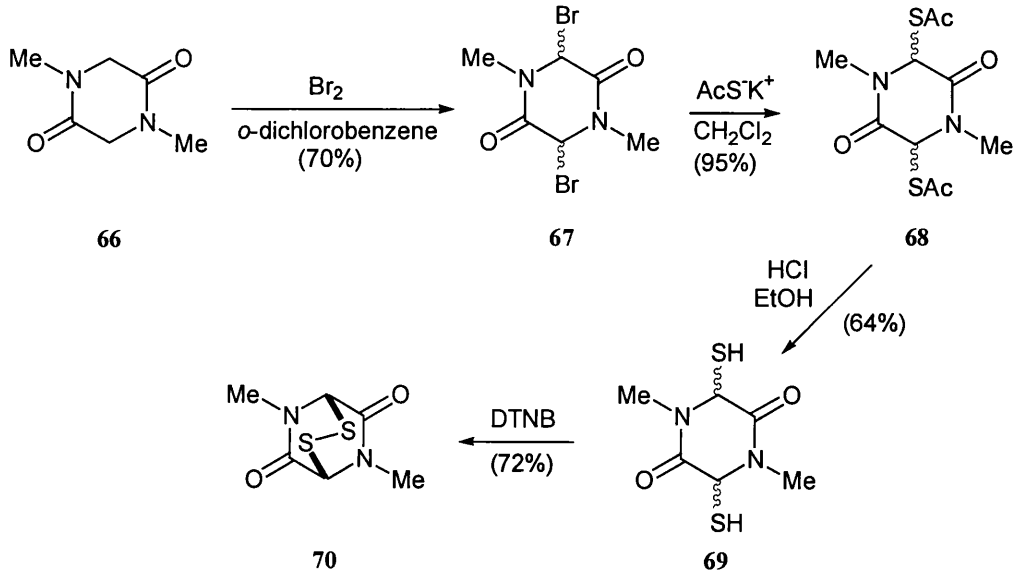
In essence however, the foregoing two sections have indicated that a vast number of fascinating structural types can be generated in nature from the 2,5-diketopiperazine core by "simple" introduction of the sulfur bridge and various oxidative transformations especially of aromatic and indole rings of the original constituent amino acid units. The very exciting possibility therefore exists that, if the synthetic organic chemist can devise a simple and effective method for introduction of disulfide bridge into any natural or unnatural cyclic dipeptide, then a host of new biologically active compounds could be prepared for testing.

1.4 Synthetic studies in the epi-3,6-dithio-2,5-diketopiperazine area

In the event however, the synthesis of epi-3,6-dithio-2,5-diketopiperazines has not be proven to be a straightforward task and over the past thirty five years, has continued to provide a formidable challenge. For ease of classification, the following synthetic studies have been subdivided into these cases where the sulfur atoms have been introduced either as a nucleophilic or as an electrophilic species.

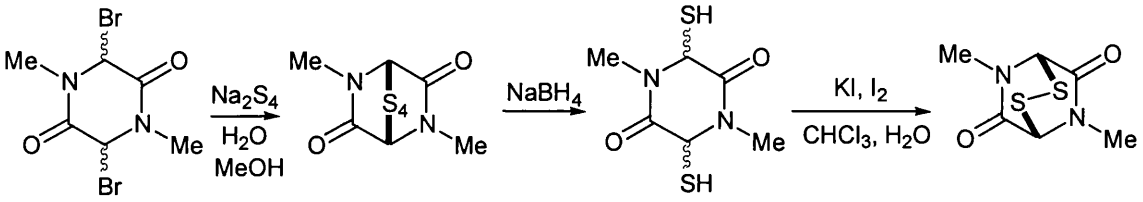
1.4.1 Methods employing sulfur nucleophiles

Epi-3,6-dithio-2,5-diketopiperazines were first synthesised by Trown *et al.*[65] In this method, sarcosine anhydride **66** was brominated to yield the dibromide compound **67** and subsequent displacement of the two bromides by potassium thioacetate followed by acidic hydrolysis to give the 3,6-dithiol-2,5-diketopiperazine **69**. Finally, epi-3,6-dithio-2,5-diketopiperazine **70** was obtained by ring closure of the dithiol **69**, with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Scheme 8).



Scheme 8

Later, in 1971, Schmidt et *al.* modified this approach, by using potassium iodide and iodine in a two phases system of water and chloroform, for the oxidation of the dithiol **69** into the disulfide **70**.[92] These authors indeed noted that a large amount of epi-3,6-tetrathio-2,5-diketopiperazine was formed when using 5,5'-dithiobis-(2-nitrobenzoic acid) as an oxidant.[93] Using Trown's dibromide **67**,[65] nucleophilic substitutions with methylthiolate and ethylthiolate were also carried out. The same group also showed that the introduction of the required *cis*-sulfide functionality into 3,6-dibromo-2,5-diketopiperazine **67**, could be achieved with sodium tetrasulfide, which after further reduction and oxidation gave the epi-3,6-dithio-2,5-diketopiperazine **70** (Scheme 9).

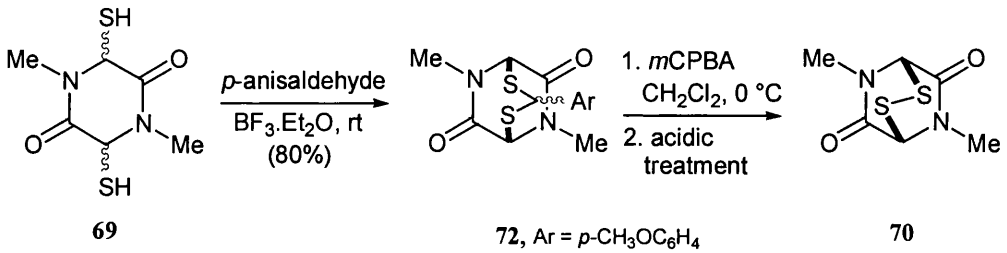


Scheme 9

Although this sulfur incorporation method was very efficient, it did not lead to the syntheses of highly functionalised epi-3,6-dithio-2,5-diketopiperazines. Indeed, the problem of competitive dehydrobromination will not permit any incorporation of substitution, at either the 3 or the 6 position of the 2,5-diketopiperazine ring. Moreover, such epi-3,6-dithio-2,5-diketopiperazines were unstable under oxidative, reductive, acidic and basic conditions. Thus, the problem of functionalisation of this unit remains unresolved.

In order to circumvent this problem, Kishi *et al.*[94] introduced the thioacetal intermediate **72**, as a protected form of the disulfide bridge of the epi-3,6-dithio-2,5-diketopiperazine (Scheme 10). Interestingly, both *cis*- and *trans*-dithiol **69** derivatives reacted with *para*-anisaldehyde in the presence of boron trifluoride etherate to yield the thioacetal **72**, which was stable under acidic, basic and reductive conditions. Consequently, anion formation could be achieved, at the 3 and 6 positions of the 2,5-diketopiperazine ring, by deprotonation with butyl

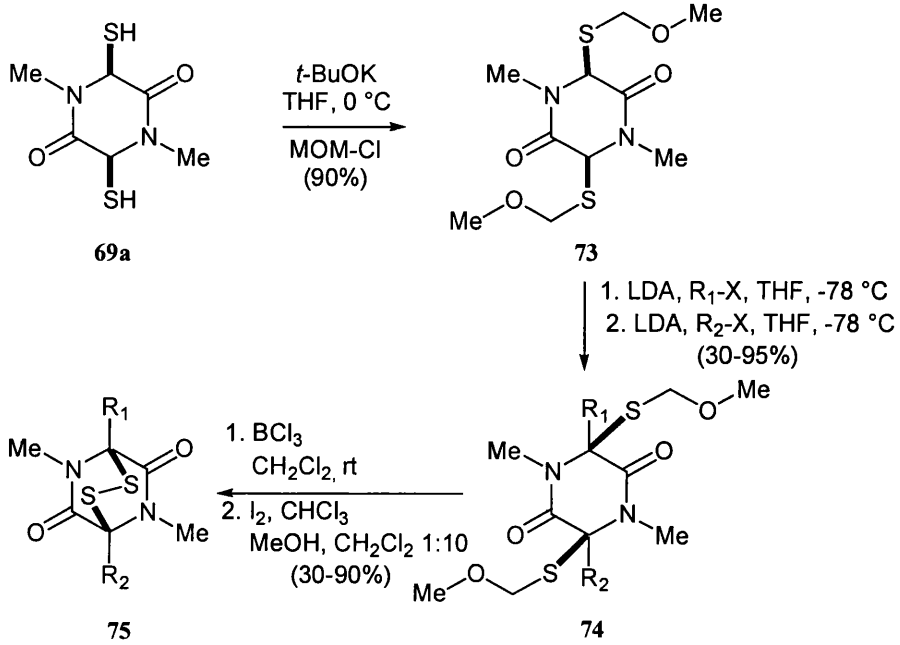
lithium and subsequently alkylation reactions could be performed. Furthermore, the disulfide bridge **70** could be regenerated by the reaction of the thioacetal **72** with *meta*-chloroperbenzoic acid to yield a sulfoxide intermediate, which could be cleaved by a protic or Lewis acid (Scheme 10). The presence of the *para*-methoxybenzene ring was proven to be crucial, as cleavage of the sulfoxide was not observed with thioacetals derived from formaldehyde, acetaldehyde or benzaldehyde.



69 **72**, Ar = *p*-$CH_3OC_6H_4$ **70**

Scheme 10

In 1976, Kishi *et al.* also outlined an alternative methodology in which they prepared a dimethoxymethylthio intermediate **73** from the *cis*-dithiol **69a** (Scheme 11).[95]



75 **74**

R_1, R_2 = Bn, Me, Et, CH_2CO_2Et or CH_2-OH

Scheme 11

After sequential alkylation, the dimethoxymethylthio compound **74** can be converted to the disulfide **75** by deprotection and then subsequent oxidation (Scheme 11).

Kishi *et al.* demonstrated that the two methoxymethylthio groups remain *cis* to one another after an alkylation reaction.[95] This stereospecificity was explained by the formation of a lithium enolate and the coordination of an oxygen lone pair to the lithium atom, leading to a stabilised intermediate. Thus, this face becomes sterically hindered and allows the approach of the alkylating agent from the opposite face (Figure 25).

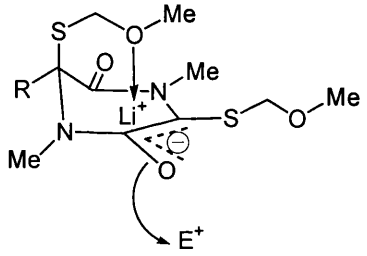
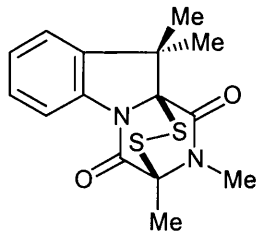


Figure 25

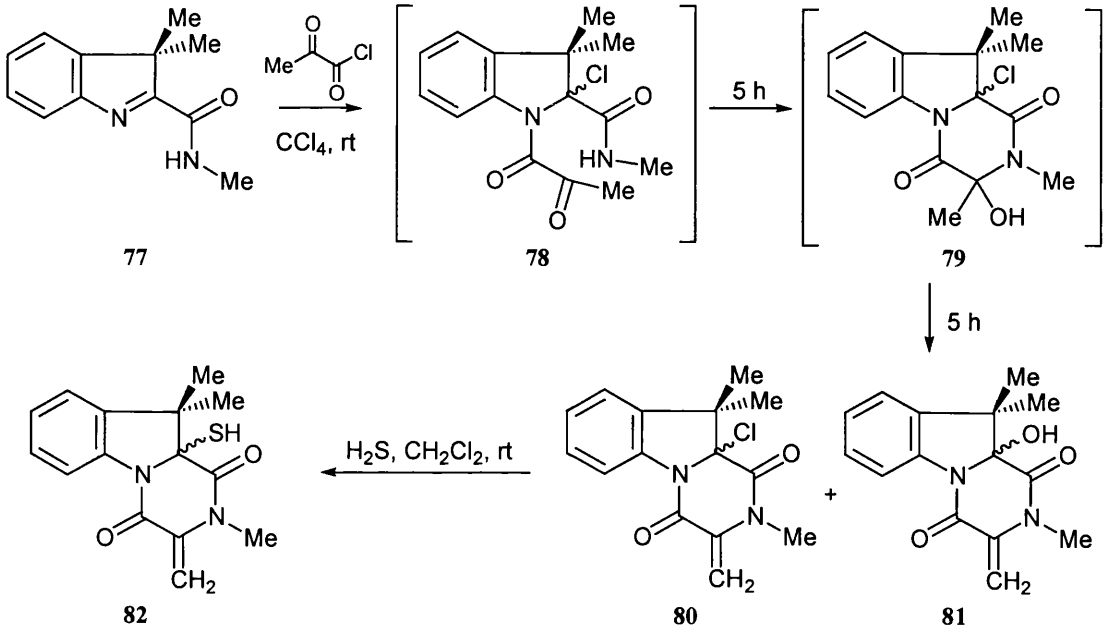
In 1975, Ottenheijm *et al.*[96,97] synthesised an analogue **76** of dehydrogliotoxin **2** (Figure 26) which was subsequently shown to have anti-reverse transcriptase activity as it inhibits normal RNA replication in some tumours viruses such as murine leukemia virus.[20] This compound was prepared in one pot by a three steps sequence. Other dehydrogliotoxin **2** analogues can also be synthesised by this method.



76

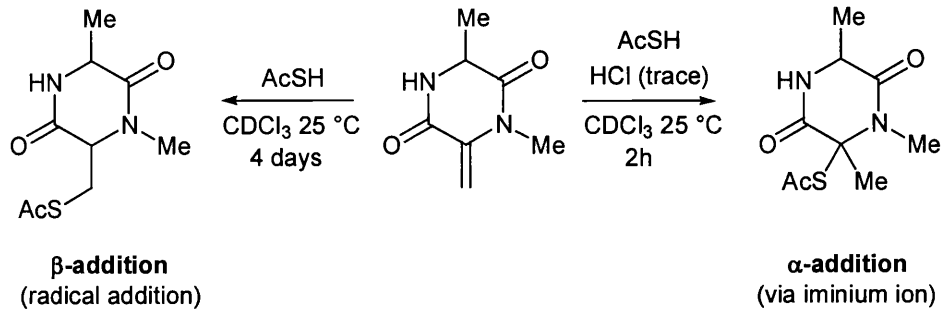
Figure 26

Indoline-2-carboxamide **77** was treated with pyruvyl chloride to form the intermediate **78** which underwent spontaneous ring closure to yield the 2,5-diketopiperazine **79** (Scheme 12). However, the 2,5-diketopiperazine **79** was relatively labile and after stirring for an additional five hours, a mixture of chloro-alkene **80** and hydroxy-alkene **81** was formed. Finally, reaction of both of these compounds with hydrogen sulfide yielded only the mercapto-alkene **82** and not the dithiol (Scheme 12).



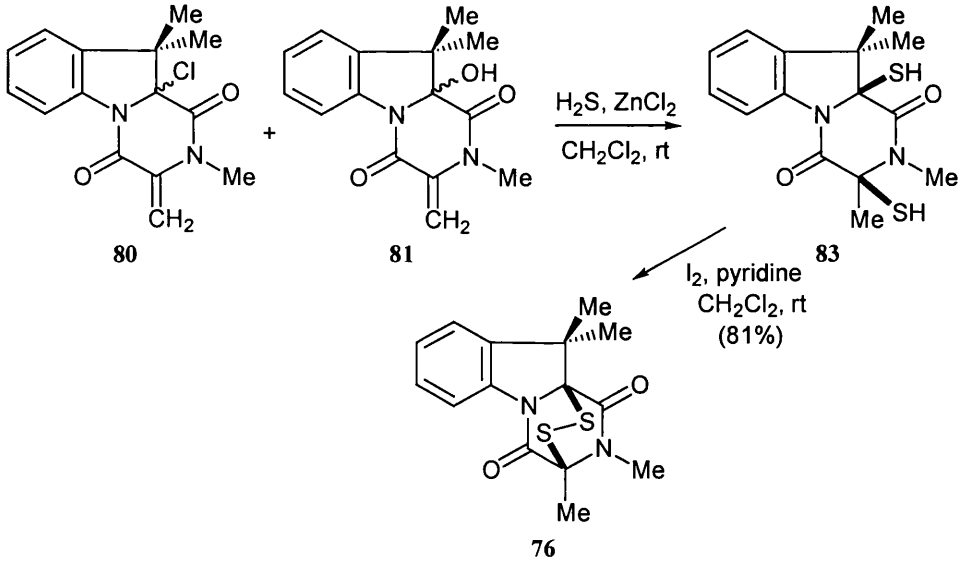
Scheme 12

However, it had been reported by Sammes *et al.* that sulfur nucleophiles can add in across the double bond of dehydrocyclodipeptides, although only in the presence of strong acids since weak acidic conditions lead to β-conjugate addition (Scheme 13).[98]



Scheme 13

Thus, Ottenheijm *et al.* proposed that hydrogen sulfide could be converted to a stronger acid through complexation with a Lewis acid and hence catalysed the α-addition of sulfur to the alkene (Scheme 14).[97] Zinc chloride was the chosen reagent.



Scheme 14

Once again, only the *cis*-dithiol **83** was detected and this regiospecific and diastereoselective addition was explained by the formation of a zinc complex with the thiol group of compound **82**, thereby directing the addition of the new thiol from the same face (Figure 27). The synthesis was then completed by oxidation of the dithiol **83** to the disulfide **76** using iodine (Scheme 14)

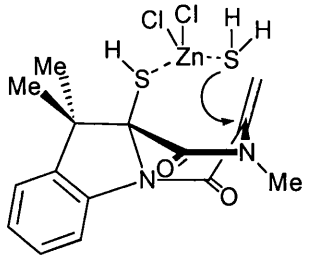
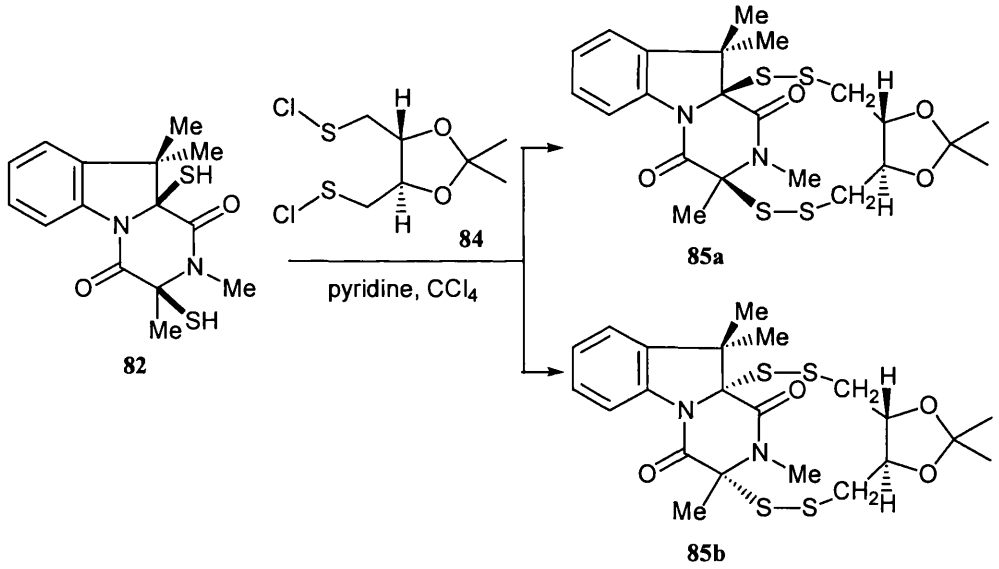


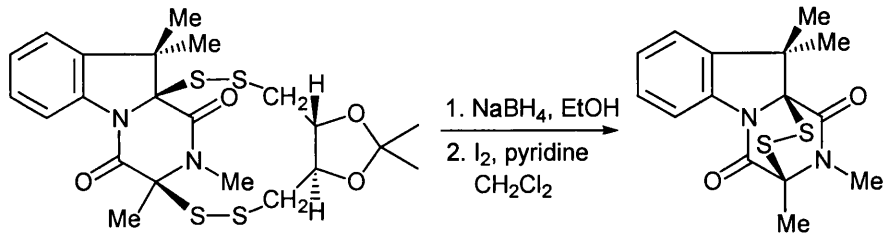
Figure 27

Although this synthesis produced a racemic product, the mixture was resolved by reducing the disulfide followed by reaction of the *cis*-dithiol **82** with a chiral *bis*-sulfenyl-chloride **84** to form two diastereoisomers[99] **85a** and **85b** (Scheme 15).



Scheme 15

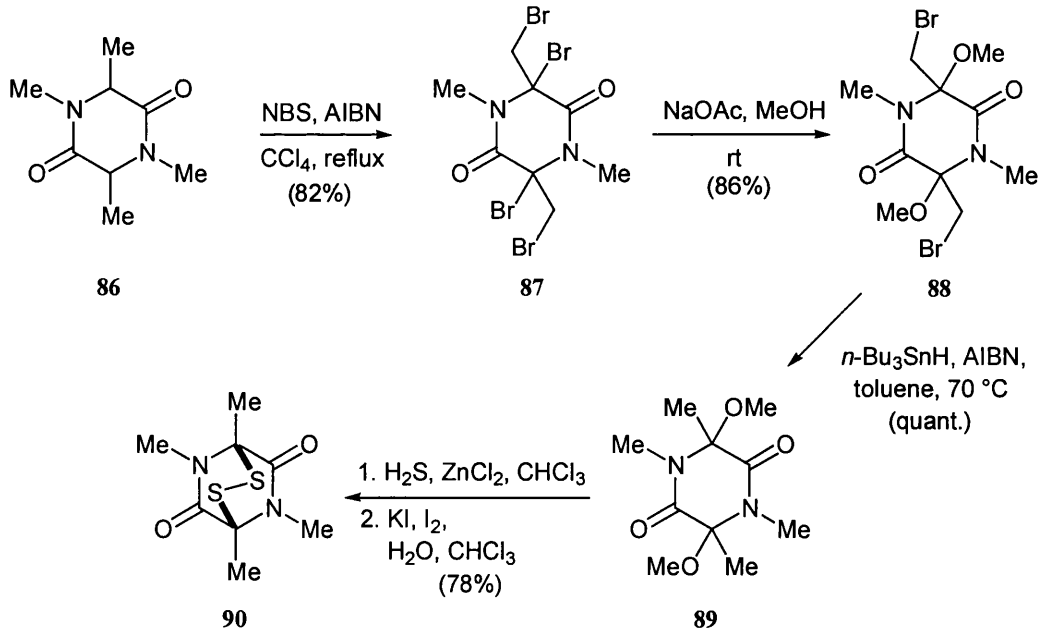
The diastereoisomer **85a** was separated from **85b** using chromatographic techniques and converted back to the optically pure disulfide **76a** (Scheme 16). Interestingly, biological testing showed that both enantiomers exhibited the same anti-reverse transcriptase activity.



85a **76a**

Scheme 16

As previously discussed, Trown's synthesis of *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine[65] **70** cannot be applied to 3,6-disubstituted analogues because of unfavourable dehydrobromination, unless the disulfide bridge is protected by selection of Kishi's thioacetal intermediate[94] and further functionalised. However, in 1975, Matsunari *et al.* solved this problem by firstly over brominating the 2,5-diketopiperazine **86** to form a tetra-bromo intermediate **87** and then displacing the 3α- and 6α- bromo groups by methoxide anion to yield the dibromo-*N,N'*-dimethoxy-2,5-diketopiperazine **88** (Scheme 17).[100]

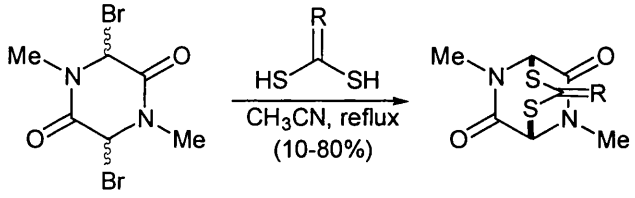


86 **87** **88** **89** **90**

Scheme 17

After the two bromide substituents were removed by free radical chain reduction with tri-*n*-butyl tin hydride, the disulfide bridge of **90** was finally introduced by bubbling hydrogen sulfide through a solution of the *N,N'*-dimethoxy-2,5-diketopiperazine **89** in the presence of zinc chloride (Scheme 17). This methodology was applied to a range of substituted 2,5-diketopiperazines and yields for the disulfide bridge incorporation varied from 2 to 56%.

The concept of a protected form of a disulfide bridge formulated by Kishi *et al.* was also taken up by Olsen *et al.*[101] The dibrominated compound **67** reacted with dithiols to yield another form of protecting bridge as product **91** (Scheme 18). However, all attempts to convert this bicyclic compound **91** into a disulfide were unsuccessful.

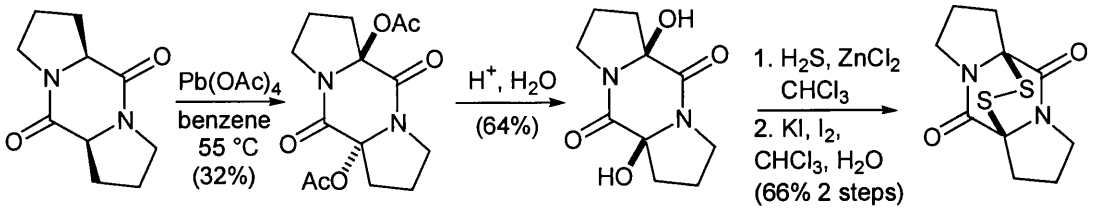


67 **91**

R = NNHPh, NPh, NEt, CHCOPh, $C(CH_2Ph)_2$, Me, *t*-Bu or O.

Scheme 18

In an alternative approach, Schmidt *et al.* proposed another method of introducing sulfur.[102] Oxidation of *cyclo*-L-proline-L-proline **92** with lead tetra-acetate yielded the 3,6-diacetoxy-*cyclo*-L-proline-D-proline **93**, which after acidic hydrolysis gave the *cis*-diol derivative **94** (Scheme 19). The disulfide bridge **95** was then obtained by Lewis-acid catalysed addition of hydrogen sulfide, followed by oxidation.[102,103] As well as hydroxyl groups, sulfones could also undergo nucleophilic displacement by sulfur.



92 **93** **94** **95**

Scheme 19

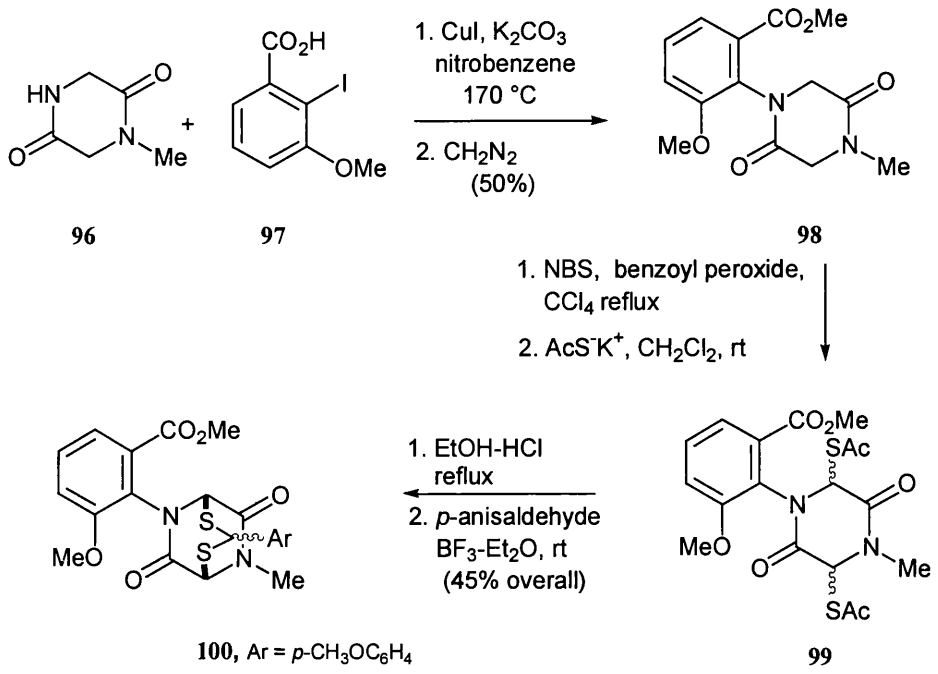
1.4.2 Total syntheses employing sulfur nucleophiles

Kishi *et al.* further applied their thioacetal methodology[94] to the total synthesis of various epi-3,6-dithio-2,5-diketopiperazines, such as dehydrogliotoxin[104] **2**, sporidesmin A[105] **3**, sporidesmin B[106] **6** and gliotoxin[95,107] **1**.

1.4.2.1 Dehydrogliotoxin

Dehydrogliotoxin **2** was the first product to be synthesised using the thioacetal intermediate **100** (Scheme 20).[104] Reaction between *cyclo*-glycine-sarcosine **96** and 2-iodo-3-methoxybenzoic acid **97**, followed by esterification using diazomethane, afforded the 2,5-diketopiperazine **98**.

Trown's methodology[65] was applied to introduce the sulfur atoms; bromination of the diketopiperazine **98** with *N*-bromosuccinimide, displacement of bromides with potassium thioacetate, followed by acidic hydrolysis and treatment with *para*-anisaldehyde in the presence of boron trifluoride etherate, yielded the required thioacetal **100** (Scheme 20).

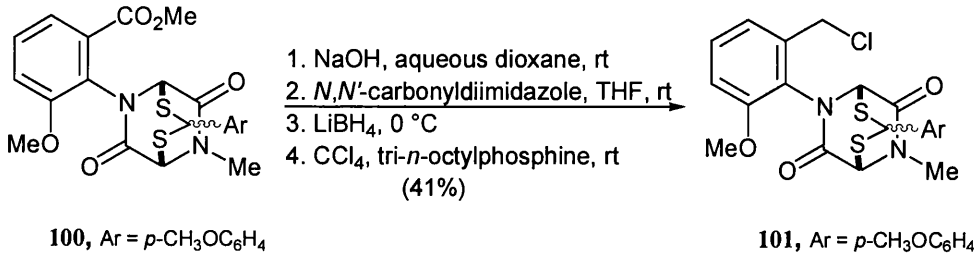


96 **97** **98**

100, Ar = *p*-$CH_3OC_6H_4$ **99**

Scheme 20

The aryl ester **100** was then converted to the benzyl chloride **101** before cyclisation onto the 2,5-diketopiperazine ring (Scheme 21). The benzyl chloride **101** was obtained by the reaction of arylester **100** with sodium hydroxide in aqueous dioxane, followed by treatment with *N,N'*-carbonyldiimidazole to yield a mixed anhydride. Reduction of the mixed anhydride with lithium borohydride produced the benzyl alcohol, which was converted to the benzyl chloride **101** by treatment with carbon tetrachloride and tri-alkylphosphine.

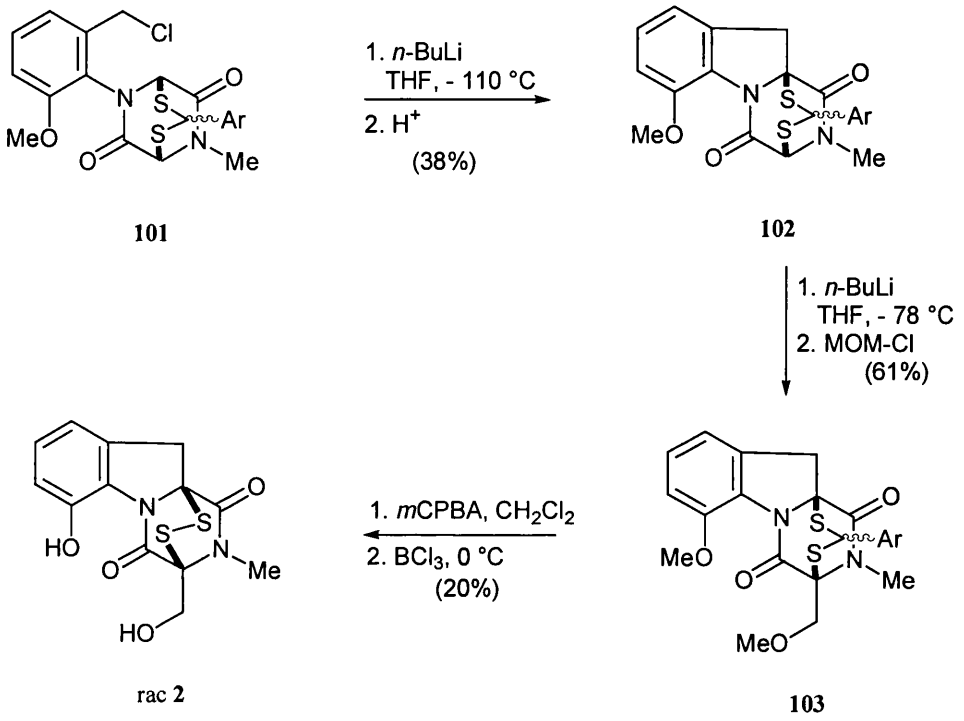


100, Ar = *p*-$CH_3OC_6H_4$ **101,** Ar = *p*-$CH_3OC_6H_4$

Scheme 21

Finally, the precursor **101** was treated with butyllithium to yield the indoline moiety **102**, which after formation of an anion at the 6 position of the 2,5-diketopiperazine **102**, and quenching by methoxymethyl chloride led to the

alkylated compound **103** (scheme 22). The disulfide bridge of **2** was then regenerated using boron trichloride with concurrent deprotection of the methyl ethers. Racemic dehydrogliotoxin **2** was obtained in 1.04% yield from the *cyclo*-glycine-sarcosine **96** (14 synthetic steps).

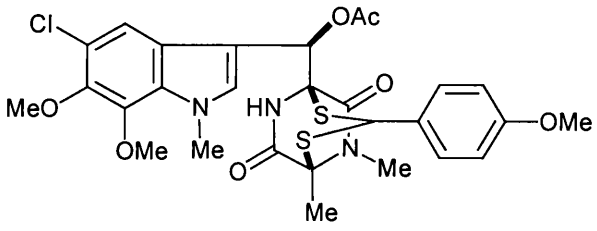


101 **102** **103** rac **2**

Scheme 22

1.4.2.2 Sporidesmins

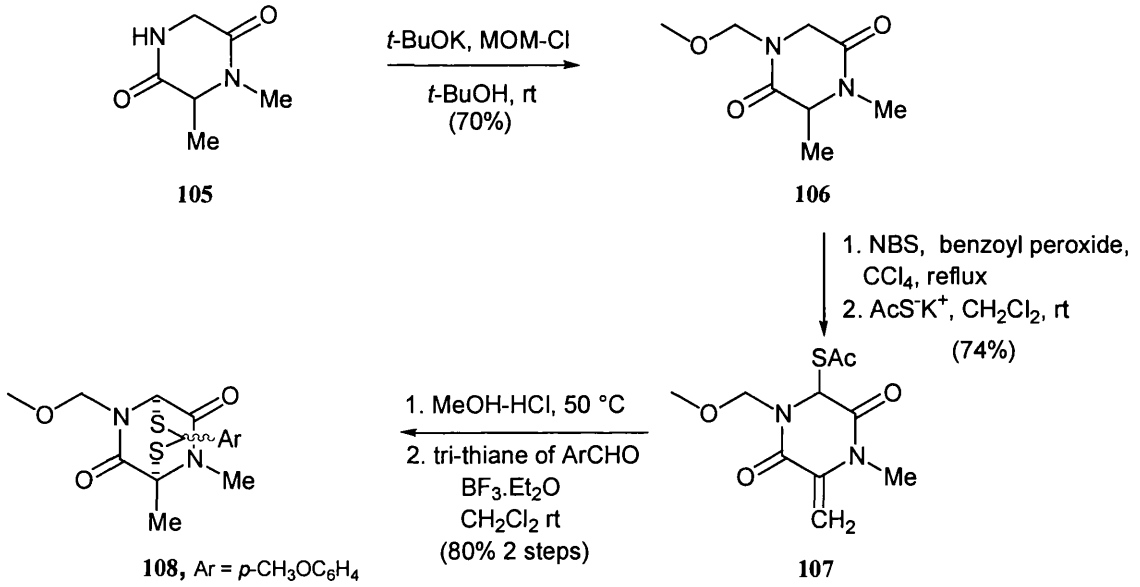
Sporidesmins A[105] **3** and B[106] **6** were also synthesised by Kishi et *al.* from a common thioacetal intermediate **104** (Figure 28) which was synthesised in a convergent manner.



104

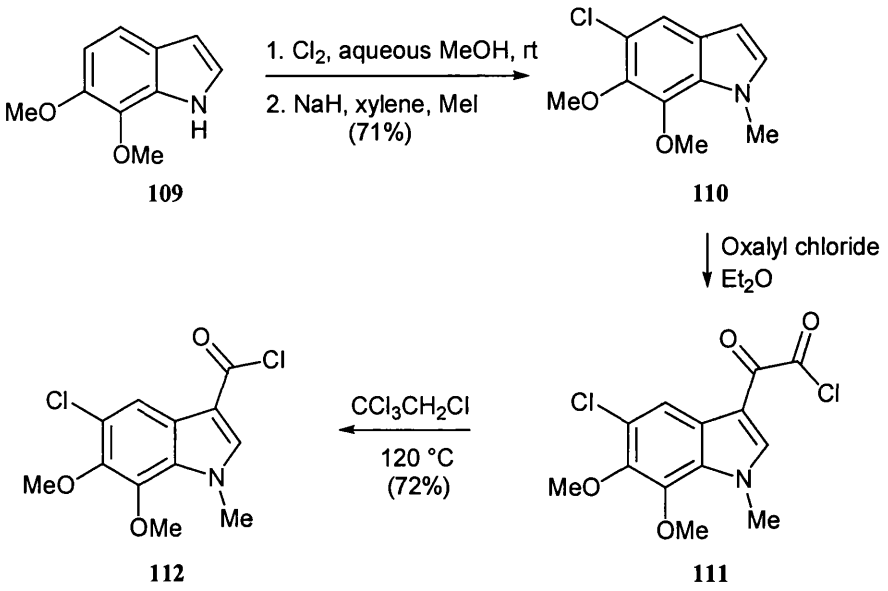
Figure 28

The thioacetal **104** used in the syntheses of sporidesmins A and B was prepared by a different approach to that used by Kishi in the total synthesis of dehydrogliotoxin.[104] Protection of the secondary amide of the 2,5-diketopiperazine **105** with a methoxy methyl group, followed by bromination of the 2,5-diketopiperazine **106** led to formation of an exo-methylene group at the 3 position of the 2,5-diketopiperazine ring **107**, due to dehydrobromination (Scheme 23). Consequently, the thioacetal **108** was prepared using the tri-thiane derivative of anisaldehyde, which was first isolated by Baumann and Fromm, in 1891 (Scheme 23).[108] A *syn*, *anti* mixture with respect to the anisaldehyde and methoxymethyl residues was formed in this reaction.



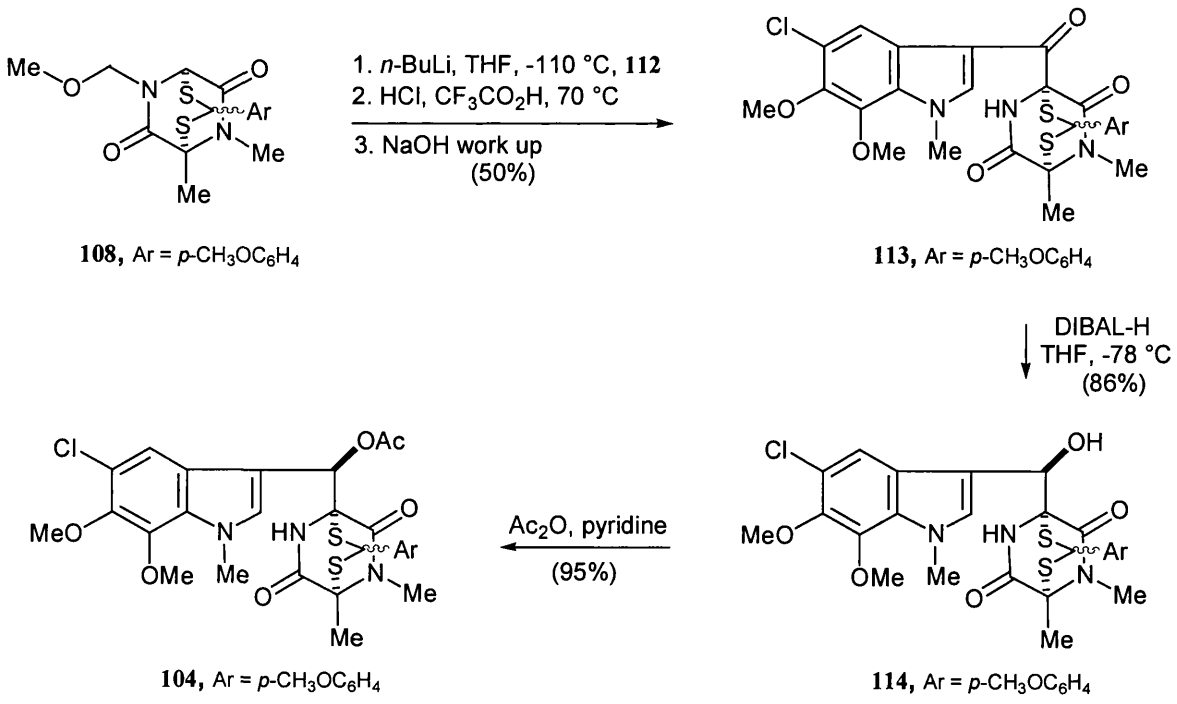
Scheme 23

The indole acyl chloride **112** was prepared from substituted indole **109** by sequential chlorination, *N*-methylation and reaction with oxalyl chloride to afford the oxamic acid chloride **111** which led in turn to acyl chloride **112** on pyrrolysis (Scheme 24).



Scheme 24

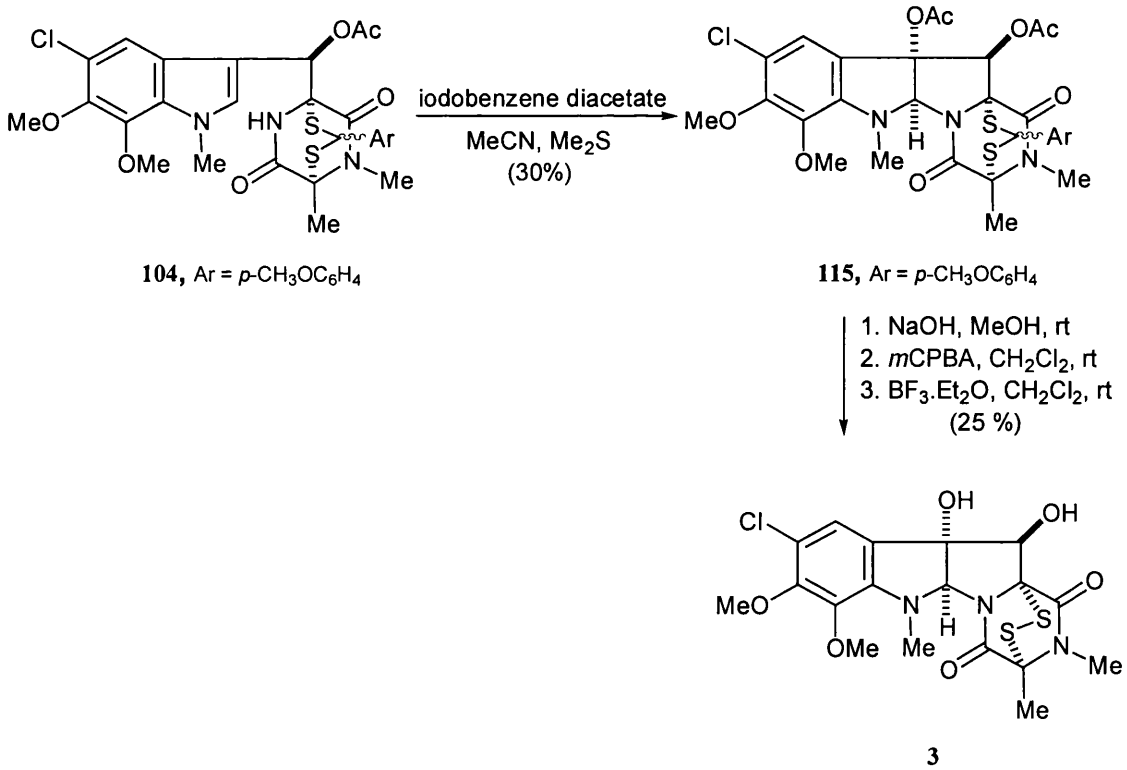
After condensation of the thioacetal **108** with the acyl chloride **112**, the methoxymethyl protecting group was removed (scheme 25). The ketone **113** was then reduced to the alcohol **114** and converted to the acetate **104**. All of these reactions proceeded efficiently to produce intermediate **104**.



Scheme 25

The relative stereochemistry of the acetate was expected by the authors to be that desired as they postulated the formation of a complex between the amide group of **113** and the reducing agent, followed by an intramolecular hydride transfer occurring from the α-face of the molecule due to the β-face being more sterically hindered by the presence of the thioacetal group.

This intermediate acetate **104** was then used in the syntheses of both sporidesmin A **3** and sporidesmin B **6**. For the formal total synthesis of sporidesmin A, Kishi *et al.* used an oxidative cyclisation reaction effected by iodobenzene diacetate, to yield the diacetate **115** in which two new stereocentres were in *trans* relationship (scheme 26).[105] Conversion of the resultant thioacetal **115** in the established manner then afforded sporidesmin A **3** in 7.5% overall yield from acetate **104** (Scheme 26).



104, Ar = *p*-$CH_3OC_6H_4$

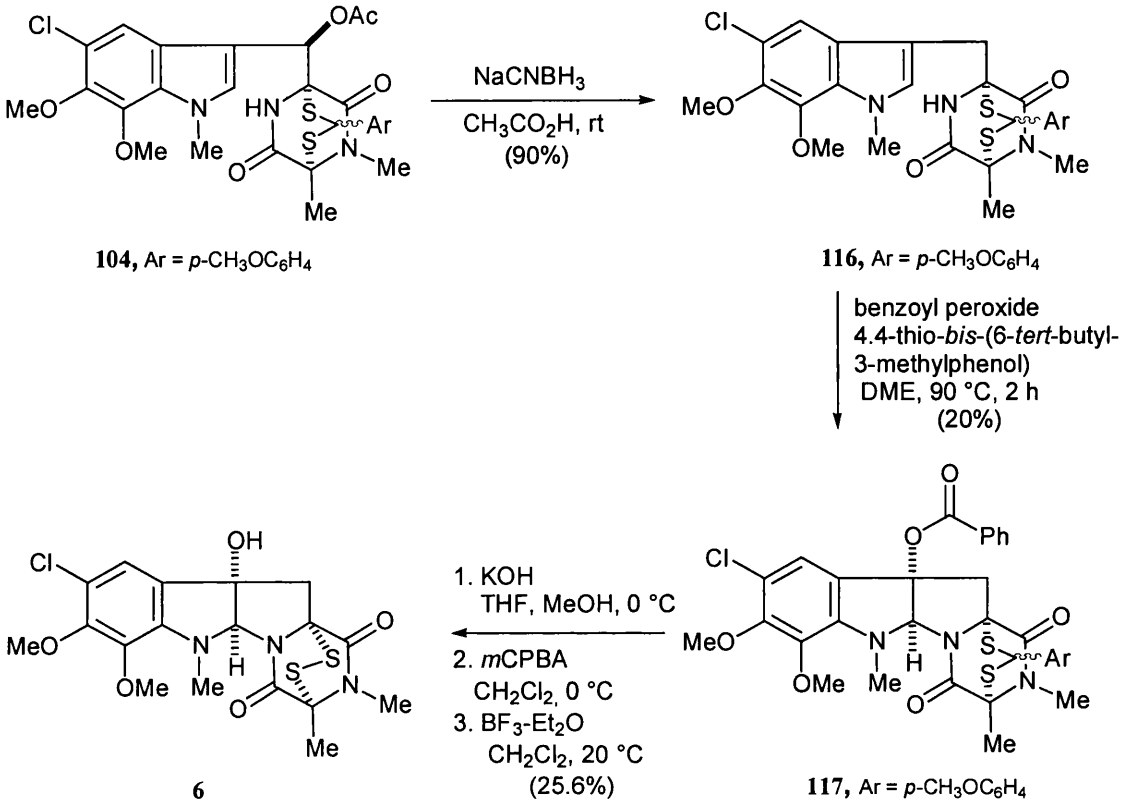
115, Ar = *p*-$CH_3OC_6H_4$

3

Scheme 26

The synthesis of sporidesmin B **6** involved the removal of the acetate group of **104** before the oxidative cyclisation, which, in this case, was effected by benzoyl

peroxide with a trace of 4,4'-thio-*bis*-(6-*tert*-butyl-3-methylphenol) to produce **117**.[106] The final steps to sporidesmin B **6** are shown in Scheme 27 and the yield from acetate **114** was 4.6%.



104, Ar = *p*-$CH_3OC_6H_4$ **116**, Ar = *p*-$CH_3OC_6H_4$

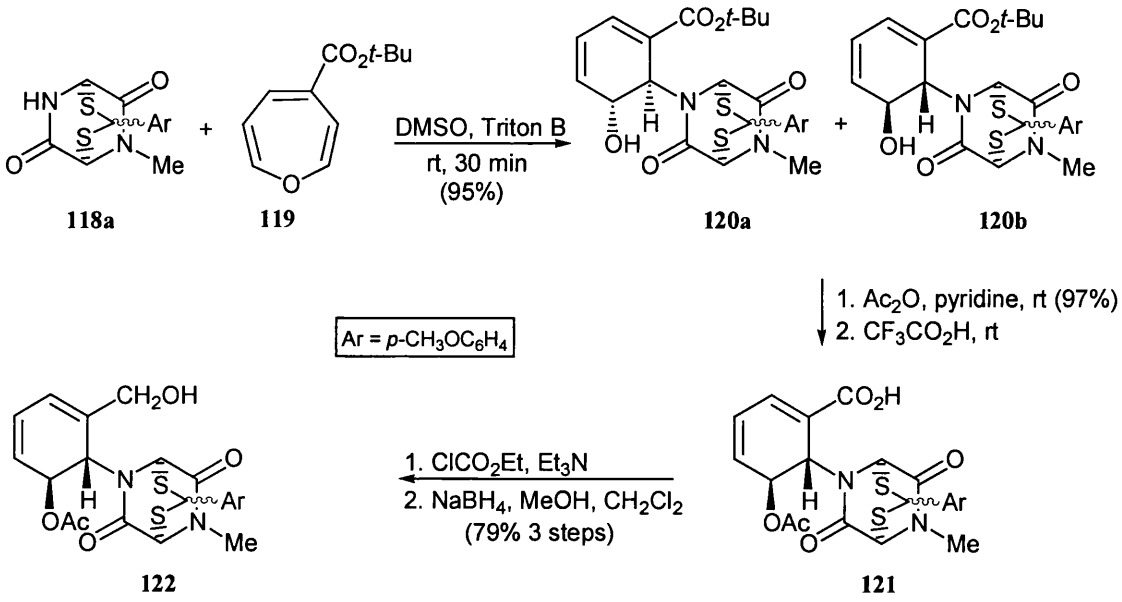
6 **117**, Ar = *p*-$CH_3OC_6H_4$

Scheme 27

1.4.2.3 Gliotoxin

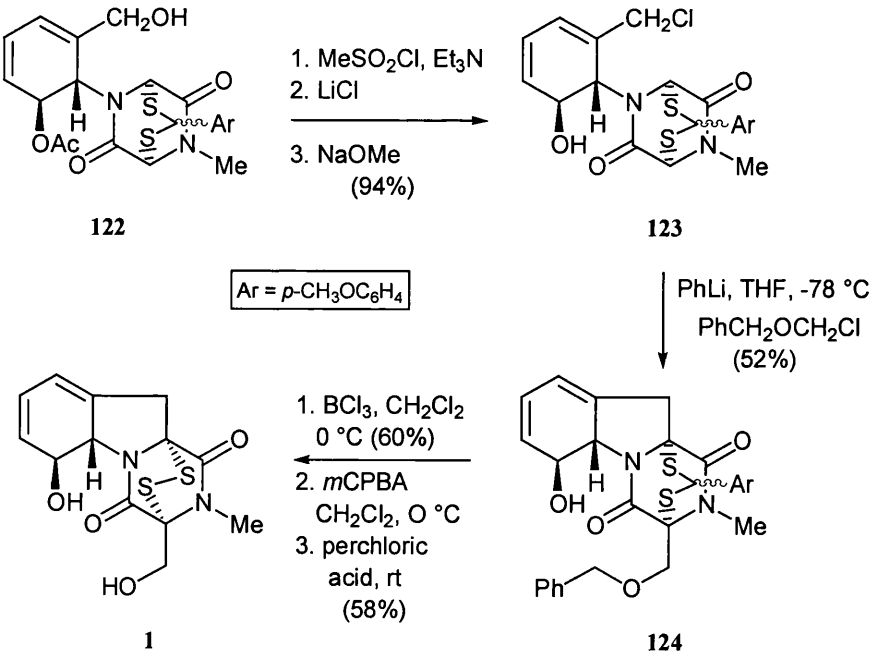
The total synthesis of gliotoxin[95,107] **1** constituted a much more challenging objective due to the necessity for incorporation of four asymmetric centres and creation of the fragile dihydro-aromatic ring in the structure. In their synthesis of racemic gliotoxin, Kishi *et al.* used a Michael reaction followed by ring opening of an arene oxide derived from an oxepin ring as their key steps.[95,107] The thioacetal **118a** was prepared in the usual manner and treated with 4-carbo-*tert*-butoxy-oxepin **119**, in the presence of Triton B, at room temperature to produce two Michael adducts **120a** and **120b** (Scheme 28). Their relative ratio was dependent on the solvent used and the most effective solvent was dimethyl sulfoxide giving a 3:1 ratio in favour of the required diastereoisomer **120b**. The alcohol **120b** was

then acetylated and the *tert*-butyl ester group was converted to the alcohol **122** *via* the acid **121** (Scheme 28).



Scheme 28

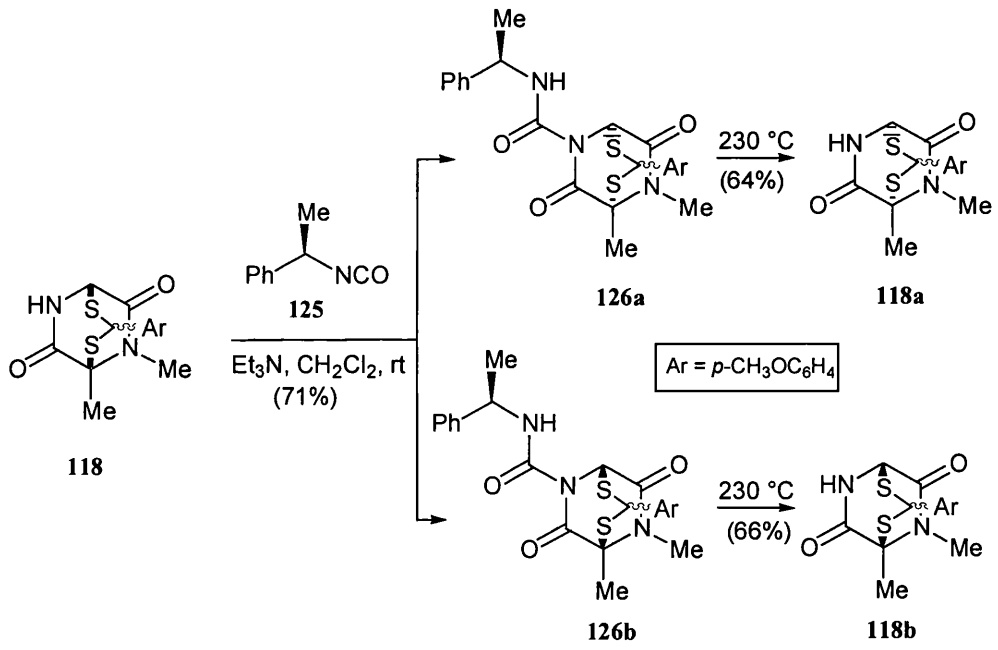
The alcohol **122** was converted into the chloride **123** (Scheme 29). The cyclisation and alkylation steps were then accomplished by the addition of phenyl lithium to the chloride **123** followed by benzyl chloro-methyl ether (Scheme 29).



Scheme 29

A free alcohol was required at this stage to prevent aromatisation, which would occur when the base was added to the thioacetal derivative **123**. Finally deprotection of **124** yielded gliotoxin **1** (scheme 29).

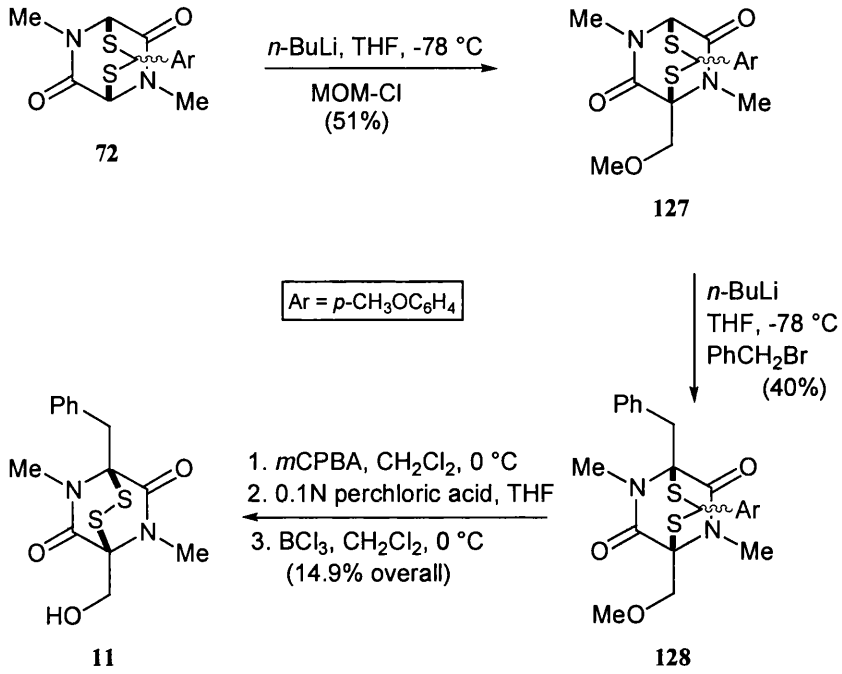
Optically pure gliotoxin **1** was synthesised after resolving the initial thioacetal **118**. Thus, treatment of **118** with (-)-α-phenylethyl-isocyanate **125** and triethylamine, at room temperature, in dichloromethane, gave diastereoisomeric ureas **126a** and **126b**, which were then readily separated by chromatographic techniques (Scheme 30). Pyrolysis of the ureas gave optically active thioacetals **118a** and **118b** in 64% and 66% yields (based on each enantiomer in racemic **118**), respectively (scheme 30).



Scheme 30

1.4.2.4 Hyalodendrin

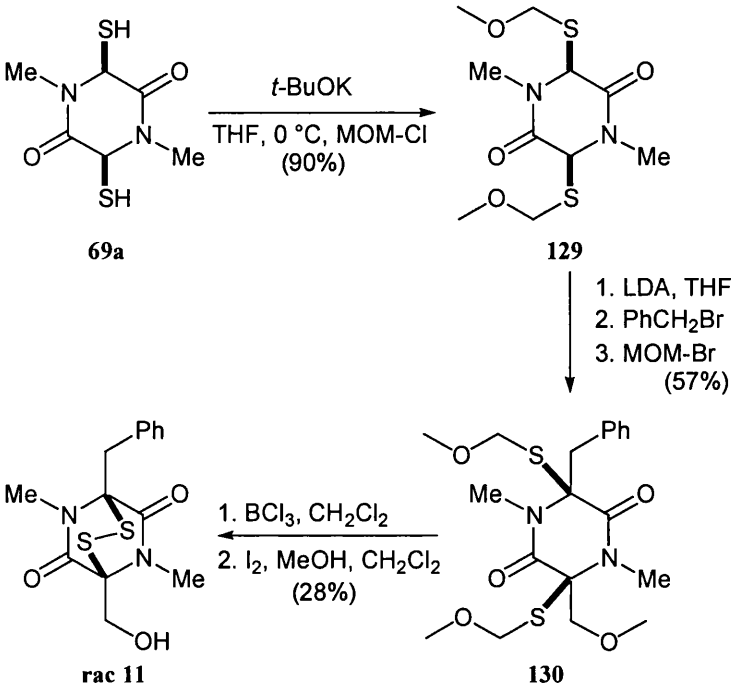
Kishi's thioacetal methodology has also been used by other researchers.[95] In 1974, Strunz and Kakushima used this strategy in the first total synthesis of (±) hyalodendrin **11** (Scheme 31).[109]



Scheme 31

However, Kishi *et al.* also published their own successful synthesis of (±) hyalodendrin **11** using their dimethoxythiomethyl derivative based methodology[95] (Scheme 32).

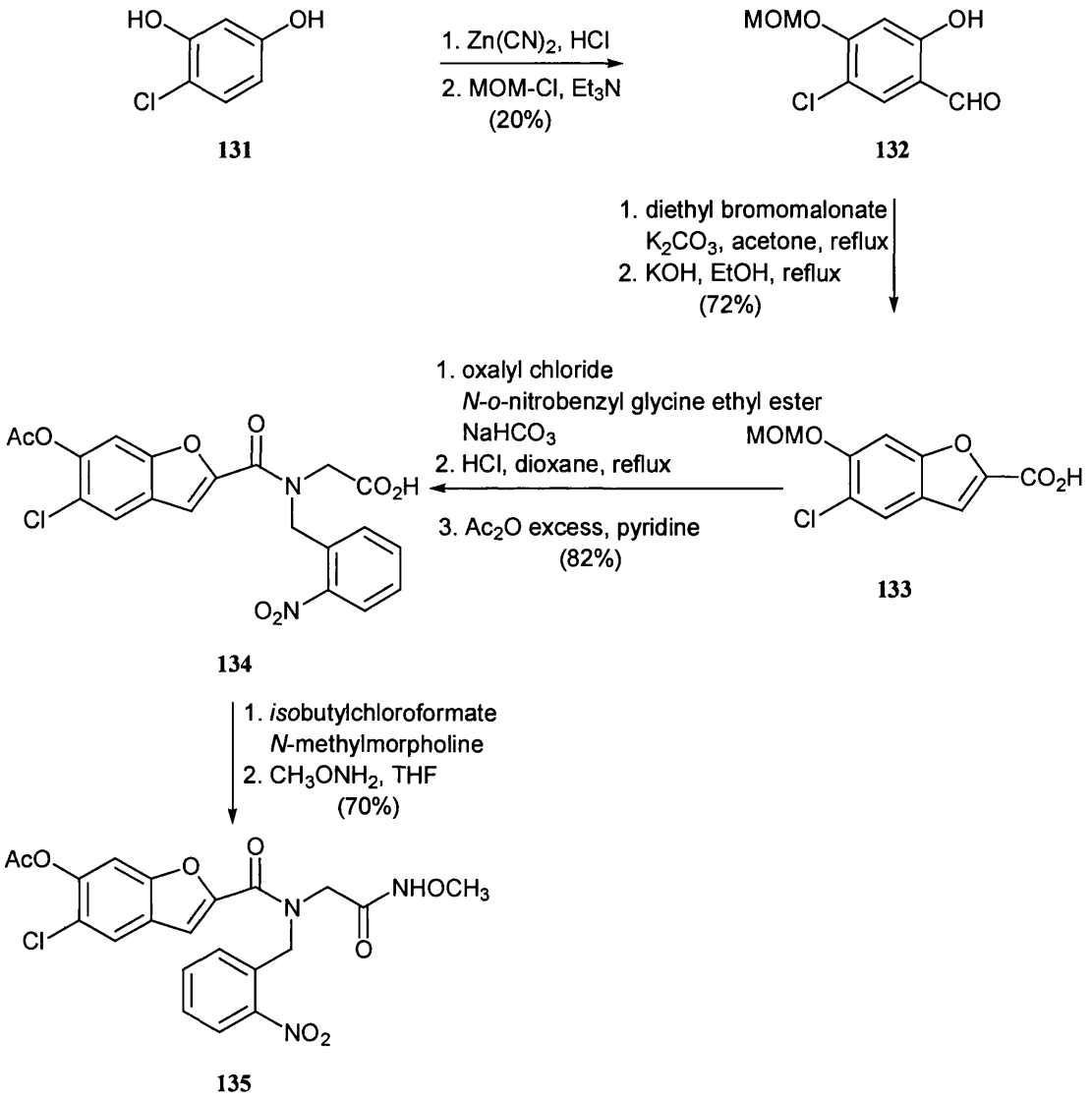
As previously discussed, the *cis*-dithiol **69a** was accordingly protected by methoxymethyl groups and the diketopiperazine **129** was deprotonated with lithium diisopropylamide to generate anions, which were then quenched with benzylbromide to yield exclusively the *cis*-product **130** (Scheme 32). The disulfide bridge **11** was then regenerated by treatment with boron trichloride followed by oxidation with iodine (Scheme 32).



Scheme 32

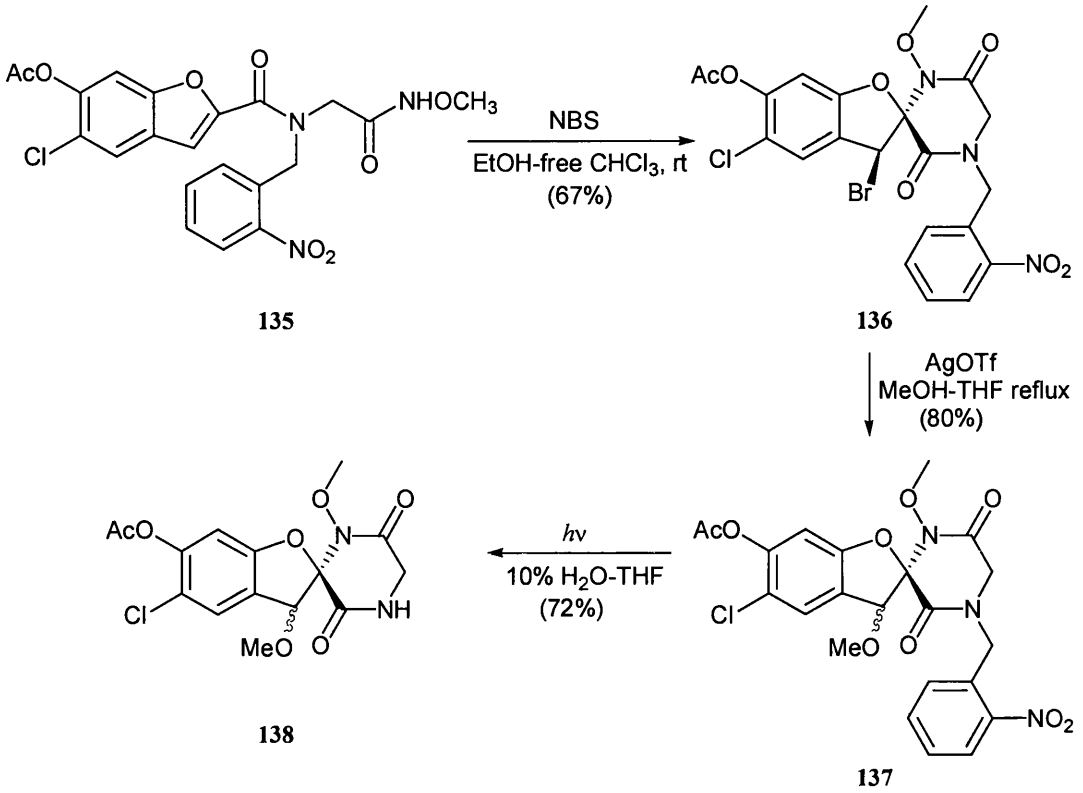
1.4.2.5 Aspirochlorine

The first total synthesis of (±) aspirochlorine **52** was published by Williams *et al.*[110] The synthesis began with Gatterman formylation of the commercially available 4-chloro-resorcinol **131** using zinc cyanide in the presence of hydrogen chloride, followed by selective protection of the 4-hydroxyl group of **131** using chloromethyl methyl ether in the presence of triethylamine to give **132** (Scheme 33). Subsequent treatment of **132** with diethylbromomalonate and potassium carbonate followed by hydrolysis under basic conditions yielded the coumarilic acid **133** in 72% yield. Coupling between the acid **133** and *N-o*-nitrobenzyl glycine ethyl ester, followed by acidic hydrolysis and acetylation afforded the compound **134**. Finally the hydroxamic acid derivative **135** was obtained by coupling **134**, *via* the mixed anhydride method, with methoxylamine (Scheme 33).



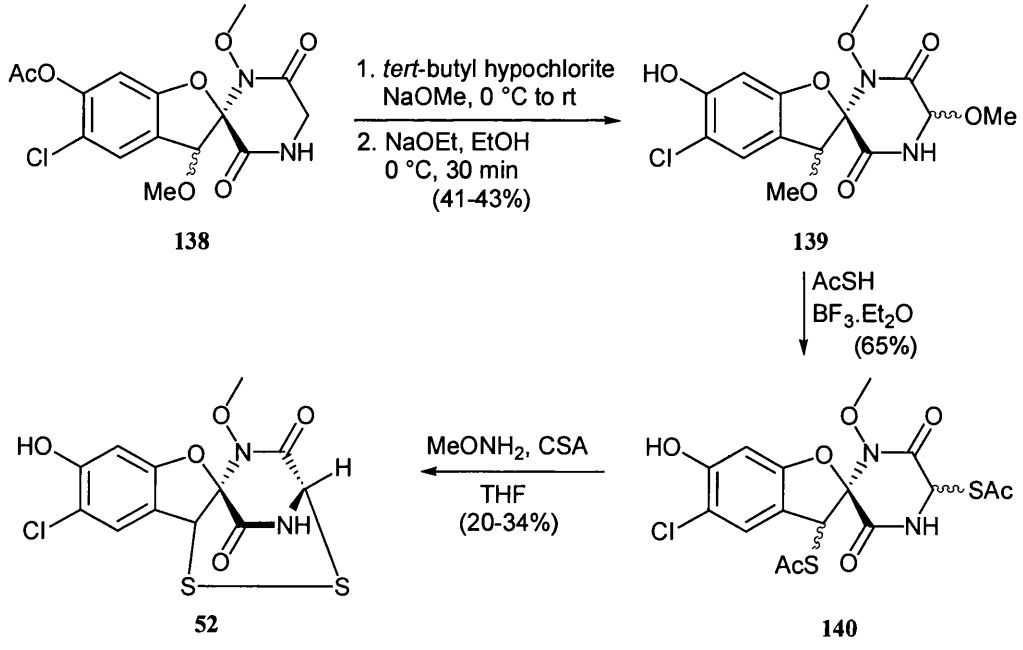
Scheme 33

Treatment of **135** with *N*-bromosuccinimide in ethanol-free chloroform yielded **136** as a single diastereoisomer possessing the desired relative stereochemistry (*i.e. trans*) (Scheme 34). Displacement of the bromide with methanol in the presence of silver triflate produced **137**, as a mixture of *cis* and *trans* products. Photolytic deprotection of the *o*-nitrobenzyl protecting group afforded **138** in 72% yield (Scheme 34).



Scheme 34

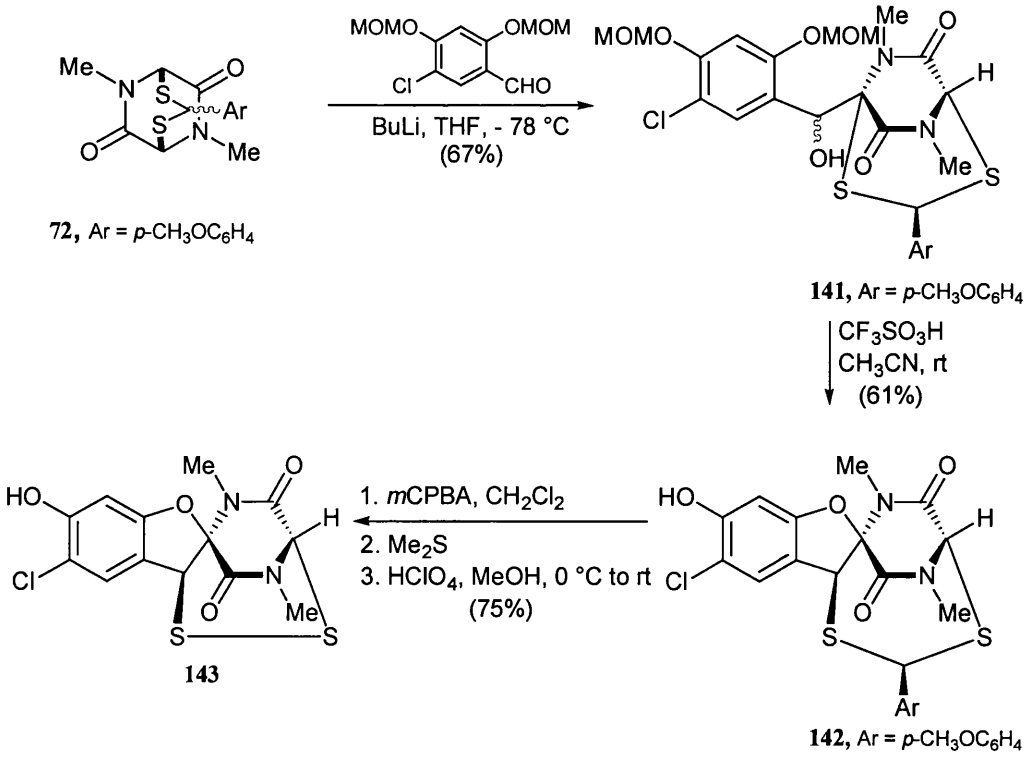
The sulfur was then introduced by firstly treating the 2,5-diketopiperazine **138** with *tert*-butylhypochlorite and sodium methoxide (Scheme 35).



Scheme 35

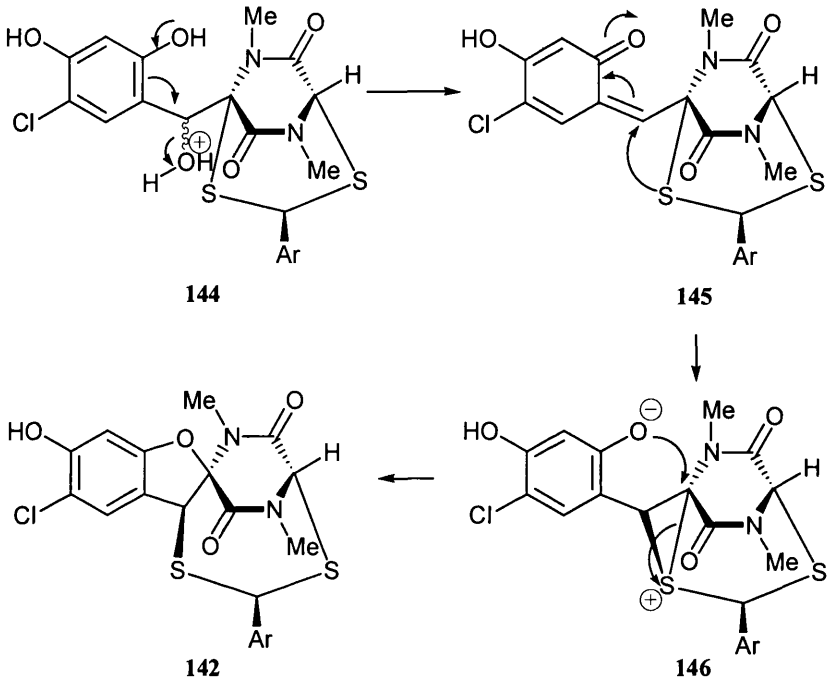
The precursor **139** formed reacted with an excess of thioacetic acid in the presence of a Lewis acid to yield **140** (Scheme 35). The disulfide bridge was finally obtained by aminolysis and produced aspirochlorin **52** in a diastereoselective fashion in 20-34% yield.

In 2000, Danishefsky *et al.*[111] published the synthesis of an analogue of aspirochlorin **52**. Their synthesis also commenced with the *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine protected with Kishi's thioacetal,[94] thus suggesting that this method is, even today, the most powerful way to introduce sulfur into a 2,5-diketopiperazine. Treatment of **72** with butyl lithium followed by addition of a highly functionalised aldehyde led to the alcohol **141**, which was treated with trifluorosulfonic acid to produce the rearranged product **142** as a single diastereoisomer (Scheme 36). Application of the thioacetal deprotection sequence yielded the analogue **143** different from aspirochlorine only in the substituents on the nitrogen atoms.



Scheme 36

From a mechanistic viewpoint, it is of interest to understand the stereochemistry of this reaction. The rearrangement occurs firstly through the protonation of the hydroxyl function to produce the intermediate **144** (Scheme 37). Elimination of water, which is aided by the phenolic oxygen atoms as shown, leads to **145** and subsequent stereospecific sulfur migration produces **142** (Scheme 37).



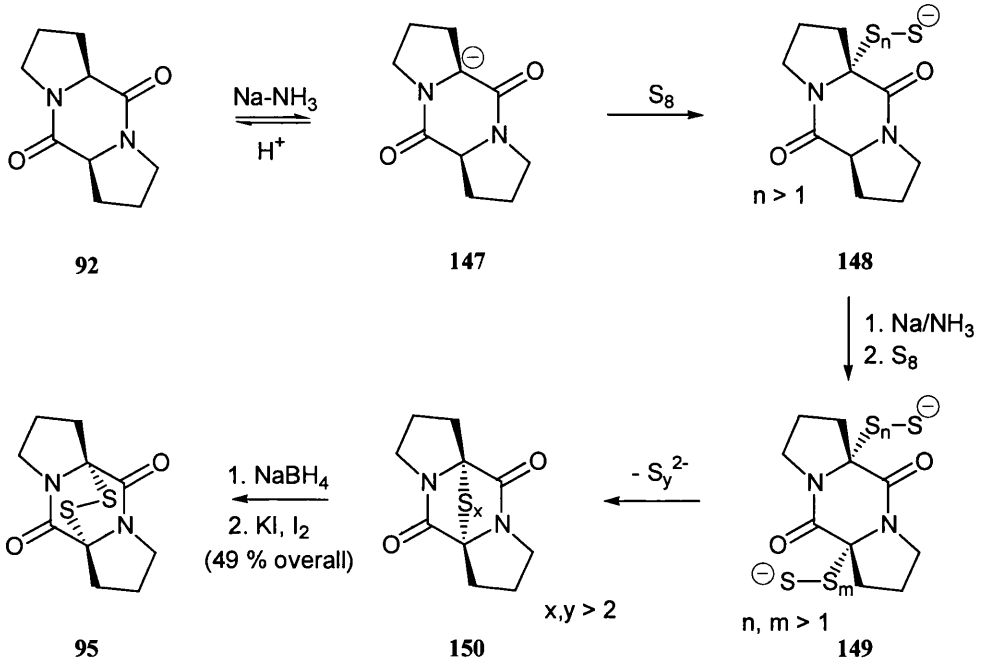
Scheme 37

1.4.3 Methods employing sulfur electrophiles

The alternative strategy for assembly of a disulfide bridge features the use of electrophilic sulfur reagents, and although less common than the foregoing nucleophilic approach, it has been applied on a number of occasions as indicated below.

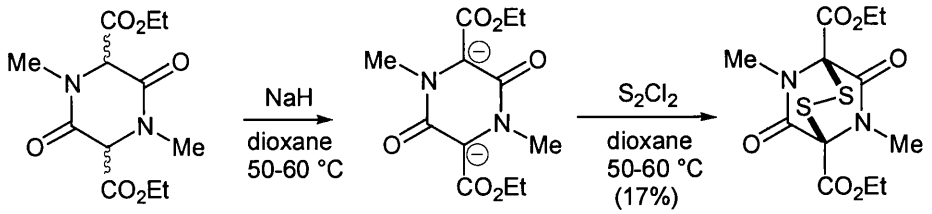
Thus, in a method introduced by Schmidt *et al.*, the disulfide bridge was incorporated into a 2,5-diketopiperazine moiety *via* reaction with a strong base and selection of atomic sulfur as the electrophile.[112,113] By way of example, deprotonation of *cyclo*-L-proline-L-proline **92** with a strong base such as sodamide

or lithium diisopropylamide afforded the anion **147**, which could be reprotonated without loss of optical activity (Scheme 38). Reaction of this anion **147** with elemental sulfur afforded the polysulfide thiolate anion **148**, and on repeating this reaction a polysulfide bridge **150** was obtained. This polysulfide bridge was then reduced and further oxidised to give the optically active epi-3,6-dithio-2,5-diketopiperazine **95** (Scheme 38).



Scheme 38

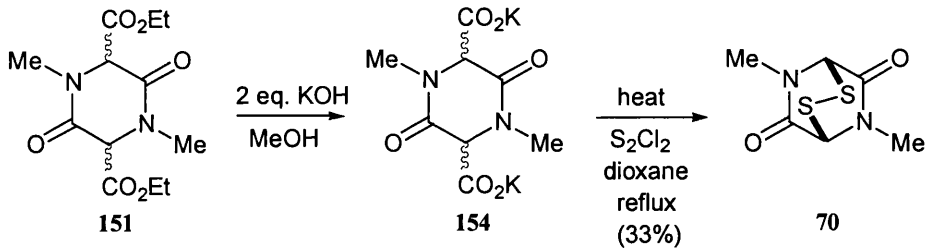
However, deprotonation can occur more easily if the 2,5-diketopiperazine possesses even more powerful electron-withdrawing substituents at the 3 and 6 positions. Hino and Sato demonstrated that 3,6-diethoxycarbonyl-2,5-diketopiperazine **151** could be deprotonated by sodium hydride to produce the dianion **152** and the disulfide bridge was then incorporated, in a single step, but in poor yield (17%) using sulfur monochloride as an alternative electrophile (Scheme 39).[114] Tetrasulfide and small amounts of tri- and mono-sulfides were also isolated along with the disulfide **153**.



151 **152** **153**

Scheme 39

The same group also demonstrated that formation of the disulfide bridge could be achieved with concomitant decarboxylation in a series of experiments involving dicarboxylate salts.[114] Thus, the potassium salts **154** of the diacid were decarboxylated in the presence of sulfur monochloride, and the disulfide bridge was formed in 33% yield (Scheme 40). Interestingly, decarboxylation did not occur in boiling dioxane alone, indicating that the sulfur reagent was involved in the decarboxylation process.



151 **154** **70**

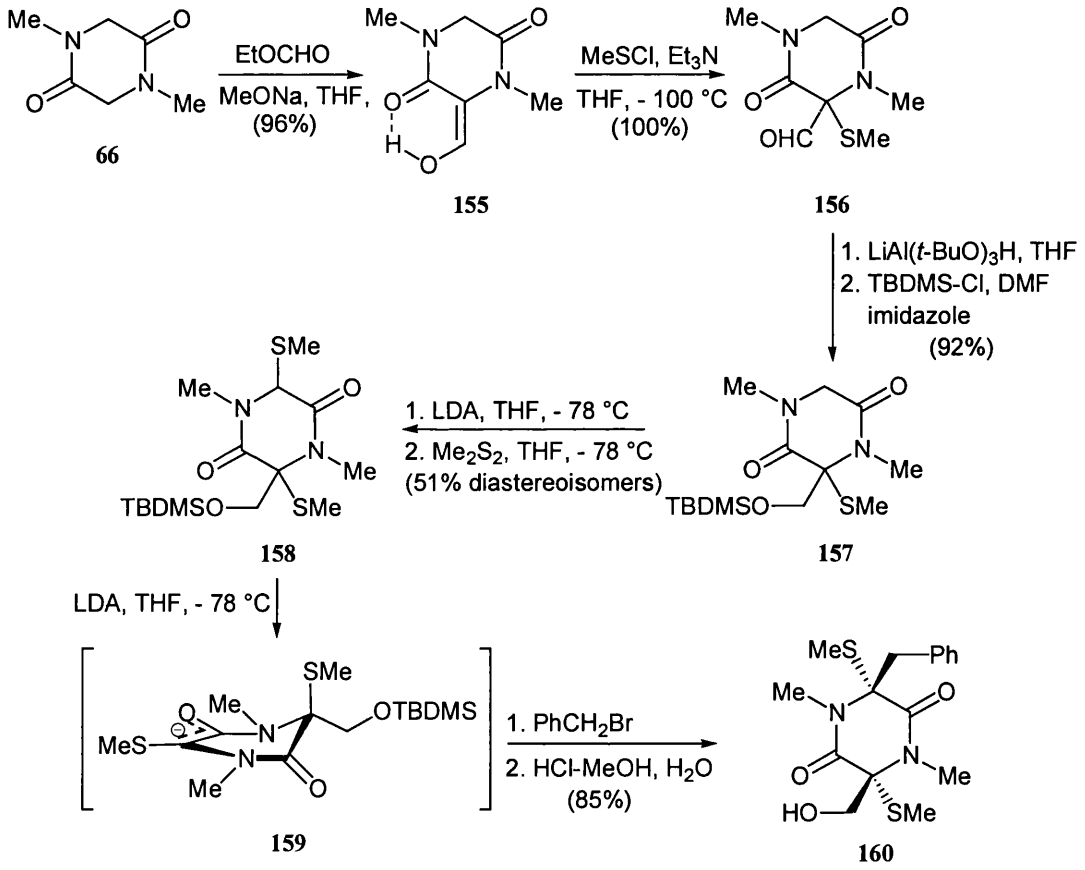
Scheme 40

1.4.4 Total syntheses employing sulfur electrophiles

1.4.4.1 Hyalodendrin

Using an electrophilic sulfur strategy, Rastetter and Williams synthesised racemic hyalodendrin **11** as well as a related metabolite, gliovictin[115] **160** (Scheme 41). Their key step was an enolate sulfenylation reaction to introduce sulfide functionality as shown in Scheme 41. Monoformylation of sarcosine anydride **66** yielded the enolic aldehyde **155**, which was sulfenylated in excellent yield with methyl sulfenyl chloride, thus introducing the first thioether group. The aldehyde

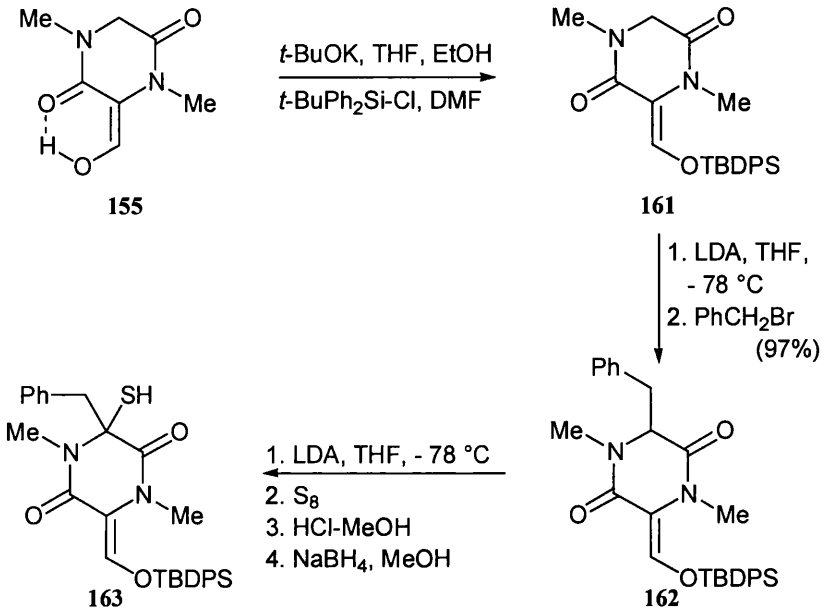
156 was then reduced to the alcohol and protected as its *tert*-butyl-dimethylsilyl ether. The second thioether moiety was then introduced at position 6 of the 2,5-diketopiperazine **157** by quenching of the lithio anion with dimethyl disulfide. On further deprotonation, the planar delocalised anion **159** was produced and electrophilic attack by benzyl bromide therefore occurred from the least hindered face (Scheme 41). After deprotection of the silyl ether, racemic gliovictin **160** was produced in 38% overall yield from sarcosine anydride **66** (Scheme 41).



Scheme 41

In order to apply this strategy to the total synthesis of (±) hyalodendrin **11**, thiol groups, and not thioethers, have of course to be introduced for subsequent oxidation to the required disulfide bridge. Accordingly, the previously described enolic aldehyde was protected as the silyl ether **161** before being deprotonated at position 6 and the benzyl side chain introduced (Scheme 42). The resulting 2,5-

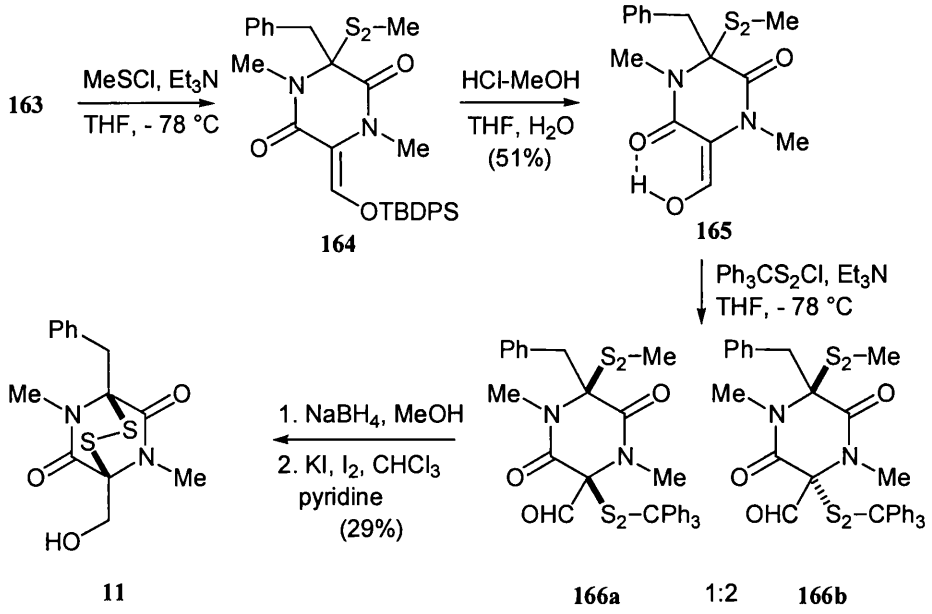
diketopiperazine **162** was again deprotonated and the anion quenched with elemental sulfur to form a polysulfide chain which was reduced to the thiol **163** (Scheme 42).



Scheme 42

Treatment of **163** with methanesulfenyl chloride led to a mixed disulfide **164** and a second disulfide unit was then introduced at position 3 of **165** by generating the free enolic aldehyde followed by reaction with triphenylmethyl-disulfenyl chloride (Scheme 43).

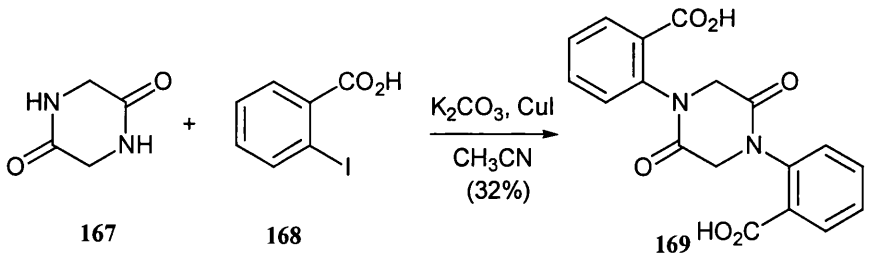
Unfortunately, two diastereoisomers **166a** and **166b** were obtained at this stage, in a ratio of 1:2 in favour of the undesired isomer. Despite this setback, the synthesis of (±) hyalodendrin **11** was completed by reduction of the dithiol, concurrently with that of the aldehyde, followed by oxidation to the disulfide (Scheme 43).



Scheme 43

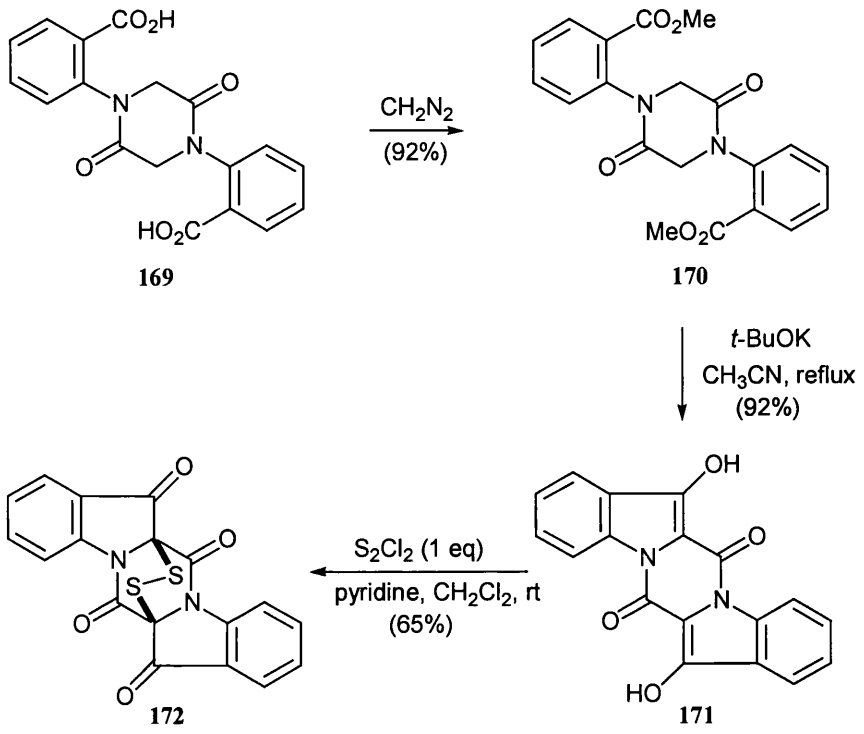
1.4.4.2 Aranotin analogue

The only synthesis, to date, of an aranotin analogue was carried out by Coffen *et al.*[116] who prepared the aromatic analogue **172** (Scheme 45). *N,N'*-Diaryl-2,5-diketopiperazine **169** was prepared using an Ullmann type condensation, similar to that used by Kishi *et al.* in their synthesis of dehydrogliotoxin **2** (Scheme 44).



Scheme 44

Esterification followed by Dieckmann condensation afforded the bis-indole **171** (Scheme 45). Reaction of **171** with sulfur monochloride proceeded efficiently in this instance and led to the disulfide **172** (Scheme 45).



Scheme 45

1.5 Characteristics of the disulfide bridge

From the preceding sections in this overview it will be apparent that the disulfide bridge of epi-3,6-dithio-2,5-diketopiperazines provides an exciting challenge for organic chemists both in terms of synthesis and biosynthesis. Even more importantly perhaps, in terms of understanding possible mode of action for this biologically active class of molecules, is the fact that removal of this bridge results in a loss of activity.[117]

In consequence, a variety of physical techniques have been employed to study the detailed physical characteristics of this unusual sulfur-sulfur bond and to reveal the features which make it very different from simpler acyclic disulfides. These are detailed below.

1.5.1 X-Ray studies

The characteristic conformation of this disulfide bridge has been demonstrated by X-ray studies to involve a helical twist wherein each sulfur atom is in closer proximity to the adjacent carbonyl group than to the adjacent nitrogen atom (Figure 29). The C-S-S-C dihedral angles vary from 8° (chaetocin **32**) to 18° (acetylaranotin **8**), the chirality of the asymmetric centres preordaining the left or right handed nature of the twist (Figure 29). The conformation of the disulfide bridge of epi-3,6-dithio-2,5-diketopiperazines therefore differs very considerably from the twisted geometry of a simple acyclic disulfide bond, such as HSSH or H_3CSSCH_3, which have dihedral angles of approximately 90°.[118] The conformation of the disulfide bridge may be considered to involve intramolecular electrostatic attraction between sulfur lone-pair electrons and the electrophilic carbon atom of the amide bond. Repulsion between sulfur and nitrogen atom lone pairs may be also implicated.

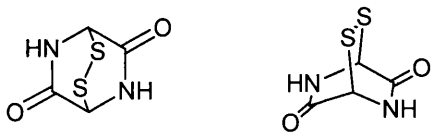
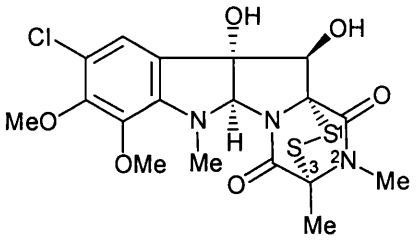


Figure 29

1.5.2 Infra-red and Raman spectra studies

Infra-red studies of sporidesmin A **3** show amide carbonyl stretching frequencies at 1655 and 1680 cm^{-1}. However, when the disulfide bridge of sporidesmin A **3** is opened and alkylated as in sporidesmin D **173**, the carbonyl stretching frequencies of these derivatives move to 1675 and 1700 cm^{-1} thereby revealing a relaxation of the disulfide bridge strain (Figure 30).[119]



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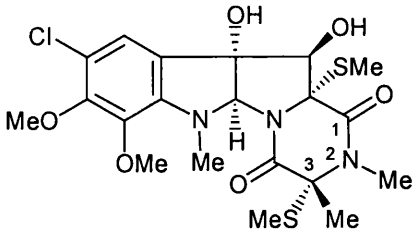


173

Figure 30

Similarly, Raman spectral studies of *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70**, chaetocin **32**, acetylaranotin **8** and gliotoxin **1** reveal S-S stretching frequencies between 486 and 510 cm^{-1} whereas normal aliphatic disulfide ν(S-S) frequencies of 1,2-dithiane derivatives are between 509 and 532 cm^{-1}, also confirming the strained nature of the disulfide bond in the epi-3,6-dithio-2,5-diketopiperazine ring.[120]

1.5.3 NMR studies

NMR studies carried out by Ronaldson also revealed the strain of the epi-3,6-dithio-2,5-diketopiperazine system through comparison of the chemical shifts of both sporidesmin A **3** and D **173** (Figure 30).[121,122] Indeed, ^{1}H NMR studies of sporidesmin D **173** showed that the proton resonance of the 3-methyl group moved upfield by 0.16 ppm (in $CDCl_3$ or 0.23 in CD_3COCD_3) compared to sporidesmin A **3** (Figure 30). Moreover, ^{13}C NMR studies of sporidesmin D **173** revealed that the 3-methyl resonance moved downfield by 7.3 ppm and that the quaternary carbon atoms bonded to sulfur moved upfield by 4.6-4.8 ppm, when compared with sporidesmin A **3**.

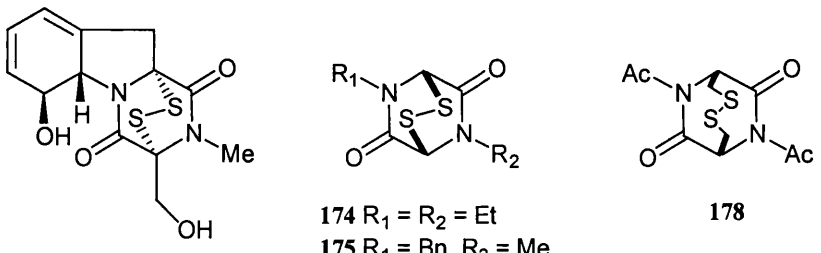
1.5.4 Circular dichroism studies

Circular dichroism (CD) studies on gliotoxin **1** showed the 1,3-cyclohexadiene long-wavelength band had an unexpected positive band at *ca.* 270 nm, which is opposite to the negative band found for simple diene systems. The same anomaly was observed in aranotin's **7** CD spectrum and could result from an interaction

between the disulfide bridge and the diene chromophore, present in both gliotoxin **1** and aranotin **7**.[123] Despite the fact that sporidesmin A **3** has the same absolute configuration of the disulfide bridgehead carbons as gliotoxin **1**, its CD spectrum showed a positive band at *ca.* 360 nm, this being the opposite to the negative band of gliotoxin **1**. Similarly, chaetocin **32** has a negative CD band although its disulfide bridge chirality is opposite to that of gliotoxin **1**. These differences can be explained by the disulfide dihedral angle being smaller (10° and 8° for sporidesmin A **3** and chaetocin **32** respectively) than that of gliotoxin **1** (15°). Therefore, the disulfide transition band will be smaller and its sign easily inversed by interaction with the amide groups and with the common indolinyl chromophores.

1.5.5 Electrochemical studies

In 1999, Chai *et al.* published the first electrochemical studies of epi-3,6-dithio-2,5-diketopiperazines,[124] by comparing the voltammetric behaviour of gliotoxin **1**, synthetic analogues **174-177,** and 1,4-diacetylcystine anhydride **178** (Figure 31). The polarograms of gliotoxin **1** and compounds **174-177** displayed a wave with two components whereas 1,4-diacetylcystine anhydride **178** was unaffected.



1

174 $R_1 = R_2 = Et$
175 $R_1 = Bn$, $R_2 = Me$
176 $R_1 = R_2 = Bn$
177 $R_1 = R_2 = p$-MeOBn

178

Figure 31

Moreover, coulometric measurements on compound **174** demonstrated a one-electron reduction of the epi-3,6-dithio-2,5-diketopiperazine compound overall suggesting that the first wave was characteristic of an adsorption phenomenon whilst the second resulted from a reduction process. The most significant conclusion from this study is that the disulfide bridge of epi-3,6-dithio-2,5-

diketopiperazines can be more easily reduced than simple dialkyl disulfide bonds which exhibit a two-electron polarographic process. This can once again be correlated with their atypical conformation and their more strained S-S bond when compared with acyclic analogues (S-S bond length 2.08 Å *vs.* 2.04 Å).

The summation of these detailed physical measurements clearly reveals the ability of the surrounding molecular architecture in these natural products to transform a disulfide linkage into a highly reactive entity which is much more easily reduced by single electron transfer than any normal sulfur-sulfur bond.

In conclusion, from every possible aspect, ranging through synthesis, biosynthesis, structure and activity, it would appear that this unusual family of natural products has many more challenges to offer for the organic chemist.

Chapter 2

Results and Discussion

Objectives of the present research programme.

The foregoing introduction has hopefully highlighted the varied and significant biological activity that may be found within the epi-3,6-dithio-2,5-diketopiperazine family and also demonstrated that the unusual properties of the disulfide bridge are clearly responsible for activity. The synthetic overview has also indicated that the method of Trown,[65] as applied and modified by Kishi *et al.*,[95] has been, to date, the most effective one for installation of the disulfide bridge of the epi-3,6-dithio-2,5-diketopiperazines.

From the outset, our primary concern was to understand the mode of action of epi-3,6-dithio-2,5-diketopiperazines in relation to their unusual disulfide bridge. For this reason, initial studies concentrated on the synthesis of simple models of natural epi-3,6-dithio-2,5-diketopiperazines using the previously discussed methodologies in order to test these molecules both by *in vitro* and *in vivo* experiments. A second ongoing challenge throughout our studies was to investigate new synthetic methodologies for disulfide bridge construction. For the purpose of clarity, although several of the above objectives were under investigation at the same time, the present chapter has been subdivided into synthetic and mechanistic sections.

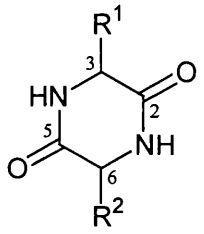
2.1 Preparation of epi-3,6-dithio-2,5-diketopiperazines.

In common with most research groups, we decided, in the first instance, that the synthesis of epi-3,6-dithio-2,5-diketopiperazines naturally begins with the preparation of 2,5-diketopiperazines as they constitute the heterocyclic skeleton of our target molecules. The subsequently introduction of sulfur using previously mentioned synthetic approaches was also considered as the simplest strategy to prepare representative models of this class of compounds for our studies.

2.1.1 2,5-Diketopiperazines.

2.1.1.1 Introduction

2,5-Diketopiperazines are the smallest cyclic peptides and a common feature in several natural products usually isolated from cultures of yeast, lichens and fungi (Figure 32).[125] They are also formed as undesired or degradation products both in solution and solid phase synthesis of linear peptides.[126]

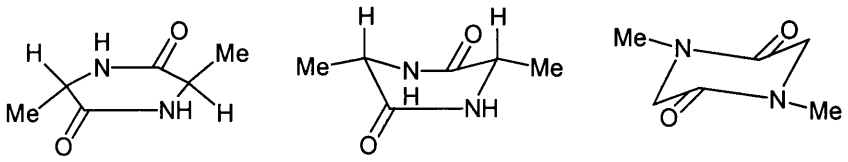


R^1, R^2 = H or amino acid side chains

Figure 32

Their existence as a special group of compounds was first recognized around 1900.[127] Fisher managed to synthesize many of the simplest members of this family in the early 1900's.[128] The parent compound, often referred to as *cyclo*-glycine-glycine, was prepared in 1888.[129] The simple 2,5-diketopiperazines are often water-soluble but many are only sparingly soluble.

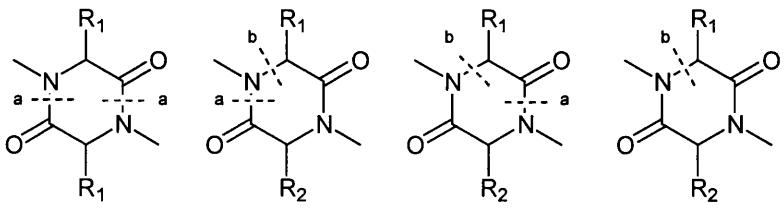
X-ray studies demonstrated that the conformation adopted by the 2,5-diketopiperazine ring can vary as a function of the nature of the substituents. Indeed *cyclo*-glycine-glycine[130] is planar as is *cyclo*-D-alanine-L-alanine[131] **179a**, but *cyclo*-L-alanine-L-alanine[131] **179b** adopts a slightly twisted boat conformation with the methyl groups in quasi-equatorial positions, whereas *cyclo*-sarcosine-sarcosine[132] **66** exhibits a flattened chair conformation (Figure 33).



179a **179b** **66**

Figure 33

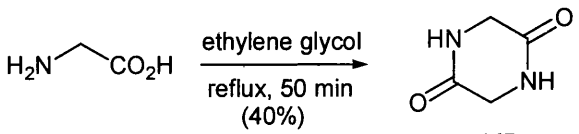
Since natural product 2,5-diketopiperazines are potential drug candidates, a number of solution and solid state syntheses have emerged and have been extensively reviewed.[1,133,134] In order to synthesise these cyclic dipeptides, synthetic strategies most commonly take advantage of the wide range of natural and unnatural amino acids as enantiomerically pure starting materials and therefore require sequential amide bond formation as in I (Scheme 46). Although formation of the amide bond (a) is dominant, alternative strategies involving C-N bond formation (b) either in a first intramolecular step or in an intramolecular cyclisation have also been used as implied in II, III and IV (Scheme 46). Thus many disconnections become feasible as shown in the representative examples.



I **II** **III** **IV**

Scheme 46

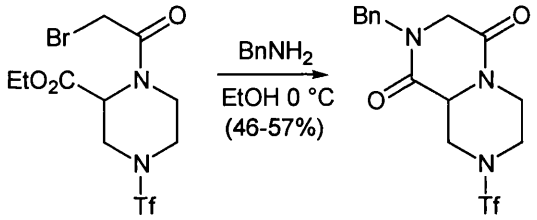
Firstly, the most convenient method for preparation of a symmetrically substituted 2,5-diketopiperazine is to heat the corresponding amino acid or its ester derivative in a high boiling point solvent. This one-pot reaction involves sequential formation of two amide bonds and was used by Schott *et al.* for the synthesis of *cyclo*-glycine-glycine.[135] Thus, glycine **180** was heated in ethylene glycol at reflux for 50 minutes and converted into the parent 2,5-diketopiperazine **167** in 40% yield (Scheme 47).



180 **167**

Scheme 47

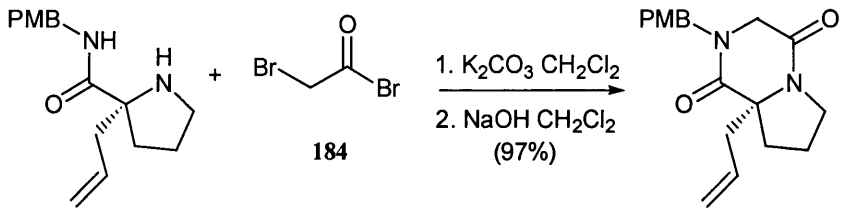
Formation of two amide bonds remains an efficient strategy for synthesis of 2,5-diketopiperazines. The tandem reaction acylation-alkylation of an amine with a suitable halo-ester allows the formation of the 2,5-diketopiperazine in a one-pot sequence. The reaction can be carried out with ammonia[136] or with primary amines, such as methylamine[137] or benzylamine,[138] as exemplified by Gubert *et al.* for the synthesis of the 2,5-diketopiperazine **182** starting from the bromo-ester **181** (Scheme 48).



181 **182**

Scheme 48

From a different disconnection approach, an α-amino-amide can react with a bifunctional electrophile. Williams *et al.* obtained 2,5-diketopiperazine **185** by reaction of the precursor **183** with bromoacetylbromide **184** (Scheme 49).[139]

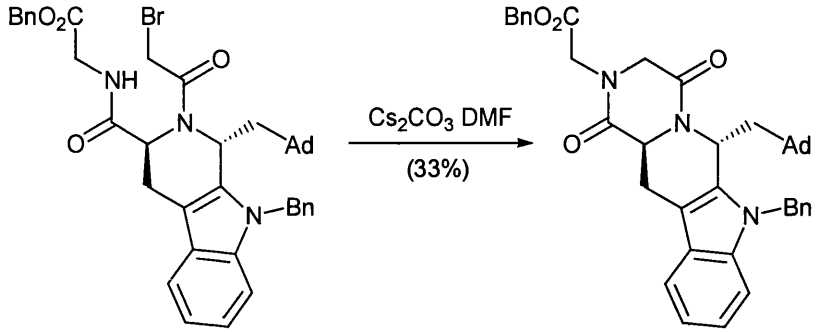


183 **184** **185**

Scheme 49

The same bifunctional electrophile **184** was used in a two-step sequence of acylation and intramolecular C-N bond formation under basic conditions.

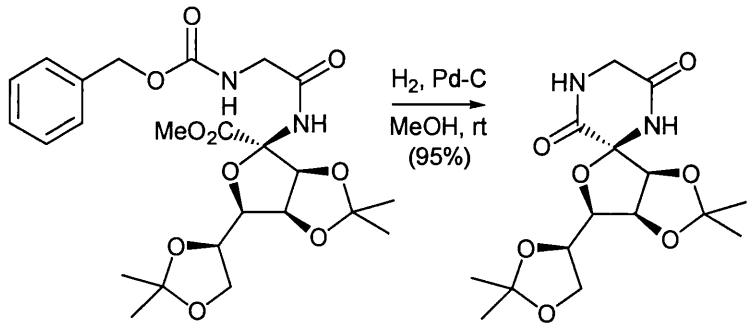
McDonald *et al.* reported this transformation in the synthesis of the 2,5-diketopiperazine **187**, which was obtained from ring closure of the precursor **186** in the presence of caesium carbonate (Scheme 50).[140] Overall however, this method is rarely used.



186 **187**

Scheme 50

For ease of installing chiral centres at the 3 and 6 positions of their ring, 2,5-diketopiperazines are most often prepared by intramolecular cyclisation of the corresponding dipeptide. After coupling of a *N*-protected amino acid to an amino ester, the dipeptide formed is *N*-deprotected and ring closure proceeds efficiently *in situ* or at elevated temperature.[141] Fisher's method[128b] of hydrogenation of benzyloxycarbonyl dipeptide methyl ester **188** in methanol over a palladium on charcoal catalyst followed by spontaneous cyclisation, leads to the formation of the 2,5-diketopiperazine **189** (Scheme 51).[141]



188 **189**

Scheme 51

An alernative is the use of *tert*-butoxycarbonyl as protecting group. Treatment of a *tert*-butoxycarbonyl-protected dipeptide methyl ester with formic acid and cyclisation by heating the derived formate salts in neutral solvents, such as toluene, produce the corresponding 2,5-diketopiperazine.

The overwhelming problem is that in order to form 2,5-diketopiperazines, the dipeptide precursors have to adopt a cisoid conformation rather than the more favoured transoid conformation. The strong contribution from the resonance form of the amide bond in the favoured transoid form is the simplest way of expressing the idea that rotation around the C-N bond is energetically demanding (Figure 34).[142] Because dipeptides containing proline are forced to adopt the cisoid conformation by the presence of the pyrrolidine ring, they are much more prone to cyclise to form the 2,5-diketopiperazine ring. Thus, it is not surprising to find quite a large number of proline derivates in this group of natural products.[134]

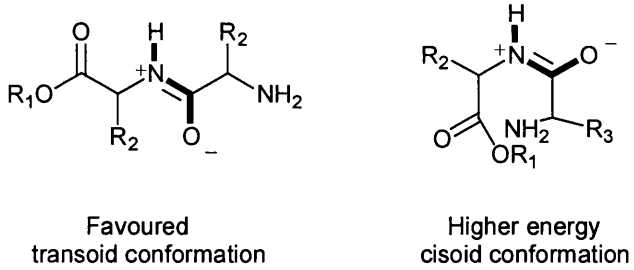
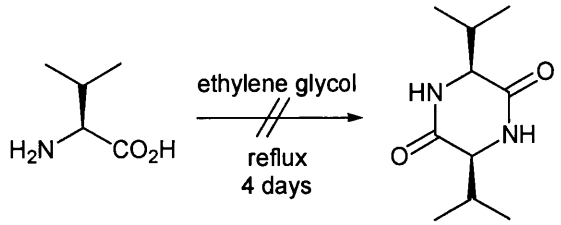


Figure 34

2.1.1.2 Symmetrical 2,5-diketopiperazines

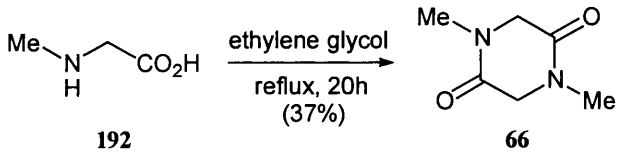
Our own first attempt in the 2,5-diketopiperazine area was the synthesis of *cyclo*-L-valine-L-valine **191** using the method described by Schott (Scheme 52).[135] The corresponding amino acid **190** was heated in ethylene glycol at reflux (Scheme 52). However L-valine **190** remained insoluble even at 180 °C and did not condense to form the desired 2,5-diketopiperazine **191**.



190 **191**

Scheme 52

Nevertheless, the preparation of *cyclo*-sarcosine-sarcosine **66** was achieved successfully using the same method.[135] The unnatural amino acid **192** was soluble in boiling ethylene glycol, and after 20 hours under reflux, the 2,5-diketopiperazine **66** crystallised from the reaction mixture on cooling to room temperature (Scheme 53).



192 **66**

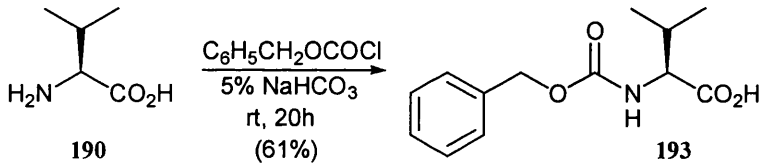
Scheme 53

Although this one-pot reaction represents an attractive strategy in principle for the synthesis of symmetrical 2,5-diketopiperazine it relies on the solubility of the amino acid during the reaction and on its concentration. Indeed, theoretically, a dilute solution helps the dimerisation whereas a concentrated solution of the amino acid can lead to acyclic polymers. On the practical front however, if the solution is too dilute, neither the linear dipeptide nor the 2,5-diketopiperazine is formed, and hence a compromise needs to be found for each amino acid between dilute and concentrated solutions.

Moreover, not only was extraction of the 2,5-diketopiperazine from ethylene glycol difficult but decolouration of the organic phase with charcoal was necessary, thus giving low yields. At this stage, more general methods, which could be applied to both symmetrical and unsymmetrical 2,5-diketopiperazines, were therefore required.

A report from Ueda *et al.* described the syntheses of various 2,5-diketopiperazines based on cyclisation of a dipeptide in refluxing methanol.[143] They wanted to find a facile synthesis of cyclic dipeptides, which would improve yield, and especially optical purity of the products. They prepared *cyclo*-L-valine-L-valine as well as *cyclo*-L-valine-D-valine and other *cyclo*-dipeptides from different amino acids such as alanine, serine and proline. As they obtained good results, we decided to follow this procedure for the synthesis of *cyclo*-L-valine-L-valine **191**.

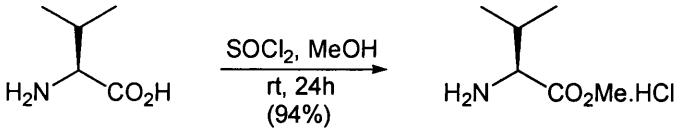
Thus the synthesis of *cyclo*-L-valine-L-valine **191** involved firstly preparing the *N*-benzyloxy-carbonyl-L-valine **193** by treating L-valine **190** with an aqueous solution of sodium bicarbonate and benzylchloroformate (Scheme 54). After stirring at room temperature, the *N*-protected L-valine **193** was obtained in 61% yield.



190 **193**

Scheme 54

L-Valine methyl ester hydrochloride **194** was also prepared independently by stirring L-valine **190** with thionyl chloride in methanol, which remains the simplest efficient method to prepare an amino acid methyl ester hydrochloride (Scheme 55).

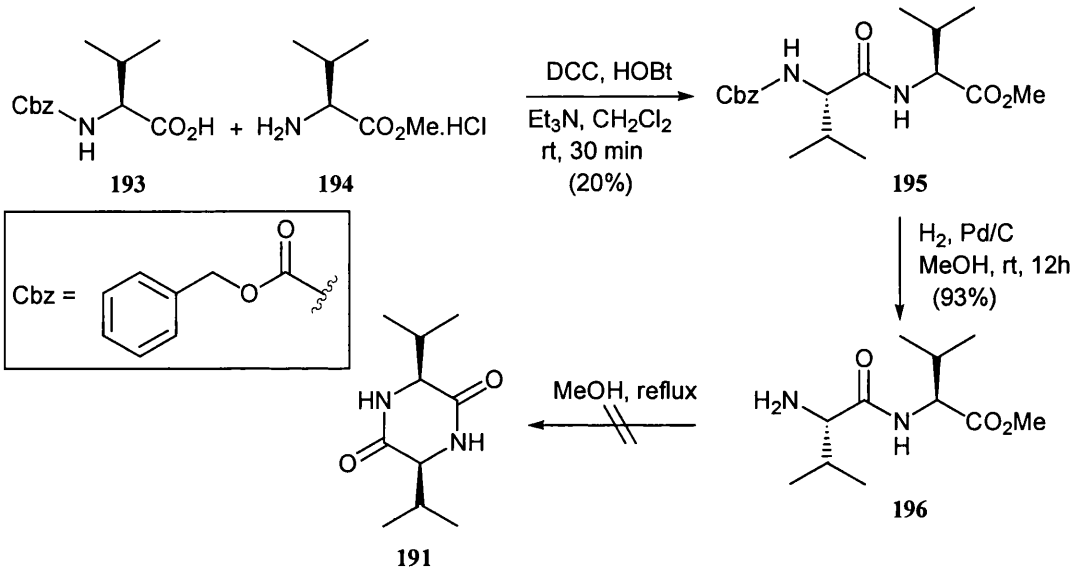


190 **194**

Scheme 55

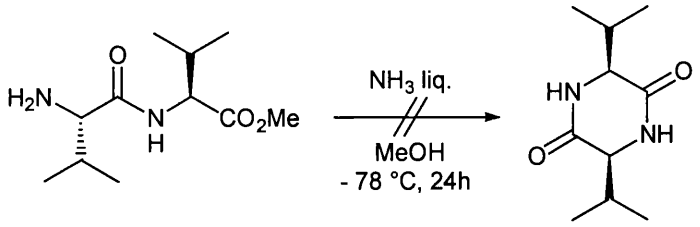
Peptidic coupling between compounds **193** and **194** using dicyclohexylcarbodiimide and 1-hydroxy-benzotriazole afforded the desired dipeptide **195,** which was subsequently hydrogenated to remove the *N*-benzyloxy-carbonyl protecting group and the resulting dipeptide methyl ester **196** was expected to cyclise to the 2,5-diketopiperazine **191** (Scheme 56). Although the

cyclisation of this dipeptide was reported to occur spontaneously, in our experiments formation of the 2,5-diketopiperazine **191** was not observed. Thus, the dipeptide **196** in solution in methanol was heated under reflux following the method of Ueda *et al.* but the expected 2,5-diketopiperazine **191** was not formed and the starting material was recovered.[143]



Scheme 56

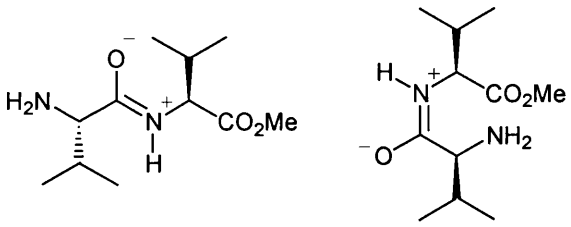
In the method described by Fisher,[128b] the basic medium created by ammonia allowed the intramolecular C-N cyclisation. This strategy was investigated with the dipeptide methyl ester **196**. The final step of the 2,5-diketopiperazine formation involved an intramolecular cyclisation in a solution of ammonia in methanol. Therefore, the dipeptide **196** was solubilised in methanol and cooled to – 78 °C to condense ammonia (Scheme 57). The resulting mixture was stirred for 24 hours and the solvent was removed, but the crude product did not contain *cyclo*-L-valine-L-valine **191** and, once again, the starting material was recovered (Scheme 57).



196 **191**

Scheme 57

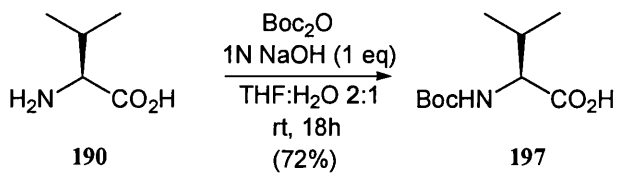
From a conformational standpoint, the preferred form of the dipeptide **196** is the transoid conformation **196a** rather than the required cisoid conformation **196b** as the dipeptide contains a secondary amide group, thus its cyclisation becomes more unlikely to occur at room temperature or in a low boiling point solvent (Figure 35).



196a **196b**

Figure 35

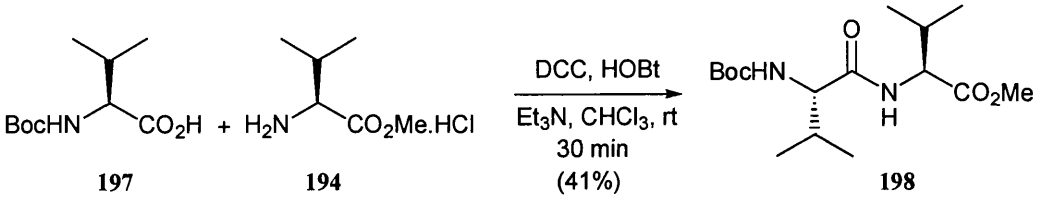
Therefore high boiling point solvents were required to force its cyclisation. The method reported by Nitecki *et al.* used a mixture of toluene and *sec*-butyl alcohol at reflux.[144] This method was also used by Ueda *et al.* for the synthesis of *cyclo*-L-valine-L-valine.[143] Thus according to this method L-valine **190** was converted into *N*-*tert*-butoxycarbonyl-L-valine **197** using di-*tert*-butyldicarbonate and an aqueous solution of sodium hydroxide in a mixture of tetrahydrofuran and water (Scheme 58).



190 **197**

Scheme 58

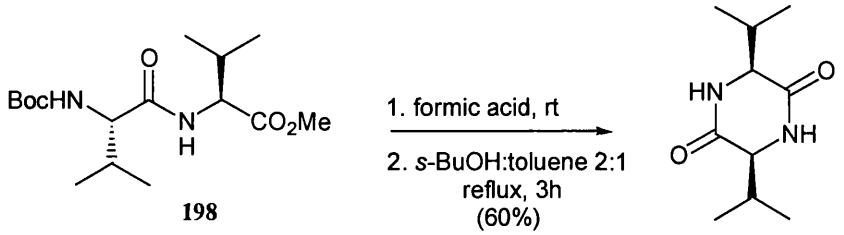
Peptidic coupling between the previously synthesised L-valine methyl ester hydrochloride **194** and *N-tert*-butoxycarbonyl-L-valine **197** using dicyclohexylcarbodiimide and 1-hydroxy-benzotriazole yielded the dipeptide **198** in 41% yield (Scheme 59).



197 **194** **198**

Scheme 59

The *N-tert*-butoxycarbonyl protecting group of **198** was then removed using formic acid at room temperature and the resulting dipeptide methyl ester formate salt heated in a mixture of toluene and *sec*-butylalcohol, under reflux (Scheme 60). *Cyclo*-L-valine-L-valine **191** was obtained in 60% yield, a result which is in good agreement with that reported by Ueda (58% yield).[143] These observations clearly imply that either formation and/or breakdown of the tetrahedral intermediate in the cyclisation may be catalysed by protons available in the formate salt.



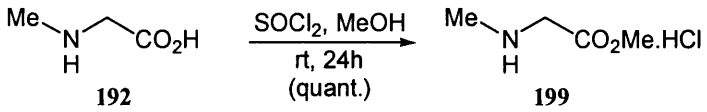
198 **191**

Scheme 60

2.1.1.3 Unsymmetrical 2,5-diketopiperazines

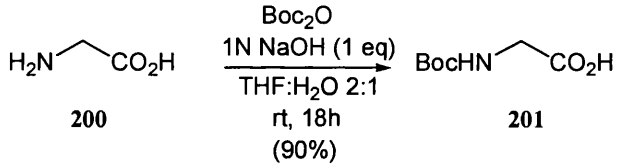
The above method was also successfully applied to the preparation of an unsymmetrical 2,5-diketopiperazine. Esterification of sarcosine **192** by reaction with thionyl chloride in methanol afforded the methyl ester hydrochloride salt **199** in a quantitative yield (Scheme 61).

with thionyl chloride in methanol afforded the methyl ester hydrochloride salt **199** in a quantitative yield (Scheme 61).



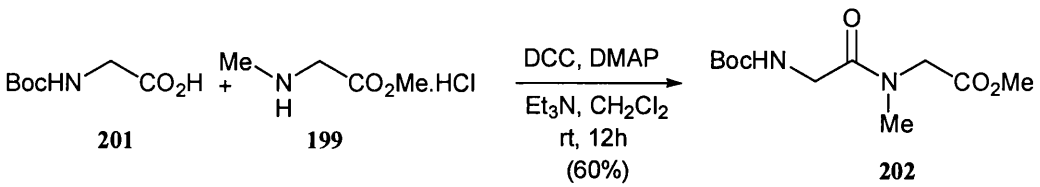
Scheme 61

Glycine **200** was *N*-protected using the *N-tert*-butoxycarbonyl protecting group (Scheme 62). *N-tert*-Butoxycarbonylglycine **201** was obtained by reaction of glycine **200** with di-*tert*-butyldicarbonate and aqueous sodium hydroxide in a mixture of tetrahydrofuran and water in 90% yield (Scheme 62).



Scheme 62

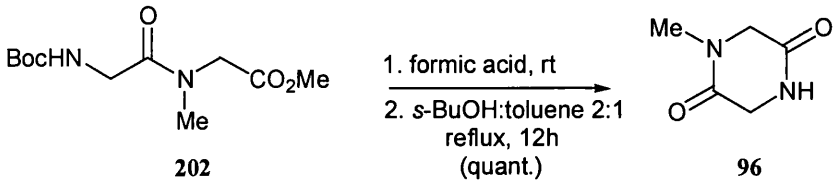
Peptidic coupling between *N-tert*-butoxycarbonylglycine **201** and sarcosine methyl ester hydrochloride **199** using dicyclohexylcarbodiimide and dimethylaminopyridine gave the desired dipeptide **202** in 60 % yield (Scheme 63).[145]



Scheme 63

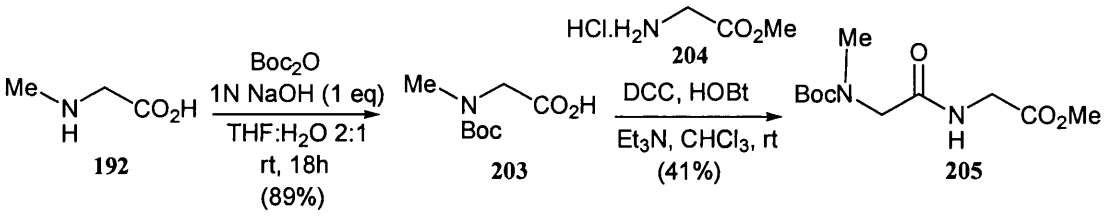
The method of Nitecki *et al.* was used for the cyclisation of the dipeptide **202**.[144] Deprotection of dipeptide **202** with formic acid yielded *in situ* a dipeptide formate salt which was heated in a mixture of toluene and *sec*-butylalcohol to give 2,5-diketopiperazine **96** in a quantitative yield (Scheme 64). The high yield obtained in this reaction may be explained by the conformation of the dipeptide wherein the

additional methyl group of sarcosine helps to favour the necessary cisoid conformation for the intramolecular cyclisation.



Scheme 64

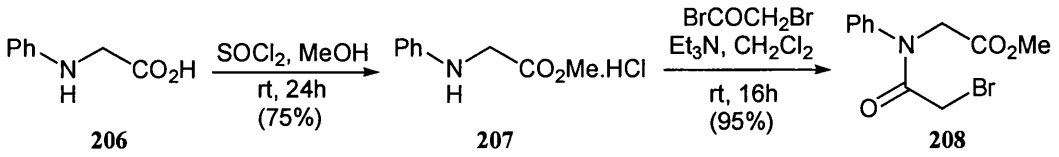
Interestingly, the dipeptide *N-tert*-butoxycarbonyl-sarcosine-glycinemethyl ester **205** synthesised in 41% yield did not yield the 2,5-diketopiperazine **96** under the previously employed conditions for cyclisation (Scheme 65). In this instance, the methyl group is no longer present on the nitrogen atom of the amide bond and, as a result, the dipeptide **205** is more likely to adopt the transoid conformation. Furthermore the secondary amine group will be more bulky and hence also make cyclisation more difficult.



Scheme 65

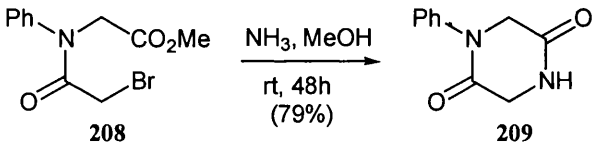
Unsymmetrical 2,5-diketopiperazines were also synthesised by the tandem reaction of acylation-alkylation with ammonia. Esterification of *N*-phenylglycine **206** with thionyl chloride and methanol gave *N*-phenylglycine methyl ester hydrochloride **207** in 75% yield (Scheme 66). The yield was slightly lower than those previously reported for esterification but, in this instance, decomposition of the starting material under these acidic conditions was apparent as the reaction mixture became dark brown after stirring for several hours. *N*-Phenylglycine methyl ester hydrochloride **207** was then converted into the bromo-ester **208** in 95% yield

(Scheme 66). The product **208** was stored under nitrogen until required as decomposition in air was observed.



Scheme 66

Ammonia was then bubbled through a solution of the bromo-ester **208** in methanol at 0 °C and the solution was left under an atmosphere of ammonia at room temperature for several hours to yield the *N*-phenyl-*cyclo*-glycine-glycine **209** in 79% yield (Scheme 67). This cyclisation is of course aided by the presence of the phenyl group on the nitrogen atom.

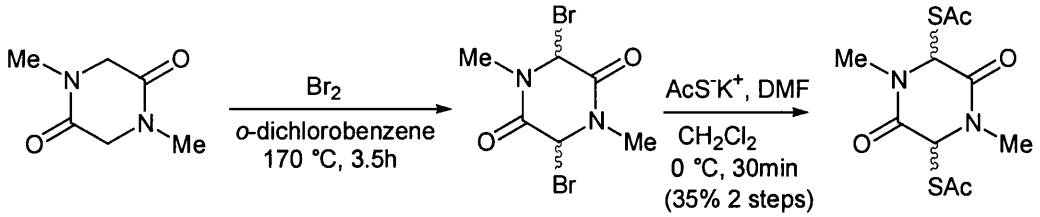


Scheme 67

2.1.2 Epi-3,6-dithio-2,5-diketopiperazines

With some suitable 2,5-diketopiperazines in hand, the stage was then set for introduction of the disulfide bridge. Sulfur was then introduced using Trown's methodology[65] with *cyclo*-sarcosine-sarcosine **66** (Scheme 68). The 2,5-diketopiperazine **66** was solubilised in *ortho*-dichlorobenzene and a solution of bromine in *ortho*-dichlorobenzene was added over 1 hour. After heating the reaction mixture at 170 °C for 3 hours, the solution was cooled and the mixture was poured into light petroleum and left at 0 °C to crystallise the product. The formation of compound **67** was confirmed by ^{1}H NMR, which showed the disappearance of the methylene group of the 2,5-diketopiperazine **66** at 3.96 ppm and the presence of a new signal at 5.85 ppm corresponding to the methine proton in **67** (Scheme 68). The crude product **67** was then used for the next step wherein a

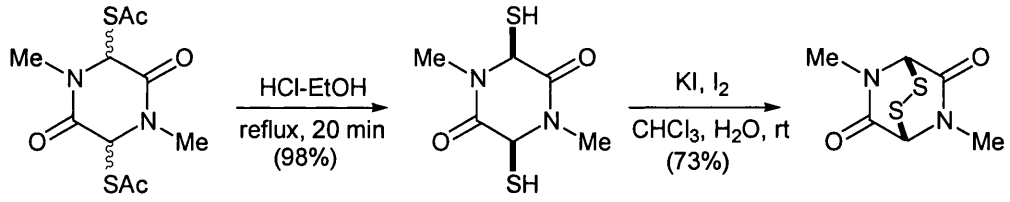
solution of potassium thioacetate in dimethylformamide was added to displace the two bromide groups and produce the compound **68** in 35% overall yield (Scheme 68).



66 **67** **68**

Scheme 68

The 3,6-di(acetylthio)-1,4-dimethyl-2,5-diketopiperazine **68** was hydrolysed under acidic conditions to yield *cis*-3,6-dithiol-1,4-dimethyl-2,5-diketopiperazine **69a** in 98% yield (Scheme 69). The epi-3,6-dithio-2,5-diketopiperazine **70** was then obtained by oxidation of the dithiol of **69a** in a two phase mixture of iodine and potassium iodide in water and chloroform in 73% yield (Scheme 69).

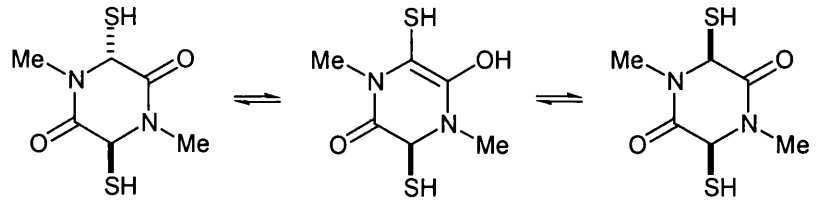


68 **69a** **70**

Scheme 69

To our great surprise and in contrast to the results of Kishi *et al.*[95] who reported that a mixture of *cis*- and *trans*-3,6-dithiol-1,4-dimethyl-2,5-diketopiperazines **69a** and **69b** was produced, the *cis*-3,6-dithiol-1,4-dimethyl-2,5-diketopiperazine **69a** was the only product observed in our experiments (Scheme 70). It was therefore of considerable interest to consider possible mechanisms for this epimerisation, which occurs under strongly acidic conditions. Three alternatives may be envisaged. Firstly, sequential keto-enol tautomerism between the *trans*-3,6-dithiol-1,4-dimethyl-2,5-diketopiperazine **69b** formed and the enol **69c**, followed by a second

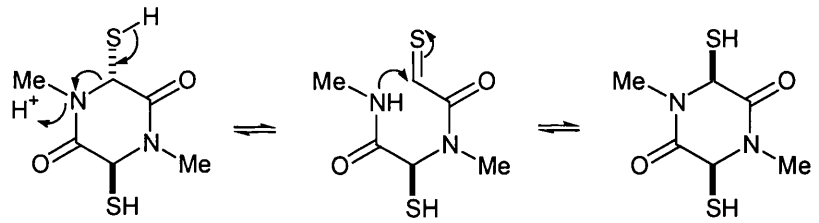
equilibrium between the same enol **69c** and *cis*-3,6-dithiol-1,4-dimethyl-2,5-diketopiperazine **69a**, may provide a feasible explanation (Scheme 70).



69b **69c** **69a**

Scheme 70

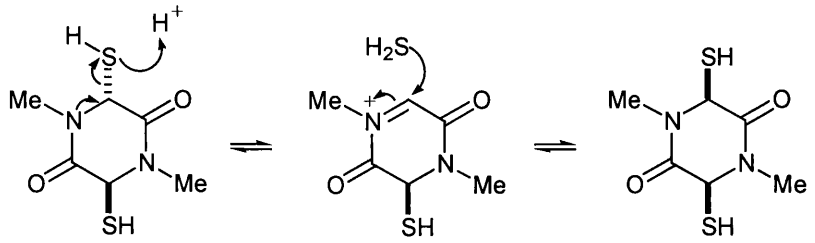
A second hypothesis involves ring opening of the *trans*-3,6-dithiol-1,4-dimethyl-2,5-diketopiperazine **69b** concurrently with *in situ* formation of a thioaldehyde **210** as a high energy intermediate (Scheme 71). The nitrogen atom of the amide function can then react intramolecularly with the thioaldehyde function to produce *cis*-3,6-dithiol-1,4-dimethyl-2,5-diketopiperazine **69a** (Scheme 71).



69b **210** **69a**

Scheme 71

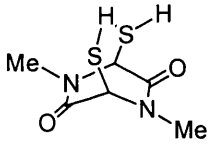
Finally, under strongly acidic conditions, protonation of the thiol group of **69b** could result in loss of hydrogen sulfide and production of an acyl iminium intermediate **211** (Scheme 72). Addition of hydrogen sulfide from the same face as the second thiol function in **211** could then provide the observed *cis*-3,6-dithiol-1,4-dimethyl-2,5-diketopiperazine **69a** (Scheme 72).



69b **211** **69a**

Scheme 72

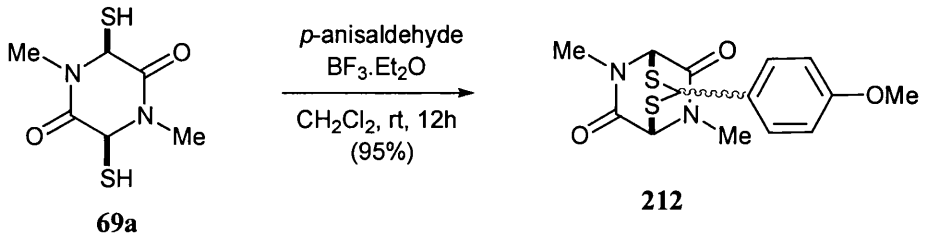
The observation that the *cis* product **69a** is the more stable isomer may be explained by intramolecular hydrogen bonding between the two thiol groups with the conformation of *cis*-3,6-dithiol-1,4-dimethyl-2,5-diketopiperazine **69a** being boat-like (Figure 36) rather than the flattened chair of *cyclo*-sarcosine-sarcosine **66** (Figure 33). Unfortunately, no X-ray crystallographic study of a 3,6-dithiol-1,4-dimethyl-2,5-diketopiperazine **69a** which could prove our hypothesis has been reported to date, and all of our many attempts to grow suitable crystals were unsuccessful.



69a

Figure 36

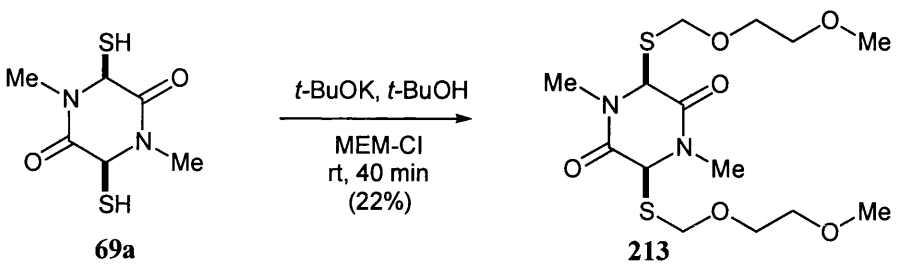
The *cis*-3,6-dithiol-1,4-dimethyl-2,5-diketopiperazine **69a** was then protected using the Kishi[95] protocol of reaction with *para*-methoxybenzaldehyde in the presence of boron trifluoride etherate in dichloromethane to produce the protected disulfide bridge **212** in 95% yield (Scheme 73).



69a **212**

Scheme 73

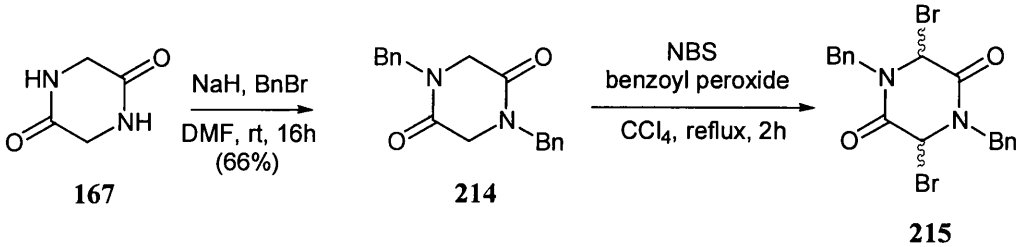
The second method of protection was to use the *N*-methoxymethyl protecting group. Due however to the toxicity of chloromethyl methyl ether, we first elected to use the derivative chloromethyl ethoxy methyl ether. This gave, on reaction with *cis*-3,6-dithiol-1,4-dimethyl-2,5-diketopiperazine **69a**, the diprotected compound **213** in 22% yield (Scheme 74).



69a **213**

Scheme 74

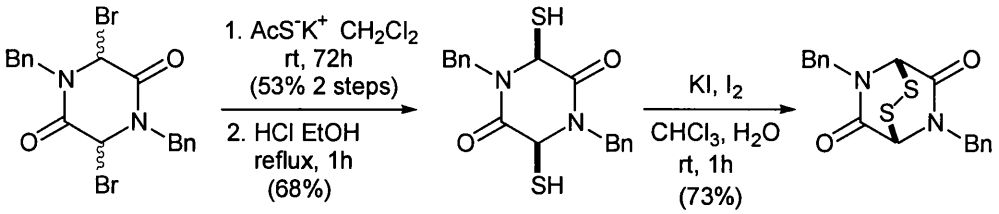
The same strategy was also applied to the 1,4-dibenzyl-2,5-diketopiperazine **214**,[146] which was prepared from the reaction of commercially available *cyclo*-glycine-glycine **167** with sodium hydride and benzylbromide (Scheme 75). For this case, dibromination was carried out using *N*-bromosuccinimide, less toxic than bromine, in the presence of a radical initiator in carbon tetrachloride. After heating the reaction mixture under reflux for 2 hours, the insoluble succinimide was filtered, the solvent was removed and the crude product **215** was used immediately for the following reaction (Scheme 75). The formation of compound **215** was however confirmed by ^{1}H NMR, which showed the disappearance of the methylene group of the 2,5-diketopiperazine **214** at 3.95 ppm and the presence of a new signal at 6.08 ppm corresponding to the methine protons in **215**.



167 **214** **215**

Scheme 75

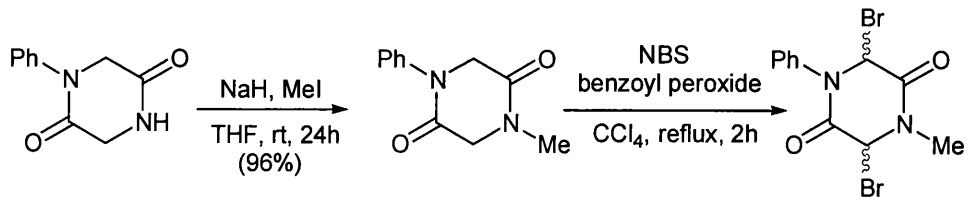
The subsequent construction of the disulfide bridge followed the same protocol as previously discussed. Displacement of the two bromides of **215** by potassium thioacetate, followed by acidic hydrolysis gave, once again, only *cis*-3,6-dithiol-1,4-dibenzyl-2,5-diketopiperazine **216** which was oxidised to yield the epi-3,6-dithio-1,4-dibenzyl-2,5-diketopiperazine **217** (scheme 76).



215 **216** **217**

Scheme 76

The introduction of the disulfide bridge was also carried out from *N*-phenyl-*cyclo*-glycine-glycine **209** (Scheme 77). Thus, 2,5-diketopiperazine **209** was first methylated using sodium hydride and methyl iodide to yield *N*-phenyl-*cyclo*-glycine-sarcosine **218**, which was then converted into the 1,4-dibenzyl-3,6-dibromo-2,5-diketopiperazine **219** by reaction with *N*-bromosuccinimide and benzoylperoxide as radical initiator (Scheme 77). The crude product was analysed by ^{1}H NMR to confirm the formation of **219** and was used without further purification for the next reaction.

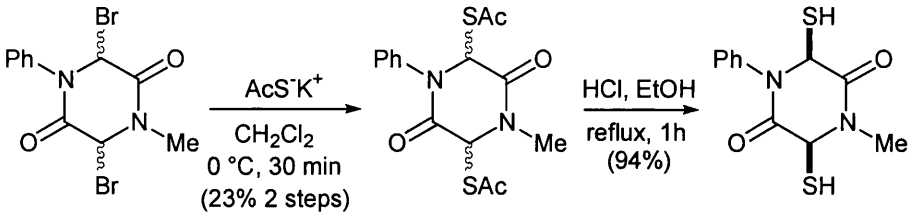


209 **218** **219**

Scheme 77

Displacement of the two bromides with potassium thioacetate yielded the compound **220** in 23% overall yield (Scheme 78). Purification of this compound was more difficult than in the case of other analogues as an oilier brown residue was present in the crude product. Indeed, when the reaction was carried out on less

than a 500 mg scale of 2,5-diketopiperazine **218,** the product **220** could not be isolated either after column chromatography or by crystallisation. After acidic hydrolysis under the previously described conditions the *cis*-dithiol **221** was obtained in 94 % yield (Scheme 78). On this occasion the *cis*-dithiol **221** was not oxidised as only a small amount was obtained and required for future experiments.

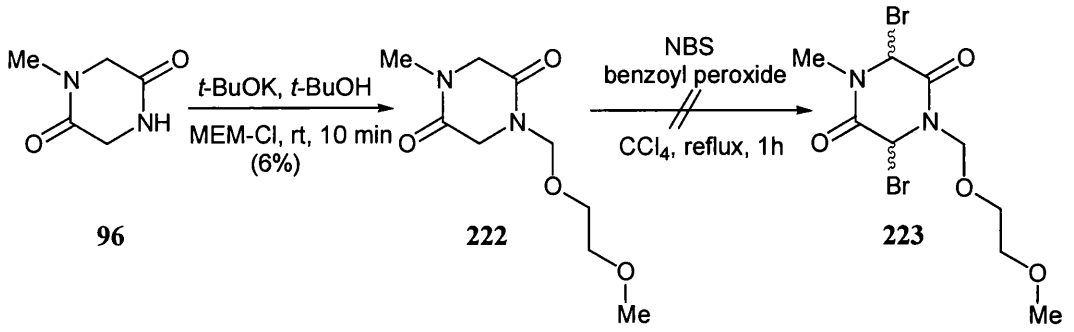


219 **220** **221**

Scheme 78

In order to synthesise even more highly functionalised epi-3,6-dithio-2,5-diketopiperazines both on the carbon atoms and also on the nitrogen atoms of the 2,5-diketopiperazine skeleton, we decided to introduce the disulfide bridge into an *N*-protected 2,5-diketopiperazine as reported by Kishi *et al.*[95] in their total synthesis of gliotoxin.

Rather that use the methyl methoxy ether protecting group we turned our attention once again to the methyl ethoxy methyl ether derivative. The *N*-protected 2,5-diketopiperazine **222** was synthesised from *cyclo*-glycine-sarcosine **96** in modest yield as a consequence of a problematic purification process (Scheme 79). Because of its high polarity, the crude oil obtained was not subjected to column chromatography, but was distilled under reduced pressure of 0.1 mm of mercury at 220 °C. A subsequent problem arose however on attempted bromination of the *N*-protected 2,5-diketopiperazine using the conditions described above, since the desired product **223** was not identified (Scheme 79).



96 **222** **223**

Scheme 79

Examination of the ^{1}H NMR spectrum of the crude reaction mixture suggests that the desired bromination may occur but is also accompanied by competing bromination at the carbon atoms of the methyl ethoxy methyl ether protecting group. Several brominated derivatives, such as the products shown in Figure 37, had been formed, as evidenced by the ^{1}H NMR spectrum which revealed numerous peaks in the region between 5.50 and 6.50 ppm.

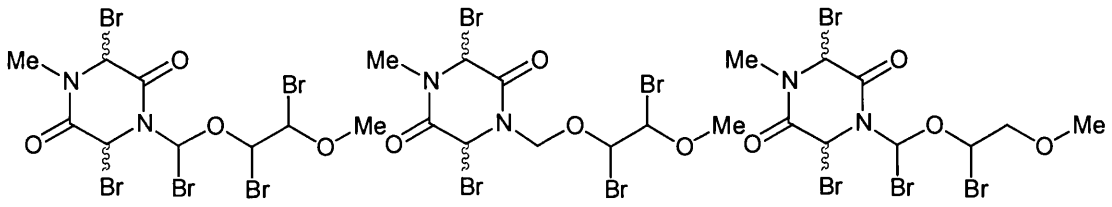
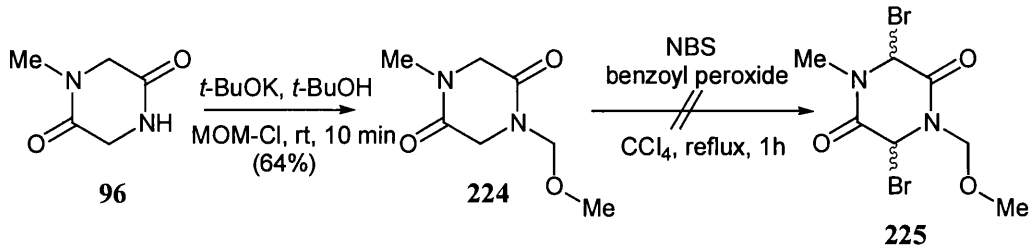


Figure 37

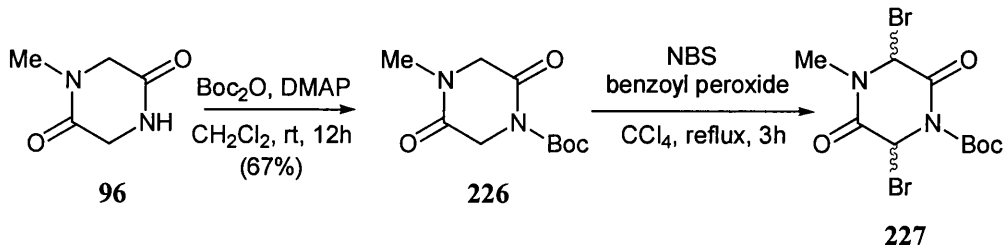
From this experiment, it was therefore concluded that this protecting group was not appropriate for use with this bromination strategy. The reactions of Kishi *et al.*[95] were thus repeated for the synthesis of *N*-methoxymethyl-*N'*-methyl-2,5-diketopiperazine **225** which was obtained in 64% yield (Scheme 80). On attempted bromination however, we observed once again that bromine had been incorporated not only into the 2,5-diketopiperazine **224** but also on the methyl methoxy ether protecting group as revealed by the ^{1}H NMR spectrum which also shows numerous peaks in the region between 5.50 and 6.50 ppm (Scheme 225). In order to purify compound **225**, an excess of potassium thioacetate was added into a solution of the crude product in dichloromethane at 0 °C and the resulting mixture was stirred at 0 °C for 30 min. After filtration, the solvent was removed under reduced pressure

and the crude brown oil was subjected to column chromatography. Unfortunately, the desired product was not isolated.



Scheme 80

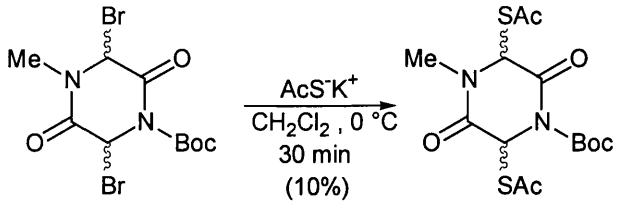
In total contrast to the work of Kishi *et al.*[95] we were therefore unable to incorporate sulfur into the *N*-methoxymethyl-*N'*-methyl-2,5-diketopiperazine **224** because of the bromination problems occurring with the methoxy methyl ether protecting group. In view of our success with other bromination reactions, we cannot fully explain this disparity. After these unfortunate results, an alternative protecting group was sought, which could easily be removed after the introduction of the disulfide bridge and the carbamate group was accordingly chosen. Therefore, 2,5-diketopiperazine **96** was protected with the *tert*-butoxycarbonyl group in 67% yield and bromination occurred quantitatively as judged by the ^{1}H NMR spectrum which showed the disappearance of the two methylene groups of the 2,5-diketopiperazine **226** at 3.97 and 4.21 ppm and the presence of two new signals at 6.00 and 6.58 ppm corresponding to the two methine groups in **227** (Scheme 81).



Scheme 81

The double displacement of the two bromides **227** with potassium thioacetate was a disappointing reaction (Scheme 82). Although this reaction was repeated several times at varying concentrations of thioacetate and under a variety of solvents and

temperatures, the best yield obtained was 10%. Thus, the bulky electron-withdrawing *tert*-butoxycarbonyl protecting group was deemed to be inappropriate for the disulfide synthesis and further investigations are required.



227 **228**

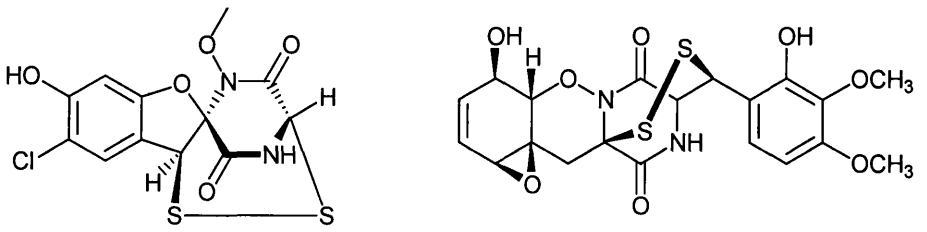
Scheme 82

2.2 Novel approaches for the introduction of the disulfide bridge

2.2.1 Introduction

As we have seen in the previous section, difficulties may be encountered in the syntheses of epi-3,6-dithio-2,5-diketopiperazines and new and more general methods are therefore required for incorporation of the disulfide bridge in a more efficient manner.

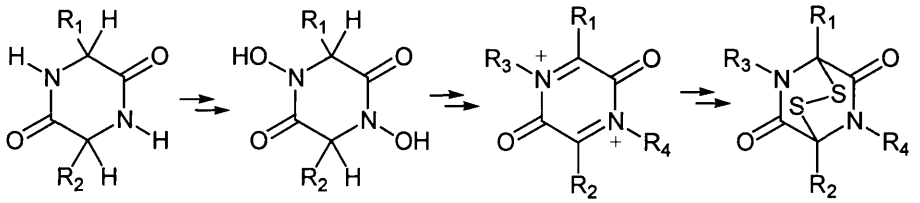
We were particularly intrigued by the structures of aspirochlorin **52** and gliovirin **53,** which appeared to indicate that the hydroxamic acid moiety could also be produced during the biosynthetic process (Figure 38).



52 **53**

Figure 38

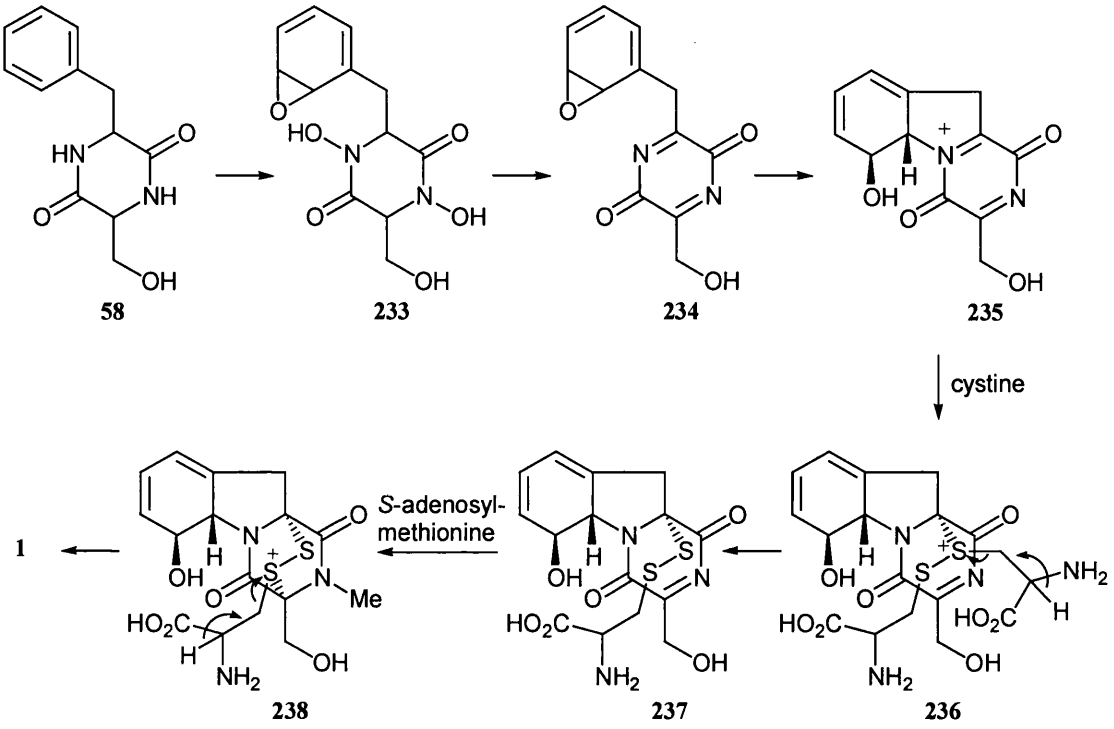
Moreover, studies by Ottenheijm *et al.* using a biomimetic approach to the synthesis of epi-3,6-dithio-2,5-diketopiperazines suggested that *N,N'*-dihydroxy-2,5-diketopiperazines **230** could be intermediates in the biosynthetic pathway (Scheme 83).[147,148] This group proposed that oxidation of the amide nitrogen in **229** led to hydroxamic acids of type **230**, which, either on stepwise or double dehydration to acyliminium cations **231** could then serve as excellent Michael acceptors for thiol nucleophiles (Scheme 83).



229 **230** **231** **232**

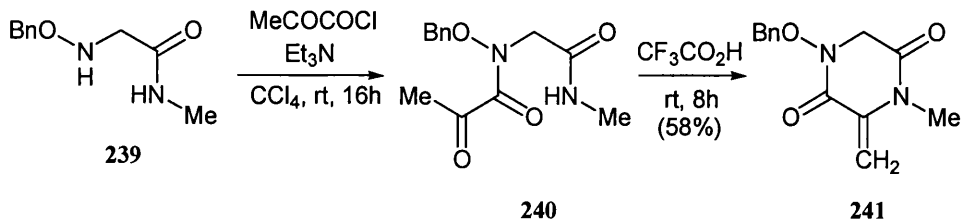
Scheme 83

They therefore suggested a possible pathway for the biosynthesis of gliotoxin **1** as shown in Scheme 84. After oxidation of *cyclo*-phenylalanine-serine **58**, the di-*N*-hydroxy-benzene oxide **233** could be dehydrated to produce the imine **234**. The nitrogen atom of the phenylalanine unit could then attack the benzene oxide to yield the acyliminium cation **235**. Incorporation of sulfur could then occur by reaction of cystine with the ion **235** to give a sulfonium ion **236**, which could undergo a β-elimination to yield the disulfide **237**. Subsequent *N*-methylation could produce a second acyliminium cation which could then be attacked by the sulfur atom of the disulfide **237** to produce the sulfonium **238**, which, upon a second β-elimination, could lead to gliotoxin **1**. With this proposal, Ottenheijm *et al.* suggested that the addition of the disulfide bridge could be stereochemically controlled.[147]



Scheme 84

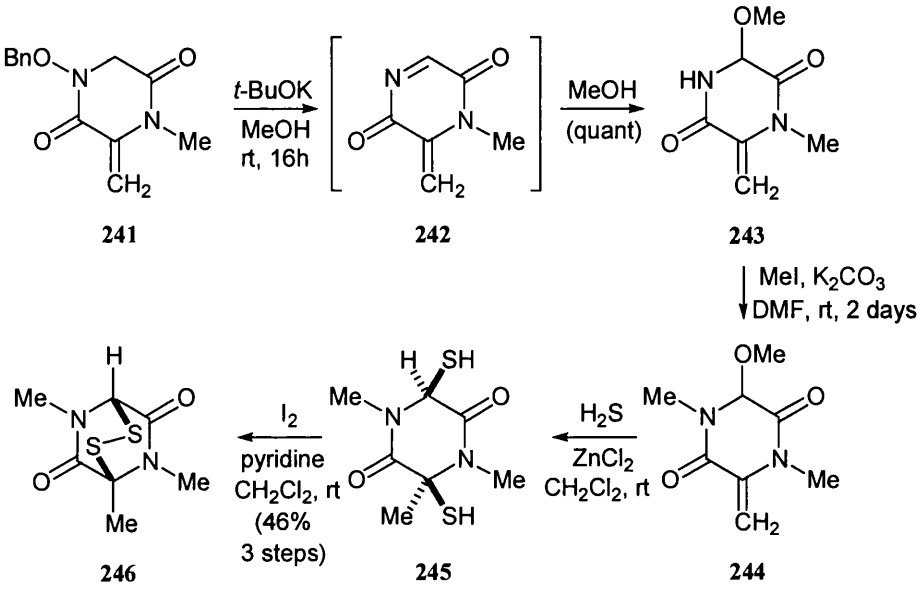
In order to test their proposal, the synthesis of an epi-3,6-dithio-2,5-diketopiperazine from an oxygenated 2,5-diketopiperazine unit incorporating a hydroxamic acid moiety was studied.[147,148] Thus, reaction of **239** with pyruvoyl chloride and triethylamine at room temperature afforded the acylated compound **240** (Scheme 85). Ring closure of **240** and subsequent dehydration catalysed by trifluoroacetic acid gave **241** in 58% yield (Scheme 85).



Scheme 85

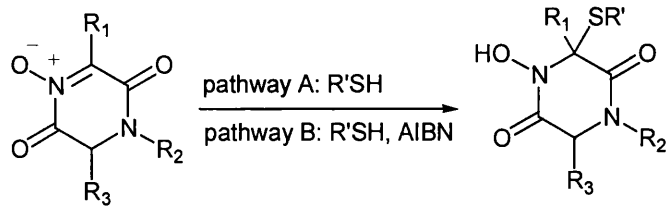
Treatment of **241** with potassium *tert*-butoxide in methanol afforded the 3-methoxy-6-methylene-2,5-diketopiperazine **243** presumably by addition of

methanol to the C=N bond of the intermediate imine **242** (Scheme 86). Compound **243** was then converted into **244** by treatment with methyl iodide and potassium carbonate in dimethylformamide (Scheme 86). Reaction of **244** with liquid hydrogen sulfide and zinc chloride, as a catalyst, yielded the *cis*-dithiol **245** and finally, oxidation with iodine in pyridine gave the epi-3,6-dithio-2,5-diketopiperazine **246** in 46% overall yield (Scheme 86).



Scheme 86

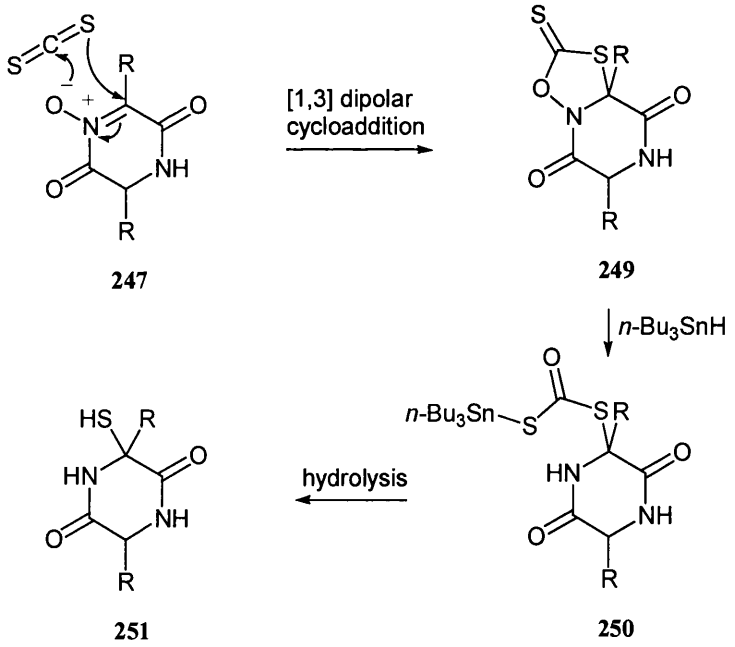
Although this strategy started from the oxygenated 2,5-diketopiperazine **241**, Ottenheijm *et al.* incorporated sulfur into intermediate **244** using their methodology that was previously described in the introduction (Section 1.4.1). Nevertheless, inspired by this approach, we turned our attention to new strategies involving oxygenated 2,5-diketopiperazines as intermediates which could undergo a range of oxidation and elimination reactions. Thus the first structural class of interest was compound **247**, which could undergo either nucleophilic reaction (pathway A) or radical reaction (pathway B), always leading to the formation of a carbon-sulfur bond to produce compound **248** (Scheme 87).



247 **248**

Scheme 87

We also envisaged that the same intermediates could be used in an alternative strategy involving a 1,3-dipolar cycloaddition reaction of **247** with carbon disulfide leading to the formation of the desired carbon-sulfur bond concurrently with the production of an oxathiazolidinethione ring in **249** (Scheme 88). Literature precedent confirms that nitrones are involved in 1,3-dipolar cycloaddition reactions with alkenes for instance for the synthesis of isooxazolidine.[149]

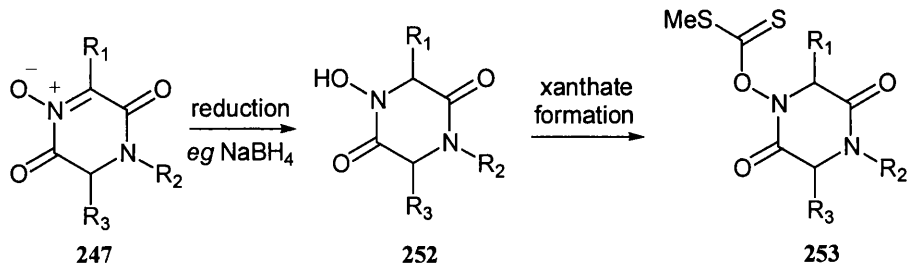


247 **249** **250** **251**

Scheme 88

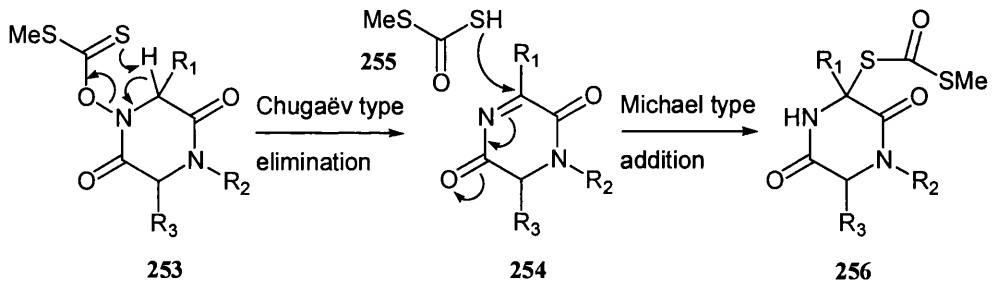
Subsequent conversion of **249** into the intermediate **250** could occur *via* reaction with tri-*n*-butylstannane by a mechanism similar to that described by Barton *et al.* (Scheme 88).[150] Finally, hydrolysis of **250** would provide the 3-thiol-2,5-diketopiperazine **251** (Scheme 88).

Alternatively, the compound **247** could be reduced into oxygenated 2,5-diketopiperazines **252** containing a hydroxamic acid moiety (Scheme 89). Compound **252** would then be used to incorporate sulfur by firstly preparing the xanthate intermediate **253** (Scheme 89).



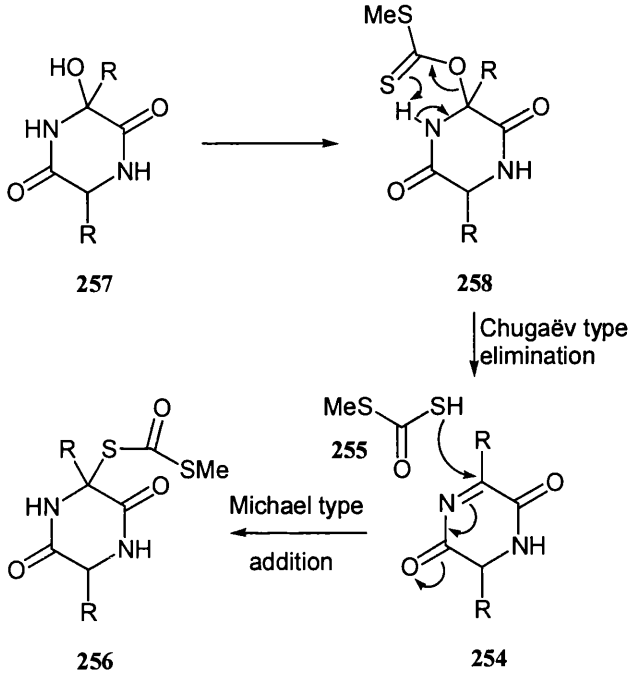
Scheme 89

This xanthate **253** could possibly undergo a Chugaëv type elimination leading to the imine derivative **254** and a dithiocarbonic acid methyl ester **255**, whose thiol function could then attack the carbon of the imine **254** in Michael fashion to form the carbon-sulfur of **256** bond in a one-pot reaction (Scheme 90).



Scheme 90

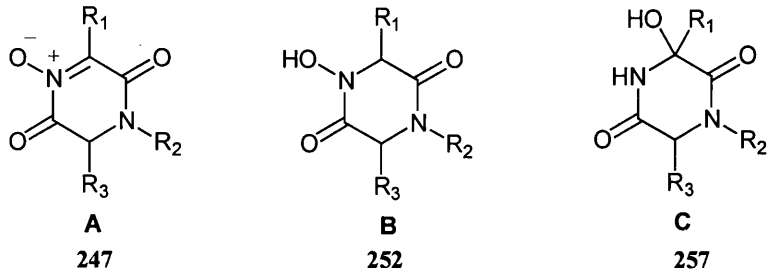
Another potential class of hydroxylated intermediate, the hydroxylated 2,5-diketopiperazines **257**, which, as we have seen, have been reported by Ottenheijm,[148] could also be used for the same strategy (Scheme 91). Thus, after formation of the xanthate **258**, compound **258** could undergo a different Chugaëv type of elimination followed by an identical Michael type addition, which would lead to the formation of the product **256** in which sulfur has been incorporated (Scheme 91).



Scheme 91

2.2.2 Oxygenated 2,5-diketopiperazines

As a consequence of the foregoing ideas, we were therefore interested in three categories of oxygenated 2,5-diketopiperazines for the development of new strategies of sulfur incorporation: type A **247**, type B **252** and type C **257** (Figure 39). Examination of the literature revealed that several groups have investigated syntheses involving these structures.

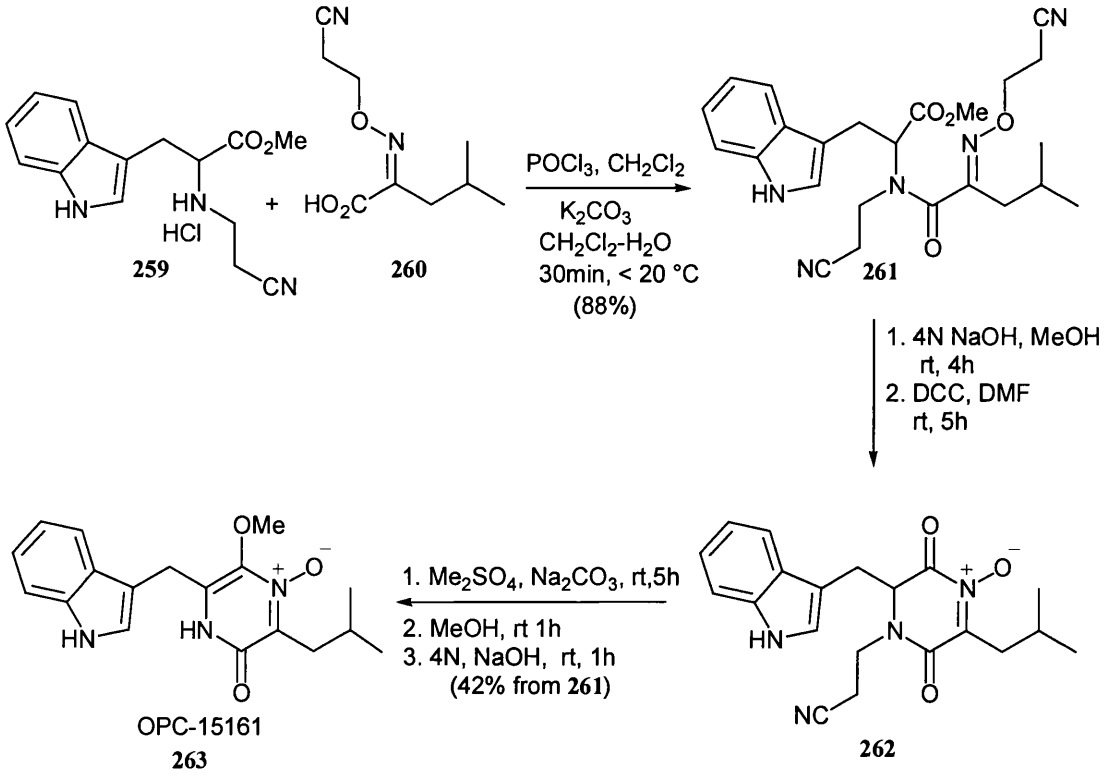


R_1, R_2, R_3, R_4 = H, alkyl, aryl

Figure 39

2.2.2.1 Synthetic approaches to substrates of type A

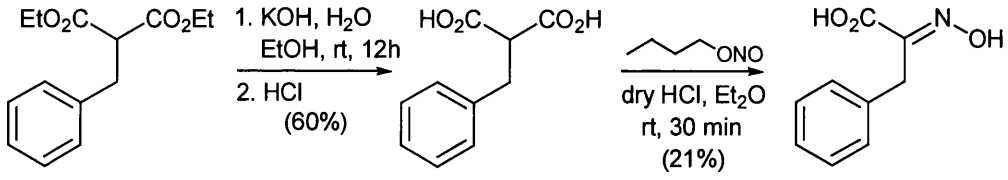
Thus, in their studies towards the synthesis of OPC-15161 **263**, an inhibitor of superoxide generation isolated from the culture of fungus *Thielavia minor* OFR-1561, Kita *et al.* described the synthesis of an oxygenated 2,5-diketopiperazine of type A based on the cyclisation of a peptide containing an oxime moiety (Scheme 92).[151,152] This methodology was later improved by Minamikawa *et al.* for plant-scale production.[153] Coupling between *N*-protected-tryptophan methyl ester hydrochloride **259** and the acid chloride of the *O*-protected-α-hydroxyimino carboxylic acid **260** gave the amide **261** in 88% yield (Scheme 92). After saponification and treatment with dicyclohexylcarbodiimide, the oxygenated 2,5-diketopiperazine **262** was obtained (Scheme 92). Finally, methylation of **262** and removal of the cyanoethyl group under basic conditions yielded OPC-15161 **263** in 42% yield (Scheme 92).



Scheme 92

We therefore attempted to adopt this strategy for the synthesis of our type A oxygenated 2,5-diketopiperazine by first synthesising the *O*-protected-α-hydroxyimino carboxylic acid **260**. Incredibly, Kita *et al.*[152] referenced for this preparation a French report[154] published in 1904 which described the synthesis of the desired α-hydroxyimino carboxylic acid in lead chambers from β-keto-esters and nitrous fumes in sulphuric acid.[155] It was both surprising and unlikely to think that Kita[152] really used this procedure for the synthesis of their starting material, and the literature was therefore investigated. α-Hydroxyimino carboxylic acids can of course be readily obtained from either malonic acids or malonate esters by standard nitrosation.[156]

Thus, for our own example, saponification of commercially available benzylmalonate ester **264** was achieved by the addition of a solution of potassium hydroxide in water to a solution of the diester **264** in ethanol (Scheme 93). The reaction mixture was stirred at room temperature for 12 hours to afford the diacid **265** in 60% yield (Scheme 93). Compound **265** was then dissolved in diethyl ether and the resulting solution was treated with *n*-butylnitrite and dry hydrogen chloride gas was bubbled through this solution for 30 minutes to yield the oxime **266** in 21% yield (Scheme 93).

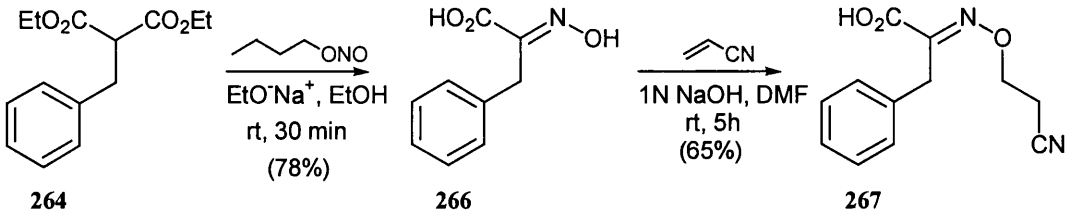


264 **265** **266**

Scheme 93

The low yield of this reaction led us to investigate an alternative method described in the same report.[156] A solution of diethyl benzylmalonate **264** in absolute ethanol was added to an alkaline solution of sodium in ethanol and *n*-butyl nitrite was added (Scheme 94). The desired product **266** was then obtained in 78% yield and the oxime function was protected with the cyanoethyl group using the Minamikawa *et al.* procedure (Scheme 94).[153] After treatment of the product **266** with an

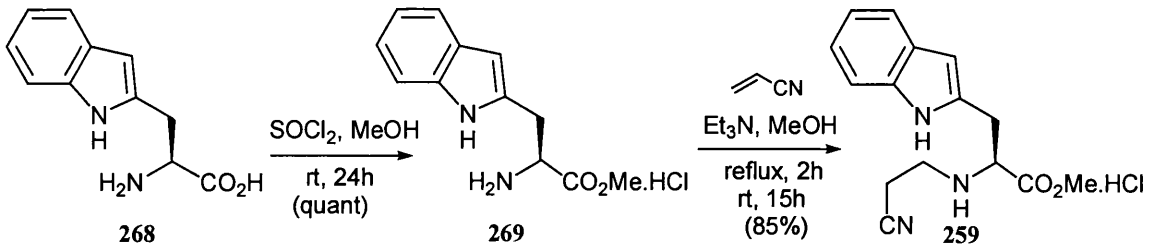
aqueous solution of sodium hydroxide and acrylonitrile the desired *O*-protected compound **267** was obtained in 65% yield (Scheme 94).



264 **266** **267**

Scheme 94

The synthesis of the second fragment involved initially the esterification of an amino acid followed by protection of the primary amine with a cyanoethyl group. These two reactions were attempted with the same amino acid used by Minamikawa *et al.*[153] Thus, L-tryptophanmethyl ester hydrochloride **269** was prepared from L-tryptophan **268** by reaction with thionyl chloride in methanol and was obtained quantitatively (Scheme 95). L-Tryptophan methyl ester hydrochloride **269** was then stirred with triethylamine and acrylonitrile, heated at reflux for 2 hours and stirred at room temperature overnight to yield the *N*-protected L-tryptophan methyl ester hydrochloride **259** in 85% (Scheme 95).

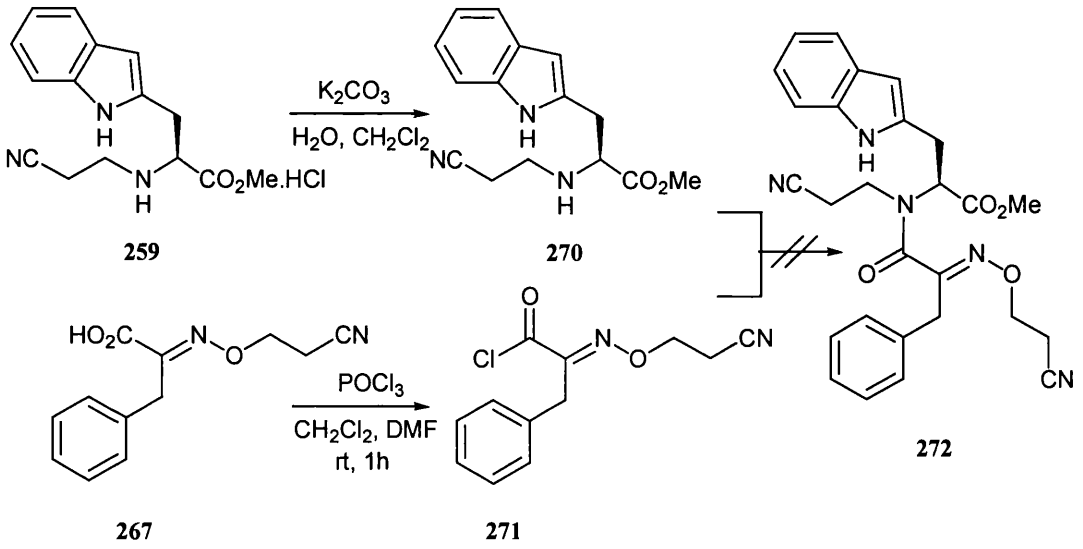


268 **269** **259**

Scheme 95

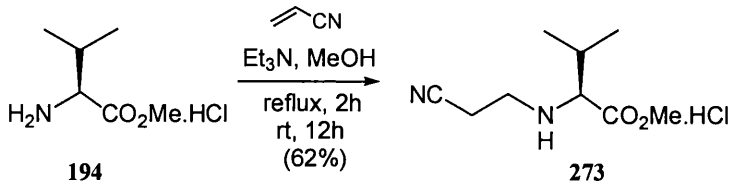
We then elected to couple **259** with **267** using the Minamikawa *et al.* procedure[153] described *vide supra* (Scheme 96). Thus *N*-protected L-tryptophan methyl ester **259** was added to a stirred solution of potassium carbonate in a mixture of water and dichloromethane to form the free *N*-protected tryptophan methyl ester **270** (Scheme 96). Simultaneously, neat phosphoryl chloride was added to a solution of the protected oxime **267** at 0 °C in a mixture of dichloromethane and

dimethylformamide and the resulting solution stirred at room temperature for 1 hour to form the acid chloride derivative **271** (Scheme 96). This acid chloride **271** solution was then added to the solution of the *N*-protected tryptophan methyl ester **270** below 20 °C and the reaction mixture stirred at room temperature for 30 minutes (Scheme 96). Since TLC analysis still indicated the presence of the starting materials, the reaction mixture was stirred for a longer period than described by Minamikawa *et al.*[153] However, although the reaction time was increased and the reaction repeated several times, only starting materials were recovered.



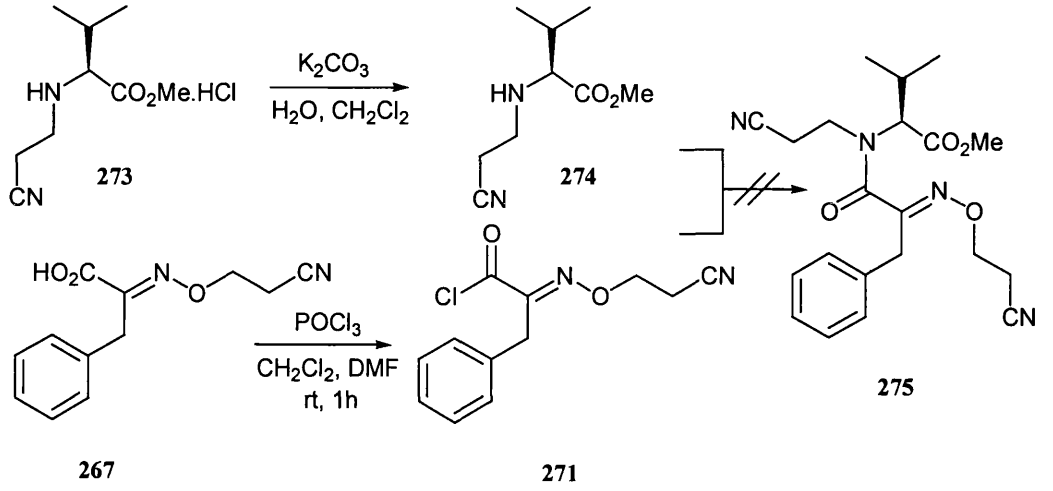
Scheme 96

In view of this result, we decided to change the *N*-protected L-tryptophan methyl ester **259** for the less sensitive *N*-protected L-valine methyl ester **194** (Scheme 97). Thus, L-valine methyl ester hydrochloride **194** was protected using the previously described methodology and *N*-cyanoethyl-L-valine methyl ester hydrochloride **273** was obtained in 62% yield (scheme 97).



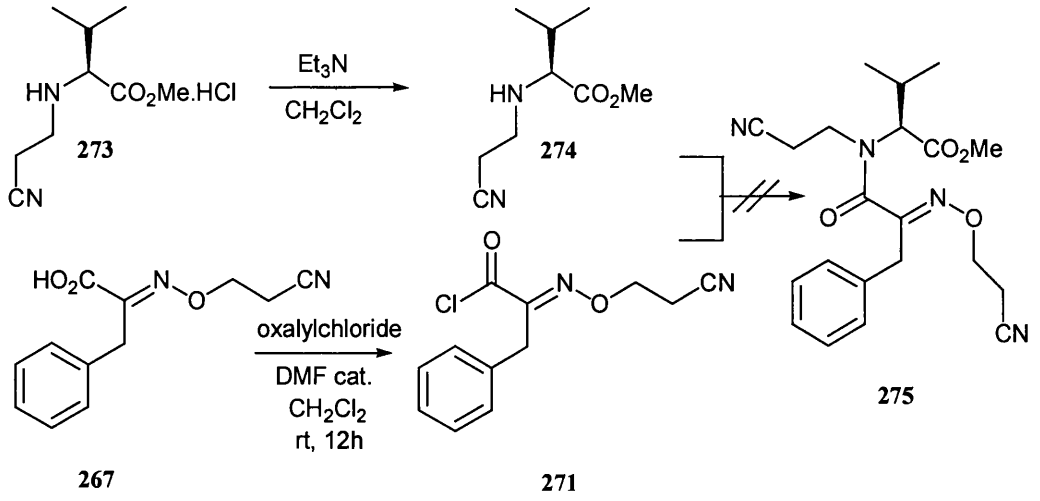
Scheme 97

N-Protected L-valine methyl ester hydrochloride **273** was liberated from its salt by potassium carbonate and the resulting free amine **274** in solution was subjected to the addition of the acid chloride **271** (Scheme 98). Unfortunately, also in this case again the formation of the amide bond was not observed and TLC analysis only showed the presence of the two starting materials **267** and **274**.



Scheme 98

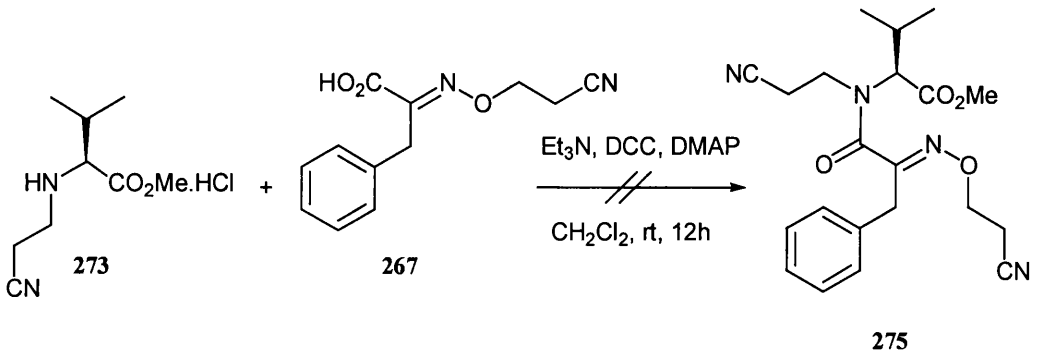
At this stage, we decided to prepare the free amine **274** and the acid chloride **271** by alternative methods and to totally avoid the presence of water during the coupling reaction between the two precursors **274** and **271**. *N*-Protected L-valine methyl ester hydrochloride **273** was treated with triethylamine in dichloromethane and the solution stirred at room temperature (Scheme 99). The acid chloride **271** was prepared by the addition of oxalylchloride to a solution of compound **267** in dichloromethane with two drops of dimethylformamide as catalyst (Scheme 99). The solution was stirred at room temperature overnight and the IR spectrum revealed that all the starting material had been consumed as evidenced by disappearance of both the O–H band at 3055 cm^{-1} and the C=O band at 1718 cm^{-1} of the acid and the presence of the C=O band of the acid chloride appearing at 1805 cm^{-1}. After removal of all the volatiles, dichloromethane was added and the acid chloride **271** solution cooled below 20 °C before being added to the solution of free amine **274**. However, after stirring for several hours, the formation of the desired product **275** was not observed (Scheme 99).



Scheme 99

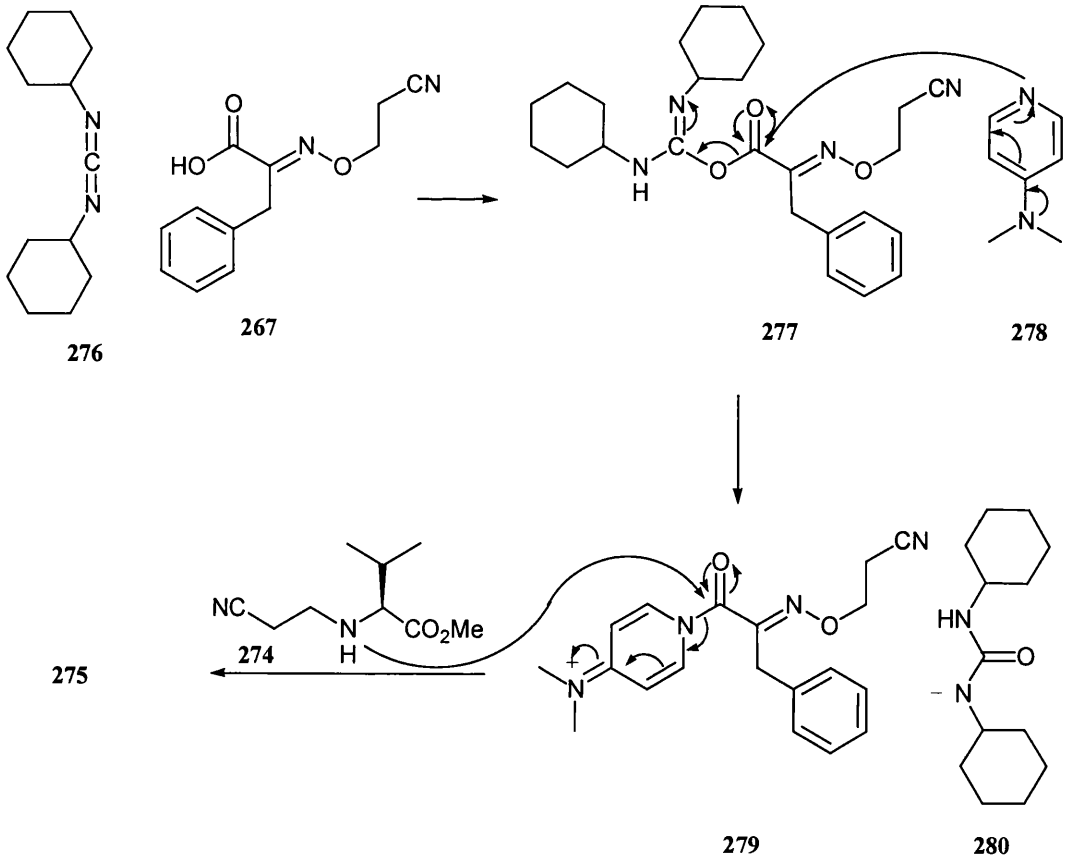
This procedure was also repeated with the *N*-protected L-tryptophan methyl ester hydrochloride **259** but again no amide bond formation was observed. The same reactions were also carried out in the presence of Hünig's base for both of the amino acids, but again formation of the product was not observed. Therefore, other peptidic coupling reactions were investigated.

N-Protected L-valine methyl ester hydrochloride **273** was stirred with triethylamine in dichloromethane and the solution was added to a solution of compound **267** in dichloromethane (Scheme 100). Dicyclohexylcarbodiimide was added followed by 4-dimethylaminopyridine and the reaction mixture was stirred for 12 hours at room temperature. However, after column chromatography only the starting materials were recovered (Scheme 100).



Scheme 100

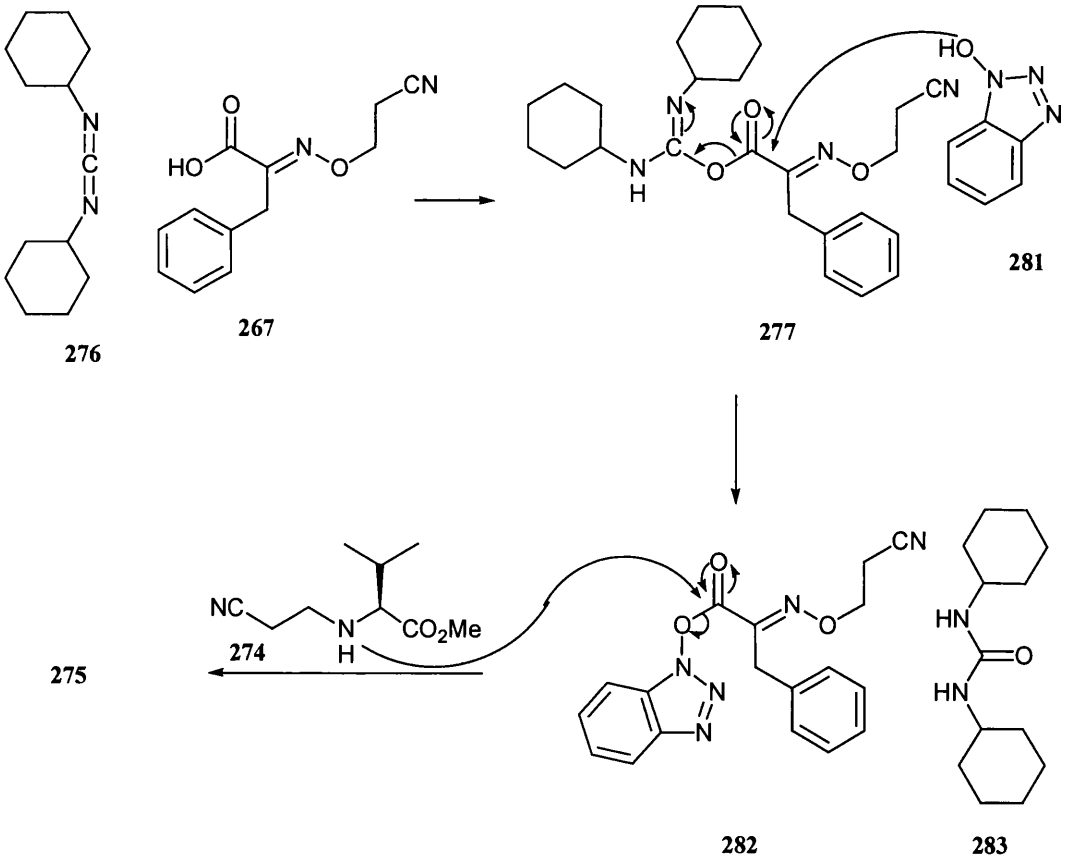
Dicyclohexylcarbodiimide **276** is used in peptidic synthesis to generate active carboxy derivatives (Scheme 101). The first step of this mechanism involves the formation of an *O*-acylisourea derivative **277** which undergoes a nucleophilic substitution by the 4-dimethylaminopyiridine additive **278** (Scheme 101). The tertiary amide **279**, formed *in situ*, is even more reactive than the *O*-acylisourea derivative **277** as it possesses a better leaving group due to the presence of the ammonium cation and the possible rearomatisation into the initial 4-dimethylaminopyiridine **278** (Scheme 101). The secondary amine **274** should have attacked the intermediate **279** to produce the desired peptide **275** but the reaction did not occur (Scheme 101). The presence of a dihydropyridine ring, a benzylic group and an *O*-cyanoethyloxime group could make such an intermediate relatively bulky towards the approach of a secondary amine such as **274**.



Scheme 101

The same procedure was then applied to the *N*-protected L-tryptophan methyl ester hydrochloride **259** and similar results were obtained implying that steric hindrance could be the problem of this peptidic coupling.

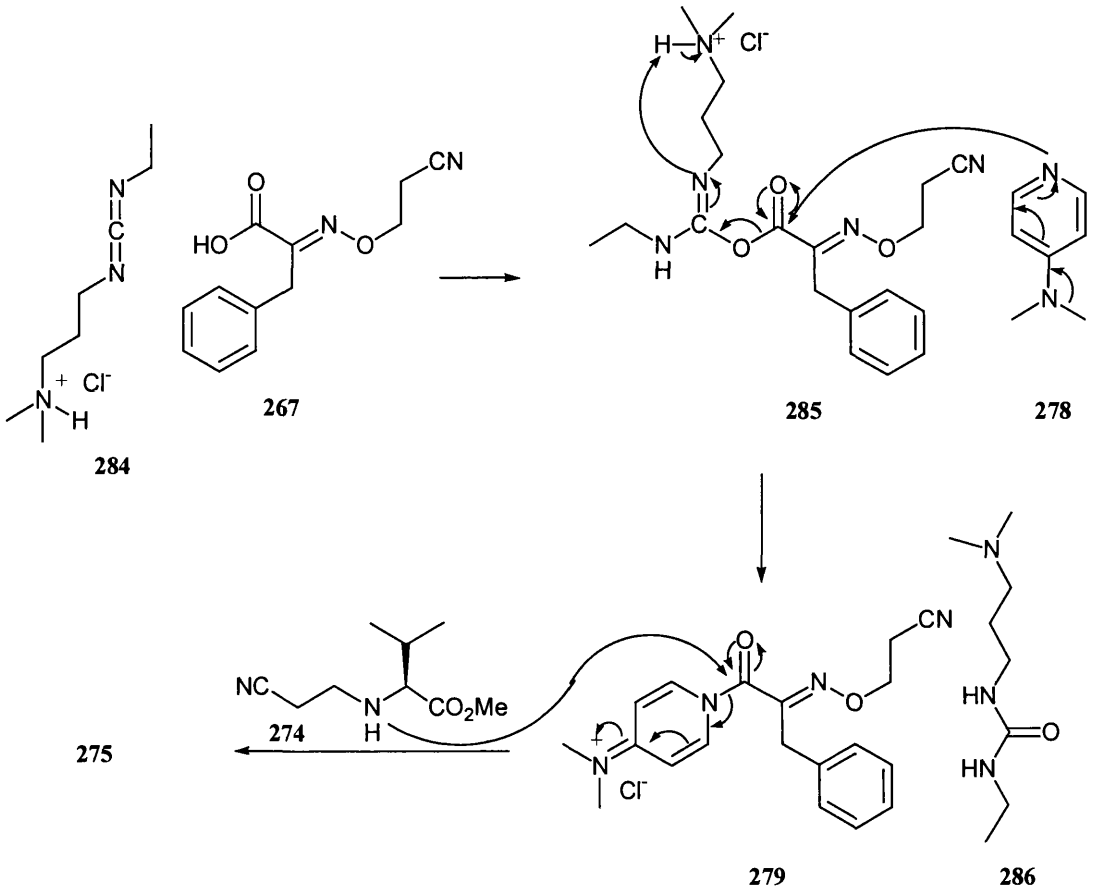
In a second attempt, *N*-hydroxylbenzotriazole **281** was chosen to replace 4-dimethylaminopyridine **278** as the catalytic additive (Scheme 102). After addition of the carboxy group **267** to the carbodiimide function **276** to produce the *O*-acylisourea **277**, *N*-hydroxybenzotriazole **281** was added and dicyclohexylurea **283** was eliminated in order to form the activated ester **282** (Scheme 102). However, once again amide bond formation did not occur to give **275**.



Scheme 102

Therefore, EDCI **284**, whose urea derivative can be easier to remove than 1,3-dicyclohexyl-urea, was used in an alternative coupling (Scheme 103). Addition of the carboxy group **267** to the carbodiimide function **284** produced the *O*-acylisourea **285**, followed by addition of the 4-dimethylaminopyridine **278** and

elimination of the urea derivative **286** produced the tertiary amide intermediate **279** (Scheme 103). However the desired peptidic product **275** was not observed.



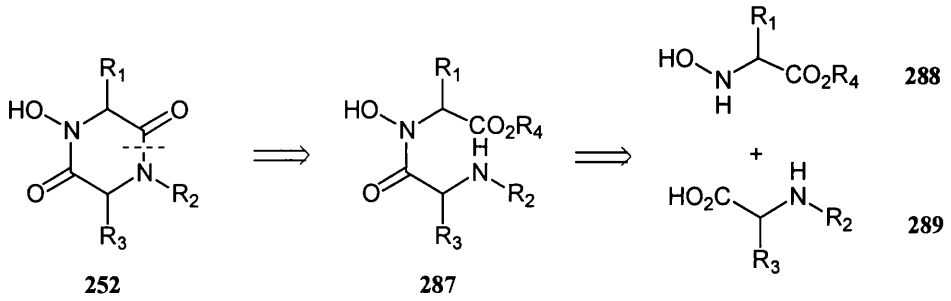
Scheme 103

Looking at the mechanisms of these reactions, it appears that although intermediates **279** and **282** possess an oxime fuction which makes the carbon of the carbonyl group more electrophilic, compounds **279** and **282** are too bulky for the attack of the secondary amine **274** or its tryptophan derivative.

2.2.2.2 Synthetic approaches to substrates of type B

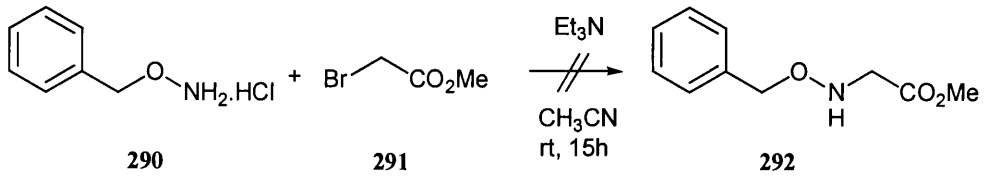
At the same time, our efforts were also concentrated on the synthesis of oxygenated 2,5-diketopiperazines of type B **252** (Scheme 104). According to the classical approach to 2,5-diketopiperazines, intramolecular cyclisation through amide bond formation appeared to be a suitable strategy. Indeed, compound **287** could easily

cyclise due to the presence of the oxygen which could assist in the adoption of the required cisoid conformation (Scheme 104). Furthermore compound **287** could be obtained from the coupling between a hydroxylamine derivative **288** and an amino acid **289** (Scheme 104).



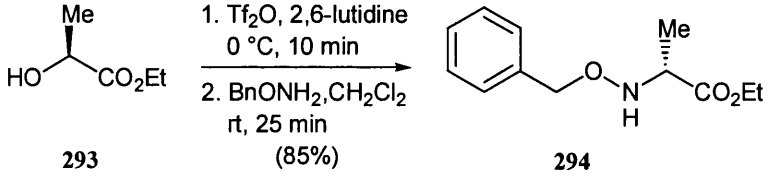
Scheme 104

Our method to synthesise the first fragment was by the reaction of commercially available *O*-benzylhydroxylamine hydrochloride salt **290** with methylbromoacetate **291** as the benzylic group of the hydroxylamine would provide not only a protecting group for the hydroxyl function but also yield a more soluble product (Scheme 105). Therefore, two equivalents of triethylamine were added to a suspension of *O*-benzylhydroxylamine hydrochloride salt **290** in acetonitrile to produce the free amine[157] and methylbromoacetate **291** was then added and the reaction mixture, which was heated under reflux for 12 hours (Scheme 105). However the free amine of benzylhydroxylamine hydrochloride salt **290** was only recovered. Nucleophiles such as benzylhydroxylamine, hydroxylamine, hydrazine or hydrogen peroxide are powerful nucleophiles due to their α-effect, the presence of an atom containing one or more unshared pairs closed to the attacking atom on the nucleophile. However, intermolecular hydrogen bonding can decrease their nucleophilicity and may explain why formation of **292** was not observed (Scheme 105).



Scheme 105

Thus, an alternative method to synthesise our *O*-benzyl-hydroxyamino acid ester **292** was required. Ottenheijm *et al.*[158] observed that α-hydroxy acid esters were successfully substituted *via* their triflates, 10^3 faster as a leaving group than a bromide, by *O*-benzylhydroxylamine to yield *O*-benzyl-hydroxyamino acid ester in high yield and thus, treatment of commercially available ethyllactate **293** with trifluoromethanesulphonic acid anydride and 2,6-lutidine, followed by addition of the free benzylhydroxylamine did produce the desired *O*-benzyl-hydroxyamino acid ester **294** in 85% yield (Scheme 106).

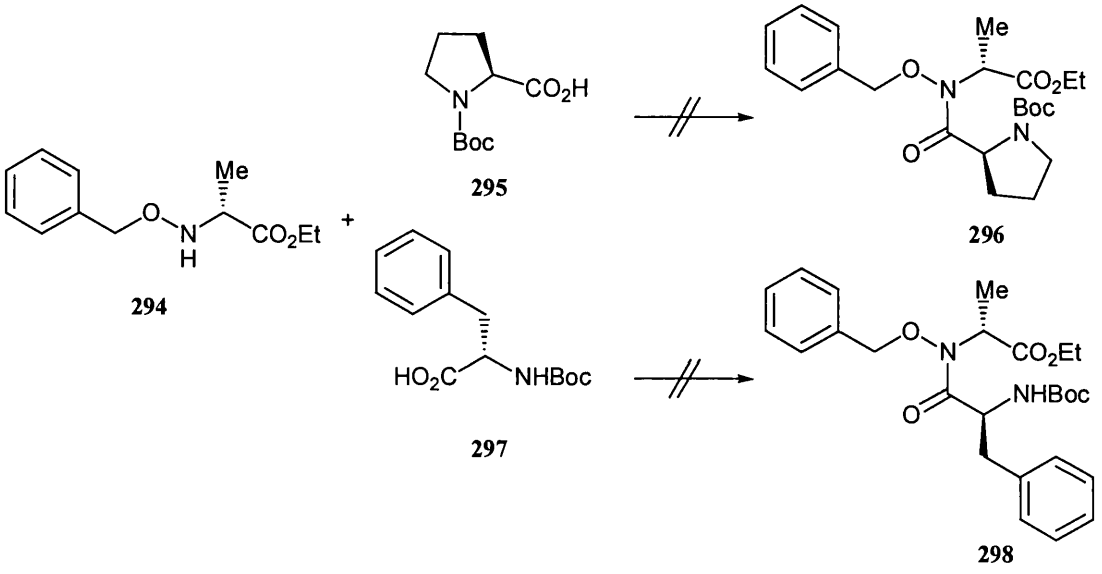


293 **294**

Scheme 106

With the *O*-benzyl-hydroxyamino acid ester **294** in hand, we then attempted to acylate with *N-tert*-butoxycarbonyl protected amino acids following a mixed anhydride method described by Akiyama *et al.*[159] Hence a solution of *N-tert*-butoxycarbonyl-proline **295** and triethylamine in tetrahydrofuran was cooled to - 15 °C and treated with a solution of *iso*-butylchloroformate in tetrahydrofuran (Scheme 107). After 15 minutes, a mixture of *O*-benzyl-hydroxyamino acid ester **294** and triethylamine in dichloromethane was added to the solution and after stirring for 3 hours at – 15 °C, the reaction mixture was kept for 45 hours at 4 °C (Entry 1, Table 1). The resulting triethylammonium chloride salt was removed and the filtrate was evaporated to give a residue, which was dissolved in ethyl acetate and the resulting solution was washed with 5% aqueous sodium carbonate. Since unchanged *O*-benzyl-hydroxyamino acid ester **294** was detected by TLC analysis, the crude product was further acylated by the above procedure, but even after repetition of the acylation process, no formation of the desired product **296** was observed. *N-tert*-Butylcarbonyl-phenylalanine **297** was also used in this sequence, but, unfortunately, this mixed anhydride method did not yield the desired product **298** (Entry 2, Table 1).

At this stage, we decided to couple compounds **294** and **297** using dicyclohexylcarbodiimide (Entry 3, Table 1). Dicyclohexylcarbodiimide was added to a solution of *N-tert*-butoxycarbonyl-L-phenylalanine **297** in dichloromethane followed by the addition of 4-dimethylaminopyridine. After the reaction mixture was stirred for 15 minutes at room temperature, a solution of *O*-benzyl-hydroxyamino acid ester **294** in dichloromethane was added dropwise and the reaction was then stirred for 12 hours at room temperature. TLC and NMR analyses of the crude product did not indicate the formation of compound **298**.



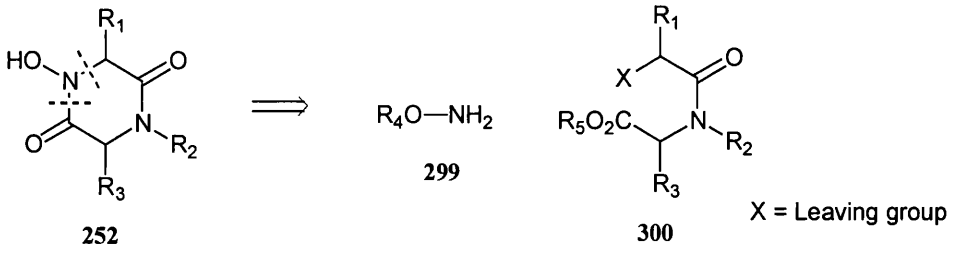
Scheme 107

Entry	*N*-Boc-amino acid	Conditions
1	*N*-Boc-L-proline **295**	Et_3N, *iso*-butylchloroformate, THF, CH_2Cl_2, - 10 °C, 48h
2	*N*-Boc- L-phenylalanine **297**	Et_3N, *iso*-butylchloroformate, THF, CH_2Cl_2, - 10 °C, 48h
3	*N*-Boc- L-phenylalanine **297**	DCC, DMAP CH_2Cl_2, rt, 12h
4	*N*-Boc- L-phenylalanine **297**	1.NaH, oxalyl chloride, rt, 18h 2. Et_3N, CH_2Cl_2, rt, 24h

Table 1

As both the mixed anhydride method and the dicyclohexylcarbodiimide-coupling did not give the expected products, we decided to synthesise the acid chloride of **297** to acylate *O*-benzyl-hydroxyamino acid ester **294** (Entry 4, Table 1). Sodium hydride was added to a solution of *N-tert*-butoxycarbonyl-L-phenylalanine **297** in dichloromethane and after 10 minutes of stirring, the solution was cooled to - 10 °C and oxalyl chloride was added dropwise. After stirring the reaction mixture overnight at room temperature, all the volatiles were removed at reduced pressure and the residue dissolved in dichloromethane. A solution of *O*-benzyl-hydroxyamino acid ester **294** and triethylamine in dichloromethane was added to the solution of the acid chloride and stirred at room temperature for 12 hours. As TLC analysis did not show the formation of product **298**, the reaction mixture was stirred for an additional 12 hours, but formation of the product **298** was not observed (Scheme 107).

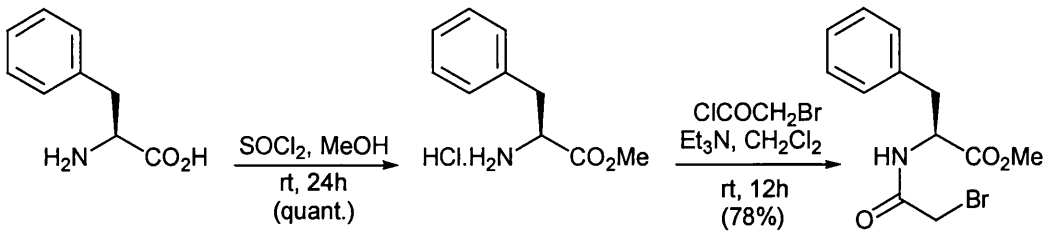
In view of these results, we decided to change our strategy and to consider another disconnection approach. As previously indicated, a synthetic approach to the of 2,5-diketopiperazine unit can be achieved by the reaction of ammonia with a halogeno-ester derivative, and, in principle, the same strategy can be applied to the oxygenated 2,5-diketopiperazine type B **252** through selection of hydroxylamine or of an *O*-protected-hydroxylamine[160,161,162] **299** as the nucleophile (Scheme 108).



Scheme 108

L-Phenylalanine **55** was converted quantitatively into L-phenylalanine methyl ester hydrochloride **301** in the presence of thionyl chloride in methanol at room temperature in 24 hours (Scheme 109). Compound **301** was then acylated in the presence of triethylamine and bromoacetylchloride in dichloromethane at room temperature over 12 hours to afford **302** in 78% yield (Scheme 109). The same

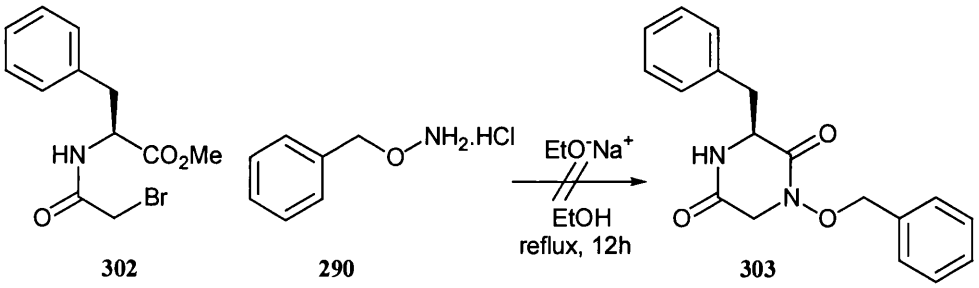
yield was observed when L-phenylalanine methyl ester hydrochloride **301** was treated with bromacetylbromide under identical conditions.



55 **301** **302**

Scheme 109

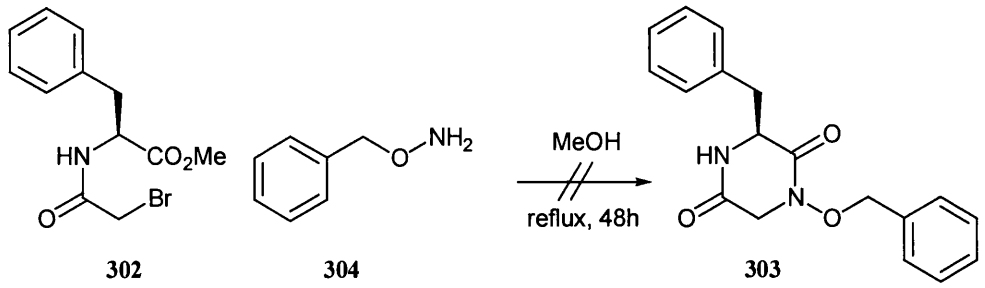
After preparing a solution of sodium ethanoate in ethanol, commercially available *O*-benzylhydroxylamine hydrochloride **290** was added to this solution and the reaction mixture stirred for 30 minutes before the addition of the bromo-ester **302** (Scheme 110). The reaction mixture was then heated at reflux for 12 hours, however the formation of product **303** was not observed and TLC analysis revealed the presence of the two starting materials **302** and the free base of **290** (Scheme 110).



302 **290** **303**

Scheme 110

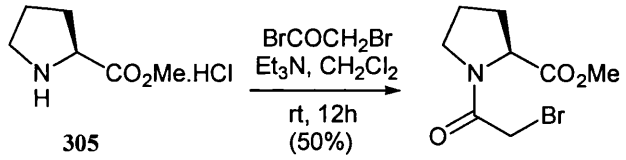
Thus, the bromo-ester **302** was dissolved in methanol at 0 °C and a solution of 2.5 equivalents of free benzylhydroxylamine **304** in methanol was added (Scheme 111). The reaction mixture was stirred and heated at reflux for 48 hours but TLC analysis indicated once again only the presence of the starting materials.



302 **304** **303**

Scheme 111

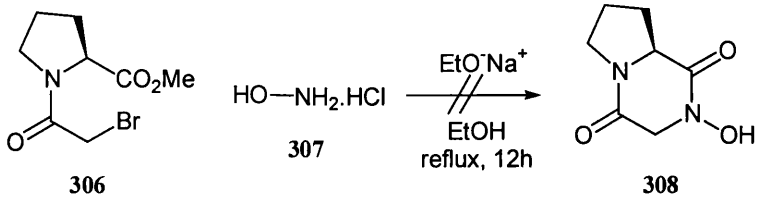
These strategies were also attempted with a proline derivative. Cyclisation should be easier since this amino acid already possesses already the "folded" conformation. Commercially available L-proline methyl ester hydrochloride **305** was therefore acylated with bromacetylbromide in the presence of triethylamine to afford the bromo-ester derivative **306** in 50% yield (Scheme 112).



305 **306**

Scheme 112

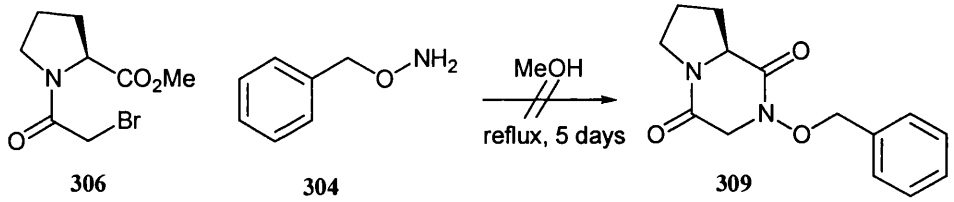
Compound **306** was added to a solution of hydroxylamine made from hydroxylamine hydrochloride **307** and freshly prepared sodium ethoxide in ethanol and the reaction mixture was heated under reflux for 12 hours (Scheme 113). TLC analysis indicated the formation of many products. The reaction solution was then concentrated under reduced pressure and the crude product gave a deep violet colouration with methanolic ferric chloride, demonstrating the presence of the hydroxamic acid moiety.[162] However, attempts to isolate the product **308** either by crystallisation in acidic aqueous solution or column chromatography using a variety of solvent mixtures such as dichloromethane and methanol, were unsuccessful (Scheme 113).



306 308

Scheme 113

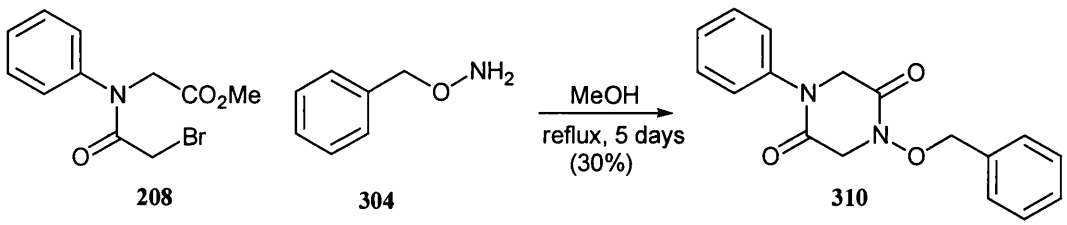
A solution of free *O*-benzylhydroxylamine **304** in methanol was then added to a solution of bromo-ester **306** in methanol at 0 °C and the reaction mixture heated under reflux for 48 hours (Scheme 114). However, in this case again we did not observe any formation of the desired product **309** and the bromo-ester **306** and *O*-benzylhydroxylamine **304** were recovered (Scheme 114).



306 304 309

Scheme 114

To our surprise however another experiment with the aniline derived bromo-ester **208** was successful and led to the synthesis of the desired oxygenated 2,5-diketopiperazine **310** (Scheme 115). Compound **208** was dissolved in methanol and a solution of 2.5 equivalents of free *O*-benzylhydroxylamine **304** in methanol was added at 0 °C, before heating the reaction mixture at reflux for 5 days (Scheme 115). After cooling, the product **310** was obtained as white crystals in 30% yield (Scheme 115).

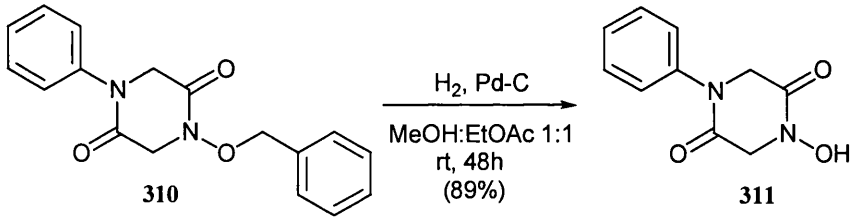


208 304 310

Scheme 115

Given the precedent for ammonia and methylamine to react with such bromo-esters to afford 2,5-diketopiperazines, the failure of α-effect hydroxylamine based nucleophiles in the foregoing sections is somewhat surprising, especially when "favoured" amino acids such as proline are selected to assist cyclisation. The successful cyclisation of the *N*-phenylglycine precursor **208** may be due to the inductively withdrawing nature of the phenyl group which therefore confers more ketonic character on the α-bromo amide unit hence favours the initial nucleophilic displacement.

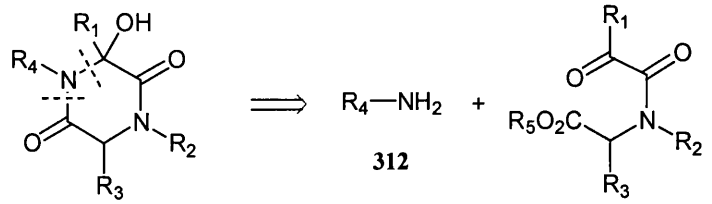
With our desired *O*-benzyl-*N*-hydroxy-2,5-diketopiperazine **310** in hand we then attempted our final step of deprotection. Compound **310** was added to a mixture of ethyl acetate and methanol and subjected to a positive pressure of hydrogen, in the presence of palladium on carbon (Scheme 116). The reaction mixture was stirred at room temperature for 48 hours to yield 1-hydroxy-4-phenyl-2,5-diketopiperazine **311** in 89% yield as the necessary substrate representative of B (Scheme 116).



Scheme 116

2.2.2.3 Synthetic approaches to substrates of type C

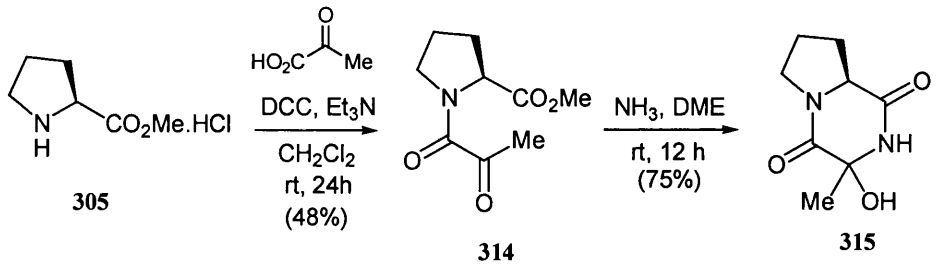
Oxygenated 2,5-diketopiperazines of type C **257** also consitute a group of interesting precursors for our methodology and can be synthesised from ring closure of pyruvoyl amino acids **313** in the presence of an amine **312** or ammonia (Scheme 117). This method has been described using a proline derivative by Bycroft *et al.*[163] in 1975 and more recently by Sanz-Cervera *et al.*[164]



257 313

Scheme 117

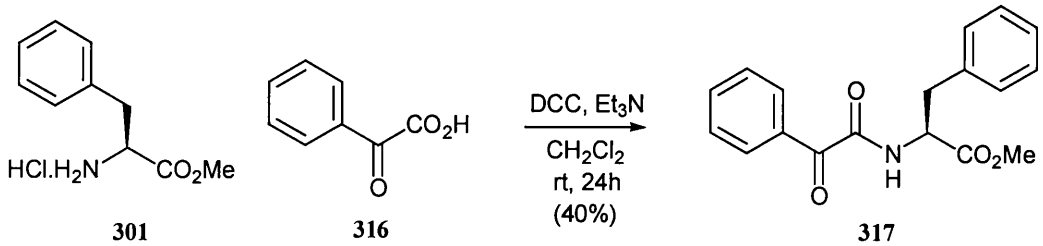
Accordingly, L-proline methyl ester hydrochloride **305** was reacted with pyruvic acid in the presence of dicyclohexylcarbodiimide and triethylamine to give the *N*-pyruvate **314** in 48% yield (Scheme 118). This compound was stirred in dimethoxyethane under an atmosphere of ammonia at room temperature and led to the formation of the *C*-hydroxy-2,5-diketopiperazine **315** as a mixture of diastereosiomers in 75% yield (Scheme 118).



305 314 315

Scheme 118

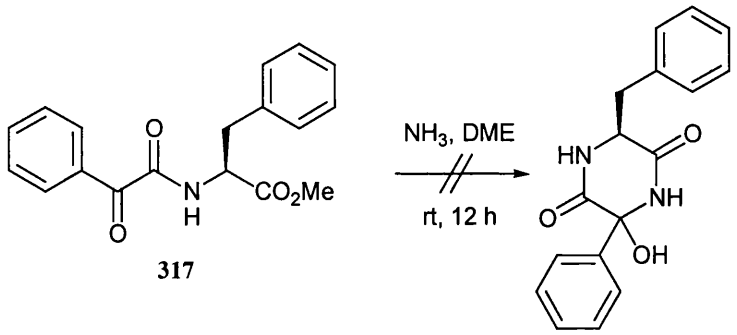
In order to obtain a more soluble member of this class, L-phenylalanine methyl ester hydrochloride **301** was coupled with benzoyl formic acid **316** in the presence of triethylamine and dicyclohexylcarbodiimide to produce compound **317** in 40% yield (Scheme 119).



301 316 317

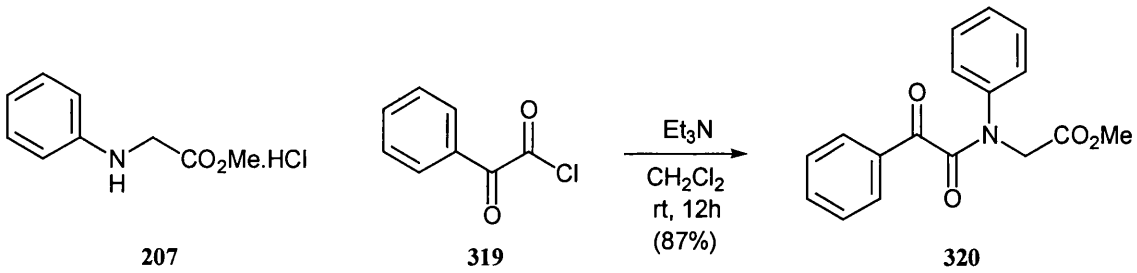
Scheme 119

The precursor **117** was then dissolved in dimethoxyethane and treated with ammonia as previously described. The reaction mixture was stirred at room temperature for 12 hours, but TLC analysis showed the presence of starting material **117** only (Scheme 120).



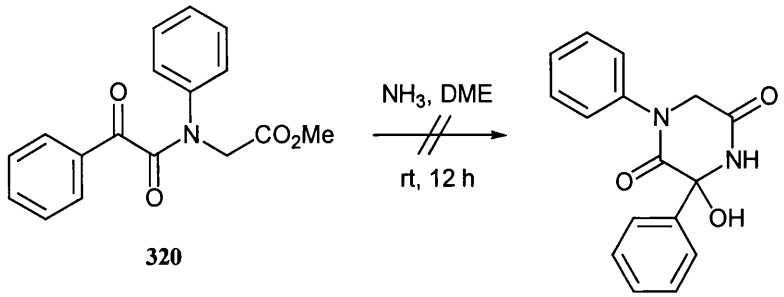
Scheme 120

As previously discussed (Section 2.1.1.3), precursors containing a tertiary amide have been demonstrated to cyclise more easily. Compound **320** was hence assembled by coupling between *N*-phenyl-glycine methyl ester hydrochloride **207** and benzoyl formic acid chloride **319** in the presence of triethylamine in 87% yield (Scheme 121).



Scheme 121

However, once again, stirring compound **320** in dimethoxyethane under an atmosphere of ammonia, at room temperature for 12 hours did not produce the desired *C*-hydroxyl-2,5-diketopiperazine **321** as the starting material **320** was entirely recovered (Scheme 122). Thus, we concluded that the benzoyl group may be too bulky for this ring closure.

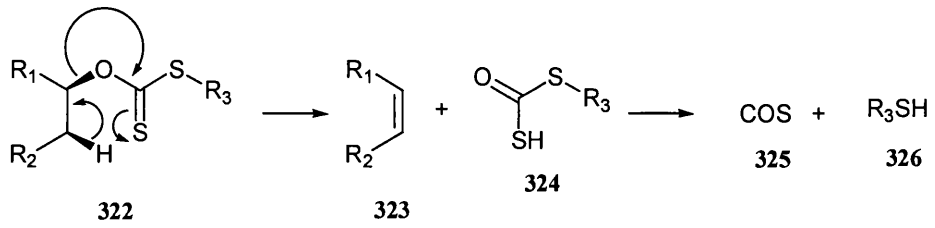


320 **321**

Scheme 122

2.2.3 Xanthate methodology

With our oxygenated 2,5-diketopiperazines of type B **311** and type C **315** in hand we then attempted to develop a novel method for the incorporation of the disulfide bridge into 2,5-diketopiperazines. As previously discussed, the Chugaëv elimination reaction represented the key step. In 1899, Chugaëv discovered that pyrrolysis of a xanthate **322** led to a *cis*-elimination to form an olefin **323** and a dithiocarbonic acid ester **324** which decomposed into carbon oxysulfide **325** and a mercaptan **326** (Scheme 123).[165] This reaction has been applied to xanthate esters of primary, secondary and tertiary alcohols.[166]

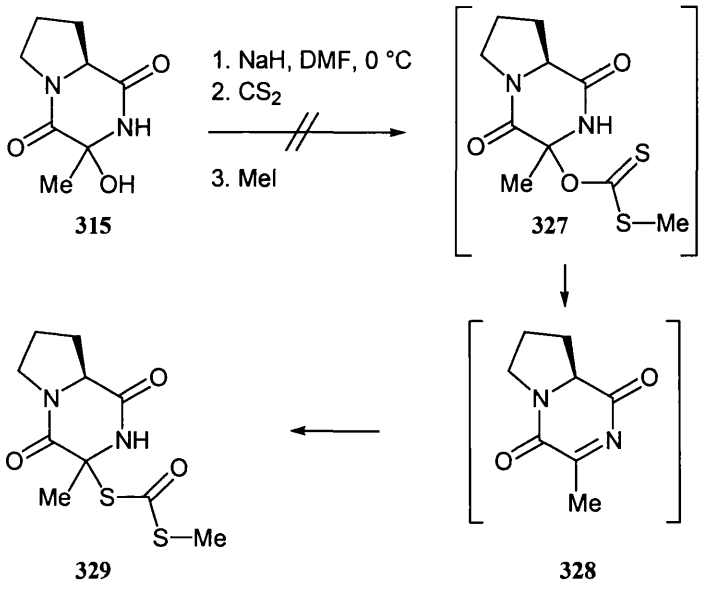


322 **323** **324** **325** **326**

Scheme 123

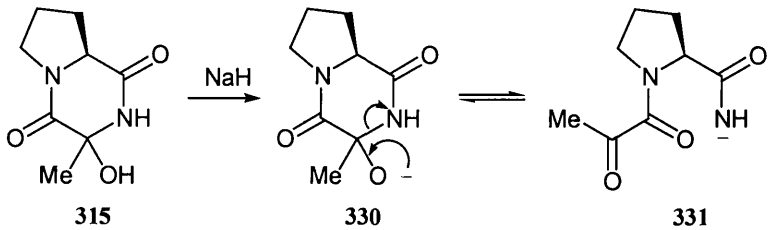
Xanthates are traditionally formed readily from alcohols by firstly adding sodium hydride, to form the alcoholate, followed by addition of carbon disulfide and methyl iodide. The reactions are usually carried out in tetrahydrofuran.[167,168] However, dimethylformamide[169] is an alternative solvent used for insoluble starting materials such as our oxygenated 2,5-diketopiperazines **311** and **315**.

Thus, compound **315** was added in dimethylformamide to give an opaque solution to which sodium hydride was then added at 0 °C and the reaction mixture was stirred until hydrogen evolution ceased (Scheme 124). Carbon disulfide was then added and the solution was stirred for 5 minutes before adding methyl iodide. The volatiles were then removed under reduced pressure and the crude product was subjected to column chromatography. In the event however, an extremely complex of mixtures of products was formed in this reaction and no evidence was adduced for formation of either the xanthate **327**, the elimination intermediate **328** or the desired adduct **329** (Scheme 124).



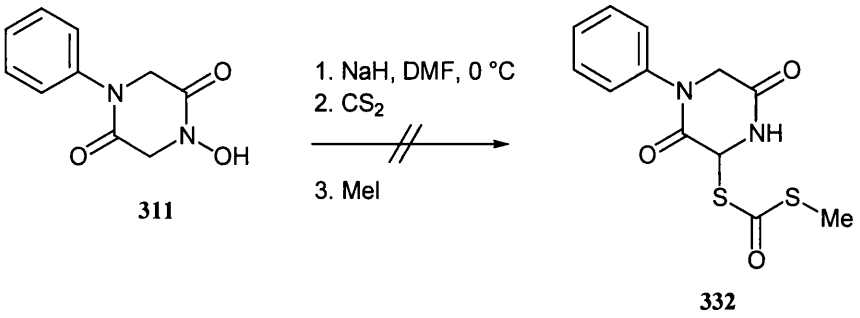
Scheme 124

In retrospect, a possible reason for the failure of this reaction could be that the alkoxide **330** formed may induce opening of the 2,5-diketopiperazine ring to form intermediate **331**, which could then react with either carbon disulfide or methyl iodide (Scheme 125).



Scheme 125

In similar fashion, this methodology was then applied to the cyclic hydroxamic acid derivative of type B **311** which was sparingly soluble in dimethylformamide (Scheme 126). To our disappointment however, attempted xanthate formation using a similar method to that outlined above yielded a very complex mixture of products from which no mechanistic insights of value could be deduced.



Scheme 126

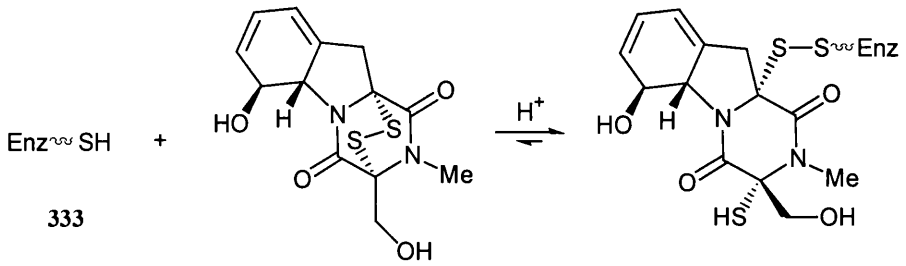
2.3 Mechanistic studies towards an understanding of the mode of action of epi-3,6-dithio-2,5-diketopiperazines

2.3.1 Introduction

Within the family of epi-3,6-dithio-2,5-diketopiperazines, it is certain that nature has constructed a unique disulfide bridge. It is therefore tempting to speculate that the wide ranging biological activity of this class of compounds stems from a common mode of action which takes advantage of the unusual physical and electronic characteristics of this particular sulfur-sulfur bond, and that the surrounding molecular architecture created by oxidation of the two amino acid units is involved in specific recognition phenomena. As we have seen in the introductory overview (Section 1.5) the strain created by the affinity of the sulfur lone pairs for the two carbonyl groups of the 2,5-diketopiperazine ring leads to a weaker disulfide bond which should be especially predisposed to ring opening.

At the present time, to the best of our knowledge, no definitive mechanism of action has been proposed for an epi-3,6-dithio-2,5-diketopiperazine. Within the

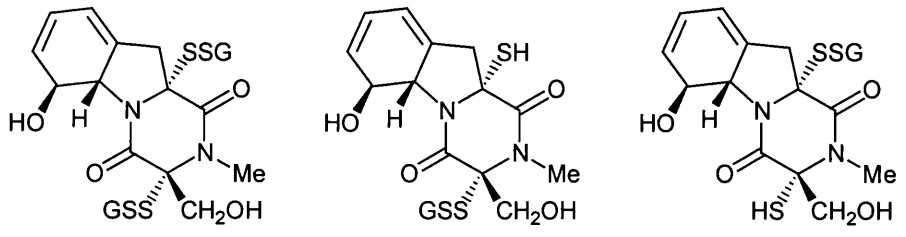
context of gliotoxin however, which has not been studied, a collaborative effort between the group of Waring and Chai has suggested two alternatives.[24] Firstly, gliotoxin **1** could initially form a disulfide bond via the well known thio-disulfide interchange mechanism and involving a thiol group on the enzyme **333** (Scheme 127).



333 **1** **334**

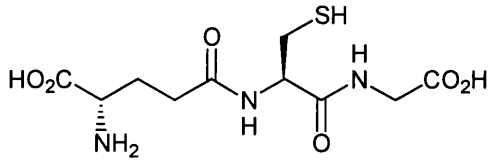
Scheme 127

Support for the ease of thio-disulfide interchange was subsequently demonstrated by the same group who studied the formation of gliotoxin-glutathione adducts **335**, **336**, and **337** (Scheme 128) in a phosphate buffer at pH 7.[170] Such adducts may also be intermediates in cell systems treated with gliotoxin.



335 **336** **337**

G = Glutathione =

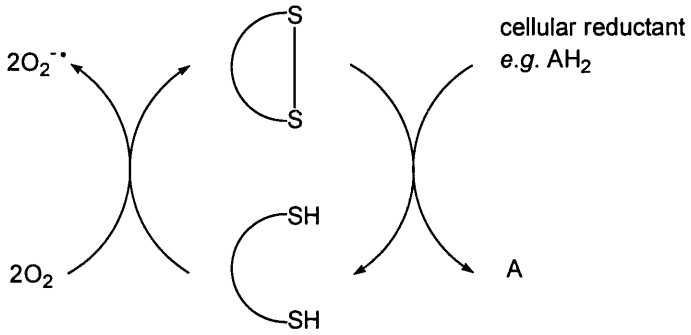


338

Scheme 128

Two surprising observations relating to the thiol function of the two mono mixed disulfide **336** and **337** were however made in this study. The first of these is that

both derivatives are prone to S_N2 reaction to regenerate gliotoxin **1**, whilst the second was detection of a molecular ion in a mass spectral study corresponding to a mono mixed disulfide + oxygen, which the authors suggest in a footnote may be the desired sulfinic acid. The second hypothesis proposed by the Australian groups[14c,124] was less specific and suggested that free radical species such as superoxide radical anion species could be produced during a redox cycle which could cause oxidative damage (Scheme 129).



Scheme 129

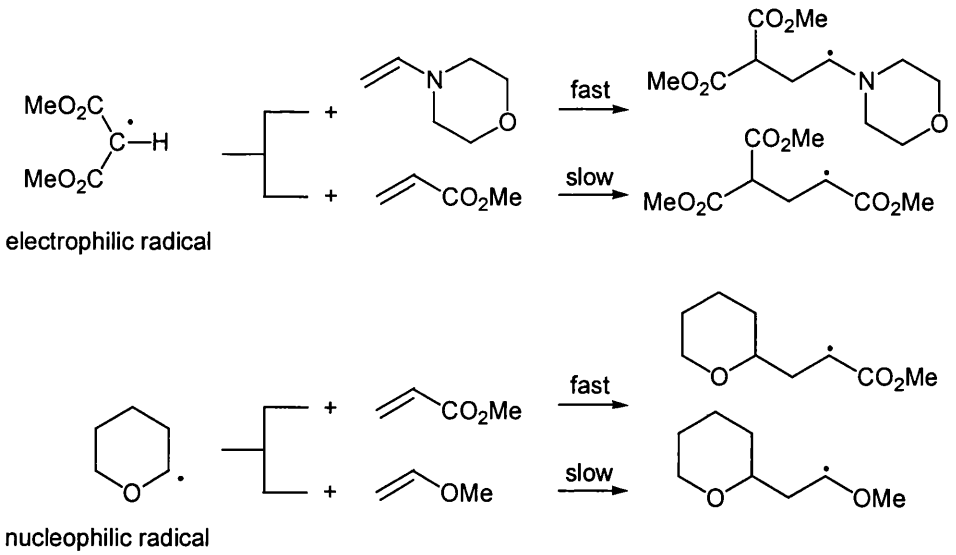
Moreover, gliotoxin **1** has been shown to cause DNA damage on cellular and plasmid DNA[171,172] and once again, speculation centres around the idea that epi-3,6-dithio-2,5-diketopiperazine core is believed to be involved in redox cycles in the presence of an appropriate reducing agent and that this process leads to the production of "reactive oxygen species" leading to fragmentation of DNA. Clearly, at the molecular level which would satisfy an organic chemist, much remains to be understood.

At this stage, since our own hypothesis as to a possible mode of action also involved the intermediate generation of thiyl radicals (*vide infra*), it is appropriate to provide a brief overview of the behaviour of sulfur radicals in general, and particularly in biological systems.

2.3.2 The reactivity and character of thiyl radicals in chemistry and chemical biology

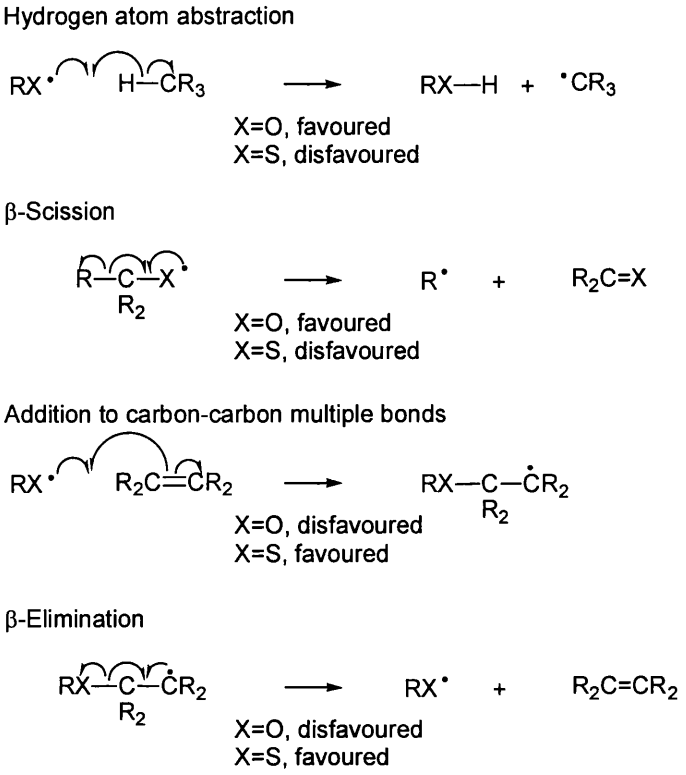
The use of free radicals reactions for organic synthesis has witnessed explosive growth over the last twenty five years, primarily as a consequence of the fact that these neutral reactive intermediates tolerate a wide variety of functional groups and that highly selective reactions can be designed through detailed understanding of kinetic phenomena and the nucleophilic or electrophilic "character" of the radicals involved.[173]

Thus for example, within the subset of carbon centered radicals, the nature of the groups attached to the carbon centre dominates the relative rates of reaction and hence determines the chemoselective behaviour of these radicals in addition to carbon-carbon double bonds (Scheme 130).



Scheme 130

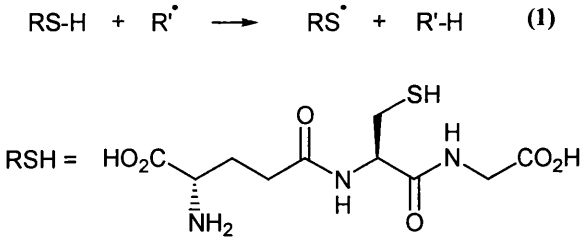
In the first instance however the chemical reactivity of a radical is dominated by the nature of the atom containing the unpaired electron, and this can be clearly illustrated by the comparison between simple alkyl substituted alkoxyl and thiyl radicals shown in Scheme 131.



Scheme 131

Thus as a consequence of the greater electronegativity of oxygen relative to sulfur, alkoxy radicals are even more electrophilic in character than thiyl radicals.

Although simplistic in nature, the above generalisations, when taken in conjunction with bond dissociation energies, help to explain why thiols are generally considered as excellent hydrogen atom donors (*e.g.* bond dissociation energy $D°(CH_3S–H)$ = 365.7 kJ.mol^{-1}) whereas alkoxy radicals behave as powerful hydrogen abstractors ($D°(CH_3O–H)$ = 433.8 kJ.mol^{-1}). Within biological systems for example, the powerful reducing agent, glutathione **338**, a thiol found in all cells is responsible for the "repair" reaction (Eqn 1) of carbon-centered radicals derived from biomolecules (Scheme 132).[174]



338

Scheme 132

In light of the foregoing analysis, it may therefore seem curious that thiyl radicals are believed to be the reactive intermediates in several biological mechanisms such as ribonucleotide reductase and DNA damage (*vide infra*), both of which require hydrogen atom abstraction from a C–H bond of a ribose ring by a thiyl radical as the primary reaction step.

However, as we have illustrated above for carbon centered radicals, the electrophilic or nucleophilic nature of a particular radical can also be "fine-tuned" by alteration of the groups attached to the radical centre. Since the potential reactivity of thiyl radicals derived from epi-3,6-dithio-2,5-diketopiperazines was of especial relevance to us, it was therefore of interest to examine in detail the exact structural features of those thiyl radicals which have shown hydrogen atom abstraction characteristics.

Within this area, our attention naturally turned to the studies of Roberts *et al.* on the concept of polarity reversal catalysis.[175] This is best illustrated by consideration of the effect of thiol catalysis of the free radical chain reduction of alkyl halides by organosilanes. The two steps of the standard propagation sequence are set out in Scheme 133 and involve halogen atom abstraction by the silicon radical followed by hydrogen atom capture from the silane.[175b]

$$R\text{-}X + R'_3SiH \longrightarrow RH + R'_3Si\text{-}X$$

Propagation Sequence

Halogen atom abstraction

$$R'_3Si^{\bullet} + X\text{-}R \longrightarrow R'_3Si\text{-}X + R^{\bullet} \qquad k \sim 10^6\ dm^3mol^{-1}s^{-1}$$

Hydrogen atom abstraction

$$R^{\bullet} + H\text{-}SiR'_3 \longrightarrow R\text{-}H + R'_3Si^{\bullet} \qquad k \sim 10^3\ dm^3mol^{-1}s^{-1}$$

Scheme 133

Since the silicon-hydrogen bond in a triorganosilane ($D°(R_3Si–H)$ 400 kJ.mol^{-1}) is typically 65 kJ.mol^{-1} stronger than the Sn–H bond in the often used organostannanes reactions with silanes often have shorter chain lengths and require higher temperatures as a consequence of the slower hydrogen abstraction.[175b]

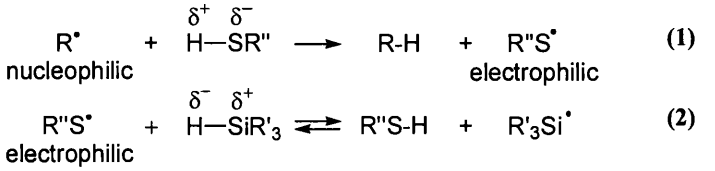
This problem can be overcome however by the addition of an alkane thiol, since the alkyl radical will abstract the hydrogen atom from an alkane thiol R"SH almost 1000 times faster than from the silane (Scheme 135).

$$R^{\bullet} + H\text{-}SR'' \longrightarrow R\text{-}H + R''S^{\bullet} \qquad k \sim 10^6\ dm^3mol^{-1}s^{-1}$$

Scheme 134

Although the above reaction is clearly favoured in thermodynamic terms since it leads to carbon-hydrogen bond formation (($D°(C–H) \approx 410$ kJ.mol^{-1}) at the expense of the weaker sulfur-hydrogen bond ($D°(R"S–H)$ 370 kJ.mol^{-1}), the faster rate of hydrogen atom capture by the thiol relative to the silane is not related to the bond energies since the silicon-hydrogen bond is just about stronger than the sulfur-hydrogen bond by 20-30 kJ.mol^{-1}.

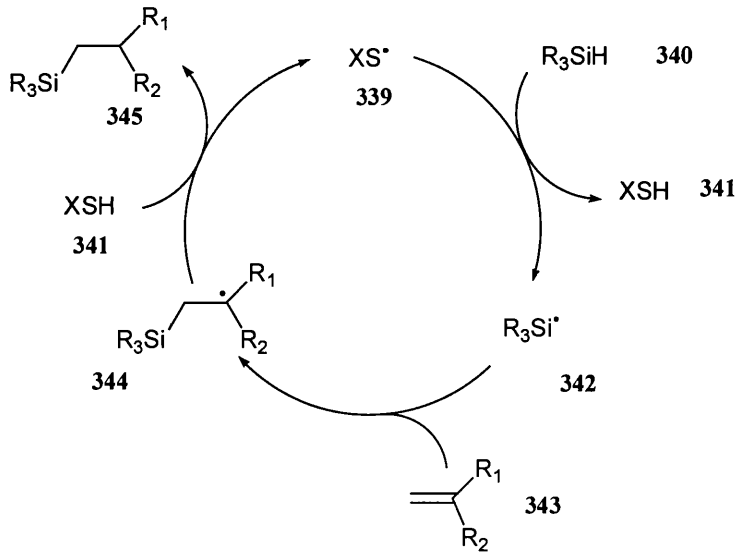
The best explanation of this phenomenon resides in consideration of the polarity of the radicals involved. Thus as shown in Scheme 135, the nucleophilic alkyl radical is abstracting an electron poor hydrogen atom from a thiol to give an eletrophilic sulfur radical (Eqn 1) whereas the hydrogen atom in the silane is relatively electron rich by virtue of being attached to an electropositive metalloid silicon atom (Eqn 2).



Scheme 135

Even though the second reaction is reversible and not exothermic, the silyl radical can then undergo competitive halogen atom abstraction to form a strong Si–X bond, thus driving the reaction to completion. In essence therefore, polarity reversal catalysis involves replacement of a slow single propagation step by two fast consecutive reactions whose rates are favoured by considerations of matching polarity.

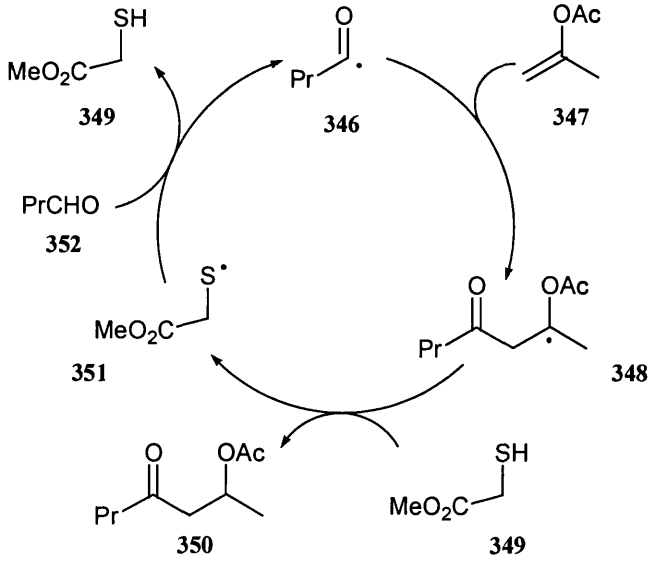
In similar vein, Roberts *et al.* also demonstrated that thiols are efficient catalysts for the hydrosilylation of alkenes.[176] Thus the thiyl radical **339** formed abstracts the hydrogen of silane **340** to produce the silyl radical **342**. Addition of this radical onto the double bond of compound **343** then produces the *C*-centered radical **344**, which provides the hydrosilylated compound **345** and regenerates the starting thiyl radical **339** (Scheme 136).



Scheme 136

Tert-dodecanethiol was used as catalyst, but methyl thioglycolate and triphenylsilanethiol were demonstrated to be more efficient catalysts. Homochiral thiols such as 1-thio-β-D-glucopyranose and 1-thio-β-D-mannopyranose were not only efficient catalysts but also gave high enantiomeric excess in addition of silanes to prochiral alkenes.

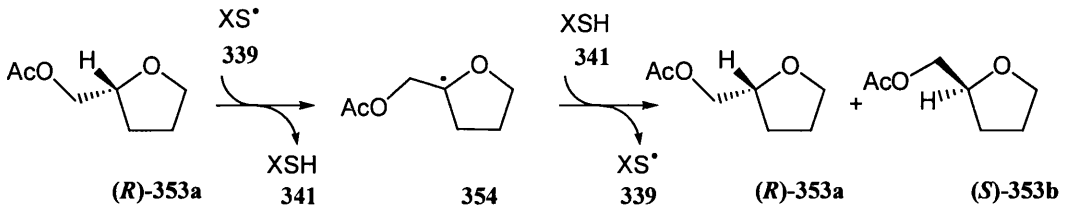
In terms of hydrogen atom abstraction from an aldehydic C–H bond, Roberts *et al.* also described that addition of butanal **352** to isopropenyl acetate **347** at 60 °C, in the presence of *trans*-di-*tert*-butyl hyponitrite as radical initiator, and methyl thioglycolate **349** as catalyst yielded the product **350** in 80% yield[177] (Scheme 137). The same reaction carried out without the thiol **349** gave the product in a low 8% yield, since hydrogen abstraction from butanal by the radical **348** is very inefficient (Scheme 137).



Scheme 137

Finally and of especial interest to us, was another study carried out by Cai and Roberts in which thiyl radicals with electron-withdrawing groups were demonstrated to be capable of abstracting hydrogen from the tetrahydrofurfuryl ester **353a** (Scheme 138).[178] The radical initiator *trans*-di-*tert*-butylhyponitrite (TBHN) produced *tert*-butoxyl radicals, which in turn provided the thiyl radicals **339** (Scheme 138). Hydrogen abstraction of (*R*)-tetrahydrofurfuryl acetate **353a** by

the thiyl radical **339** gave **354** which was "repaired" by the thiol **341** to yield either the (*R*)- or (*S*)-tetrahydrofurfurylacetate **353a** and **353b** (Scheme 138). Once again, thiols which possess electron-withdrawing groups were better catalysts and therefore triphenylsilanethiol, 1-thio-β-D-glucopyranose tetraacetate and 2,2,2-trifluoroethanethiol were the most efficient catalysts. Methyl thioglycolate, dodecan-1-thiol and *tert*-dodecanethiol were less effective catalysts and L-cysteine ethyl ester was found to be totally ineffective.



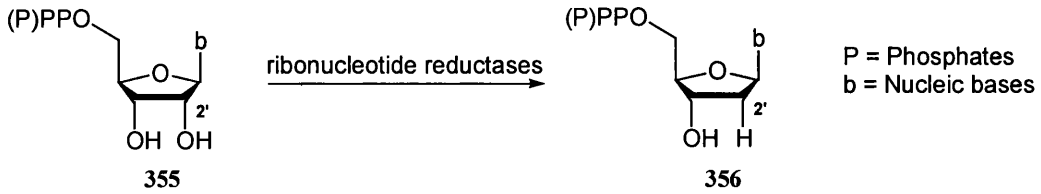
Scheme 138

The relevance of the above results for DNA strand cleavage in the oxygen deficient environment found in tumour cells was recognised by Roberts and the suggestion was made that sufficiently eletrophilic thiols might therefore be capable of amplifying radiation damage in such cases.

In fact thiyl radicals are believed to be the reactive intermediates in two of the most fundamental biological mechanisms, DNA damage and ribonucleotide reduction, both of which involve hydrogen atom abstraction from a C–H bond of a ribose ring by a thiyl radical as the primary step. Some of the more recent studies in these two areas are outlined below and shed further light on the causative factors responsible for efficient hydrogen atom abstraction.

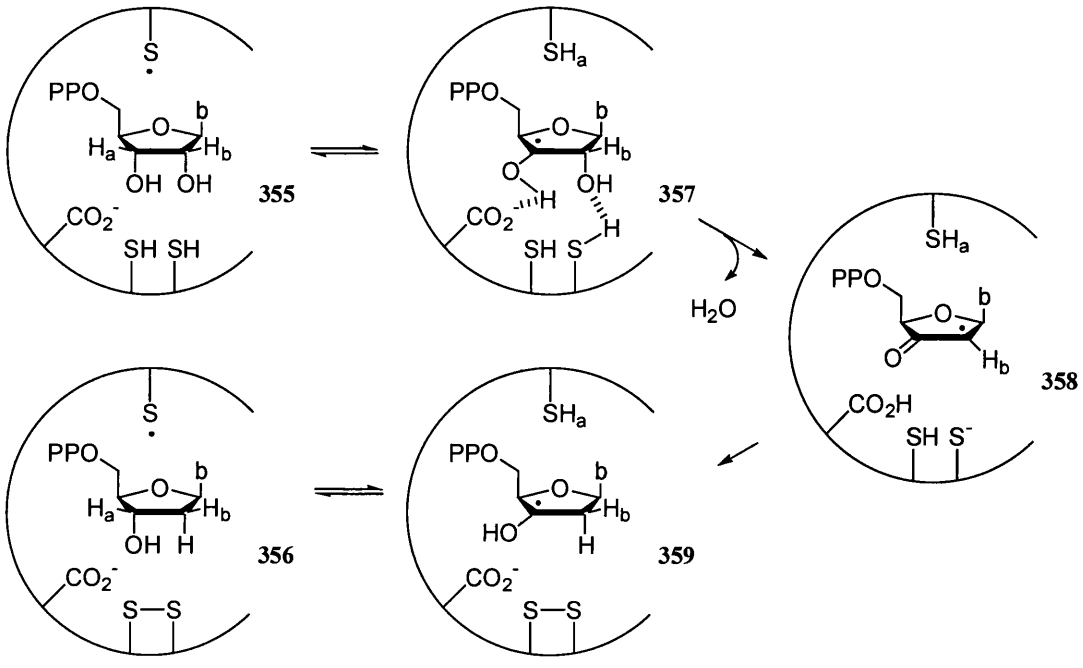
2.3.2.1 Ribonucleotide reductase

Recently thiyl radicals have been found, by Stubbe *et al.*, to be involved in the mechanism of reduction of ribonucleotides 5'-(di or tri)phosphates **355** into 2'-deoxyribonucletotides **356** catalysed by ribonucleotide reductases[179] (Scheme 139). These enzymes play a key role in all living organisms as they provide the monomeric precursors **356** required for DNA biosynthesis.



Scheme 139

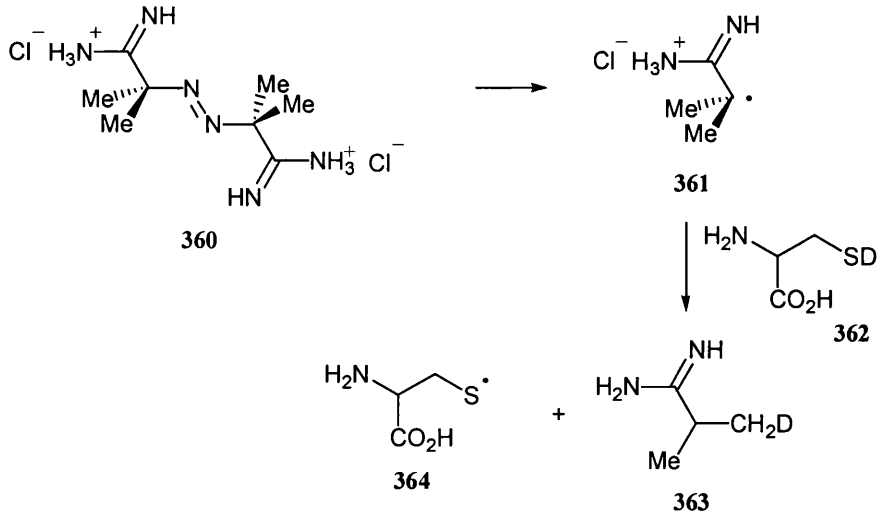
A common radical-based reaction mechanism has been postulated for all ribonucleotide reductases. After binding of the diphosphate substrate **355** into the active site of the enzyme, hydrogen atom abstraction at the C-3' position by a thiyl radical **357** derived from a cysteine residue affords the C-3' radical **358** (Scheme 140). The "repair" reaction does not occur as the reaction is then driven by the irreversible elimination of water, which, after subsequent protonation of the 2'-hydroxyl group of **357** by one of the two redox-active cysteine residues, leads to the 2'-deoxy-3'-ketonucleotide radical **358** (Scheme 140). This radical **358** is then reduced to the C-3' radical **359** by the thiol/thiolate pair and in the final step, the initially abstracted hydrogen atom H_a is transferred back to the C-3'position forming **356** with concomitant regeneration of the protein radical (Scheme 140).



Scheme 140

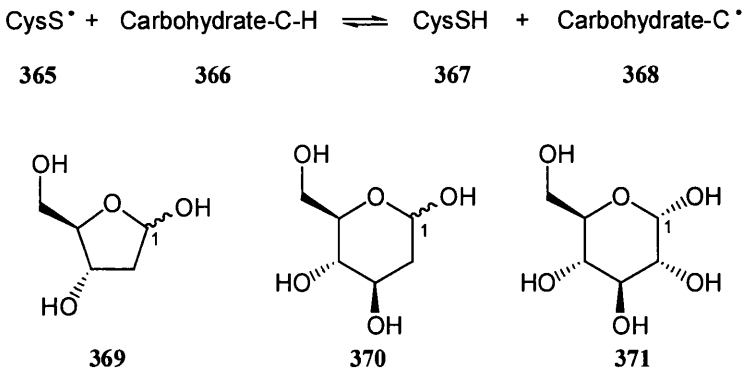
After this mechanism was postulated, other researchers demonstrated its feasibility and concentrated on showing that thiyl radicals were indeed capable of hydrogen abstraction at the C-3' position. Thus, Stubbe *et al.* demonstrated that thiyl radicals do exist as an intermediate in the nucleotide reduction process through electron paramagnetic resonance spectra of ribonucleoside triphosphate reductase containing specifically deuterated cysteine residues.[180]

Additionally, radiation chemical studies optimised by an NMR method allowed Schöneich *et al.* to show that thiyl radicals can abstract hydrogen atoms from carbohydrates.[181] Thiyl radicals were produced from azo*bis*(2-methyl-propionamidine)dihydrochloride **360**, which yields 2-methyl-2propionamidinyl radical **361** which abstracts selectively the deuterium atom of the cysteine **362** to produce the required thiyl radical **364** (Scheme 141).



Scheme 141

They measured the rate constants for the hydrogen transfer reaction between selected carbohydrates, 2-deoxy-D-ribose **369**, 2-deoxy-D-glucose **370**, α-D-glucose **371** and thiyl radicals **365** (Scheme 142). They demonstrated that thiyl radicals attack the C^1-H bond of the carbohydrates preferentially with rate constants in the range of (1-3) × 10^4 $M^{-1}s^{-1}$. The rate constants for the reverse reaction carried out with model compounds for carbohydrates (alcohols and ethers) are between 10^7 and 10^8 $M^{-1}s^{-1}$.

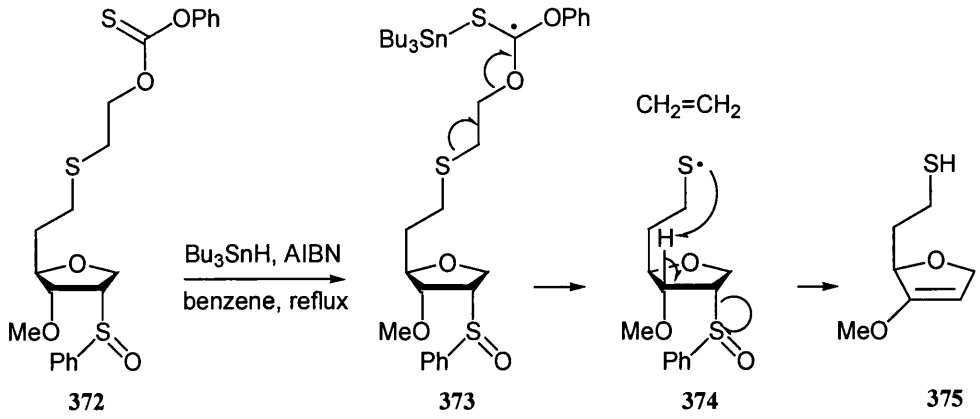


369 **370** **371**

Scheme 142

It is also of interest to note that Schöneich *et al.* demonstrated that thiyl radicals, derived from cysteamine, abstract a hydrogen atom from the $^{\alpha}$C-H bond of amino acids with rate constants which are influenced by the conformation of the amino acid.[182] In the enzyme, the thiyl radical should abstract preferentially the hydrogen from the $^{\alpha}$C-H bond of the protein backbone as the bond energy of the $^{\alpha}$C-H is lower than that of the C-H bond of carbohydrates. However, the ribonucleotide reductase mechanism shows that the selectivity of hydrogen abstraction reaction by thiyl radicals towards the C-3' bond of ribonucleotides may be driven by conformational and dynamic factors.

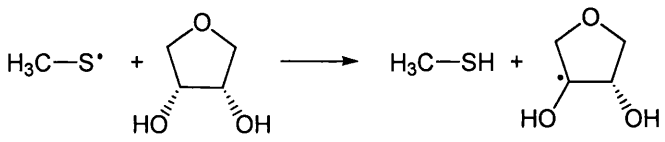
In another study, Robins *et al.* obtained chemical evidence for the first two steps of the ribonucleotide reductase mechanism by generating a primary aliphatic thiyl radical **373** in a tetrahydrofuran model, substituted at the C-2' position which resulted in hydrogen atom abstraction at the C-3' position (Scheme 143).[183] Thus, attack of the tributylstannyl radical on **372** gave **373**, which underwent a double homolytic β-elimination to generate the thiyl radical **374** and ethylene. Intramolecular [1,5]-hydrogen transfer and susequent elimination of a phenylsulfinyl radical from **374** produced **375** (Scheme 143). In this instance, the thiyl radical does not possess any strong electrophilic character and the hydrogen atom abstraction step is clearly aided by the intramolecular nature of the process.



372 373 374 375

Scheme 143

In their computational studies related to the ribonucleotide reductase mechanism, Zipse *et al.* also demonstrated that hydrogen transfer reactions between methanol, ethylene glycol or *cis*-3,4-dihydroxytetrahydrofuran **377** and methyl thiyl radical **276** were endothermic processes (Scheme 144).[184] Moreover, the smallest endothermicity and the lowest reaction barrier, according to density functional calculations, were observed for the tetrahydrofuran substrate **377**.



376 377 378 379

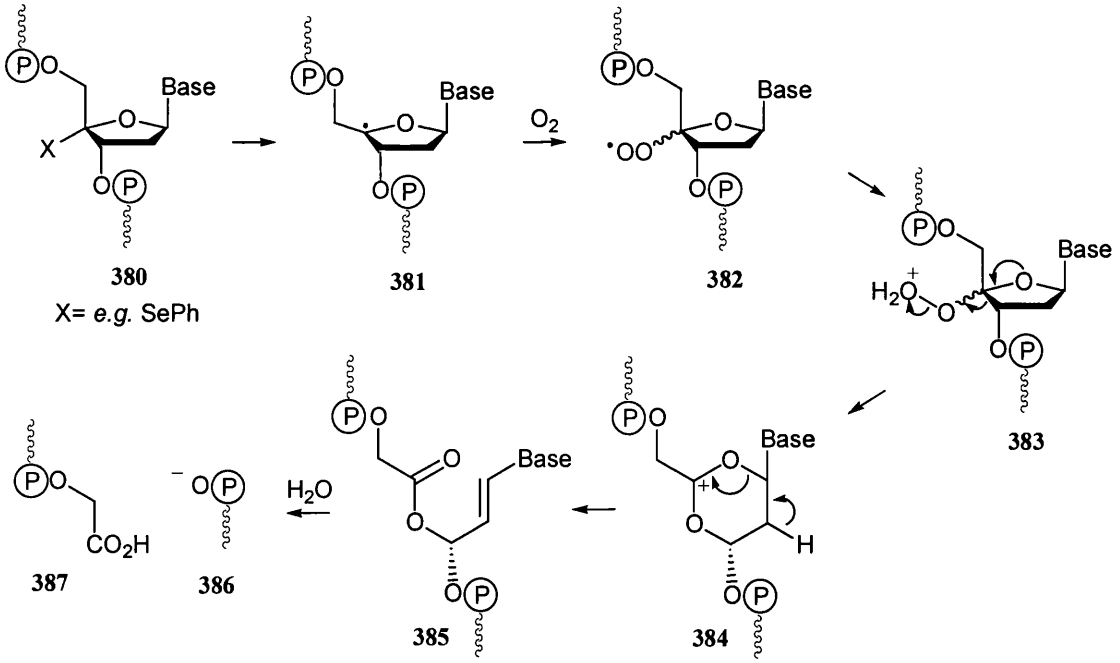
Scheme 144

2.3.2.2 DNA damage

The second related area of intense current research interest within chemical biology centres around DNA damage. Jain *et al.* have reported that glutathione **338** can induce DNA cleavage in the presence of cupric ion, and suggested that thiyl radicals could be involved since no hydroxyl radicals were present in the reaction media.[185]

DNA damage is believed to occur *via* the formation of DNA radicals[186] and in recent years, compelling evidence for the central role of the 4'-DNA radical has emerged in an interesting series of studies especially from the groups of Giese[187] and Crich.[188] Thus through classical independent generation of 4'-DNA radical **381** in a series of model oligomers, it was possible to examine the fate of such intermediates (Scheme 145).

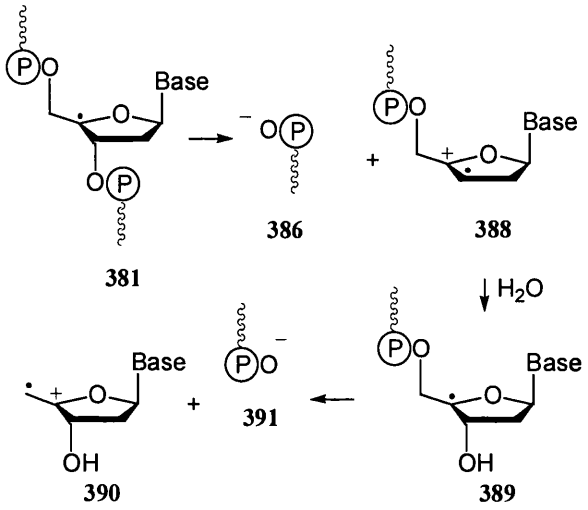
In the presence of oxygen, trapping of the carbon-centered radical **381** to give a hydroperoxy radical **382** is very rapid and the resultant hydroperoxide **383** can then undergo proton induced ring expansion to give the low energy cation **384** (Scheme 145). Subsequent elimimation and hydrolysis steps lead to the 3'-phosphate **386** and 5'-phosphoglycolate **387** as cleavage products (Scheme 145).[189,190]



Scheme 145

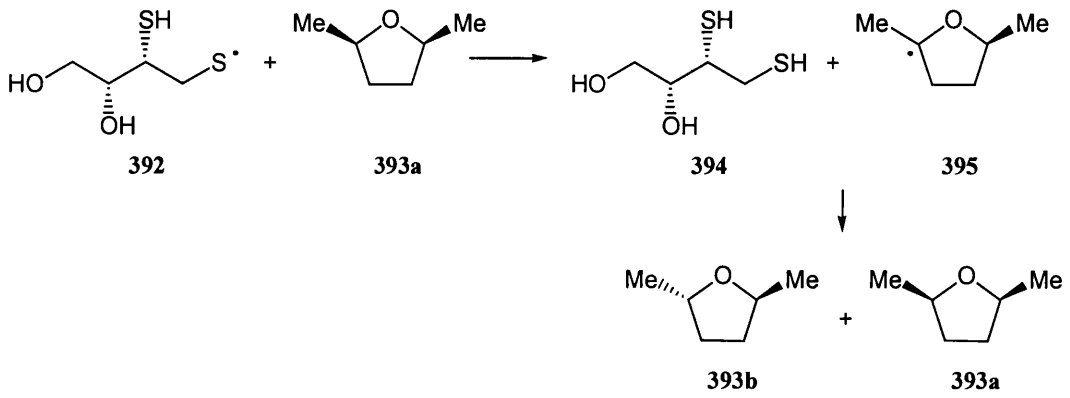
However the 4'-DNA radical **381** does not require the presence of oxygen to induce DNA damage.[191] Thus, strand scission of **381** can also occur by spontaneous heterolytic β-C,O bond cleavage (Scheme 146). This leads to 3'-phosphate anion **386** and concomitant generation of a DNA radical cation **388**,

which is then trapped by water and yields 5'-phosphate anion **391** in a second β-C,O bond scission reaction (Scheme 146).



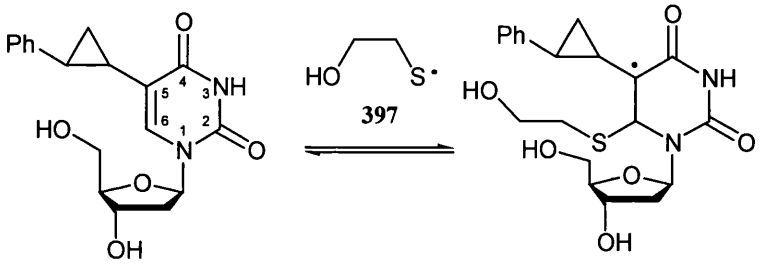
Scheme 146

Prior to the recognition of the importance of polarity effects in radical reactions by Roberts[175,178] (*vide supra*), Von Sonntag *et al.*[192] also hypothesised that thiyl radicals could abstract the hydrogen atom at the C-4' position of the DNA ribose ring, to produce 4'-DNA radicals which would induce DNA damage. Therefore they showed that thiyl radicals derived from 1,4-dithiothreitol **392**, reacted with *cis*-2,5-dimethyltetrahydrofuran **393a**, a model for the sugar moiety of DNA, by abstracting a hydrogen atom in the α-position to the ether functionality (Scheme 147). The planar radical **395** thus formed was then "repaired" by the thiol **394** thereby regenerating either the *cis*- or *trans*-2,5-dimethyltetrahydrofuran **393a** or **393b** (Scheme 147). Therefore cellular thiols may be involved in the cellular repair process of DNA radicals or even induce DNA damage.



Scheme 147

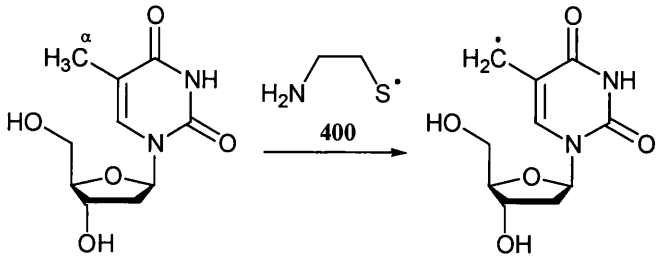
Alternative sites of attack by thiyl radicals in model DNA systems have also been investigated. Thus according to recent literature reports, thiyl radicals can add in classical fashion to a nucleobase such as the pyrimidine ring of DNA. By way of example, Greenberg, Schiesser *et al.* have produced chemical evidence that thiyl radical **397** derived from β-mercaptoethanol can add at the C-6 position of the double bond of a pyrimidine nucleoside model **396**, thereby giving an alternative pathway for DNA damage generated by thiyl radicals (Scheme 148).[193]



396 **398**

Scheme 148

On the other hand, Schöneich *et al.* have demonstrated that cysteamine thiyl radicals **400** can abstract the allylic hydrogen atom from the $^{\alpha}$C-H bond of the nucleobase **399** with rate constants similar to those found for the hydrogen atom abstraction reactions from carbohydrates (Scheme 149).[194]



399 **401**

Scheme 149

2.3.2 Hypothesis

From the foregoing overview of the reactivity of thiols both in organic synthesis and in chemical biology several relevant conclusions can be drawn, particularly in

terms of their ability to participate in hydrogen atom abstraction and "repair" reactions from the 4'-position of the ribose ring in DNA models and related tetrahydrofuran derivatives.

Thus in the first instance, although thiyl radicals are inherently less electrophilic than alkoxy radicals, the incorporation of additional electron withdrawing substituents can certainly enhance this character. This has been clearly demonstrated in the studies of Von Sonntag who found that dithiothreitol was more effective than glutathione in catalysing the epimerisation of the 2,5-dimethyl tetrahydrofuran (*vide supra*). In this instance the presence of the β-oxygen atoms in dithiothreitol relative to glutathione may increase the electrophilicity of the thiyl radical as well as the strength of the S–H bond. Even more so, the range of thiols including silane thiols, which were examined as catalysts for epimerisation of a tetrahydrofurfuryl ester by Roberts *et al.* clearly define the importance of the electron withdrawing substituents on the thiol. [178]

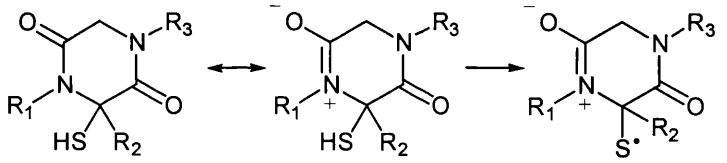
The second factor of importance in terms of allowing fragmentation of a 4'-DNA radical to occur is that sufficient time can elapse for this reaction to occur relative to the rate of hydrogen atom abstraction from the thiol which would result in the effective "repair" of the system. For this reason the strength of the S–H bond in the thiol is also an important consideration, *e.g.* the polar effect in ensuring that repair is slow.

Finally, as demonstrated by the elegant experiment carried out by Robins *et al.* in their intramolecular variant, even thiyl radicals which do not possess electron withdrawing groups can carry out efficient hydrogen atom abstraction leading to fragmentation.[183] The conclusion to be drawn from this model study, as pointed out in reference 178, is that if a thiol could be suitably bound to DNA then the reaction could effectively become intramolecular and hence benefit from huge rate accelerations over free solution values.

In all the above experiments, oxygen is absent and hence it is unnecessary to take into account either the capture of thiyl radicals to give sulfonyl radicals or the

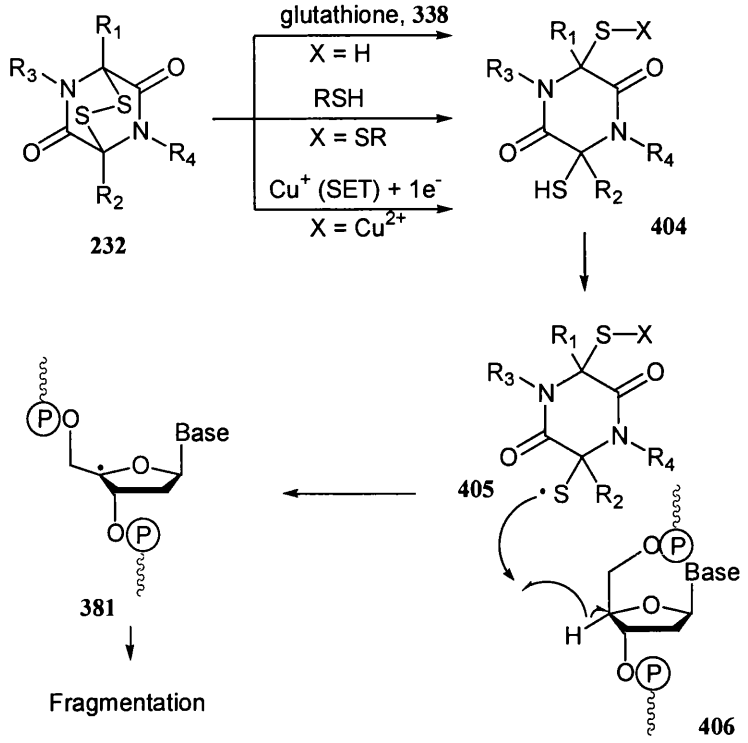
capture of carbon centered radicals to give hydroperoxy radicals. Such anaerobic situations however are still of considerable interest to the biomedical scientists since the concentration of oxygen inside the DNA helix of a tumour cell could be very low.

In light of such information on thiyl radical reactivity and also taking into account the studies of Giese[187] and Crich[188] (*vide supra*) which establish that ready fragmentation of DNA fragments can occur after generation of a 4'-DNA radical, we were naturally interested to examine the reactivity of thiyl radicals derived from the epi-3,6-dithio-2,5-diketopiperazines core. As encapsulated in Scheme 150, we reasoned that the combination of the adjacent carbonyl group of the amide and the relative electronegative nitrogen on the second amide grouping could provide a sufficiently eletrophilic thiyl radical **403** for hydrogen atom abstraction.



Scheme 150

As shown in scheme 151, the free thiol **404** required for generation of the thiyl radical **405** can be produced by opening of the disulfide bridge **232** under a variety of conditions (Scheme 151). These include reduction to the disulfide bridge **232** using glutahione **338**, thiol disulfide interchange, or even a metal induced single electron transfer process which would involve the direct formation of a radical anion (Scheme 151). Thereafter, hydrogen atom abstraction from the 4'-position of **405** could be followed by fragmentation *via* the radical cation **381** as established by Giese[187] and Crich[188] (Scheme 151).



Scheme 151

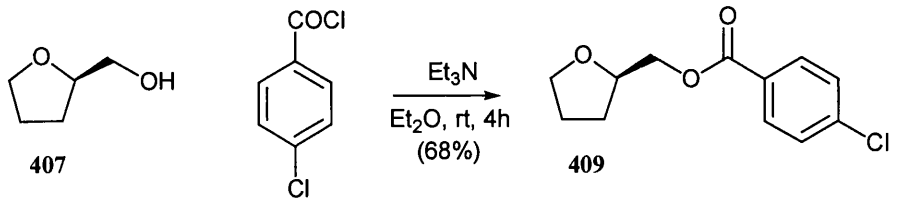
Since the nature of thiyl radicals with such adjacent functionality was unknown, we therefore decided to examine their reactivity and also to gain some insight into the strength of the S–H bond by carrying out a series of experiments using two key reactions studied by Roberts *et al.*[176,178] In the first instance, as a matter of convenience, we also elected to select the derived dithiols as the precurors for thiyl radical generation.

2.3.3 Probing the thiyl radical reactivity and the strength of the S–H bond in epi-3,6-dithio-2,5-diketopiperazine derived dithiols by two reactions: tetrahydrofurfuryl ester racemisation and hydrosilylation.

As previously discussed (*vide supra*) the seminal demonstration by Roberts *et al.*[178] that alkane thiols with electron withdrawing groups can act as polarity reversal catalysts to promote the radical chain racemisation of (*R*)-tetrahydrofurfuryl acetate provides a highly relevant model for the radical induced strand cleavage of DNA. We therefore decided to study the possible racemisation of this compound using

our 3,6-dithiol-2,5-diketopiperazines as catalysts in order to provide strong presumptive evidence that the derived thiyl radicals could abstract a hydrogen atom from the C-4' position of the ribose ring of DNA.

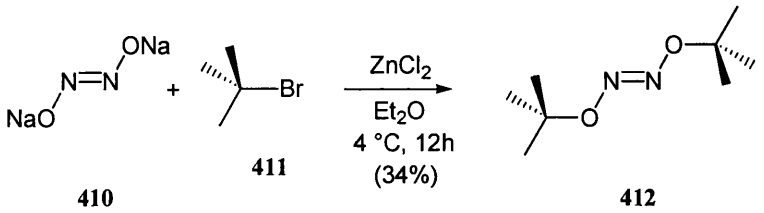
In the first instance, we were however plagued by the fact that the chiral-stationnery-phase GLC column (Supelco β-DEX 120, 30 m x 0.25 mm bore capillary column; 0.25 μm coating containing permethylated β-cyclodextrin) no longer provided, even after several runs, the required separation of the two enantiomers of tetrahydrofurfuryl acetate. Following advice from the Roberts group, we therefore used the *para*-chlorobenzoate ester of tetrahydrofurfuryl alcohol **409**, which allowed us to monitor the enantiomeric excess of the remaining ester using chiral HPLC (chiralcel© OD column) equipped with a UV detector (Scheme 152). Addition of a solution of (*R*)-tetrahydrofurfuryl alcohol **407** and triethylamine in diethyl ether to a solution of the acid chloride **408** in diethyl ether yielded the ester **409** in 68% yield (Scheme 152).



407 **408** **409**

Scheme 152

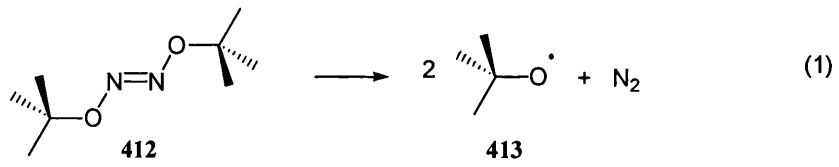
The radical initiator, *trans*-di-*tert*-butylhyponitrite **412**, can be synthesised either from *tert*-butyl halide and dry silver *trans*-hyponitrite[195] or from *tert*-butyl halide and sodium *trans*-hyponitrite in the presence of a Lewis acid[196] (scheme 153). Thus, in our own case, sodium *trans*-hyponitrite hydrate **410** was treated with *tert*-butyl bromide **411** and zinc chloride in diethyl ether to produce the desired initiator **412** in 34% yield (Scheme 153).



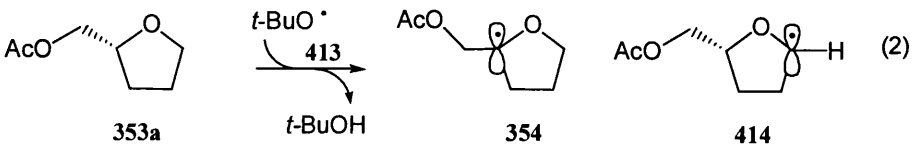
410 **411** **412**

Scheme 153

Trans-di-*tert*-butylhyponitrite **412** generates, on heating, *tert*-butoxyl radicals **413** (Eqn 1) and its half life at 60 °C is *ca.* 55 min (Scheme 154).[195] It has also been demonstrated by Roberts *et al.* that when the *trans*-di-*tert*-butylhyponitrite **412** alone is stirred with tetrahydrofurfuryl acetate **353a** in benzene and heated at 60 °C for 3 hours, *ca.* 10% of the ester **353a** was consumed and the % ee of the remaining ester **353a** remained unchanged (Scheme 154). The explanation of the authors was that the *tert*-butoxyl radicals **413**, in the absence of other reagents, abstract hydrogens from **353a** to give the radicals **354** and **414**, but that these radicals do not abstract hydrogen from **353a** at a significant rate (Eqn 2).[178]



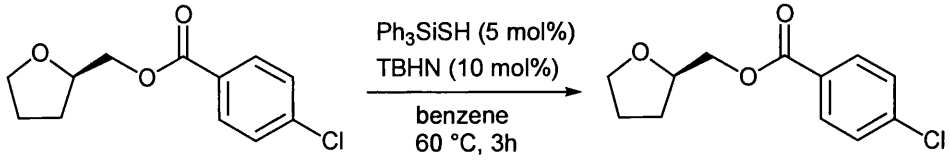
412 **413**



353a **354** **414**

Scheme 154

With the DNA model **409** and the radical iniator **412** in hand, we carried out a control racemisation reaction of **409** using triphenylsilanethiol as catalyst (Scheme 155). Hence (*R*)-tetrahydrofurfuryl-*p*-chlorobenzoate **409** was stirred with *trans*-di-*tert*-butylhyponitrite (10 mol% based on **409**) and triphenylsilanethiol (5 mol% based on **409**) in benzene, at 60 °C for 3 hours (Scheme 155). Gratifyingly, racemisation was efficiently complete (3% ee) after this time.

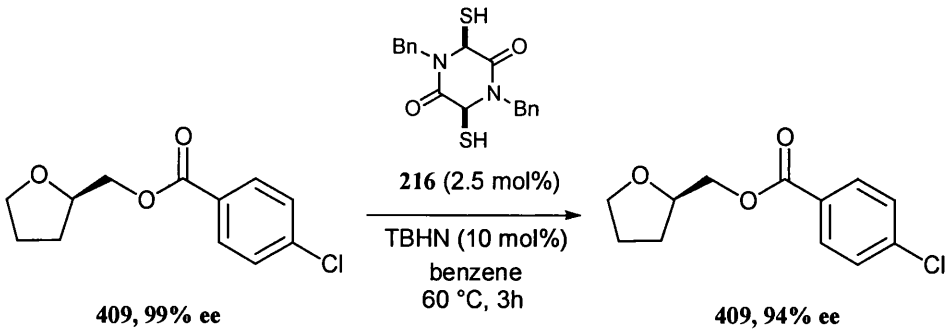


409, 99% ee **409, 3% ee**

Scheme 155

We also performed a control experiment without *trans*-di-*tert*-butylhyponitrite **412** to confirm that triphenylsilanethiol does not racemise the ester **409** under the same reaction conditions. Thus, (*R*)-tetrahydrofurfuryl-*p*-chlorobenzoate **409** was stirred with triphenylsilanethiol (5 mol% based on **409**) in benzene at 60 °C for 3 hours and, as expected, the % ee of the ester **409** remained unchanged.

We then carried out the first key experiment in the presence of *N,N'*-dibenzyl-3,6-dithiol-2,5-diketopiperazine **216** as a catalyst. (*R*)-tetrahydrofurfuryl-*p*-chlorobenzoate **409** was stirred with *trans*-di-*tert*-butylhyponitrite (10 mol% based on **409**) and *N,N'*-dibenzyl-3,6-dithiol-2,5-diketopiperazine **216** (2.5 mol% based on **409**) in benzene at 60 °C for 3 hours (Scheme 156). However as the % ee of the remaining ester was 94%, no definitive conclusions could be made. At this stage, we recognised that the two benzylic groups attached to nitrogen could have provided potentially competitive sites for intramolecular hydrogen atom abstraction.

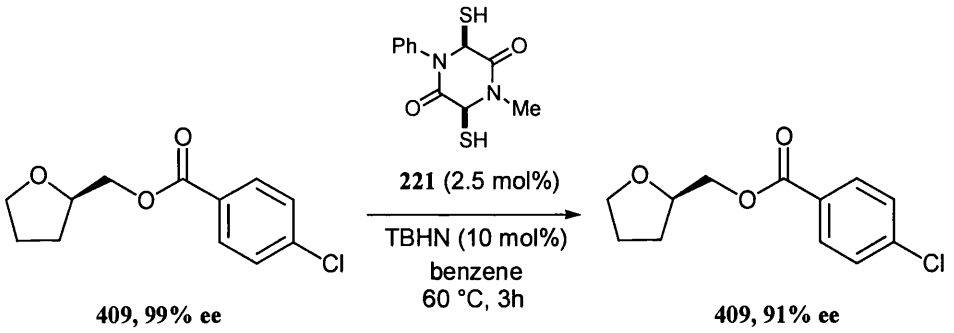


409, 99% ee **409, 94% ee**

Scheme 156

Consequently, we decided to use a dithiol derivative containing no benzylic groups. The racemisation reaction was therefore repeated using *N*-methyl-*N'*-phenyl-3,6-

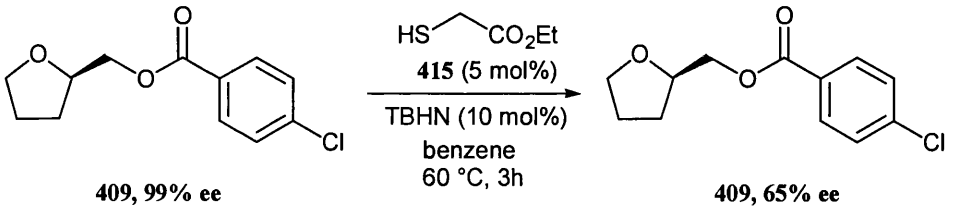
dithiol-2,5-diketopiperazine **221** (Scheme 157). Once again however the % ee obtained was 91% under the same reaction conditions.



409, 99% ee **409, 91% ee**

Scheme 157

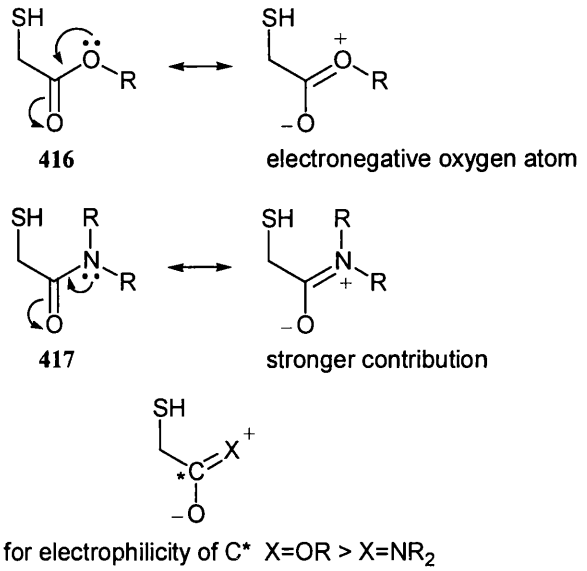
At this stage, in view of the apparent lack of reactivity of the *cis*-3,6-dithiol-2,5-diketopiperazines **216** and **221**, we decided to reproduce an experiment carried out by Roberts and Cai[178] using ethyl thioglycolate **415** as the polarity reversal catalyst, but with the *para*-chlorobenzoate ester **409** as substrate rather than tetrahydrofurfuryl acetate (Scheme 158).



409, 99% ee **409, 65% ee**

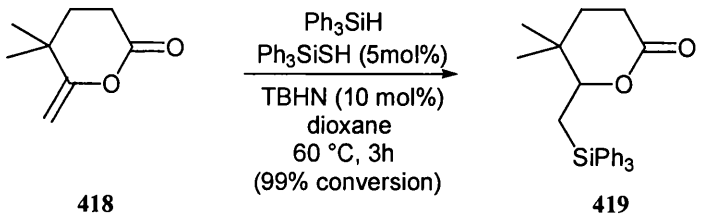
Scheme 158

In this instance, significant racemisation occurred and an enantiomeric excess of 65% was measured in line with the value obtained by Roberts *et al.*[178] Although, as implied in Scheme 159, the electron withdrawing nature of the amide group **417** is certainly less than that of an ester **416** by virtue of the stronger contribution of amide resonance and the resultant decrease in electrophilicity of the carbonyl carbon atom, we were nevertheless surprised that this change was sufficient to make the *cis*-3,6-dithiol-2,5-diketopiperazines **216** and **221** effectively unreactive.



Scheme 159

In order to demonstrate that the generation of a thiyl radical from a *cis*-3,6-dithiol-2,5-diketopiperazine derivative was indeed possible, and also to gain additional insight into the possible nature of such radicals we then decided to examine a second reaction pioneered by Roberts *et al.* which also involves polarity reversal catalysis, the hydrosilylation of the enol lactone, 5,5-dimethyl-6-methylene-tetrahydro-pyran-2-one **418** (Scheme 160). Once again an appropriate control experiment using triphenylsilanethiol as catalyst allowed us to reproduce the reported literature experiment in essentially quantitative yield.

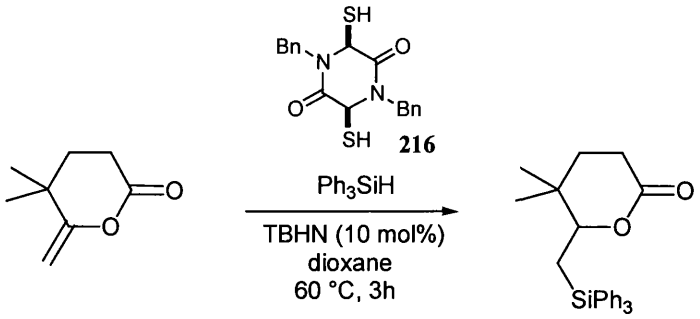


418 **419**

Scheme 160

The same reaction was then repeated but with *N,N'*-dibenzyl-3,6-dithiol-2,5-diketopiperazine **216** as the catalyst (Scheme 161). Two different amounts of catalyst were examined (Table 2). In the first experiment, when 10 mol% of catalyst **216** was used, the percentage of conversion of the lactone **418** into the hydrosilylated product **419** was only 40% (Entry 1) (Table 2). Interestingly

however, when 2.5 mol% of **216** was used, 70% of the product **419** was formed (Entry 2).



418 **419**

Scheme 161

Entry	Catalyst	Mol%	Conversion[a]
1	**216**	10	40%
2	**216**	2.5	70%

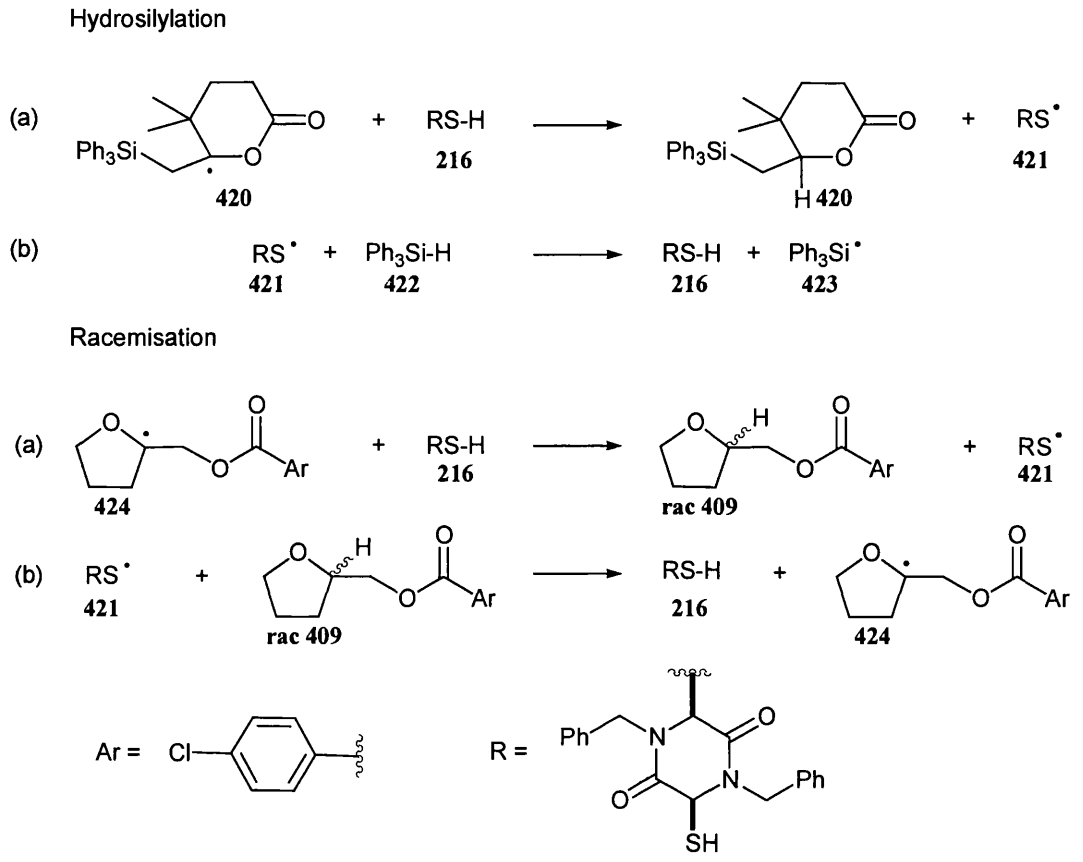
[a] Based on ^{1}H NMR analyses

Table 2

The observation that *cis*-3,6-dithiol-2,5-diketopiperazines could indeed act as a polarity reversal catalyst was a very encouraging one, indicating that thiyl radicals could indeed be generated and function, albeit less effectively than triphenylsilane thiol, in such a reaction.

In terms of understanding the success of the hydrosilylation reaction and the failure of our key racemisation reaction, it is interesting to compare the propagation steps in each of these two sequences as shown in Scheme 162. Hydrogen atom capture from the thiol **216** (step a) in both the hydrosilylation and in the racemisation reaction should be a relatively efficient process. In terms of hydrogen atom abstraction by the thiyl radical **421** (step b), consideration of bond dissociation energies clearly reveals that thermodynamic factors are not important since the bond dissociation energy of triphenylsilane **422** ($D°(Ph_3Si–H)$ = 371.1 kJ.mol^{-1})[197] should be comparable to that of the α-C–H bond of the tetrahydrofuran system ($D°$(tetrahydrofuran C–H) = 385 kJ.mol^{-1}).[198] In terms of polarity however the two reactions of step (b) are very different with the more "hydridic" character of the

silane **422** induced by the electropositive silicon atom providing a much more favoured path. The inability of a thiyl radical **421** derived from an *cis*-3,6-dithiol-2,5-diketopiperazine **216** to abstract the hydrogen atom from the C-4' position of the ribose model **409** could also, of course, involve simple steric factors.



Scheme 162

Nevertheless, in terms of the electrophilicity of the thiyl radical, we considered the possibility that the selection of a *cis*-3,6-dithio-2,5-diketopiperazine could have led to a less reactive radical as a consequence of intramolecular hydrogen bonding (Figure 40).

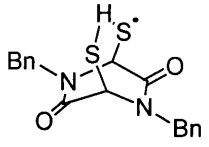
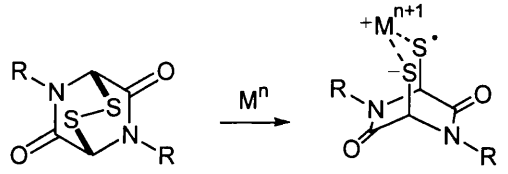


Figure 40

However, infrared dilution studies on the parent *N,N'*-dibenzyl-3,6-dithiol-2,5-diketopiperazine **216** failed to reveal any evidence for the presence of an intramolecular hydrogen bond.

At the same time as the above studies were underway, some preliminary experiments involving alternative potential pathways for the generation of thiyl radicals from epi-3,6-dithio-2,5-diketopiperazines were also underway.

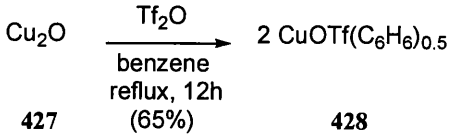
These included the proposal that single electron transfer from a metal to the disulfide would lead to the formation of a ring opened complex with radical anion characteristics (Scheme 163).



425 **426**

Scheme 163

We elected to use a copper(I)-triflate-benzene complex for this purpose.[199] This complex was prepared from the reaction of Cu_2O **427** and trifluoromethane sulfonic anhydride in benzene in 65% yield (Scheme 164). As the benzene is weakly coordinated, the complex decomposes very rapidly upon exposure to air and was used immediately after preparation.

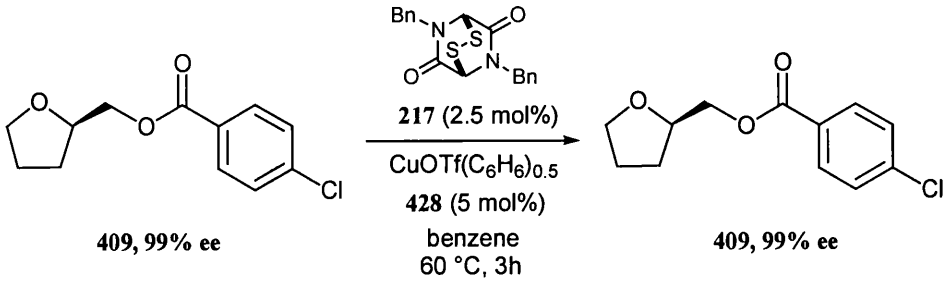


427 **428**

Scheme 164

A solution of the tetrahydrofurfuryl ester **409** in benzene was then treated with *N,N'*-dibenzyl-epi-3,6-dithio-2,5-diketopiperazine **217** (5 mol%) and copper(I)-triflate-benzene complex **428** (10 mol%) (Scheme 165). The reaction mixture was

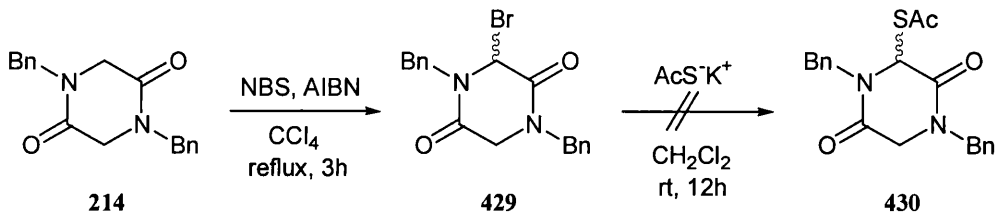
heated at 60 °C for 3 hours and a sample was analysed by chiral HPLC. Unfortunately however no trace of racemisation was observed.



Scheme 165

In a second approach, since we were concerned that a thiyl radical derived from a *cis*-3,6-dithiol-2,5-diketopiperazine might be less reactive because of intramolecular hydrogen bonding, we attempted to prepare a simple monothiol analogue.

Thus, *N,N'*-dibenzyl-2,5-diketopiperazine **214** was treated with one equivalent of *N*-bromosuccinimide in the presence of a catalytic amount of AIBN in carbon tetrachloride (Scheme 166). The reaction mixture was heated under reflux for 3 hours, all volatiles were then removed at reduced pressure, and the crude material containing **429** used for the next step (Scheme 166). Potassium thioacetate was added to a solution of the crude product **429** in dichloromethane at 0 °C and the reaction mixture was stirred overnight at room temperature. After filtration and removal of the solvent, the new crude product **430** was finally subjected to column chromatography (Scheme 166). The desired product **430** was not however isolated and only a small amount of the dithioacetate was obtained (Scheme 430). Although this reaction was repeated, the mono-thioacetate **429** could not be isolated either by crystallisation or by column chromatography.

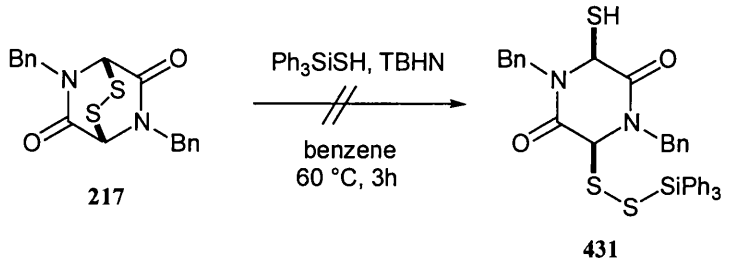


Scheme 166

An alternative strategy for the synthesis of a "monothiol" was also attempted by opening the disulfide bridge of epi-3,6-dithio-2,5-diketopiperazine using a thiol-disulfide exchange reaction as we have noted in the introduction (*vide supra*). Chai *et al.* demonstrated the formation of gliotoxin-glutathione adducts **335**, **336** and **337** in a phosphate buffer solution at pH 7 and these adducts may of course be intermediates in cell systems treated with gliotoxin (Scheme 128).[170]

The surprising observations of Chai that the thiol function in the two mono mixed disulfides is very reactive and prone to an intramolecular S_N2 reaction to regenerate gliotoxin and that this thiol function could also be oxidised (as observed from a mass spectrum where a molecular ion corresponded to a mono mixed disulfide + O_2) were also intriguing. Therefore it was of interest not only to synthesise a mono mixed disulfide of an epi-3,6-dithio-2,5-diketopiperazine but also to verify if the thiol function formed could be oxidised by O_2, especially given the stability of the *cis*-3,6-dithiol-2,5-diketopiperazines derivatives.

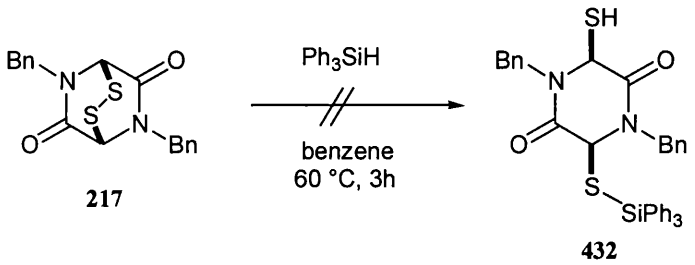
A radical process was therefore envisaged to open the disulfide bridge and *N,N'*-dibenzyl-epi-3,6-dithio-2,5-diketopiperazine **217**, triphenylsilanethiol and *trans*-di-*tert*-butyl-hyponitrite were accordingly stirred in benzene at 60 °C for 3 hours. It was anticipated that triphenylsilanethiyl radical formed by reaction of triphenylsilanethiol with the *tert*-butoxy radical of TBHN would open the disulfide bridge of **217** to yield product **431** (Scheme 167). Although TLC analysis revealed the formation of a product, our attempts to purify and identify this product were unsuccessful.



217 **431**

Scheme 167

Finally, we also attempted to open the disulfide bridge with a silane to obtain product **432** (Scheme 168). Thus, *N,N'*-dibenzyl-epi-3,6-dithio-2,5-diketopiperazine **217** and triphenylsilane were stirred in benzene at 60 °C for 3 hours (Scheme 168). Irrespective of the presence or absence of radical initiator (*e.g.* AIBN or TBHN), no reaction was observed and starting material was recovered.



217 **432**

Scheme 168

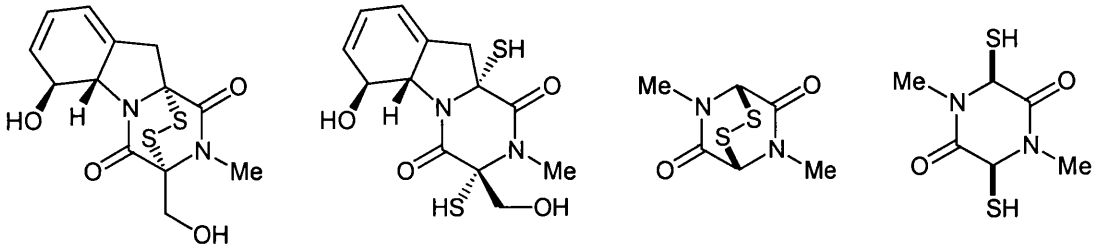
Unfortunately, at this stage, time constraints prevented further studies of thiol disulfide interchange possibilities using simple epi-3,6-dithio-2,5-diketopiperazines and thiols.

2.4 *In vivo* investigations

In parallel with the foregoing studies, we were also interested in exploring the mode of action of epi-3,6-dithio-2,5-diketopiperazines with DNA. Consequently, *in vivo* experiments were carried out with DNA using some simple epi-3,6-dithio-2,5-diketopiperazines which had been prepared.

2.4.1 Previous biological tests

As discussed in the introduction, gliotoxin **1** has interested many scientists because of its biological properties. Waring, Müllbacher *et al.* have reported that DNA damage, on both eukariotic and unicellular DNA, was one of the properties of gliotoxin **1** as well as the simpler *N,N'*-dimethyl-2,5-diketopiperazine **70** and their related reduced forms **433** and **69** (Figure 41).[200]

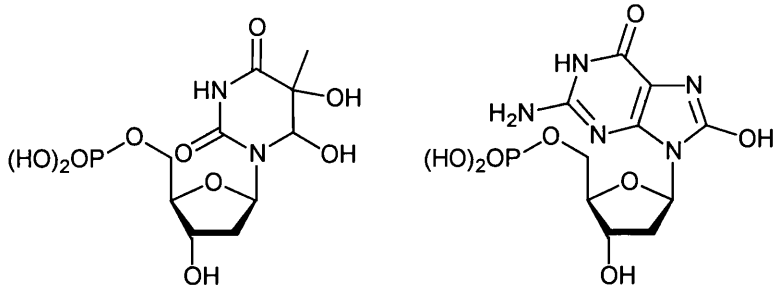


1 **433** **70** **69**

Figure 41

In their experiments, gliotoxin **1** was active only in the presence of reducing agents such as dithiothreitol, glutathione **338**, or reduced pyridine nucleotides. This implies that glutathione **338**, which is an intracellular reducing agent, may be responsible for initiating the redox cycle after gliotoxin **1** enters the cell. Moreover, they also demonstrated that the reduced form of gliotoxin **433** caused single and double stranded breaks in plasmid DNA in the presence of metal ions such as Fe(III) and Cu(II), and that these breaks were enhanced when complexed by EDTA (ethylenediamine tetraacetic acid). According to the authors, the requirement of metal ions for these experiments suggests a process involving a hydroxyl radical or a superoxide radical anion species. Indeed, during the DNA damage experiment, they observed the formation of hydrogen peroxide and autooxidation of the reduced form of gliotoxin **1**.

Recently, Steinberg *et al.* also reported DNA damage by gliotoxin and demonstrated, by examining eukariotic DNA residues, that hydroxyl radicals were produced during the degradation process. DNA adducts, β-hydro-5,6-dihydroxythymidine monophosphate **434** and 8-hydroxy-2'-deoxyguanosine **435** monophosphate, were detected by ^{32}P DNA labelling (Figure 42). It was therefore proposed that these adducts resulted from the reaction of the DNA bases, thymine and guanine, with hydroxyl radicals generated by gliotoxin **1**.[201]



434 **435**

Figure 42

2.4.2 Biological tests without oxygen

2.4.2.1 The role of glutathione

Glutathione **338** is found in all cells and provides a strong reducing environment. It is believed that H-donor compounds such as glutathione **338** can act as antioxidants against free radical damage. However, this compound can also act as a pro-oxidant and cause plasmid DNA damage in the presence of Cu(II)[202] or Fe(III).[203] It is commonly proposed that transition metals such as Cu(II) or Fe(III) interact with thiols to form Cu(I) or Fe(II) and free thiyl radicals (Eqn 1), which dimerise to the disulfide (Eqn 2) (Scheme 169).

$$Cu(II) + GSH \leftrightarrows Cu(I) + GS^{\bullet} + H^{+} \quad (1)$$

$$2\ GS^{\bullet} \rightarrow GSSG \quad (2)$$

Scheme 169

Moreover, the presence of scavengers of oxygen species clearly indicates that hydroxyl radicals are involved in the DNA degradation process[202] and, as shown in Scheme 170, oxygen radicals can be generated by Fe(II), in contact with oxygen, producing a superoxide radical anion (Eqn 1), which then generates hydrogen peroxide (Eqn 2). Subesequently, Fe(II) and H_2O_2 lead to the formation of the reactive hydroxyl radical species (Eqn 3) (Scheme 170).

$$Fe(II) + O_2 \rightarrow Fe(III) + O_2^{\bullet-} \quad (1)$$

$$HO_2^{\bullet} + O_2^{\bullet-} + H^+ \rightarrow O_2 + H_2O_2 \quad (2)$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\bullet}OH + OH^- \quad (3)$$

Scheme 170

Prütz demonstrated that glutathione **338** interacted with Cu(II) and suggested the formation of a DNA-Cu(I) complex responsible for DNA damage under aerobic conditions.[204] This complex can undergo oxidative damage generated by peroxide to produce oxidising radicals such as hydroxyl radicals (Scheme 171).

$$DNA\text{-}Cu(I) + H_2O_2 \rightarrow DNA^{\bullet}OH + Cu(II) + OH^-$$

$$\downarrow$$

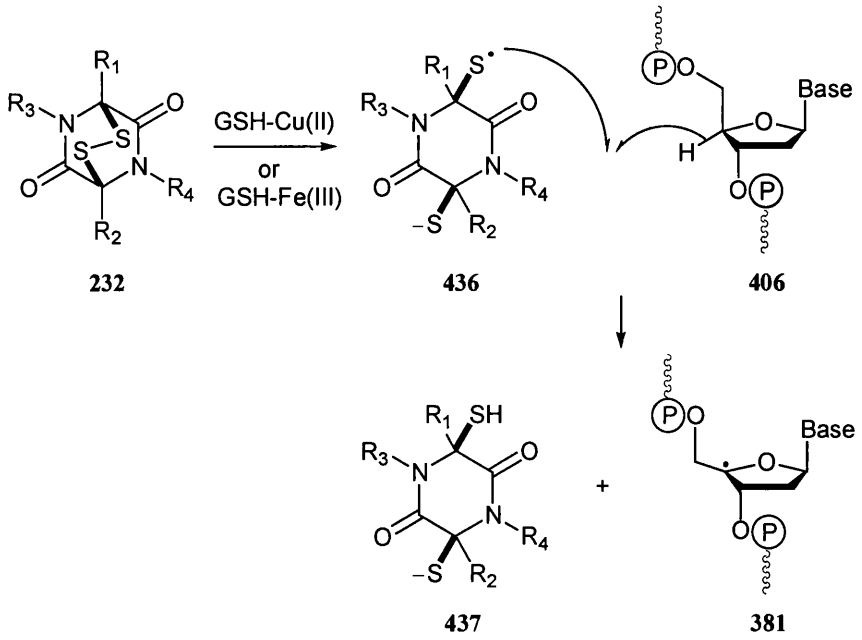
DNA damage

Scheme 171

Although Prütz showed DNA damage by the system glutathione-Cu(II), he did not propose the generation of free thiyl radicals by Cu(II). Interestingly, Hadi *et al.* observed the same DNA degradation in the absence of any oxygen species. Therefore, since involvement of hydroxyl radicals was impossible in this case, the authors suggested thiyl radicals as the major cleaving species.[205]

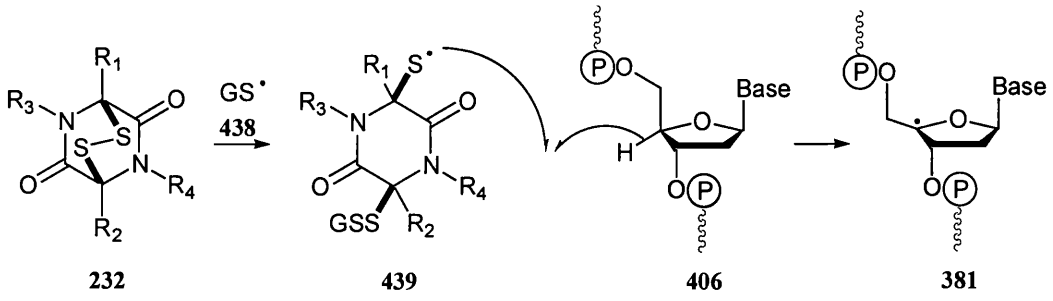
2.4.2.2 Tests

In terms of our interest in the role of the disulfide bridge of epi-3,6-dithio-2,5-diketopiperazines, it was possible to envisage that, under anaerobic conditions, the system glutathione-metal ion, Cu(II) or Fe(III), would provide Cu(I) or Fe(II), which would be able to reduce the disulfide bridge of the epi-3,6-dithio-2,5-diketopiperazine **232** by donating an electron to produce the compound **436** (Scheme 172). The radical anion **436** would then react with the ribose ring of DNA **406** to form the 4'-DNA radical **381**, causing DNA degradation (Scheme 172).



Scheme 172

Thus thiyl radicals **438** produced by the glutathione-metal ion system could easily add to the disulfide bridge of **232** to produce the mixed disulfide-thiyl radical **439**, whose products, as we have previously discussed, have been isolated (Scheme 173). This radical would then abstract the hydrogen at the C-4'position of **406** and produce the related DNA radical **381** leading to further DNA damage (Scheme 173).



Scheme 173

We therefore elected to find appropriate conditions to test our *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70** in the presence of metal ions, Fe(III) or Cu(II) and glutathione with plasmid DNA in the complete absence of oxygen and hence to provide proof that a thiyl radical derived from an epi-3,6-dithio-2,5-

diketopiperazine could be formed under the correct conditions and be a sufficiently reactive species for DNA degradation.

Thus, the reactivity of epi-3,6-dithio-2,5-diketopiperazine with supercoiled plasmid DNA, from unicellular systems, was tested. Supercoiled plasmid DNA consists of two intermingled circular strands, so-called covalently closed form **I** and can undergo a single strand break resulting in the open circular form **II** or a double strand break, giving the linear form **III** (Figure 43). All of these forms can be separated and determined by gel electrophoresis followed by photography under UV light.

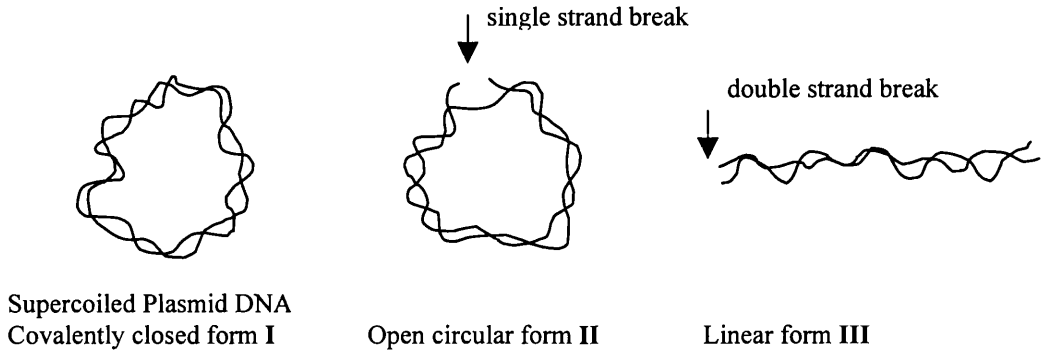


Figure 43

All of the sample mixtures containing plasmid DNA in an aqueous buffer solution of Tris-HCl (pH 7.4) were prepared in a glove box under an atmosphere of nitrogen. In addition, all of the reagents were dissolved and diluted in doubly distilled water until the desired concentrations were reached. Due to the insolubility of the *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70** in water, this reagent was dissolved in the minimum amount of dimethylsulfoxide before dilution to the working concentrations. Gliotoxin **1** was dissolved in ethanol. After incubation of the samples at 37 °C for 2 hours, electrophoresis was performed and the stained gel which revealed the DNA fragments under ultraviolet light was photographed for analysis.

2.4.2.2.1 N,N'-*Dimethyl-epi-3,6-dithio-2,5-diketopiperazine*

In order to determine the correct conditions, the first experiments carried out consisted of controls for all the reagents that were to be used for the DNA degradation experiments: glutathione **338**, Cu(II), Fe(III), EDTA, epi-3,6-dithio-2,5-diketopiperazine **70** alone and DMSO.

Dimethylsulfoxide and *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70** were separately tested with plasmid DNA in order to confirm that they did not degrade DNA. Thus incubation of plasmid DNA with *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70** (lane 2) and separately with dimethylsulfoxide (lane 3) showed only the initial form of plasmid DNA (Figure 44).

As previously discussed, the system glutathione **338** GSH-Cu(II) or GSH-Fe(III) has been reported to induce DNA damage but at the correct concentrations, it can also provide electrons without altering DNA. Therefore, incubation of covalently closed plasmid DNA with the system GSH **338** (200 μM) and Cu(II) (300 μM) indicated no strand breaks (lane 1) (Figure 44).

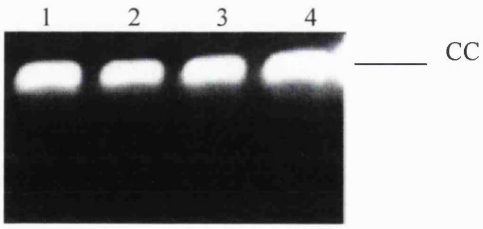


Figure 44. Agarose gel electrophoresis: (A), **lane 1**: $CuCl_2$ (300 μM) and GSH **338** (200 μM); **lane 2**, *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70** (25 μM); **lane 3**, DMSO; **lane 4**, no additions. *CC*, covalently closed circular form of plasmid DNA.

Although the system GSH-Cu(II) resulted in no strand breaks, we also needed to test the system GSH-Fe(III). Therefore we elected to carry out a comparative study between the two systems. GSH **338** alone did not induce any DNA damage (lane 1), neither did the mixture of Fe(III) and EDTA (lane 6) (Figure 45). As the presence of EDTA has been demonstrated to enhance DNA damage,[200] a sample

mixture of GSH **338** Cu(II) and EDTA was also incubated with plasmid DNA. However, no strand breaks were observed by electrophoresis (lane 3) and the same result was obtained when plasmid DNA was treated with a mixture of GSH **338**, Cu(II) and EDTA (lane 4) (Figure 45). The mixture of GSH **338** and Cu(II) did not alter plasmid DNA (lane 4). However, the mixture GSH **338** and Fe (III) caused single and double strand breaks (lane 5) (Figure 45).

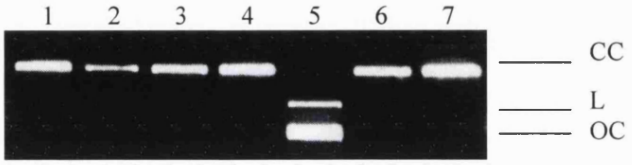


Figure 45. Agarose gel electrophoresis: (B), **lane 1**: GSH **338** (200 μM); **lane 2**, $CuCl_2$ (300 μM), EDTA (20 μM) and GSH **338** (200 μM); **lane 3**, $FeCl_3$ (300 μM), EDTA (20 μM) and GSH **338** (200 μM); **lane 4**, $CuCl_2$ (300 μM) and GSH **338** (200 μM); **lane 5**, $FeCl_3$ (300 μM) and GSH **338** (200 μM); **lane 6**, $FeCl_3$ (300 μM) and EDTA (20 μM); **lane 7**, no addition. *CC*, *L* and *OC*, covalently closed circular, linear and open circular forms of plasmid DNA.

Thus, the system GSH-Fe(III) was obviously too reactive for our needs, whereas, GSH-Cu(II) seemed appropriate as no DNA degradation was observed. It was surprising that the presence of EDTA in the mixture GSH **338** and Fe(III) did not provide any strand breaks and consequently we decided to remove it from our subsequent experiments.

With our glutathione-Cu(II) solutions in hand, we attempted to test *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70** while simultaneously using DMSO alone as the control experiment. *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70** in DMSO (lane 1) and DMSO alone (lane 8) did not cause any DNA damage and the same results were observed with the mixtures *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine-GSH (lane 2), DMSO-GSH (lane 7) and DMSO-Cu(II) (lane 6). (Figure 46).

However the incubation of plasmid DNA with *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70** and Cu(II) led to single strand break (lane 3). Moreover this

damage was enhanced by the addition of glutathione **338** in the sample mixture as single and double strand breaks were observed (lane 4) on the photograph of the gel electrophoresis (Figure 46). Additionally, the same breaks were observed for the mixture glutathione **338**, Cu(II) and DMSO (lane 5) (Figure 46).

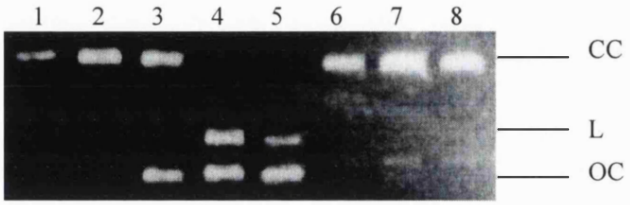


Figure 46. Agarose gel electrophoresis: **(C), lanes 1-4**: *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70** (200 μM) in DMSO; **lanes 5-8**: control with DMSO; **additions**: lanes 2 and 7 GSH **338** (200 μM); lanes 3 and 6 $CuCl_2$ (300 μM); lanes 4 and 5 GSH **338** (200 μM) and $CuCl_2$ (300 μM). *CC*, *L* and *OC*, covalently closed circular, linear and open circular forms of plasmid DNA.

From these results, it appears that the epi-3,6-dithio-2,5-diketopiperazine **70** is associated with Cu(II) in the redox processes which induce DNA damage. These processes are probably enhanced by the presence of glutathione **338**, but, unfortunately the control experiment with DMSO does not allow any conclusions towards the mode of action of epi-3,6-dithio-2,5-diketopiperazine with GSH-Cu(II) to be made. Nonetheless it was of interest to explore the reactivity of the mixture of an epi-3,6-dithio-2,5-diketopiperazine and glutathione **338**.

2.4.2.2.2 Gliotoxin

In order to avoid any trace of DMSO which, as demonstrated in the previous experiments, alters DNA in the presence of GSH and Cu(II), we used the commercially available gliotoxin **1**, isolated from cultures of *Aspergillus fumigatus*, soluble in ethanol. Interestingly the mixture of gliotoxin **1**, GSH **338** and Cu(II) caused single and double strand breaks (lane 1) but in the case of the mixture of gliotoxin 1 and Cu(II) no DNA damage was observed (lane 2) (Figure 47). Incubation of plasmid DNA with gliotoxin **1** and GSH **338** did not cause any DNA

damage and the same results were obtained with gliotoxin **1** alone (lane 4), GSH alone **338** (lane 6) and Cu(II) alone (lane 7). However, surprisingly, in this set of experiments we observed single and double strand breaks caused by the mixture GSH-Cu(II) (lane 5) (Figure 47). Thus, in light of this, it cannot be concluded that gliotoxin **1** was responsible for the DNA damage in lane 1.

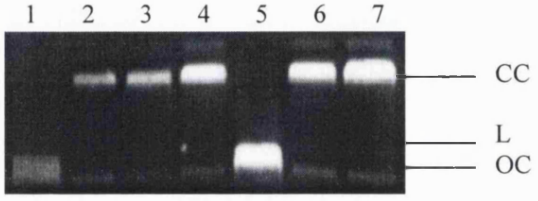
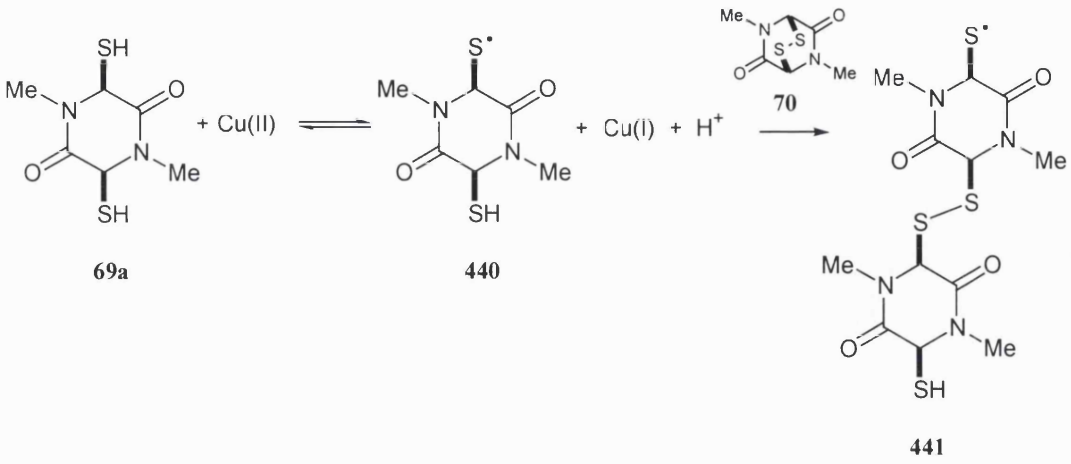


Figure 47. Agarose gel electrophoresis: (**D**), **lane 1**: Gliotoxin **1** (200 μM), GSH **338** (200 μM) and CuCl2 (300 μM); **lane 2**, Gliotoxin **1** and $CuCl_2$; **lane 3**, Gliotoxin **1** and GSH **338**; **lane 4**, Gliotoxin **1**; **lane 5**, GSH **338** and $CuCl_2$; **lane 6**, GSH **338**; **lane 7**, $CuCl_2$. *CC*, *L* and *OC*, covalently closed circular, linear and open circular forms of plasmid DNA.

2.4.2.2.3 N,N'-3,6-dithiol-2,5-diketopiperazine

Waring, Müllbacher *et al.* reported that the reduced form **69a** of *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70** induced DNA damage in the presence of Cu(II) under aerobic conditions. Under anaerobic conditions compound **69a** could have the same properties as glutathione **338** and thus could provide the radical **440**, which could cause DNA damage (Scheme 174).

It was therefore of interest to test *N,N'*-dimethyl-3,6-dithiol-2,5-diketopiperazine **69a** under anaerobic conditions with plasmid DNA and in the presence of the *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70**, as radical **440** which could attack the disulfide bridge to form a mixed disulfide **441** radical which could also be a reactive species (Scheme 174).



Scheme 174

Incubation of plasmid DNA with *N,N'*-dimethyl-3,6-dithiol-2,5-diketopiperazine **69a**, *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70** and Cu(II) led to single strand break (lane 1) and the same result was obtained with the mixture *N,N'*-dimethyl-3,6-dithiol-2,5-diketopiperazine **69a** and Cu(II) (lane 4). Blank experiments with *N,N'*-dimethyl-3,6-dithiol-2,5-diketopiperazine **69a** and *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70** (lane 2) and *N,N'*-dimethyl-3,6-dithiol-2,5-diketopiperazine **69a** (lane 3) were negative as no DNA damage was observed (Figure 48).

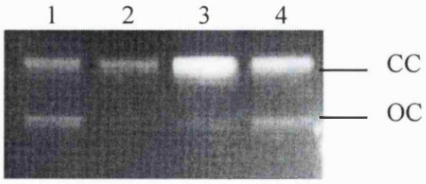


Figure 48. Agarose gel electrophoresis: **(E)**, **lane 1**: *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70** (200 μM), *N,N'*-dimethyl-3,6-dithiol-2,5-diketopiperazine **69a** (200 μM) and $CuCl_2$ (20 μM); **lane 2**: *N,N'*-dimethyl-epi-3,6-dithio-2,5-diketopiperazine **70** (200 μM) and *N,N'*-dimethyl-3,6-dithiol-2,5-diketopiperazine **69a**; **lane 3:** *N,N'*-dimethyl-3,6-dithiol-2,5-diketopiperazine **69a** (200 μM); **lane 4**: *N,N'*-dimethyl-3,6-dithiol-2,5-diketopiperazine **69a** (200 μM) and $CuCl_2$ (20 μM). *CC* and *OC*, covalently closed circular and open circular forms of plasmid DNA.

Thus, from this experiment, we concluded that *N,N'*-dimethyl-3,6-dithiol-2,5-diketopiperazine **69a** caused DNA damage in the presence of Cu(II) without any oxygen species in the reaction mixture, and that the reactive species involved would most probably be the thiyl radical **440**.

Chapter 3

Conclusions and Perspectives

Overall conclusions and perspectives

The foregoing discussion of our results has involved several interesting themes of epi-3,6-dithio-2,5-diketopiperazine chemistry.

As emphasised in the introduction, and indeed confirmed by our own studies, the construction of this key core remains as a significant challenge to synthetic organic chemists, and to this day, no simple and effective method is available for the introduction of the disulfide bridge into a 2,5-diketopiperazine, save for the bromination sequence introduced by Trown in 1968,[65]and also by Kishi[95] for elaboration of his dithioacetal building block.

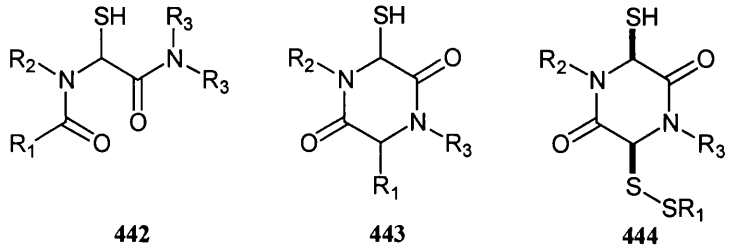
In the event, our own requirements for epi-3,6-dithio-2,5-diketopiperazine also came to rely on Trown methodology,[65] and hence, in the first instance on preparation of 2,5-diketopiperazines. In spite of the fact that these cyclic dipeptides are often formed as unwanted products during linear peptide assembly, many of these numerous methods depend on the solubility and concentration of the starting materials as well as the products and hence cannot be applied to a range of substrates. However, intramolecular cyclisation of a dipeptide formate salt in a high boiling point solvent or cyclisation *via* a tandem acylation-alkylation with ammonia, both at a precise concentration were eventually proven to be the most general and powerful methods in our hands both for symmetrical and unsymmetrical 2,5-diketopiperazines.

The subsequent application of the Trown methodology[65] to symmetrical *N,N'*-dimethyl- or *N,N'*-dibenzyl-epi-3,6-dithio-2,5-diketopiperazines **70** and **217** was successful and interestingly, and in contrast to literature precedent led only to the desired *cis*-dithiol intermediates as opposed to the previously reported *cis-trans* mixtures. Unfortunately, the hypothesis that the *cis*-3,6-dithiol-2,5-diketopiperazine is a thermodynamic product as a consequence of intramolecular hydrogen bonding could not be proven in spite of repeated efforts to grow suitable crystals for X-Ray diffraction. The limitations of the bromination-thioacetate displacement sequence were apparent however when various *N*-protected-2,5-

diketopiperazines were examined and accordingly led us to consider more general methods for disulfide bridge construction.

Three classes of 2,5-diketopiperazines were examined, an aromatic *N*-oxide **247**, a hydroxamic acid **252** and a *C*-hydroxylated derivative **257**. Of these, the hydroxamic acid unit was introduced *via* displacement of an appropriate acyclic bromo ester derivative with *O*-benzylhydroxylamine followed by deprotection whilst the *C*-hydroxy derivative **257** was obtained from the reaction of a pyruvyl derivative with ammonia. In spite of literature precedent however, we were unable to achieve the synthesis of an unsaturated *N*-oxide derivative **247**. Moreover, attempted formation of xanthate derivatives followed by a tandem Chugaëv elimination and Michael addition sequence led, in preliminary studies to intractable mixtures and was also plagued by solubility problems. Further work in this area will require the preparation of organic soluble precursors which are more amenable to chromatography and characterisation.

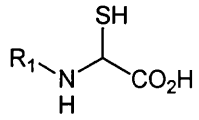
Nevertheless, with a range of epi-3,6-dithio-2,5-diketopiperazines in hand, together with their *cis*-dithiol precursors it was possible to study the behaviour of the thiyl radical in such systems. The *in vitro* experiments using *N,N'*-dibenzyl-3,6-dithiol-2,5-diketopiperazine **216** as a polarity reversal catalyst for the hydrosilylation of the enol lactone clearly demonstrated that such radicals can be generated. The observation that the thiyl radicals derived from the two *cis*-dithiol **216** and **221** are effectively unreactive in promoting racemisation of the chiral tetrahydrofurfuryl benzoate ester was of course disappointing, and this result may be attributed either to polarity and/or to steric effects. Nevertheless, given the possibility that the adjacent thiol group may influence the reactivity of the thiyl radical causing it to become less reactive, it is certainly of interest to pursue systems such as acyclic **442**, monocylic **443** and mixed disulfide **444** systems in order to probe for increased electrophilic character (Figure 49). The influence of metal cations both on bridged disulfides and thiols also warrants further study.



442 **443** **444**

Figure 49

During our studies towards the mode of action of epi-3,6-dithio-2,5-diketopiperazines, the use of *N,N'*-benzyl-3,6-dithiol-2,5-diketopiperazine **216** as polarity-reversal catalyst for the hydrosilylation reaction demonstrated that the thiyl radical of the related *N,N'*-benzyl-3,6-dithiol-2,5-diketopiperazine **216**, until now unknown, did actually exist. However, the *in vitro* tests performed on the DNA model did not allow us to conclude that this thiyl radical was sufficiently electrophilic to abstract a hydrogen from the C-4' position of the ribose ring of DNA. The same hydrogen bonding which allowed us to obtain exclusively the *cis*-3,6-dithio-2,5-diketopiperazine may also stabilise the thiyl radical, causing it to become less reactive. Therefore it would be of interest to test a monothiol **443** or mixed disulfide monothiol **444** (Figure 49) or α-thiol amino acid **445** (Figure 50) in order to prove that a thiyl radical of this type are sufficiently electrophilic to abstract a hydrogen from a C-H bond.



445

Figure 50

In terms of further *in vitro* experiments, it is interesting to speculate that, in selecting a disulfide, nature intended that both sulfur atoms should be used. Thus as shown in Figure 51, if one of the thiol groups were to add to the pyrimidine base of DNA, as precedented by the work of Greenberg and Schiesser,[193] then the other could be involved in thiyl radical formation and cleavage of DNA might be

possible. The overall process would be intramolecular and benefit from rate accelerations as demonstrated by the model study of Robins[183] on ribonucleotide reductase.

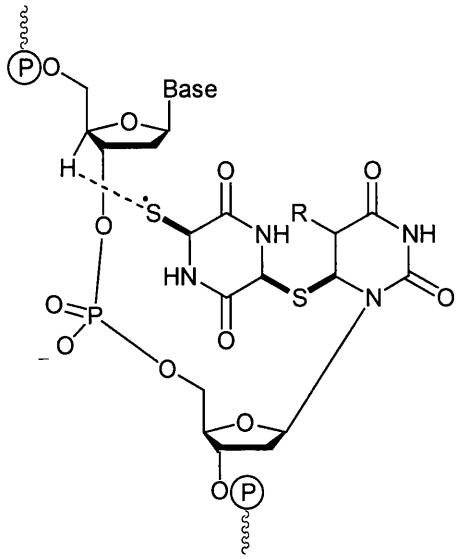


Figure 51

Finally, the initial biological experiments were promising as DNA damage was observed using an epi-3,6-dithio-2,5-diketopiperazine with glutathione **338** and $CuCl_2$. However, insolubility problems required us to use dimethylsulfoxide as a solvent and unfortunately the blank experiment carried out with this solvent was positive in showing DNA damage. Consequently, no conclusions as to the cause of the plasmid DNA damage could be made. For these studies, it is highly recommended to synthesise water soluble epi-3,6-dithio-2,5-diketopiperazines, which would be easier to handle in a buffer solution.

When gliotoxin **1**, soluble in ethanol, was tested with plasmid DNA, DNA damage was observed under anaerobic conditions. However strand breaks were also noted with the couple, GSH-Cu(II), which had been previously inactive towards plasmid DNA. Consequently, we are not able to confirm that gliotoxin **1**, itself, caused the DNA damage. This result led us to use the reduced 3,6-dithiol-2,5-diketopiperazine in order to avoid the reducing agent glutathione **338** and we demonstrated that *N*,*N*'-dimethyl-3,6-dithiol-2,5-diketopiperazine **69a** induced DNA degradation in the presence of Cu(II) under anaerobic conditions. Thus, it is tempting to conclude

that this DNA damage was caused by the formation of a thiyl radical produced by reaction of *N*,*N*'-dimethyl-3,6-dithiol-2,5-diketopiperazine **69a** with $CuCl_2$.

As a consequence, it would be of interest to carry out further biological experiments with epi-3,6-dithio-2,5-diketopiperazines, in order to prove not only that the disulfide bridge can be opened to give a thiyl radical but also to demonstrate that this thiyl radical is a reactive species. Clearly, the epi-3,6-dithio-2,5-diketopiperazines will continue to challenge both synthetic organic chemists and chemical biologists for many years to come.

Chapter 4

Experimental

4.1. Experimental

4.1.1. General experimental procedures

Melting points were determined using a Reichert hot stage and are uncorrected. Boiling points for Kugelröhr bulb to bulb distillations refer to uncorrected air temperatures. Pressure was measured using a standard Gallenkamp manometer. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter (using the sodium D line; 589 nm) and $[\alpha]_D^T$ are given in units of 10^{-1} deg dm^2 g^{-1}. Infrared spectra were recorded as thin films on NaCl plates or as KBr disks on a Perkin-Elmer FT-IR 1605 instrument. Mass spectra were recorded either under electron impact (EI), electrospray (ESP), chemical ionisation (CI) or fast atom bombardment (FAB) at the University College London Chemistry Department. Accurate mass measurements and elementary analyses were performed at University College London.

^1H NMR spectra were recorded on a Bruker AMX-300 or a Bruker AMX-400 at 300 MHz or 400 MHz respectively. ^{13}C NMR spectra were recorded on the same instruments at 75 MHz or 100 MHz. ^{13}C NMR spectra assignments were supported by DEPT spectra. Proton and carbon NMR chemical shifts (δ) are quoted in parts per million (ppm) and are referenced to the residual solvent peak. Coupling constants *J* are measured in Hertz. The abbreviations used to indicate multiplicity are s, singlet; d, doublet; t, triplet; q, quartet; h, heptuplet, m, multiplet; br, broad; or a combination of these.

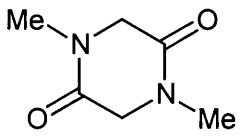
Reactions were monitored by thin layer chromatography performed on Merck Kieselgel $60F_{264}$ plates. The components were visualised with U.V. light (254 nm), and by staining with iodine, basic potassium permanganate, ethanoic anisaldehyde or ninhydrin, all followed by heat. Flash chromatography was carried out using BDH flash silica gel (40-60 μm). Chiral HPLC was performed using an Chiralcel© OD column with a Shimatzu LC-10AS pump, a Shimatzu SPD-6A UV detection

system measuring the absorbance at 254 nm and a Shimatzu C-R6A Chromatopac channel integrator.

All reactions using dry solvents were carrried out in oven dried glassware, under a positive atmosphere of nitrogen. Solvent transfer was performed by syringe. Molecular sieves were activated by microwave irradiation. Diethyl ether and tetrahydrofuran were distilled from sodium-benzophenoneketyl. Dichloromethane was distilled from calcium hydride. Benzene was distilled from sodium. Methanol was distilled from magnesium turnings and iodine. Dimethylformamide was distilled from calcium hydride, at reduced pressure, and stored over 4Å molecular sieves, under nitrogen. *tert*-Butanol was distilled and stored over 4Å molecular sieves, under nitrogen. Dimethoxyethane was distilled from lithium aluminium hydride. Triethylamine was pre-dried with anhydrous potassium hydroxide and distilled under nitrogen. Acetyl chloride and thionyl chloride were distilled under nitrogen before use. *N*–Bromosuccinimide was recrystallised from water and dried at reduced pressure. Benzoyl peroxide was recrystallised from a 1:1 mixture of chloroform and light petroleum (bp 40–60 °C) and dried at reduced pressure.

4.1.2. Synthesis of 2,5-diketopiperazines

1,4-Dimethyl-piperazine-2,5-dione[206] **66**



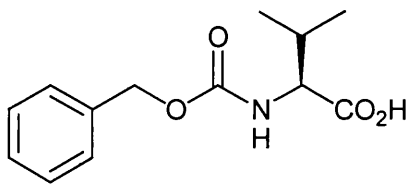
$C_6H_{10}N_2O_2$

142.16 g.mol^{-1}

Sarcosine **192** (40.0 g, 0.44 mol) was suspended in ethylene glycol (40 mL) and heated under reflux for 20 h. The yellow solution was allowed to cool and the crystals formed were filtered to yield 1,4-dimethyl-2,5-dioxopiperazine **66** as colourless prisms (24 g, 37%). The resulting solution was extracted with dichloromethane (3 x 60 mL), treated with charcoal, heated and filtered until complete decolouration. The clear solution was then dried over anhydrous magnesium sulfate and the solvent was removed under reduced pressure to yield only a small amount of the desired product **66** (720 mg, 1%).

Mp 149–152 °C (lit.,[206] 149–150 °C); **IR** (KBr)/cm^{-1}: ν_{max} 1712 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 3.96 (4 H, s, CH_2), 2.96 (6 H, s, CH_3); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 163.4 (*C*=O), 51.8 (CH_2), 33.4 (CH_3); **LRMS** (+FAB) *m/z* 143 (100%, M^+).

(2S)-3-Methyl-2-[(*N*-phenylmethoxycarbonyl)amino]butanoic acid[207] **193**



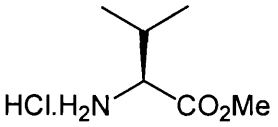
$C_{13}H_{17}NO_4$

251.25 g.mol^{-1}

To a stirred solution of L-valine **190** (5.0 g, 42 mmol) in 5% aqueous $NaHCO_3$ (250 mL) was added benzylchloroformate (7.2 mL, 50 mmol), and the resulting mixture stirred, at room temperature, for 20 hours. The reaction mixture was washed with diethyl ether (80 mL). The resulting aqueous layer was acidified with 20% hydrochloric acid (10 mL) and extracted with dichloromethane (3 x 80 mL). The organic extracts were combined, dried and evaporated to give a transparent solid **193** (6.3 g, 61%).

Mp 55–58 °C (lit.,[207] not specified, lit.,[208] 62 °C); $[\alpha]_D^{25}$ + 4.3 (*c.* 4 in $CHCl_3$) [lit.,[207] $[\alpha]_D^{20}$ + 6.3 (*c.* 4 in $CHCl_3$)]; **IR** (KBr)/cm^{-1}: ν_{max} 3140 (O–H), 2963 (N–H), 1659 (C=O) ; **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.28 (5 H, m, C*H*, Ph), 5.14 (1 H, d, *J* 8.8 Hz, N*H*), 5.05 (2 H, s, CH_2–O), 4.27 (1 H, dd, *J* 4.7 and 8.8 Hz, C*H*–NH_2), 2.17 (1 H, m, C*H*–CH_3), 0.93 (3 H, d, *J* 6.7 Hz, CH_3), 0.87 (3 H, d, *J* 6.7 Hz, CH_3); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 175.3 (CO_2H), 155.2 (*C*O–NH), 134.9 (*C*q, Ph), 127.3 (*C*H, Ph), 127.0 (*C*H, Ph), 126.9 (*C*H, Ph), 65.9 (CH_2–O), 64.6 (*C*H–NH), 29.8 (*C*H–CH_3), 17.7 (CH_3–CH), 16.1 (CH_3–CH) ; **LRMS** (+FAB) *m/z* 252 (20%, M^+), 91 (100).

L-Valine methyl ester hydrochloride[209] 194



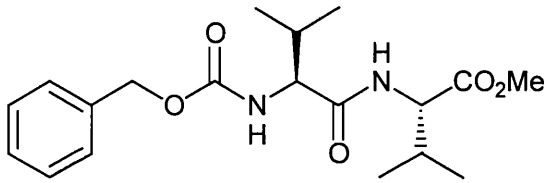
$C_6H_{13}NO_2.HCl$

168.62 $g.mol^{-1}$

Thionyl chloride (5.4 mL, 74 mmol) was carefully added dropwise to a stirred suspension of L-valine **190** (5.5 g, 47 mmol) in methanol (100 mL) at 0 °C. The reaction was allowed to warm to room temperature and stirred for 24 hours, after which the solution was evaporated at reduced pressure to give L-valine methyl ester hydrochloride **194** (6.7 g, 94%).

Mp 105–110 °C (lit.,[209] 155–160 °C); $[\alpha]_D^{29}$ + 15.6 (*c.* 2 in H_2O) [lit.,[209] $[\alpha]_D^{25}$ + 15.7 (*c.* 2 in H_2O)]; **IR** (KBr)/cm^{-1}: ν_{max} 2966 (N–H), 1738 (C=O); **^{1}H NMR** (300 MHz; D_2O): δ_H 3.96 (1 H, d, *J* 4.8 Hz, C*H*–NH_2), 3.78 (3 H, s, CH_3–O), 2.27 (1 H, dh, *J* 4.8 and 7.0 Hz, C*H*–CH_3), 0.96 (3 H, d, *J* 7.0 Hz, CH_3–CH), 0.95 (3 H, d, *J* 7.0 Hz, CH_3–CH); **^{13}C NMR** (75 MHz; D_2O): δ_C 169.2 (*C*=O), 57.3 (*C*H–N), 52.3 (*C*H_3–O), 28.2 (*C*H–CH_3), 16.1 (*C*H_3), 15.9 (*C*H_3); **LRMS** (+CI) *m/z* 186 (47%, $M^+ + CH_4$), 133 (77, $M^+ - HCl$), 100 (80), 88 (68), 72 (100).

_N_-Benzyloxycarbonyl-L-valine-L-valine-methyl ester[143] 195



$C_{19}H_{28}N_2O_5$

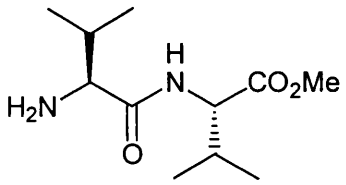
364.40 g.mol^{-1}

A mixture of *N*-benzyloxycarbonyl-L-valine **193** (1.5 g, 6.0 mmol), dicyclohexylcarbodiimide (1.3 g, 6.3 mmol) and 1-hydroxybenzotriazole (972 mg, 7.19 mmol) in dichloromethane (20 mL) was stirred, under nitrogen, at 0 °C, for 1 hour and then at room temperature for 1 hour. A mixture of L-valine methyl ester hydrochloride **194** (1.0 g, 6.0 mmol) and triethylamine (837 μL, 6.01 mmol) in dichloromethane (10 mL) was then added and after 30 minutes the insoluble dicyclohexyl-urea by-product was filtered off. The filtrate was evaporated to dryness and the residue was purified by column chromatography (silica, dichloromethane–methanol, 10:1) to give the desired product **195** (440 mg, 20%) as a pale yellow solid.

Mp 115–120 °C (lit.,[143] 145–147 °C); R_f [light petroleum (bp 40–60 °C)–ethyl acetate, 1:1] 0.68; $[\alpha]_D^{25}$ – 27.3 (*c.* 1 in MeOH) [lit.,[143] $[\alpha]_D^{20}$ – 28.0 (*c.* 1 in MeOH)]; **IR** (KBr)/cm^{-1}: ν_{max} 3301 (N–H), 2952 (N–H), 1735 (C=O), 1691 (C=O),

1648 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.22–7.28 (5 H, m, C*H*, Ph), 6.27 (1 H, d, *J* 8.6 Hz, N*H*), 5.28 (1 H, d, *J* 6.5 Hz, N*H*), 5.04 (2 H, s, CH_2), 4.46 (1 H, q, *J* 4.9 and 8.6 Hz, C*H*–NH), 3.96 (1 H, t, *J* 6.5 and 8.3 Hz, C*H*–NH), 3.65 (3 H, s, CH_3–O), 1.99–2.14 (2 H, m, C*H*–CH_3), 0.90 (3 H, d, *J* 6.8 Hz, CH_3), 0.87 (3 H, d, *J* 6.8 Hz, CH_3), 0.84 (3 H, d, *J* 6.8 Hz, CH_3), 0.82 (3 H, d, *J* 6.8 Hz, CH_3); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 172.5 (*C*=O), 171.7 (*C*=O), 156.8 (*C*=O), 136.7 (*C*q, Ph), 128.8 (*C*H, Ph), 128.4 (*C*H, Ph), 128.3 (*C*H, Ph), 67.3 (*C*H_2), 60.7 (*C*H–NH), 57.5 (*C*H–NH), 52.4 (*C*H_3–O), 31.5 (2 *C*H–CH_3), 19.5 (*C*H_3), 19.2 (*C*H_3), 18.2 (*C*H_3), 18.1 (*C*H_3); **LRMS** (+CI) *m/z* 365 (33%, M^+), 91 (100).

L-Valine-L-valine methyl ester <u>196</u>



$C_{11}H_{22}N_2O_3$

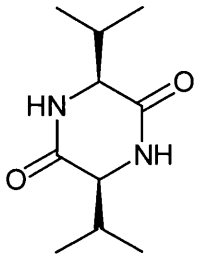
230.30 g.mol^{-1}

N-Benzyloxycarbonyl-L-valine-L-valine-methyl ester **195** (219 mg, 0.60 mmol) was dissolved in methanol (5 mL) and hydrogenated overnight under an atmosphere of hydrogen in the presence of palladium black. After the catalyst was removed by filtration on celite, the filtrate was evaporated under reduced pressure to yield L-valine-L-valine methyl ester **196** (130 mg, 93%). The product has been previously characterised as the hydrobromide salt.[210]

$[\alpha]_D^{25}$ – 29.1 (*c.* 1 in EtOH); **IR** (thin film)/cm^{-1}: ν_{max} 3331 (N–H), 1739 (C=O), 1652 (C=O); **^{1}H NMR** (300 MHz; D_2O): δ_H 4.33 (1H, d, *J* 6.3 Hz, C*H*–NH_2), 3.82 (3 H, s, CH_3–O), 3.30 (1 H, d, *J* 6.3 Hz, C*H*–NH), 2.18–2.32 (1 H, m, C*H*–CH_3), 1.91–2.02 (1 H, m, C*H*–CH_3), 1.11 (3 H, d, *J* 6.3 Hz, CH_3), 1.07 (3 H, d, *J* 6.3 Hz, CH_3), 1.07 (3 H, d, *J* 6.3 Hz, CH_3), 1.06 (3 H, d, *J* 6.3 Hz, CH_3); **^{13}C NMR** (75

MHz; D_2O): δ_C 177.2 (*C*=O), 174.4 (*C*=O), 60.2 (*C*H–NH), 59.0 (*C*H–NH), 52.9 (*C*H_3–O), 32.3 (*C*H–CH_3), 30.2 (*C*H–CH_3), 18.7 (*C*H_3), 18.5 (*C*H_3), 17.9 (*C*H_3), 17.4 (*C*H_3); **LRMS** (+CI) *m/z* 231 (21%, M^+), 199 (100), 72 (38). **Microanalysis** Found: C, 58.13; H, 9.54; N, 12.41%. $C_{11}H_{22}N_2O_3$ requires C, 57.37; H, 9.63; N, 12.16%.

Attempted syntheses of 3,6-diisopropyl-piperazine-2,5-dione[143] 191



$C_{10}H_{18}N_2O_2$
198.25 g.mol^{-1}

Ethylene glycol method:

L-Valine **190** (53 g, 0.4 mol) was suspended in ethylene glycol (60 mL) and heated under reflux for 20 h. The dark brown reaction mixture was cooled to room temperature and placed in a refrigerator, at 4 °C for 12 hours. As the desired product did not crystallise, the brown solution was extracted with dichloromethane (4 x 60 mL), and the combined extracts were treated with charcoal, heated and filtered until complete decolouration. The resulting clear solution was dried over magnesium sulfate, filtered and the solvent was removed at reduced pressure to yield a small amount of a pale yellow solid, which from ^1H and ^{13}C NMR was the starting material **190**.

Cyclisation of the free amine dipeptide method:

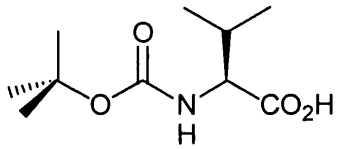
A solution of L-valine-L-valine methyl ester **196** (130 mg, 0.56 mmol) in methanol (10 mL) was heated at reflux for 5 days. TLC analysis did not show formation of any product. The reaction mixture was cooled to room temperature and the solvent

was removed at reduced pressure. ^{1}H NMR analysis confirmed that only the starting material **196** was present.

Ammonia method:

A solution of L-valine-L-valine methyl ester **196** (100 mg, 0.43 mmol) in methanol (5 mL) was cooled to – 78 °C and liquid ammonia (approximately 5 mL) was condensed into the flask. The reaction mixture was stirred at – 78 °C for 24 hours and at room temperature for 2 hours. The solvent was then removed and the crude product was analysed by ^{1}H and ^{13}C NMR to reveal the presence of starting material **196** and no cyclised product.

_N_-(*tert*-Butoxycarbonyl)-L-valine[211] 197



$C_{10}H_{19}NO_4$

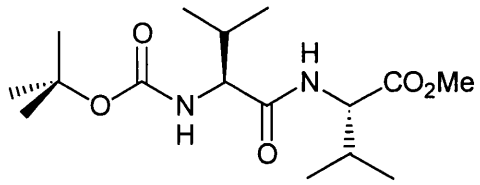
217.23 g.mol^{-1}

To a stirred solution of L-valine **190** (1.0 g, 8.5 mmol), in a mixture of 2:1 tetrahydrofuran-water (30 mL), was added 1 N sodium hydroxide (8.5 mL, 8.5 mmol) and di-*tert*-butyldicarbonate (1.8 g, 8.5 mmol). The resulting solution was stirred at room temperature for 18 hours. After the reaction mixture was washed with ethyl acetate (20 mL) and acidified with hydrochloric acid until pH 4, the aqueous layer was extracted with ethyl acetate (3 x 50 ml). The organic extracts were dried over anhydrous magnesium sulfate, filtered and evaporated under reduced pressure to give **197** as a dense oil (1.3 g, 72%) which crystallised on standing.

Mp 85–88 °C (lit.,[211] 77–78 °C); $[\alpha]_D^{25}$ – 6.0 (*c.* 1 in CH_3CO_2H) [lit.,[211] $[\alpha]_D^{20}$ – 6.5 (*c.* 1 in CH_3CO_2H)]; **IR** (KBr)/cm^{-1}: ν_{max} 3302 (O–H), 2984 (N–H), 1703 (C=O),

1643 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 9.79 (1 H, br, COO*H*), 5.04 (1 H, d, *J* 8.3 Hz, N*H*), 4.27 (1 H, dd, *J* 4.4 and 8.3 Hz, C*H*), 2.21 (1 H, m, C*H*), 1.46 (9 H, s, CH_3–C), 1.02 (3 H, d, *J* 6.8 Hz, CH_3–CH), 0.95 (3 H, d, *J* 6.8 Hz, CH_3–CH); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 177.4 (*C*=O), 156.2 (*C*=O), 80.4 (*C*q), 58.8 (*C*H–NH), 31.3 (*C*H–CH_3), 28.6 (3 *C*H_3), 19.4 (*C*H_3), 17.8 (*C*H_3); **LRMS** (+CI) *m/z* 218 (32%, MH^+), 162 (34), 116 (55), 72 (42), 58 (100).

***N*-(*tert*-Butoxycarbonyl)-L-valine-L-valine methyl ester[212] 198**



$C_{16}H_{30}N_2O_5$

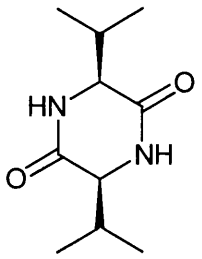
330.39 g.mol^{-1}

A mixture of *N*-(*tert*-butoxycarbonyl)-L-valine **197** (1.0 g, 4.6 mmol), dicyclohexylcarbodiimide (949 mg, 4.60 mmol) and 1-hydroxybenzotriazole (622 mg, 4.60 mmol) in chloroform (60 mL), was stirred, under nitrogen, at 0 °C, for 1 hour, and then at room temperature for 1 hour. A mixture of L-valine methyl ester hydrochloride **194** (776 mg, 4.6 mmol) and triethylamine (641 μL, 4.60 mmol) in chloroform (20 mL) was then added, after 30 minutes, the insoluble dicyclohexyl-urea by-product was removed by filtration. The filtrate was evaporated under reduced pressure and the residue was purified by column chromatography (silica, ethyl acetate) to yield **198** (639 mg, 41%) as a pale yellow solid.

Mp 127–130 °C (lit.,[212] 109–110 °C); R_f [light petroleum (bp 40–60 °C)–ethyl acetate, 1:1] 0.31; $[\alpha]_D^{25}$ – 37.7 (*c.* 1 in MeOH) [lit.,[212] $[\alpha]_D^{20}$ – 44.2 (*c.* 1 in MeOH)]; **IR** (KBr)/cm^{-1}: ν_{max} 3323 (N–H), 2954 (N–H), 1745 (C=O), 1683 (C=O), 1645 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 6.31 (1 H, br, N*H*), 4.97 (1 H, br, N*H*), 4.44–4.49 (1 H, m, C*H*–NH), 3.81–3.86 (1 H, m, C*H*–NH), 3.66 (3 H, s,

C*H*$_3$–O), 2.03–2.15 (2 H, m, C*H*–CH$_3$), 1.37 (9 H, s, C*H*$_3$–Cq), 0.90 (3 H, d, *J* 7.0 Hz, C*H*$_3$–CH), 0.87 (6 H, d, *J* 7.0 Hz, C*H*$_3$–CH), 0.84 (3 H, d, *J* 7.0 Hz, C*H*$_3$–CH); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 172.4 (*C*=O), 171.9 (*C*=O), 156.1 (*C*=O), 80.2 (*C*q), 60.6 (*C*H–NH), 57.4 (*C*H–NH), 52.4 (*C*H$_3$–O), 31.6 (*C*H), 30.9 (*C*H), 28.6 (*C*H$_3$–Cq), 19.6 (*C*H$_3$), 19.2 (*C*H$_3$), 18.2 (*C*H$_3$), 18.1 (*C*H$_3$); **LRMS** (+ES) *m/z* 353 (100%, MNa^+), 331 (35), 275 (100), 231 (84).

3,6-Diisopropyl-piperazine-2,5-dione[143] 191



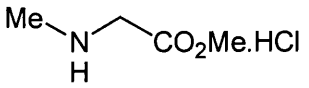
$C_{10}H_{18}N_2O_2$

198.25 g.mol^{-1}

N-(*tert*-butoxycarbonyl)-L-valine-L-valine methyl ester **198** (200 mg, 0.60 mmol) was dissolved in formic acid (20 mL) and the solution stirred, at room temperature, for 2 hours. After removal of the excess formic acid at reduced pressure, at temperature not exceeding 30 °C, the residue was dissolved in *sec*-butyl alcohol (10 mL) and toluene (5 mL). The solution was refluxed for 3 hours. On cooling, the solvents were removed at reduced pressure and the residue was washed with diethyl ether to yield white crystals of **191** (71 mg, 60%).

Mp 270–272 °C (lit.,[143] 272–273 °C); $[\alpha]_D^{27}$ – 62.8 (*c.* 0.5 in AcOH) [lit.,[143] $[\alpha]_D^{20}$ – 62.0 (*c.* 0.5 in AcOH)]; **IR** (KBr)/cm^{-1}: ν_{max} 3454 (N–H), 1659 (C=O); **^{1}H NMR** (300 MHz; DMSO–d_6): δ_H 7.92 (2 H, br, N*H*), 3.69 (2 H, m, C*H*–NH), 2.13–2.19 (2 H, m, C*H*–CH$_3$), 0.96 (6 H, d, *J* 7.1 Hz, C*H*$_3$), 0.84 (6 H, d, *J* 7.1 Hz, C*H*$_3$); **^{13}C NMR** (75 MHz; DMSO-d_6): δ_C 167.7 (*C*=O), 59.5 (*C*H–NH), 31.4 (*C*H–CH$_3$), 19.0 (*C*H$_3$), 17.6 (*C*H$_3$); **LRMS** (+FAB) *m/z* 198 (100%, M^+), 72 (28).

Sarcosine methyl ester hydrochloride[213] **199**



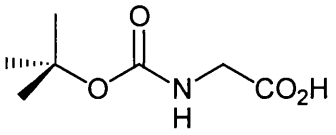
$C_4H_9NO_2.HCl$

139.58 $g.mol^{-1}$

Thionyl chloride (24.4 mL, 0.33 mol) was added dropwise to a stirred suspension of sarcosine **192** (10.0 g, 0.11 mol) in methanol (250 mL), at 0 °C. The reaction was allowed to warm to room temperature and stirred for 24 hours, after which the solvent was removed at reduced pressure to give sarcosine methyl ester hydrochloride **200** (15.5 g, quant.).

Mp 121–122 °C (lit.,[213] 121.5–122.5 °C); **IR** (KBr)/cm^{-1}: ν_{max} 2969 (N–H), 1746 (C=O); **^{1}H NMR** (300 MHz; D_2O): δ_H 4.05 (2 H, s, C*H*$_2$), 3.88 (3 H, s, C*H*$_3$–O), 2.84 (3 H, s, C*H*$_3$–NH), **^{13}C NMR** (75 MHz; D_2O): δ_C 166.4 (C=O), 52.1 (*C*H$_3$–O), 47.4 (*C*H$_2$), 31.6 (*C*H$_3$–N); **LRMS** (+CI) *m/z* 157 (12%, $M^+ + CH_4$), 104 (70, M^+ – HCl), 44 (100).

***N*-(*tert*-Butoxycarbonyl)-glycine**[214] **201**



$C_7H_{13}NO_4$

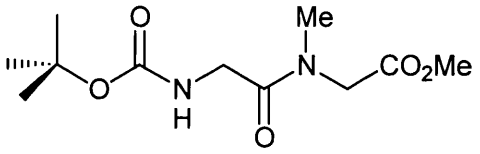
175.00 $g.mol^{-1}$

To a stirred solution of glycine **200** (10.0 g, 0.13 mol) in a mixture of 2:1 tetrahydrofuran–water (250 mL) was added 1 N sodium hydroxide (134 mL, 0.13 mol) and di-*tert*-butyldicarbonate (32.0 g, 0.14 mol) and the resulting mixture was stirred, at room temperature, for 18 hours. The aqueous solution was then washed with ethyl acetate (200 mL), acidified with hydrochloric acid to pH 2 and extracted

with ethyl acetate (3 x 200 mL). The combined organic extracts dried over anhydrous sodium sulfate and evaporated to give **201** as white crystals (21g, 90%).

Mp 89 °C (from ethyl acetate), (lit.[214] 87–88 °C); **IR** (KBr)/cm^{-1}: ν_{max} 3130 (O–H), 2966 (N–H), 1472 (C=O), 1672 (C=O), 1537 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 5.06 (1 H, br, N*H*), 3.87 (2 H, m, CH_2), 1.38 (9 H, s, CH_3–C); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 174.9 (*C*=O), 156.4 (*C*=O), 80.8 (*C*q), 42.6 (*C*H_2), 28 (*C*H_3); **LRMS** (+FAB) *m/z* 176 (37%, M^+), 120 (100), 76 (34), 57 (84).

_N_-(*tert*-Butoxycarbonyl)-glycine-sarcosine methyl ester[145] 202



$C_{11}H_{20}N_2O_5$

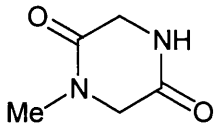
260.25 g.mol^{-1}

A mixture of *N*-(*tert*-butoxycarbonyl)-glycine **201** (2.7 g, 15 mmol), dicyclohexylcarbodiimide (3.2 g, 15 mmol) and dimethylaminopyridine (306 mg, 1.54 mmol) in dichloromethane (30 mL), was stirred, under nitrogen, at 0 °C, for 1 hour. A mixture of sarcosine methyl ester hydrochloride **199** (2.1 g, 15 mmol) and triethylamine (2.2 mL, 15 mmol) in dichloromethane (20 mL) was then added and after stirring, for 12 hours, the insoluble dicyclohexyl-urea by-product was removed by filtration. After the filtrate was evaporated to dryness at reduced pressure, the residue was recrystallised from ethyl acetate to yield **202** as transparent crystals (2.4 g, 60%).

Mp 74–75 °C (lit.,[145] 72–73 °C); R_f [light petroleum (bp 40–60 °C), ethyl acetate 1:1] 0.32 ; **IR** (KBr)/cm^{-1}: ν_{max} 2976 (N–H), 1746 (C=O), 1703 (C=O), 1652 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H two conformers in a ratio 4:1, 5.49 (1 H, br, N*H*), 4.20 (2 H, s, CH_2–N, *major*), 4.08 (2 H, d, *J* 4.3 Hz, CH_2–NH, *major*) 4.06

(2 H, s, C*H*$_2$–N, *minor*), 3.94 (2 H, d, *J* 4.3 Hz, C*H*$_2$–NH, *minor*), 3.83 (3 H, s, C*H*$_3$–O, *minor*), 3.80 (3 H, s, C*H*$_3$–O, *major*), 3.09 (3 H, s, C*H*$_3$–N, *major*), 3.06 (3 H, s, C*H*$_3$–N, *minor*), 1.51 (18 H, s, C*H*$_3$–Cq, *major and minor*); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 169.6 (*C*=O), 169.1 (*C*=O), 156.2 (*C*=O), 80.0 (*C*q), 52.9 (*C*H$_3$–O, *minor*), 52.5 (*C*H$_3$–O, *major*), 50.6 (*C*H$_2$–NH, *minor*), 49.8 (*C*H$_2$–NH, *major*), 42.6 (*C*H$_2$–N, *major*), 42.5 (*C*H$_2$–N, *minor*), 35.5 (*C*H$_3$–N, *major*), 35.3 (*C*H$_3$–N, *minor*), 28.7 (*C*H$_3$–Cq); **LRMS** (+FAB) *m/z* 261 (70%, M^+), 205 (63), 161 (100).

1-Methyl-piperazine-2,5-dione[215] 96



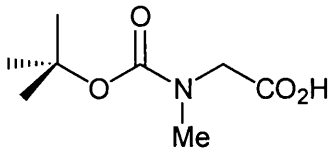
$C_5H_8N_2O_2$

128.11 g.mol^{-1}

N-(*tert*-Butoxycarbonyl)-glycine-sarcosine methyl ester **202** (1.0 g, 3.8 mmol) was dissolved in formic acid (25 mL) and the solution stirred at room temperature for 5 hours. Excess formic acid was removed, at reduced pressure and at temperature not exceeding 30 °C. The crude product was dissolved in *sec*-butylalcohol (60 mL) and toluene (30 mL) and the solution refluxed for 12 hours. After cooling, the volatiles were removed at reduced pressure and the residue was washed with diethyl ether to yield **96** as white crystals (490 mg, quant.).

Mp 139–144 °C (lit.,[215] 142–146 °C); **IR** (KBr)/cm^{-1}: ν_{max} 3171 (N–H), 1695 (C=O), 1648 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 4.57 (2 H, s, C*H*$_2$), 3.84 (2 H, s, C*H*$_2$), 2.79 (3 H, s, C*H*$_3$); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 168.4 (*C*=O), 166.7 (*C*=O), 52.6 (*C*H$_2$), 46.0 (*C*H$_2$), 34.2 (*C*H$_3$); **LRMS** (+EI) *m/z* 128 (100%, M^+), 71 (43), 43 (88), 30 (56).

_N_-(*tert*-Butoxycarbonyl)-sarcosine[216] **203**



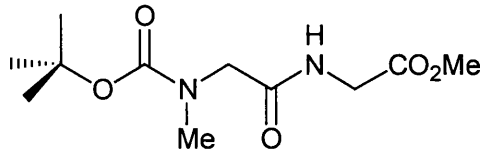
$C_8H_{15}NO_4$

189.18 g.mol^{-1}

To a stirred solution of sarcosine **192** (1.0 g, 11 mmol) in a mixture of 2:1 tetrahydrofuran–water (50 mL) was added 1 N sodium hydroxide (11.2 mL, 11.2 mmol) and di-*tert*-butyldicarbonate (2.7 g, 12 mmol). The resulting solution was stirred at room temperature for 18 hours. After washing with ethyl acetate (30 mL) and acidification with hydrochloric acid to pH 4, the aqueous layer was extracted with ethyl acetate (3 x 70 ml). The combined organic extracts were dried over anhydrous magnesium sulfate, and evaporated at reduced pressure to give the desired product **203** (1.9 g, 89%) as an oil which crystallised on standing.

Mp 89–91°C (lit.,[216] 84–86 °C); **IR** (KBr)/cm^{-1}: ν_{max} 3108 (O–H), 1752 (C=O), 1646 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H two conformers in a ratio 1:1, 4.10 (2 H, s, C*H*$_2$–N, *conformer 1*), 4.02 (2 H, s, C*H*$_2$–N, *conformer 2*), 3.01 (6 H, s, C*H*$_3$–N, *conformer 1 and 2*), 1.54 (9 H, s, C*H*$_3$–Cq, *conformer 1*), 1.52 (9 H, s, C*H*$_3$–Cq, *conformer 2*); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 175.41 (*C*=O, *conformer 1*), 175.40 (*C*=O, *conformer 2*), 156.0 (*C*=O, *conformer 1*), 155.8 (*C*=O, *conformer 2*), 81.0 (*C*q, *conformer 1 and 2*), 51.1 (*C*H$_2$–N, *conformer 1*), 50.5 (*C*H$_2$–N, *conformer 2*), 35.9 (*C*H$_3$–N, *conformer 1 and 2*), 28.6 (*C*H$_3$–Cq, *conformer 1 and 2*); **LRMS** (+CI) *m/z* 190 (5%, M$^+$), 134 (74), 90 (71), 61 (100).

_N_-(*tert*-Butoxycarbonyl)-sarcosine-glycine methyl ester 205



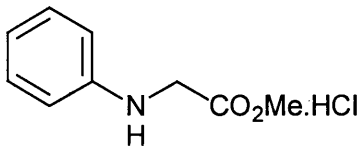
$C_{11}H_{20}N_2O_5$

260.25 g.mol^{-1}

A mixture of *N*-(*tert*-butoxycarbonyl)-sarcosine **203** (1.5 g, 7.9 mmol), dicyclohexylcarbodiimide (1.7 g, 7.9 mmol) and 1-hydroxybenzotriazole (1.1 g, 7.9 mmol) in chloroform (60 mL), was stirred, under nitrogen, at 0 °C, for 1 hour and then at room temperature for 1 hour. A mixture of glycine methyl ester hydrochloride **204** (995 mg, 7.92 mmol) and triethylamine (1.1 mL, 7.9 mmol) in chloroform (20 mL) was then added, and after 30 minutes the insoluble dicyclohexylurea–by–product was removed by filtration. After the filtrate was evaporated to dryness at reduced pressure, the residue was purified by column chromatography (silica, ethyl acetate) to yield (1 g, 41%) a white solid.

Mp 75–77 °C; R_f (ethyl acetate) 0.30; **IR** (KBr)/cm^{-1}: ν_{max} 2981 (N–H), 1749 (C=O), 1712 (C=O), 1660 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H two conformers in a ratio 4:1, 4.22 (2 H, s, C*H*$_2$–N, *major*), 4.07 (2 H, d, *J* 4.4 Hz, C*H*$_2$–NH, *major*) 4.06 (2 H, s, C*H*$_2$–N, *minor*), 3.95 (2 H, d, *J* 4.2 Hz, C*H*$_2$–NH, *minor*), 3.85 (3 H, s, C*H*$_3$–O, *minor*), 3.81 (3 H, s, C*H*$_3$–O, *major*), 3.11 (3 H, s, C*H*$_3$–N, *major*), 3.08 (3 H, s, C*H*$_3$–N, *minor*), 1.52 (9 H, s, C*H*$_3$–Cq, *major and minor*); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 169.6 (*C*=O), 169.1 (*C*=O), 156.1 (*C*=O), 79.9 (*C*q), 52.8 (*C*H$_3$–O, *major*), 52.4 (*C*H$_3$–O, *minor*), 50.6 (*C*H$_2$–NH, *minor*), 49.7 (*C*H$_2$–NH, *major*), 42.6 (*C*H$_2$–N, *major*), 42.4 (*C*H$_2$–N, *minor*), 35.5 (*C*H$_3$–N, *major*), 35.3 (*C*H$_3$–N, *minor*), 28.6 (*C*H$_3$–Cq); **LRMS** (+FAB) *m/z* 283.08 (100%, MNa$^+$); **HRMS** (+FAB) Found: M$^+$, 261.14495; $C_{11}H_{20}N_2O_5$ requires *M*, 261.14504.

Phenylamino-acetic acid methyl ester hydrochloride[217] 207



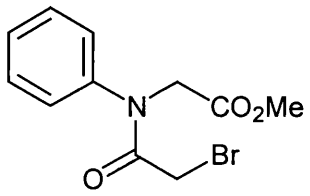
$C_9H_{11}NO_2.HCl$

201.64 g.mol^{-1}

Thionyl chloride (14.5 mL, 0.19 mol) was added dropwise to a stirred suspension of *N*-phenylglycine **206** (10.0 g, 66.1 mmol) in methanol (100 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 24 hours, after which time the precipitate formed was filtered and washed with diethyl ether to give phenylamino-acetic acid methyl ester hydrochloride **207** (10.0 g, 75%).

Mp 144–146 °C (lit.,[217] 140–143 °C); **IR** (KBr)/cm^{-1}: ν_{max} 2936 (N–H), 1743 (C=O); **^{1}H NMR** (300 MHz; D_2O): δ_H 7.37–7.55 (5 H, m, C*H*, Ph), 4.34 (2 H, s, CH_2), 3.81 (3 H, s, CH_3); **^{13}C NMR** (75 MHz; D_2O): δ_C 169.0 (*C*=O), 136.6 (*C*q, Ph), 130.7 (*C*H, Ph), 128.7 (*C*H, Ph), 121.5 (*C*H, Ph), 53.8 (*C*H$_3$), 50.5 (*C*H$_2$); **LRMS** (+EI) *m/z* 165 (100%, M$^+$ – HCl), 106 (97), 77 (100), 51 (53).

[(2-Bromo-acetyl)-phenyl-amino]-acetic acid methyl ester 208



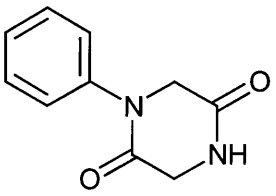
$C_{11}H_{12}NO_3Br$

286.12 g.mol^{-1}

A solution of bromoacetyl bromide (3.2 mL, 37 mmol) in dichloromethane (30 mL) was added dropwise, over 30 minutes, to a stirred solution of phenylamino-acetic acid methyl ester hydrochloride **207** (5.6 g, 34 mmol) and triethylamine (9.5 mL, 68 mmol) in dichloromethane (70 mL) at 0 °C. When addition was complete, stirring was continued for 16 hours at room temperature. After the organic solvent was removed at reduced pressure, the residue was dissolved in dichloromethane (100 mL), washed with water (100 mL), dried over anhydrous magnesium sulfate, filtered and the solvent removed at reduced pressure to give a dark brown solid. Purification by column chromatography [silica, light petroleum (bp 40–60 °C)–ethyl acetate, 3:1] gave [(2-bromo-acetyl)-phenyl-amino]-acetic acid methyl ester **208** as a white solid (9.3 g, 95%).

Mp 70–71 °C; **IR** (KBr)/cm^{-1}: ν_{max} 1752 (C=O), 1651 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.25–7.46 (5 H, m, C*H*, Ph), 4,37 (2 H, s, CH_2–Br), 3.72 (3 H, s, CH_3), 3.70 (2 H, s, CH_2), **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 169.0 (*C*=O), 166.9 (*C*=O), 141.8 (*C*q, Ph), 130.1 (*C*H, Ph), 130.0 (*C*H, Ph), 129.0 (*C*H, Ph), 127.8 (*C*H, Ph), 52.3 (*C*H_3), 51.7 (*C*H_2), 26.4 (*C*H_2); **LRMS** (+EI) *m/z* 286 (15%, M^+), 165 (54, M^+ – $COCH_2Br$), 106 (100), 77 (32); **HRMS** (+EI) Found: M^+, 284.99816; $C_{11}H_{12}NO_3Br$ requires *M*, 284.99951; **Microanalysis** Found: C, 46.70; H, 4.40; N, 4.80%. $C_{11}H_{12}NO_3Br$ requires C, 46.18; H, 4.23; N, 4.90%.

1-Phenyl-piperazine-2,5-dione[218] 209



$C_{10}H_{10}N_2O_2$

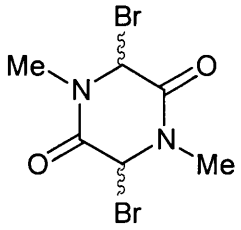
190.20 g.mol^{-1}

Ammonia gas was passed through a solution of [(2-bromo-acetyl)-phenyl-amino]-acetic acid methyl ester **208** (9.2 g, 32 mmol) in methanol (90 mL) at 0 °C until the solution became saturated. The reaction was left to stir for 48 hours and the white solid formed was filtered and washed with methanol (20 mL) to give 1-phenyl-piperazine-2,5-dione **209** as a white solid (4.8 g, 79%).

Mp 249–251 °C (lit.,[218] 251 °C); **IR** (KBr)/cm^{-1}: ν_{max} 3170 (N–H), 1655 (C=O); **^{1}H NMR** (300 MHz; DMSO–d_6): δ_H 8.06 (1 H, br, N*H*), 6.77–7.20 (5 H, m, C*H*, Ph), 4.01 (2 H, s, CH_2), 3.73 (2 H, s, CH_2); **^{13}C NMR** (75 MHz; DMSO–d_6): δ_C 166.1 (*C*=O), 165.0 (*C*=O), 141.0 (*C*q, Ph), 129.2 (*C*H, Ph), 126.9 (*C*H, Ph), 125.7 (*C*H, Ph), 52.6 (CH_2), 45.3 (CH_2); **LRMS** (+EI) *m/z* 190 (100%, M^+), 106 (65), 94 (72), 77 (24); **HRMS** (+EI) Found: M^+, 190.07391; $C_{10}H_{10}N_2O_2$ requires *M*, 190.07420.

4.1.3. Synthesis of epi-3,6-dithio-2,5-diketopiperazines

3,6-Dibromo-1,4-dimethyl-piperazine-2,5-dione[65] 67



$C_6H_8N_2O_2Br_2$

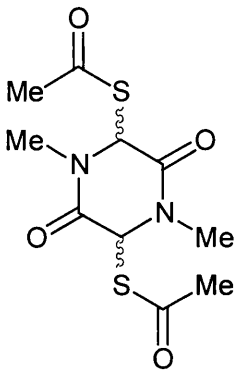
299.95 g.mol^{-1}

1,4-Dimethyl-piperazine-2,5-dione **66** (6.0 g, 42 mmol) was dissolved in *o*-dichlorobenzene (90 mL) and a solution of bromine (4.6 ml, 90 mmol) in *o*-dichlorobenzene (90 mL) was added to the vigorously stirred solution, at room temperature over 1 h. The resultant yellow suspension was heated at 170 °C for 3.5 h, cooled and the filtrate poured into light petroleum (bp 40–60 °C) (1000 ml).

After 2 days at 0 °C, the pale yellow crystals of the desired product **67** were filtered and used without further purification.

^{1}H NMR (300 MHz; $CDCl_3$): δ_H 5.85 (2 H, s, C*H*–Br), 2.92 (6 H, s, CH_3–N).

Thioacetic acid *S*-(5-acetylsulfanyl-1,4-dimethyl-3,6-dioxo-piperazin-2-yl) ester[65] 68



$C_{10}H_{14}N_2O_2S_2$

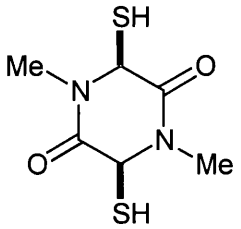
290.36 g.mol^{-1}

Potassium thioacetate (4.3 g, 38 mmol) in dimethylformamide (20 mL) was added dropwise over 45 minutes, to a stirred solution of 3,6-dibromo-1,4-dimethyl-piperazine-2,5-dione **67** (4.0 g, 13 mmol) in dichloromethane (50 mL), at 0 °C. After stirring at 0 °C for 30 minutes, the reaction mixture was poured onto ice and the organic phases were extracted with dichloromethane (2 x 50 mL), washed with water (30 mL), dried and evaporated at reduced pressure at a temperature not exceeding 40 °C. The residue was triturated with diethyl ether to give fine white needles of thioacetic acid *S*-(5-acetylsulfanyl-1,4-dimethyl-3,6-dioxo-piperazin-2-yl) ester **68** (4.3 g, 35%).

Mp 160–165 °C (decomposition), (lit.,[65] 205–208 °C); **IR** (KBr)/cm^{-1}: ν_{max} 1686 (C=O), 1690 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 5.70 (2 H, s, C*H*–S), 2.81 (6 H, s, CH_3–N), 2.41 (6 H, s, CH_3–CO); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 192.2 (S–

C=O), 164.0 (N–*C*=O), 62.9 (*C*H), 32.5 (*C*H_3–N), 30.8 (*C*H_3–CO); **LRMS** (+FAB) *m/z* 291 (63%, M^+), 215 (100, M^+ – $HSCOCH_3$), 173 (65), 141 (30), 43 (24).

Cis-3,6-dimercapto-1,4-dimethyl-piperazine-2,5-dione[65] 69a



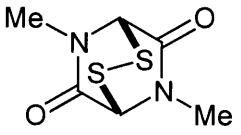
$C_6H_{10}N_2O_2S_2$

206.27 g.mol^{-1}

Thioacetic acid *S*-(5-acetylsulfanyl-1,4-dimethyl-3,6-dioxo-piperazin-2-yl) ester **68** (4.0 g, 13 mmol) was hydrolysed in refluxing ethanolic hydrogen chloride (100 mL) for 20 minutes. The solution was then evaporated and the residue triturated with a small volume of methanol to give the title compound **69a** as white crystals (2.8 g, 98%).

Mp 111–114 °C (lit.,[65] 110–113 °C); **IR** (KBr)/cm^{-1}: ν_{max} 2539 (S–H), 1681 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 4.92 (2 H, d, *J* 7.0 Hz, S*H*), 3.01 (6 H, s, CH_3), 2.41 (2 H, d, *J* 7.0 Hz, C*H*); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 165.4 (*C*=O), 59.9 (*C*H), 33.4 (*C*H_3); **LRMS** (+FAB) *m/z* 207 (21%, M^+), 173 (100, M^+ – SH), 141 (24, M^+– 2 SH), 112 (23), 42 (52).

5,7-Dimethyl-2,3-dithia-5,7-diaza-bicyclo[2.2.2]octane-6,8-dione[65] 70



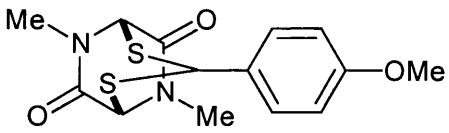
$C_6H_8N_2O_2S_2$

204.25 g.mol^{-1}

Water (150 mL) was added to a solution of *cis*-3,6-dimercapto-1,4-dimethyl-piperazine-2,5-dione **69a** (2.5 g, 12 mmol) in chloroform (600 mL). To this mixture, a solution of iodine (3.4 g, 13 mmol) and potassium iodide (9.0 g, 54 mmol) in water (60 mL) was added dropwise. The reaction was monitored visually, and the colour of the iodine disappeared on reaction with vigorous mixing of the two phases. When further addition of iodine produced permanent colouration, a few drops of sodium thiosulfate solution were added to remove the excess iodine. The chloroform phase was separated, dried over anhydrous magnesium sulfate and evaporated to give the desired product. Recrystallisation from acetonitrile afforded **70** as pale yellow prisms (1.8 g, 73%).

Mp 174–177 °C (lit.,[65] 184–185 °C); **IR** (KBr)/cm^{-1}: ν_{max} 1697 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 5.15 (2 H, s, C*H*), 3.04 (6 H, s, CH_3); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 164.5 (*C*=O), 67.7 (*C*H), 31.7 (*C*H_3); **LRMS** (+ES) *m/z* 226 (31%, MNa^+), 194 (100), 172 (93), 140 (43).

3-(4-Methoxy-phenyl)6,8-dimethyl-2,4-dithia-6,8-diaza-bicycla[3,2,2]nonane-7,9-dione[95] 212



$C_{14}H_{16}N_2O_3S_2$

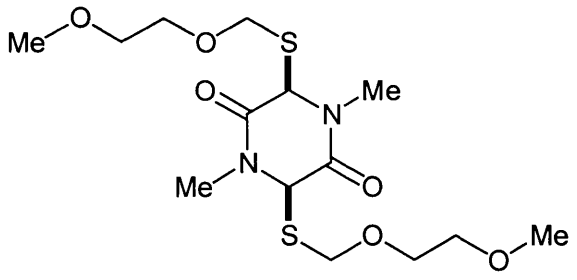
324.40 g.mol^{-1}

To a stirred solution of *cis*-3,6-dimercapto-1,4-dimethyl-piperazine-2,5-dione **69a** (600 mg, 2.90 mmol) and *para*-anisaldehyde (1.7 mL, 14 mmol) in dichloromethane was added boron trifluoride etherate (1.0 mL, 0.8 mmol). After stirring at room temperature for 12 hours, the solution was poured into a solution of saturated sodium bicarbonate solution. The aqueous layer was separated and thoroughly extracted with dichloromethane. The combined extracts were dried over

anhydrous magnesium sulfate, filtered and evaporated to give the crude product, which was washed with diethyl ether, to eliminate excess of *para*-anisaldehyde and became a white solid (900 mg, 95%).

Mp 268–269 °C (lit.,[95] 267–268 °C); **IR** (KBr)/cm^{-1}: ν_{max} 1676 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.53 (2 H, d, *J* 6.7 Hz, C*H*, Ph), 7.04 (2 H, d, *J* 6.7 Hz, C*H*, Ph), 5.33 (1 H, s, C*H*), 5.20 (1 H, s, C*H*–N), 4.93 (1 H, s, C*H*–N), 3.97 (3 H, s, CH_3–O), 3.37 (3 H, s, CH_3–N), 3.23 (3 H, s, CH_3–N); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 164.5 (*C*=O), 163.7 (*C*=O), 161.0 (*C*q–O, Ph), 130.9 (*C*H, Ph), 127.0 (*C*q–CH, Ph), 114.8 (*C*H, Ph), 66.4 (*C*H–N), 64.2 (*C*H–N), 55.7 (*C*H_3–O), 49.7 (*C*H–S), 32.9 (*C*H_3–N), 32.2 (*C*H_3–N); **LRMS** (+FAB) *m/z* 325 (67%, M^+), 205 (100), 173 (35).

3,6-*Bis*-methoxyethoxymethylsulfonyl-1,4-dimethyl-2,5-dioxopiperazine 213



$C_{14}H_{26}N_2O_6S_2$

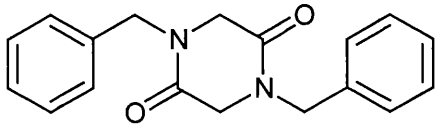
382.46 g.mol^{-1}

To an ice cold stirred solution of *cis*-3,6-dimercapto-1,4-dimethyl-piperazine-2,5-dione **69a** (600 mg, 2.90 mmol) in dry tetrahydrofuran (120 mL) was added potassium *tert*-butoxide (685 mg, 6.10 mmol), in one portion. After 1 minute, chloromethoxyethoxymethyl (2.7 mL, 24 mmol) was added and stirring continued for an additionnal 40 minutes. The reaction mixture was poured into water (20 mL). After separation, the aqueous layer was thoroughly extracted with dichloromethane (3 x 50 mL). The combined extracts were dried over anhydrous sodium sulfate, filtered and evaporated. The red residue was crystallised from

dichloromethane–methanol (10:1) and the white crystals (250 mg, 22%) of **213** obtained were washed with diethyl ether.

Mp 70–72 °C; **IR** (KBr)/cm^{-1}: ν_{max} 1670 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 4.89 (2 H, s, C*H*), 4.93 (4 H, ABq, *J* 11.7 Hz, CH_2), 3.90–3.95 (2 H, m, CH_2), 3.55–3.64 (6 H, m, CH_2), 3.26 (6 H, s, CH_3–O), 3.02 (6 H, s, CH_3–N); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 165.1 (*C*=O), 74.5 (*C*H_2), 71.4 (*C*H_2), 67.9 (*C*H_2), 62.3 (*C*H), 59.0 (*C*H_3–O), 32.0 (*C*H_3–N); **LRMS** (+FAB) *m/z* 383 (100%, M^+), 261 (78), 215 (82); **HRMS** (+CI) Found: M^+, 383.13148; $C_{14}H_{26}N_2O_6S_2$ requires *M*, 383.13105; **Microanalysis** Found: C, 43.68; H, 7.16; N, 7.24; S, 17.10%. $C_{14}H_{26}N_2O_6S_2$ requires C, 43.96; H, 6.85; N, 7.32; S, 16.77%.

1,4-Dibenzyl-piperazine-2,5-dione[146] 214



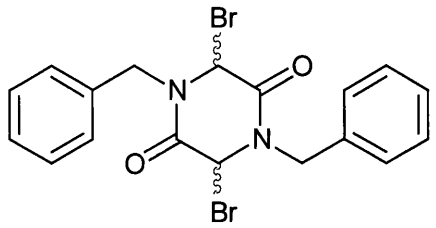
$C_{18}H_{18}N_2O_2$

294.35 g.mol^{-1}

A solution of piperazine-2,5-dione **167** (3.2 g, 27 mmol) in dimethylformamide (30 mL) was added to a stirred suspension of sodium hydride (2.4 g of a 60% dispersion in mineral oil, hexane washed, 61 mmol) in dimethylformamide (90 mL) at 0 °C. Once after the addition was complete, the mixture was allowed to warm to room temperature and stirred for one hour. Benzyl bromide (6.6 mL, 56 mmol) was then added dropwise at room temperature and the reaction mixture stirred for 16 hours. All volatiles were removed at reduced pressure and the solid residue washed with water (200 mL). Purification by recrystallisation (ethanol) gave 1,4-dibenzyl-piperazine-2,5-dione **214** as a white crystalline solid (5.4 g, 66%).

Mp 181–182 °C (lit.,[146] 166–168 °C); **IR** (KBr)/cm^{-1}: ν_{max} 1666 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.28–7.37 (10 H, m, C*H*, Ph), 4.60 (4 H, s, CH_2–Ph), 3.95 (4 H, s, CH_2–CO); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 163.1 (*C*=O), 124.8 (*C*q), 128.8 (*C*H, Ph), 128.4 (*C*H, Ph), 128.1 (*C*H, Ph), 49.2 (*C*H_2), 49.1 (*C*H_2); **LRMS** (+EI) *m/z* 294 (100%, M^+), 91 (97), 65 (57).

1,4-Dibenzyl-3,6-dibromo-piperazine-2,5-dione[146] 215



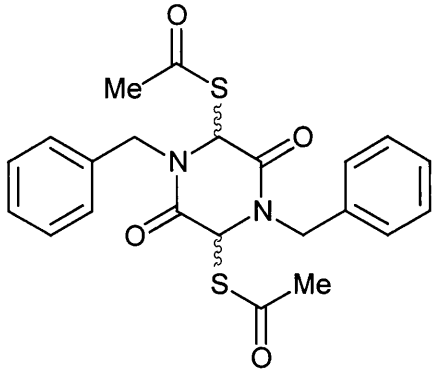
$C_{18}H_{16}N_2O_2Br_2$

452.14 g.mol^{-1}

Benzoyl peroxide (8 mg, 30 μmol) was added to a solution of 1,4-dibenzyl-piperazine-2,5-dione **214** (1.0 g, 3.5 mmol) and *N*-bromosuccinimide (1.3 g, 7.4 mmol) in carbon tetrachloride (35 mL) at reflux. When the addition was complete, heating was continued for 2 hours. After cooling, the reaction mixture was filtered and the solid residue washed with carbon tetrachloride (20 mL). All volatiles were removed at reduced pressure to give 1,4-dibenzyl-3,6-dibromo-piperazine-2,5-dione **215** as a light yellow oil (1.5 g, 97%), which was used immediately without further purification due to its instability.

^{1}H NMR (300 MHz; $CDCl_3$): δ_H 7.47–7.60 (10 H, m, C*H*, Ph), 6.08 (2 H, s, C*H*), 5.53 (2 H, d, *J* 15.0 Hz, CH_2), 4.23 (2 H, d, *J* 15.0 Hz, CH_2).

Thioacetic acid S-(5-acetylsulfanyl-1,4-dibenzyl-3,6-dioxo-piperazin-2-yl) ester[146]



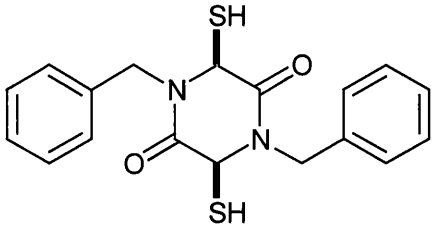
$C_{22}H_{22}N_2O_4S_2$

442.55 g.mol^{-1}

Potassium thioacetate (757 mg, 6.63 mmol) was added to a stirred solution of 1,4-dibenzyl-3,6-dibromo-piperazine-2,5-dione **219** (1.5 g, 3.3 mmol) in dichloromethane (40 mL) at 0 °C. The resulting solution was allowed to warm to room temperature and stirred for 72 hours. After filtration, the solvent was removed at reduced pressure. Purification of the crude product by recrystallisation (ethyl acetate/ hexane) gave the thioacetic acid *S*-(5-acetylsulfanyl-1,4-dibenzyl-3,6-dioxo-piperazin-2-yl) ester as a white solid (778 mg, 53%).

Mp 181–183 °C, (lit.,[146] 179–181 °C); **IR** (KBr)/cm^{-1}: ν_{max} 1717 (C=O), 1694 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.28–7.37 (10 H, m, C*H*, Ph), 5.89 (2 H, s, C*H*), 5.02 (2 H, d, *J* 15.0 Hz, CH_2), 4.03 (2 H, d, *J* 15.0 Hz, CH_2), 2.45 (6 H, s, CH_3); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 192.0 (*C*=O), 164.2 (*C*=O), 135.3 (*C*H, Ph), 129.2 (*C*H, Ph), 128.9 (*C*H, Ph), 128.6 (*C*H, Ph), 60.7 (*C*H), 48.4 (*C*H$_2$), 30.8 (CO*C*H$_3$); **LRMS** (+EI) *m/z* 443 (28%, M$^+$), 367 (80), 325 (50), 91 (100), 43 (35).

1,4-Dibenzyl-*cis*-3,6-dimercapto-piperazine-2,5-dione[146] 216



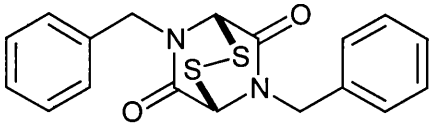
$C_{18}H_{18}N_2O_2S_2$

358.48 g.mol^{-1}

The thioacetic acid *S*-(5-acetylsulfanyl-1,4-dibenzyl-3,6-dioxo-piperazin-2-yl) ester (750 mg, 1.69 mmol) was hydrolysed in refluxing ethanolic hydrogen chloride (25 mL) for one hour. The solution was then evaporated and the crude product purified by recrystallisation (ethyl acetate/ hexane) to give 1,4-dibenzyl-3,6-dimercapto-piperazine-2,5-dione **216** as a white solid (413 mg, 68%).

Mp 155–157 °C (lit.,[146] not specified); **IR** (KBr)/cm^{-1}: ν_{max} 2507 (S–H), 1652 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.16–7.31 (10 H, m, C*H*, Ph), 5.17 (2 H, d, *J* 14.7 Hz, CH_2), 4.88 (2 H, d, *J* 6.9 Hz, S*H*), 4.08 (2 H, d, *J* 14.7 Hz, CH_2), 2.98 (2 H, d, *J* 6.9 Hz, C*H*); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 165.1 (*C*=O), 134.5 (*C*q, Ph), 129.5 (*C*H, Ph), 129.0 (*C*H, Ph), 128.9 (*C*H, Ph), 56.2 (*C*H), 47.8 (*C*H_2); **LRMS** (+FAB) *m/z* 359 (18%, M^+), 327 (100, M^+ – SH), 292 (31, M^+– 2 SH), 91 (52).

5,7-Dibenzyl-2,3-dithia-5,7-diaza-bicyclo[2.2.2]octane-6,8-dione[146] 217



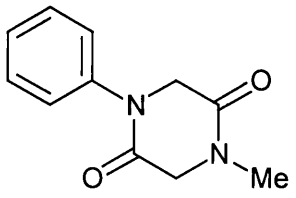
$C_{18}H_{16}N_2O_2S_2$

356.46 g.mol^{-1}

A solution of iodine (290 mg, 1.16 mmol) in chloroform (20 mL) was added to a stirred solution of 1,4-dibenzyl-*cis*-3,6-dimercapto-piperazine-2,5-dione **216** (410 mg, 1.14 mmol) in chloroform (25 mL) at room temperature. After stirring for one hour, the reaction mixture was poured into a solution of saturated sodium bicarbonate solution (100 mL) containing sodium thiosulfate (2 g) and stirring was continued until the colour, due to iodine, was disappeared. After separation, the aqueous layer was extracted with dichloromethane (2 x 100 mL) and the combined extracts were dried over anydrous magnesium sulfate, filtered and the solvent removed under reduced pressure. The crude product was purified by recrystallisation (ethanol) to give 5,7-dibenzyl-2,3-dithia-5,7-diaza-bicyclo[2.2.2]octane-6,8-dione **217** as a yellow crystalline solid (250 mg, 62%)

Mp 166–188 °C, (lit.,[146] 156–158 °C); **IR** (KBr)/cm^{-1}: ν_{max} 1678 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.40–7.54 (10 H, m, C*H*, Ph), 5.39 (2 H, s, C*H*), 4.99 (2 H, d, *J* 15.0 Hz, CH_2), 4.63 (2 H, d, *J* 15.0 Hz, CH_2); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 164.1 (*C*=O), 134.5 (*C*q, Ph), 129.5 (*C*H, Ph), 129.0 (*C*H, Ph), 128.8 (*C*H, Ph), 65.1 (*C*H), 48.1 (*C*H_2); **LRMS** (+EI) *m/z* 356 (10%, M^+), 292 (63), 91 (100).

1-Methyl-4-phenyl-piperazine-2,5-dione 218



$C_{11}H_{12}N_2O_2$

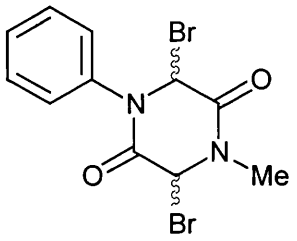
204.23 g.mol^{-1}

Sodium hydride (170 mg of a 60% dispersion in mineral oil, 4.22 mmol) was added portionwise to a stirred suspension of 1-phenyl-piperazine-2,5-dione **209** (730 g, 3.84 mmol) in tetrahydrofuran (80 mL) at 0 °C. The resulting mixture was allowed to warm to room temperature and heated at reflux for 2.5 hours. The solution was

cooled to 0 °C and methyl iodide (1.0 mL, 15.3 mmol) was added. The resulting solution was allowed to warm to room temperature and stirred overnight. All volatiles were removed under reduced pressure and the residue purified by column chromatography (silica, dichloromethane–methanol, 9:1) to give 1-methyl-4-phenyl-piperazine-2,5-dione **218** as a white solid (750 mg, 96%);

Mp 140–142 °C; **IR** (KBr)/cm^{-1}: ν_{max} 1655 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.11–7.37 (5 H, m, C*H*, Ph), 4.26 (2 H, s, CH_2), 4.06 (2 H, s, CH_2), 2.97 (3 H, s, CH_3); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 164.0 (*C*=O), 163.8 (*C*=O), 140.0 (*C*q, Ph), 129.8 (*C*H, Ph), 127.9 (*C*H, Ph), 125.5 (*C*H, Ph), 53.0 (CH_2), 52.8 (CH_2), 33.7 (CH_3); **LRMS** (+EI) *m/z* 204 (100%, M^+), 94 (96); **HRMS** (+EI) Found: M^+, 204.08969; $C_{11}H_{12}N_2O_2$ requires *M*, 204.08990.

3,6-Dibromo-1-methyl-4-phenyl-piperazine-2,5-dione 219



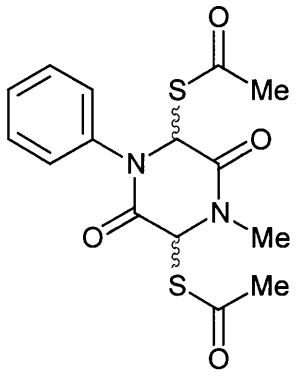
$C_{11}H_{10}Br_2N_2O_2$

362.02 g.mol^{-1}

N-Bromosuccinimide (1.3 g, 7.3 mmol) and benzoylperoxide (20 mg, 85 μmol) were added to a stirred solution of 1-methyl-4-phenyl-piperazine-2,5-dione **218** (500 mg, 2.44 mmol) in carbon tetrachloride (50 mL) at room temperature. The reaction mixture was heated at reflux for 3 hours, cooled, filtered to remove the succinimide. The filtrate was evaporated at reduced pressure and the crude 3,6-dibromo-1-methyl-4-phenyl-piperazine-2,5-dione **219** was used without further purification due to its instability.

^{1}H NMR (300 MHz; $CDCl_3$): δ_H 7.33–7.52 (5 H, m, C*H*, Ph), 6.26 (1 H, s, C*H*–Br), 6.14 (1 H, s, C*H*–Br), 3.10 (3 H, s, C*H*$_3$–N).

Thioacetic acid *S*-(5-acetylsulfanyl-1-methyl-3,6-dioxo-4-phenyl-piperazin-2-yl) ester 220



$C_{15}H_{16}N_2O_4S_2$

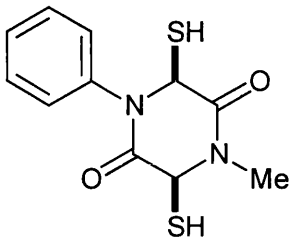
352.40 g.mol^{-1}

Potassium thioacetate (840 mg, 7.34 mmol) was added to a stirred solution of crude 3,6-dibromo-1-methyl-4-phenyl-piperazine-2,5-dione **219**, in dichloromethane (25 mL), at 0 °C. Stirring was continued, at 0 °C, for 30 minutes and the reaction mixture then poured onto ice. The mixture was extracted with dichloromethane (2 x 15 mL), and the combined extracts washed with water (15 mL), dried and evaporated at reduced pressure, at temperatures not exceeding 40 °C. The brown residue was triturated with diethyl ether to give fine white needles of thioacetic acid *S*-(5-acetylsulfanyl-1-methyl-3,6-dioxo-4-phenyl-piperazin-2-yl) ester **220** (200 mg, 23%).

Mp 144–148 °C; **IR** (KBr)/cm^{-1}: ν_{max} 1757 (C=O), 1687 (C=O), 1686 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.17–7.60 (5 H, m, C*H*, Ph), 6.13 (1 H, s, C*H*–S), 5.89 (1 H, s, C*H*–S), 3.01 (3 H, s, C*H*$_3$–N), 2.51 (3 H, s, C*H*$_3$–CO), 2.29 (3 H, s, C*H*$_3$–CO); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 191.6 (S–*C*=O), 190.0 (S–*C*=O), 164.0 (N–*C*=O), 163.1 (N–*C*=O), 137.1 (*C*q, Ph), 129.5 (*C*H, Ph), 128.7 (*C*H, Ph), 126.9 (*C*H, Ph), 63.4 (*C*H), 63.0 (*C*H), 32.3 (*C*H$_3$–N), 30.5 (*C*H$_3$–CO), 30.2 (*C*H$_3$–CO);

LRMS (+FAB) *m/z* 352 (4%, M^+), 309 (46, M^+ – Ac), 277 (100, M^+ – SAc), 235 (98, M^+ – SAc – Ac), 104 (59), 77 (70), 43 (81), 28 (56); **HRMS** (+FAB) Found: M^+, 352.05532; $C_{15}H_{16}N_2O_4S_2$ requires *M*, 352.05515.

Cis-3,6-dimercapto-1-methyl-4-phenyl-piperazine-2,5-dione 221



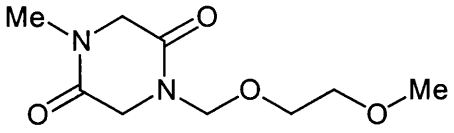
$C_{11}H_{12}N_2O_2S_2$

268.36 $g.mol^{-1}$

The thioacetic acid *S*-(5-acetylsulfanyl-1-methyl-3,6-dioxo-4-phenyl-piperazin-2-yl) ester **220** (100 mg, 0.28 mmol) was hydrolysed in refluxing ethanolic hydrogen chloride (2.5 mL) for one hour. The solution was then evaporated and the crude product triturated with a small amount of methanol to give *cis*-3,6-dimercapto-1-methyl-4-phenyl-piperazine-2,5-dione **221** as a white solid (72 mg, 94%).

Mp 140–142 °C; **IR** (KBr)/cm^{-1}: ν_{max} 2521 (S–H), 1678 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.20–7.49 (5 H, m, C*H*, Ph), 5.40 (1 H, d, *J* 5.9 Hz, S*H*), 5.10 (1 H, d, *J* 7.5 Hz, S*H*), 3.36 (1 H, d, *J* 7.5 Hz, C*H*), 3.15 (1 H, d, *J* 5.9 Hz, C*H*), 3.12 (3 H, s, C*H*$_3$–N); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 164.6 (*C*=O), 164.3 (*C*=O), 137.7 (*C*q, Ph), 129.6 (*C*H, Ph), 128.9 (*C*H, Ph), 127.1 (*C*H, Ph), 60.2 (*C*H), 59.7 (*C*H), 32.6 (*C*H$_3$); **LRMS** (+EI) *m/z* 268 (4%, M^+), 235 (100, M^+ – SH), 202 (35, M^+ – 2 SH), 174 (28), 138 (31), 104 (48), 77 (52); **HRMS** (+FAB) Found: M^+, 268.03768; $C_{11}H_{12}N_2O_2S_2$ requires *M*, 268.03347.

1-(2-Methoxy-ethoxymethyl)-4-methyl-piperazine-2,5-dione 222



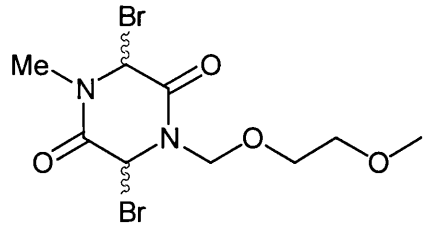
$C_9H_{16}N_2O_4$

216.21 g.mol^{-1}

To a stirred solution of 1-methyl-piperazine-2,5-dione **96** (1.0 g, 7.8 mmol) in *tert*–butanol (20 mL) was added a solution of 0.9 N potassium *tert*–butoxide in *tert*–butanol (17 mL, 15 mmol) over a period of 2 minutes, at room temperature. After the addition was complete, chloromethyl ethoxy methyl ether (1.8 mL, 15 mmol) was added and stirring was continued for 10 minutes. After the solution was treated with acetic acid (1.0 mL), the solvent was removed at reduced pressure and the residue was taken up in a dilute solution of sodium chloride (20 mL) and thoroughly extracted with dichloromethane (3 x 20 mL). The combined extracts were dried over anhydrous sodium sulfate, filtered and evaporated to give an oil, which was distilled at reduced pressure to yield 1-(2-methoxy-ethoxymethyl)-4-methyl-piperazine-2,5-dione **222** (100 mg, 6%).

Bp 220 °C, 0.1 mm Hg; **IR** (thin film)/cm^{-1}: ν_{max} 1666 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 5.00 (2 H, s, N–C*H*$_2$–O), 4.16 (2 H, s, C*H*$_2$–CO), 4.11 (2 H, s, C*H*$_2$–CO), 3.59–3.76 (4 H, m, C*H*$_2$), 3.46 (3 H, s, C*H*$_3$–O), 3.08 (3 H, s, C*H*$_3$–N); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 164.7 (*C*=O), 164.1 (*C*=O), 75.3 (*C*H$_2$), 72.0 (*C*H$_2$), 68.7 (*C*H$_2$), 59.3 (*C*H$_3$–O), 51.8 (*C*H$_2$–CO), 48.4 (*C*H$_2$–CO), 33.5 (*C*H$_3$–N); **LRMS** (+CI) *m/z* 217 (100%, M$^+$), 186 (59, M$^+$ – OCH_3), 141 (37, M$^+$ – $OCH_2CH_2OCH_3$), 113 (44); **HRMS** (+CI) Found: M$^+$, 217.11890; $C_9H_{16}N_2O_4$ requires *M*, 217.11880.

Attempted synthesis of 3,6-dibromo-1-(2-methoxy-ethoxymethyl)-4-methyl-piperazine-2,5-dione 223

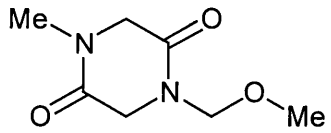


$C_9H_{14}Br_2N_2O_4$

374.03 g.mol^{-1}

Benzoyl peroxide (8 mg, 30 μmol) was added to a solution of 1-(2-methoxy-ethoxymethyl)-4-methyl-piperazine-2,5-dione **222** (80 mg, 0.37 mmol) and *N*-bromosuccinimide (164 mg, 0.92 mmol) in carbon tetrachloride (10 mL) at reflux. When the addition was complete, heating was continued for 1 hour. After cooling, the reaction mixture was filtered and the solid residue washed with carbon tetrachloride (10 mL). All volatiles were removed at reduced pressure to give a yellow oil as the crude product, which, on analysis by ^{1}H NMR, revealed that bromination occured on both the 2,5-diketopiperazine and the protecting group.

1-Methoxymethyl-4-methyl-piperazine-2,5-dione[95] 224



$C_7H_{12}N_2O_3$

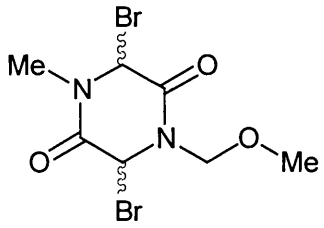
172.16 g.mol^{-1}

To a stirred solution of 1-methyl-piperazine-2,5-dione **96** (1.0 g, 7.8 mmol) in *tert*–butanol (20 mL) was added a solution of 0.9 N potassium *tert*–butoxide in *tert*–butanol (11 mL, 10 mmol) over a period of 2 minutes, at room temperature. After the addition was complete, chloromethyl methyl ether (750 μL, 10.1 mmol) was added and stirring was continued for 10 minutes. After the solution was treated

with acetic acid (1.0 mL), the solvent was removed at reduced pressure and the residue was taken up in a dilute solution of sodium chloride (15 mL) and thoroughly extracted with dichloromethane (3 x 15 mL). The combined extracts were dried over anhydrous sodium sulfate, filtered and evaporated to give **224** an orange oil (900 mg, 67%).

IR (thin film)/cm^{-1}: ν_{max} 1651 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 4.69 (2 H, s, C*H*$_2$–O), 3.92 (4 H, s, C*H*$_2$–CO), 3.25 (3 H, s, C*H*$_3$–O), 2.83 (3 H, s, C*H*$_3$–N); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 164.3 (*C*=O), 163.6 (*C*=O), 76.0 (*C*H$_2$–O), 56.3 (*C*H$_3$–O), 51.7 (*C*H$_2$–CO), 48.0 (*C*H$_2$–CO), 33.1 (*C*H$_3$–N); **LRMS** (+EI) *m/z* 172 (67%, M^+), 157 (100, $M^+ - CH_3$), 141 (59, $M^+ - OCH_3$), 127 (57, $M^+ - CH_2OCH_3$), 113 (80), 45 (100).

Attempted synthesis of 3,6-dibromo-1-methoxymethyl-4-methyl-piperazine-2,5-dione[95] 225

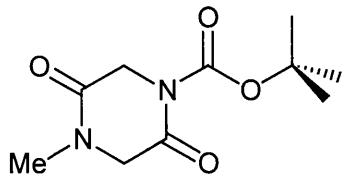


$C_7H_{10}Br_2N_2O_3$

329.97 $g.mol^{-1}$

Benzoyl peroxide (100 mg, 0.40 mmol) was added to a solution of 1-(2-methoxy-ethoxymethyl)-4-methyl-piperazine-2,5-dione **224** (700 mg, 4.06 mmol) and *N*-bromosuccinimide (1.8 g, 10 mmol) in carbon tetrachloride (85 mL) at reflux. When the addition was complete, heating was continued for 2 hours. After cooling, the reaction mixture was filtered and the solid residue washed with carbon tetrachloride (50 mL). All volatiles were removed at reduced pressure to give a yellow oil, which, on analysis by ^{1}H NMR, showed that bromination occured on both the 2,5-diketopiperazine and the protecting group.

4-Methyl-2,5-dioxo-piperazine-1-carboxylic acid *tert*-butyl ester 226



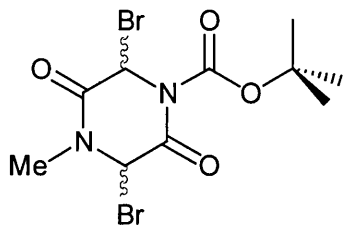
$C_{10}H_{16}N_2O_4$

228.11 g.mol^{-1}

To a solution of 1-methyl-piperazine-2,5-dione **96** (1.0 g, 7.8 mmol) and triethylamine (1.1 mL, 7.8 mmol) in dichloromethane (50 mL) at 0 °C, was added di-*tert*-butyldicarbonate (1.7 g, 7.8 mmol) and 4-dimethylaminopyridine (10 mg, 78 μmol) and the reaction mixture was stirred at room temperature for 12 hours. The solvent was then removed at reduced pressure and the crude product purified by column chromatography (silica, dichloromethane–methanol, 100:1) to yield a white solid (1.2 g, 67%).

Mp 127–129 °C; R_f (CH_2Cl_2) 0.65; **IR** (KBr)/cm^{-1}: ν_{max} 1748 (C=O), 1701 (C=O), 1643 (C=O); ^{1}**H NMR** (300 MHz; $CDCl_3$): δ_H 4.21 (2 H, s, C*H*$_2$), 3.97 (2 H, s, C*H*$_2$), 2.97 (3 H, s, C*H*$_3$–N), 1.46 (9 H, s, C*H*$_3$–C); ^{13}**C NMR** (75 MHz; $CDCl_3$): δ_C 164.6 (*C*=O), 164.5 (*C*=O), 150.1 (*C*=O), 85.0 (*C*q), 53.7 (*C*H$_2$), 48.4 (*C*H$_2$), 33.4 (*C*H$_3$–N), 28.2 (*C*H$_3$–C); **LRMS** (+FAB) *m/z* 229 (10%, M$^+$), 173 (17), 129 (100), 57 (66). **Microanalysis** Found: C, 52.66; H, 7.24; N, 12.22%. $C_{10}H_{16}N_2O_4$ requires C, 52.62; H, 7.07; N, 12.27%.

2,5-Dibromo-4-methyl-3,6-dioxo-piperazine-1-carboxylic acid *tert*-butyl ester 227



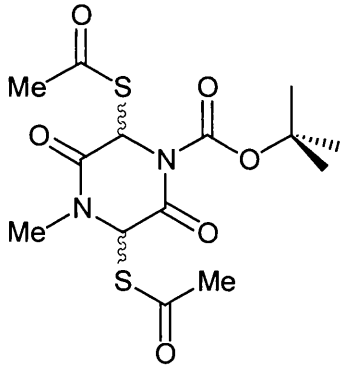
$C_{10}H_{14}N_2O_4Br_2$

386.01 g.mol^{-1}

N-Bromosuccinimide (1.8 g, 10 mmol) and benzoyl peroxide (37 mg, 0.1 mmol) were added to a stirred solution 4-methyl-2,5-dioxo-piperazine-1-carboxylic acid *tert*-butyl ester **226** (1.0 g, 4.3 mmol) in carbon tetrachloride (50 mL) at room temperature. The reaction mixture was heated, at reflux for 3 hours, cooled and filtered to remove the succinimide. The filtrate was evaporated at reduced pressure and the crude product **227** obtained was used without further purification due to its instability.

^{1}H NMR (300 MHz; $CDCl_3$): δ_H 6.58 (1 H, s, C*H*–Br), 6.00 (1 H, s, C*H*–Br), 2.99 (3 H, s, CH_3–N), 1.51 (9 H, s, CH_3–C).

2,5-*Bis*-acetylsulfanyl-4-methyl-3,6-dioxo-piperazine-1-carboxylic acid *tert*-butyl ester 228



$C_{14}H_{20}N_2O_6S_2$

376.45 g.mol^{-1}

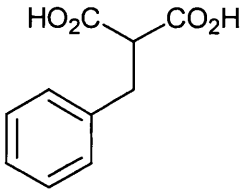
Potassium thioacetate (1.4 g, 12 mmol) in dimethylformamide (1 mL) was added, dropwise, over 45 minutes, to a stirred solution of the crude 2,5-dibromo-4-methyl-3,6-dioxo-piperazine-1-carboxylic acid *tert*-butyl ester **227** in dichloromethane (50 mL), at 0 °C. Stirring was continued at 0 °C for 30 minutes then the reaction mixture was poured onto ice. The mixture was extracted with dichloromethane (2 x 50 mL), and the combined extracts were washed with water (30 mL), dried and evaporated at reduced pressure, at temperatures not exceeding 40 °C. The brown residue was recrystallised from diethyl ether to give fine white needles of 2,5-*bis*-

acetylsulfanyl-4-methyl-3,6-dioxo-piperazine-1-carboxylic acid *tert*-butyl ester **228** (135 mg, 10%).

Mp 130–132 °C; **IR** (KBr)/cm^{-1}: ν_{max} 1741 (C=O), 1710 (C=O), 1680 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 6.47 (1 H, s, C*H*–S), 5.91 (1 H, s, C*H*–S), 2.93 (3 H, s, C*H*$_3$–N), 2.48 (3 H, s, C*H*$_3$–CO), 2.44 (3 H, s, C*H*$_3$–CO), 1.51 (9 H, s, C*H*$_3$–Cq); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 191.6 (S–*C*=O), 189.4 (S–*C*=O), 164.0 (N–*C*=O), 162.8 (N–*C*=O), 148.3 (O–*C*=O), 86.2 (*C*q), 63.7 (*C*H), 57.4 (*C*H), 31.8 (*C*H$_3$–N), 30.4 (*C*H$_3$–CO), 30.3 (*C*H$_3$–CO), 27.7 (*C*H$_3$–Cq); **LRMS** (+FAB) *m/z* 377 (19%, M^+), 277 (100, M^+ – Boc), 201 (78, M^+ – Boc – SAc); **HRMS** (+CI) Found: M^+, 377.08220; $C_{14}H_{20}N_2O_6S_2$ requires *M*, 377.08410 (M + H).

4.1.4. Synthesis of oxygenated 2,5-diketopiperazines

2-Benzyl-malonic acid[219] 265



$C_{10}H_{10}O_4$

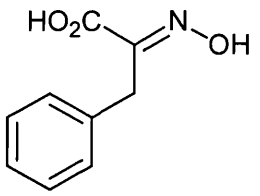
194.16 g.mol^{-1}

An aqueous solution of 1.7 M potassium hydroxide (5 mL) was added to a stirred solution of 2-benzyl-malonic acid diethyl ester **264** (10.0 mL, 42.5 mmol) in ethanol (13 mL) at room temperature. After stirring for 12 hours, water (10 mL) was added and the mixture washed with diethyl ether (15 mL). After acidification with concentrated hydrochloric acid to pH 1, the mixture was extracted with dichloromethane (3 x 20 mL). The combined organic extracts were dried over

anhydrous magnesium sulfate, filtered and the solvent removed at reduced pressure to yield a white solid (4.6 g, 60%).

Mp 117–120 °C (lit.,[219] 120 °C); **IR** (KBr)/cm^{-1}: ν_{max} 3130 (O–H), 1757 (C=O); **^{1}H NMR** (300 MHz; DMSO-d$_6$): δ_H 7.15–7.28 (5 H, m, C*H*, Ph), 3.59 (1 H, t, *J* 7.8 Hz, C*H*), 3.02 (2 H, d, *J* 7.8 Hz, CH_2); **^{13}C NMR** (75 MHz; DMSO-d$_6$): δ_C 170.1 (*C*=O), 138.4 (*C*q, Ph), 128.6 (*C*H, Ph), 128.1 (*C*H, Ph), 126.2 (*C*H, Ph), 53.2 (*C*H) 34.1 (*C*H2); **LRMS** (+CI) *m/z* 195 (6%, M$^+$), 134 (35), 90 (77), 41 (100).

2-Hydroxyimino-3-phenyl-propionic acid[156] 266



$C_9H_9NO_3$

179.15 g.mol^{-1}

Method A:

To a stirred solution of 2-benzyl-malonic acid **265** (2.8 g, 14 mmol) in diethyl ether (17 mL) in an ice-salt bath was added *n*-butyl nitrite (3.6 mL, 30 mmol) and dry hydrochloric acid was then slowly bubbled into the solution. The reaction mixture became red and after 30 minutes, the solvent was removed at reduced pressure, cold water (10 mL) was added and the precipitate formed filtered off. The white product (540 mg, 21%) was recrystallised from ethanol and washed with dichloromethane.

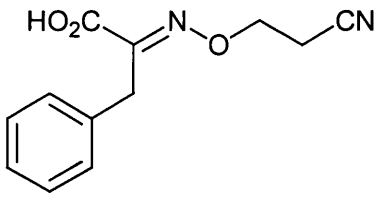
Method B:

To a cooled (10 °C) and stirred solution of sodium (2.3 g, 0.1 mol) in absolute ethanol (50 mL) was added 2-benzyl-malonic acid diethyl ester **264** (25.0 g, 0.11 mol), followed by *n*-butyl nitrite (11.6g, 0.11 mol), over a period of 15 minutes,

with vigorous stirring. After the addition was complete, stirring was continued at room temperature for 30 minutes and then ethanol and butanol were removed at reduced pressure. Crushed ice (25 g) was added to the residue and the solution was acidified with 6 N sulfuric acid and extracted with diethyl ether. The combined ethereal layers were thoroughly extracted with 10% sodium hydroxide solution, which was warmed at 80 °C for 15 minutes to ensure saponification of the oximino ester. To obtain the free oximino acid, the solution was then chilled and carefully acidified with concentrated hydrochloric acid until pH 1. The precipitated acid **266** was filtered and washed with dichloromethane to yield a white solid (14 g, 78%).

Mp 164–169 °C (lit.,[156] 165 °C); **IR** (KBr)/cm^{-1}: ν_{max} 3216 (O–H), 3073 (O–H), 1696 (C=O), 1651 (C=N); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.24–7.36 (5 H, m, C*H*, Ph), 4.03 (2 H, s, CH_2); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 167.2 (*C*=O), 152.5 (*C*=N), 138.3 (*C*q, Ph), 130.3 (*C*H, Ph), 129.7 (*C*H, Ph), 127.7 (*C*H, Ph), 31.3 (CH_2); **LRMS** (+ES) *m/z* 201 (100%, MNa$^+$).

2-(2-Cyano-ethoxyimino)-3-phenyl-propionic acid[153] 267



$C_{12}H_{12}N_2O_3$

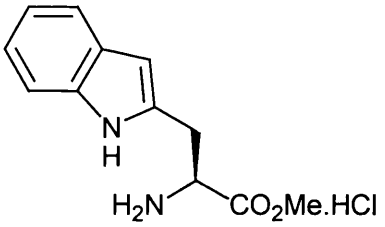
232.24 g.mol^{-1}

To a cooled (10 °C) and stirred solution of 2-hydroxyimino-3-phenyl-propionic acid **266** (1.5 g, 8.3 mmol) in dimethylformamide (4 mL) was added 1 N sodium hydroxide solution (10 mL). Acrylonitrile (661 µL, 10.0 mmol) was then added to the resulting clear solution and the mixture allowed to warm to room temperature and stirred for 5 hours. After cooling to 10 °C, 3 N concentrated hydrochloric acid was added to adjust pH to 3–4. Dilution with a mixture of ethyl acetate (10 mL)

and 10% aqueous sodium hydroxide (5 mL), followed by extraction with ethyl acetate (2 x 10 mL) afforded the combined organic extratcts, which were subsequently washed with 10% aqueous sodium hydroxide (3 x 5 mL, pH = 3) to give a clear organic layer. Evaporation, after drying over anhydrous magnesium sulfate and filtration, gave **267** as a white solid (1.3 g, 65%).

Mp 179–181 °C (lit.,[153] not specified);; **IR** (KBr)/cm^{-1}: ν_{max} 3055 (O–H), 1718 (C=O), 1647 (C=N); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.18–7.27 (5 H, m, C*H*, Ph), 4.63 (2 H, t, *J* 6.1 Hz, CH_2–O), 3.95 (2 H, s, CH_2–Ph), 2.76 (2 H, t, *J* 6.1 Hz, CH_2–C≡N); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 163.7 (*C*=O), 152.3 (*C*=N), 134.9 (*C*q, Ph), 129.5 (*C*H, Ph), 129.1 (*C*H, Ph), 127.4 (*C*H, Ph), 117.1 (*C*≡N), 70.3 (CH_2–O), 31.3 (CH_2–Ph), 18.8 (CH_2–C≡N); **LRMS** (+EI) *m/z* 232 (3%, M^+), 144 (44), 77 (55), 51 (87), 39 (92).

L-Tryptophan methyl ester hydrochloride[221] **269**



$C_{12}H_{14}N_2O_2.HCl$

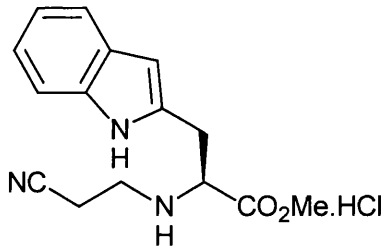
254.70 g.mol^{-1}

Thionyl chloride (6.1 mL, 83 mmol) was carefully added dropwise to a stirred suspension of L-tryptophan **268** (11.4 g, 55.8 mmol) in methanol (100 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 24 hours, after which the solution was evaporated at reduced pressure to give L-tryptophan methyl ester hydrochloride **269** (14.0 g, quant.).

Mp 198–200 °C (lit.,[221] 218–220 °C); $[\alpha]_D^{25}$ + 18.5 (*c.* 1 in MeOH) [lit.,[221] $[\alpha]_D^{20}$ + 18.0 (*c.* 1 in MeOH)]; **IR** (KBr)/cm^{-1}: ν_{max} 3279 (N–H), 2985 (N–H) 1751 (C=O);

^{1}H NMR (300 MHz; D_2O): δ_H 7.63–7.72 (2 H, m, C*H* arom.), 7.27–7.42 (3 H, m, C*H* arom.), 4.55 (1 H, t, *J* 6.1 Hz, C*H*), 3.90 (3 H, s, CH_3), 3.52 (2H, d, *J* 6.1 Hz, CH_2); **^{13}C NMR** (75 MHz; D_2O): δ_C 170.9 (*C*=O), 136.8 (*C*q arom.), 126.9 (*C*H arom.), 125.8 (*C*H arom.), 122.7 (*C*H arom.), 120.0 (*C*H arom.), 118.5 (*C*H arom.), 112.5 (*C*H arom.), 106.5 (*C*q arom.), 54.1 (*C*H), 53.8 (CH_3), 26.1 (CH_2); **LRMS** (+FAB) *m/z* 219 (100%, M^+ – HCl), 202 (44), 130 (92).

_N_-2-Cyanoethyl-L-tryptophan methyl ester hydrochloride[153] 259



$C_{15}H_{16}N_3O_2$

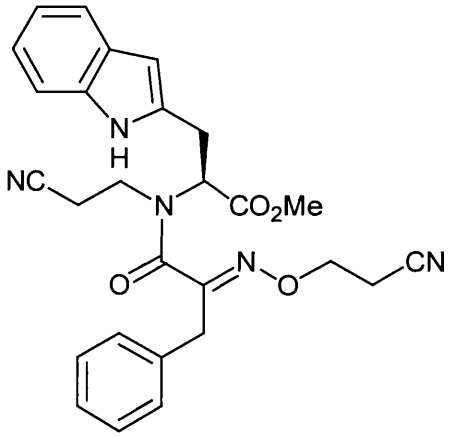
271.30 g.mol^{-1}

Triethylamine (0.7 mL, 5.1 mmol) was added to a stirred solution of L-tryptophan methyl ester hydrochloride **269** (1.3 g, 5.1 mmol) in methanol (10 mL) under nitrogen, to form a clear solution. Acrylonitrile (1.7 mL, 25 mmol) was then added slowly at room temperature and the reaction mixture refluxed for 2 hours then cooled and stirred at room temperature overnight. On concentrating the mixture at reduced pressure, at temperatures not exceeding 35 °C, a precipitate formed gradually. After filtration, the solid was suspended in isopropanol and acidified with concentrated hydrochloric acid and the mixture maintained under 10 °C for 1 hour. The crystals **259** (1.34 g, 85%) were filtered, washed with isopropanol and dried at reduced pressure.

Mp 154–155 °C (lit.,[153] 178–180 °C); $[\alpha]_D^{20}$ + 42.6 (*c.* 1 in MeOH) (lit.,[153] not specified); **IR** (KBr)/cm^{-1}: ν_{max} 3378 (N–H), 1762 (C=O); **^{1}H NMR** (300 MHz; D_2O): δ_H 7.53–7.59 (2 H, m, C*H* arom.), 7.18–7.33 (3 H, m, C*H* arom.), 4.46 (1 H,

t, *J* 6.1 Hz, C*H*), 3.53 (2 H, dd, *J* 2.7 and 6.1 Hz, CH_2), 3.46 (2 H, t, *J* 6.8 Hz, CH_2), 2.96 (2H, t, *J* 6.8 Hz, CH_2); **^{13}C NMR** (75 MHz; D_2O): δ_C 169.8 (*C*=O), 136.6 (*C*q arom.), 126.7 (*C*H arom.), 125.8 (*C*H arom.), 122.7 (*C*H arom.), 120.1 (*C*H arom.), 118.4 (*C*H arom.), 117.6 (*C*≡N), 112.5 (*C*H arom.), 105.8 (*C*q arom.), 60.9 (*C*H), 54.1 (*C*H_3), 42.3 (*C*H_2), 25.4 (*C*H_2), 15.2 (*C*H_2); **LRMS** (+FAB) *m/z* 272 (100%, M^+ – HCl), 202 (25), 154 (55), 130 (75), 102 (95).

Attempted synthesis of 2-[[2-(2-cyano-ethoxyimino)-3-phenyl-propionyl]-(2-cyano-ethyl)-amino]-3-(1H-indol-2-yl)-propionic acid methyl ester 272



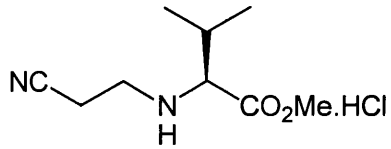
$C_{27}H_{27}N_5O_4$

485.83 g.mol^{-1}

The *N*-protected L-tryptophan methyl ester hydrochloride **259** (664 mg, 2.44 mmol) was added to a mixture of a 5M aqueous solution of potassium carbonate (6 mL) and dichloromethane (5 mL). The mixture was stirred to form a clear layer of the free *N*-protected L-tryptophan ester **270** and kept below 10 °C. Neat phosphoryl chloride (278 µL, 2.92 mmol) was added carefully to a solution of the 2-(2-cyano-ethoxyimino)-3-phenyl-propionic acid **267** (568 mg, 2.44 mmol) in dichloromethane (5 mL) containing a catalytic amount of dimethylformamide at 0 °C. The latter reaction mixture was then stirred at room temperature for 1 hour, cooled below 10 °C and added to the solution of the free *N*-protected L-tryptophan ester **270** at 0 °C. The combined reaction mixtures were then stirred at room

temperature and analysed by TLC at time intervals. After 12 hours, TLC analysis indicated the presence of starting materials **267** and **270** only.

N-2-Cyanoethyl-L-valine methyl ester hydrochloride 273



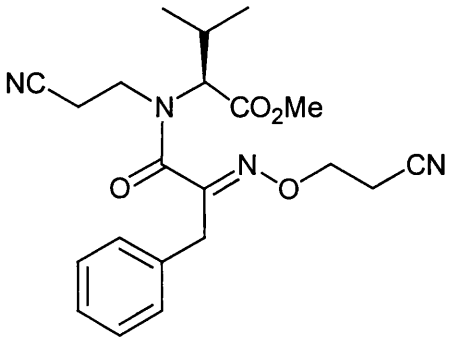
$C_9H_{16}N_2O_2.HCl$

220.70 g.mol^{-1}

Acrylonitrile (27.0 mL, 0.41 mol) was added to a solution of L-valine methyl ester hydrochloride **194** (13.8 g, 81.8 mmol) and triethylamine (11.4 mL, 81.8 mmol) in methanol (150 mL) at room temperature. The reaction mixture was then heated at reflux for 2 hours followed by stirring at room temperature for 12 hours. After filtration to remove the triethylammonium chloride salt, the solvent was evaporated at reduced pressure. Addition of isopropanol (40 mL) yielded a suspension to which was added concentrated hydrochloric acid (10 mL). After 2 hours at 8 °C, the solids were filtered and washed with isopropanol and diethyl ether to yield white crystals (11.2 g, 62%).

Mp 168–170 °C; $[\alpha]_D^{27}$ – 23.9 (*c*.1 in MeOH); **IR** (KBr)/cm^{-1}: ν_{max} 2962 (N–H), 1732 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 3.85 (3 H, s, C*H*$_3$–O), 3.11 (2 H, dt, *J* 5.8 and 6.6 Hz, C*H*$_2$–NH), 2.78 (1 H, dd, *J* 5.8 and 6.7 Hz, C*H*– NH), 2.58 (2 H, t, *J* 6.6 Hz, C*H*$_2$), 1.98 (1 H, dq, *J* 6.6 and 6.7 Hz, C*H*–CH$_3$), 1.84 (1 H, s, NH), 1.06 (3 H, d, *J* 6.6 Hz, C*H*$_3$), 1.04 (3 H, d, *J* 6.7 Hz, C*H*$_3$); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 175.4 (*C*=O), 118.9 (*C*≡N), 67.8 (*C*H$_3$–O), 51.9 (*C*H–NH), 44.5 (*C*H$_2$–NH), 31.9 (*C*H–CH$_3$), 19.6 (*C*H$_3$–CH), 19.4 (*C*H$_2$–CN), 18.6 (*C*H$_3$–CH); **LRMS** (+CI) *m/z* 185 (100%, M^+ – HCl), 144 (54), 125 (90), 70 (65). **Microanalysis** Found: C, 48.86; H, 8.12; N, 12.59; Cl, 16.37%. $C_9H_{16}N_2O_2.HCl$ requires C, 48.98; H, 7.76; N, 12.69; Cl, 16.06%.

Attempted syntheses of 2-[[2-(2-cyano-ethoxyimino)-3-phenyl-propionyl]-(2-cyano-ethyl)-amino]-3-methyl-butyric acid methyl ester 275



$C_{21}H_{26}N_4O_4$

398.46 $g.mol^{-1}$

Method A:

The *N*-protected L-valine methyl ester hydrochloride **273** (500 mg, 2.27 mmol) was added to a mixture of a 5M aqueous solution of potassium carbonate (5 mL) and dichloromethane (4 mL). The mixture was stirred to form a clear layer of the free *N*-protected L-valine ester **274** and kept below 10 °C. Neat phosphoryl chloride (258 µL, 2.72 mmol) was added carefully to a solution of the 2-(2-cyano-ethoxyimino)-3-phenyl-propionic acid **267** (528 mg, 2.27 mmol) in dichloromethane (5 mL) containing a catalytic amount of dimethylformamide (10 µL) at 0 °C. The latter reaction mixture was then stirred at room temperature for 1 hour, cooled below 10 °C and added to the solution of the free *N*-protected L-valine ester at 0 °C. The combined reaction mixtures were then stirred at room temperature and periodically analysed by TLC. After 12 hours, TLC analysis indicated the presence of starting materials only.

Method B:

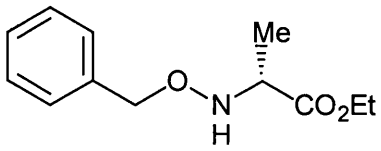
The *N*-protected L-valine methyl ester hydrochloride **273** (500 mg, 2.27 mmol) and triethylamine (317 µL, 2.27 mmol) were stirred in dichloromethane (10 mL) to form the free *N*-protected L-valine ester **274** and the mixture was kept below 10 °C. Neat oxalylchloride (198 µL, 2.27 mmol) was added to a solution of the 2-(2-cyano-ethoxyimino)-3-phenyl-propionic acid **267** (528 mg, 2.27 mmol) in

dichloromethane (5 mL) containing a catalytic amount of dimethylformamide (10 μL) at 0 °C. The latter reaction mixture was then stirred at room temperature for 12 hours, cooled below 10 °C and added to the solution of the free *N*-protected L-valine ester at 0 °C. The combined reaction mixtures were then stirred at room temperature and the reaction followed by TLC analysis.

Method C:

The *N*-protected L-valine methyl ester hydrochloride **273** (500 mg, 2.27 mmol) and triethylamine (317 μL, 2.27 mmol) were stirred in dichloromethane (20 mL) to form the free *N*-protected L-valine ester **270** and the mixture was kept below 10 °C. Dicyclohexylcarbodiimide (470 mg, 2.27 mmol) and hydroxybenzotriazole (30 mg, 0.2 mmol) were added to a solution of the 2-(2-cyano-ethoxyimino)-3-phenyl-propionic acid **267** in dichloromethane at 0 °C. Thus, the reaction mixture was then stirred at room temperature for 1 hour, cooled below 10 °C and the solution of the free *N*-protected L-valine ester was added at 0 °C. The combined reaction mixtures were then stirred at room temperature and periodically analysed by TLC.

(2R)-2-Benzyloxyamino-propionic acid ethyl ester[220] 294



$C_{17}H_{17}NO_3$

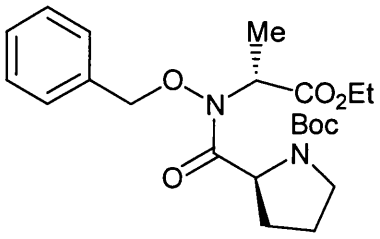
223.25 $g.mol^{-1}$

Trifluoromethanesulphonic acid anhydride (4.4 g, 25 mmol) was added all at once to a stirred solution of L-ethyl lactate **293** (3.0 mL, 25 mmol) in dry dichloromethane (40 mL) under a nitrogen atmosphere at 0 °C. After 5 minutes, 2,6-lutidine (3.2 mL, 27 mmol) was added in one portion and five minutes later, a solution of *O*-benzylhydroxylamine (3.8 g, 30 mmol) in dry dichloromethane (10 mL) was added dropwise. Subsequently, the cooling bath was removed allowing

the reaction to reach room temperature and stirring was continued for 25 minutes. The reaction mixture was concentrated at reduced pressure and the residual oil was subjected to column chromatography [silica, light petroleum (bp 40–60 °C)–diethyl ether 1:4] to yield **294** an oily product (5 g, 85%).

R_f [light petroleum (bp 40–60 °C)–ethyl acetate, 1:1] 0.84; $[\alpha]_D^{29}$ + 39.2 (*c.* 1.5 in $CHCl_3$) [lit.,[220] $[\alpha]_D^{20}$ + 51.7 (*c.* 1.5 in $CHCl_3$)]; **IR** (thin film)/cm^{-1}: ν_{max} 3275 (N–H), 1739 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.54–7.59 (5 H, m, C*H*, Ph), 4.96 (2 H, s, CH_2), 4.45 (2 H, q, *J* 7.1 Hz, CH_2–CH_3), 3.94 (1 H, q, *J* 7.1 Hz, C*H*–N), 1.53 (3 H, t, *J* 7.1 Hz, CH_3–CH_2), 1.45 (3 H, d, *J* 7.1 Hz, CH_3–CH); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 174.1 (*C*=O), 137.8 (*C*q, Ph), 128.2 (*C*H, Ph), 128.0 (*C*H, Ph), 127.7 (*C*H, Ph), 76.2 (CH_2), 60.8 (CH_2), 58.9 (*C*H), 14.8 (CH_3), 14.1 (CH_3); **LRMS** (+FAB) *m/z* 224 (100%, M^+), 149 (63), 91 (94).

Attempted synthesis of 2-[benzyloxy-(1-ethoxycarbonyl-ethyl)-carbamoyl]-pyrrolidine-1-carboxylic acid tert-butyl ester 296



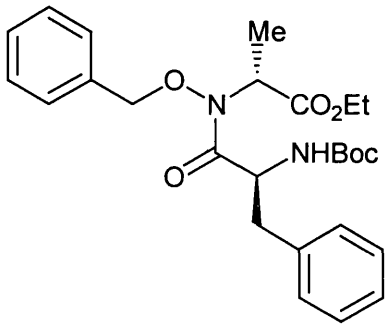
$C_{22}H_{32}N_2O_6$

420.50 $g.mol^{-1}$

A solution of isobutyl chloroformate (120 µL, 0.91 mmol) in tetrahydrofuran (1 mL) was added to a solution of *N-tert*-butoxycarbonyl-L-proline **295** (196 mg, 0.91 mmol) and triethylamine (127 µL, 0.91 mmol) in tetrahydrofuran (1.5 mL) at – 15 °C. After stiring for 15 minutes, a solution of 2-benzyloxyamino-propionic acid ethyl ester **294** (170 mg, 0.76 mmol) in dichloromethane (1 mL) was added and the resulting mixture stirred at – 15 °C for 3 hours, and kept for 45 hours at 4 °C. After

removal of the resulting triammonium salt by filtration, the solvent was removed at reduced pressure and the residue dissolved in ethyl acetate (5 mL). The resulting solution was washed with a 5% aqueous solution of $NaHCO_3$ (5 mL). Since unchanged 2-benzyloxyamino-propionic acid ethyl ester was detected by TLC in the extract, it was further acylated by the above procedure. However, repetition of the reaction did not give the desired product.

Attempted synthesis of 2-[benzyloxy-(2-tert-butoxycarbonylamino-3-phenyl-propionyl)-amino]-propionic acid ethyl ester 298



$C_{26}H_{34}N_2O_6$

470.56 $g.mol^{-1}$

Mixed anhydride method:

A solution of isobutyl chloroformate (95 µL, 0.7 mmol) in tetrahydrofuran (1 mL) was added to a solution of *N-tert*-butoxycarbonyl-L-phenylalanine **297** (185 mg, 0.69 mmol) and triethylamine (98 µL, 0.7 mmol) in tetrahydrofuran (1 mL) at – 15 °C. After stirring for 15 minutes, a solution of 2-benzyloxyamino-propionic acid ethyl ester **294** (130 mg, 0.58 mmol) in dichloromethane (1 mL) was added and the resulting mixture stirred at – 15 °C for 3 hours, and kept for 45 hours at 4 °C. After removal of the resulting triammonium salt by filtration, the solvent was removed at reduced pressure and the residue obtained dissolved in ethyl acetate (5 mL). The resulting solution was washed with a 5% aqueous solution of $NaHCO_3$ (5 mL). Since unchanged 2-benzyloxyamino-propionic acid ethyl ester **294** was detected by

TLC in the extract, it was further acylated by the above procedure. However, repetition of the reaction did not give the desired product **298**.

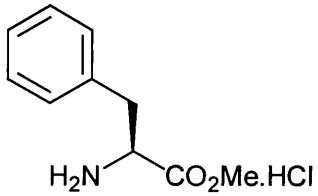
DCC coupling method:

Dicyclohexylcarbodiimide (1.1 g, 5.4 mmol) and 4-dimethylaminopyridine (66 mg, 0.4 mmol) were added to a solution of *N-tert*-butoxycarbonyl-L-phenylalanine **297** (1.4 g, 5.4 mmol) in dichloromethane (40 mL) at 0 °C. After stirring for 15 minutes, a solution of 2-benzyloxyamino-propionic acid ethyl ester **294** (1.0 g, 4.5 mmol) in dichloromethane (10 mL) was added and the reaction mixture was stirred at room temperature for 12 hours. On analysis of the reaction mixture by TLC, the presence of the starting materials **294** and **297** was shown.

Acid chloride method:

Sodium hydride (45 mg, 1.9 mmol) was added to a solution of *N-tert*-butoxycarbonyl-L-phenylalanine **297** (500 mg, 1.88 mmol) in dichloromethane (10 mL) at 0 °C and the mixture was stirred for 15 minutes. Oxalylchloride (164 μL, 1.88 mmol) was then added dropwise at 0 °C and the reaction mixture was stirred at room temperature for 2 hours. After removal of all the volatiles at reduced pressure, the residue was dissolved in dichloromethane (5 mL) and the solution was added dropwise to a mixture of 2-benzyloxyamino-propionic acid ethyl ester **294** (418 mg, 1.88 mmol) and triethylamine (262 μL, 1.88 mmol) in dichloromethane (5 mL). After stirring for 1 hour, TLC analysis showed that only the starting materials were present, thus stirring was continued for 12 hours, however only the starting materials **294** and **297** were recovered.

L-Phenylalanine methyl ester hydrochloride[221] 301



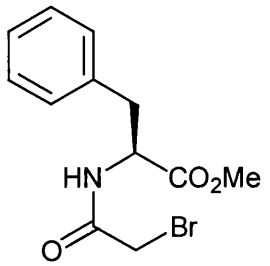
$C_{10}H_{13}NO_2.HCl$

214.65 $g.mol^{-1}$

Thionyl chloride (26.5 mL, 0.36 mol) was added dropwise to a stirred suspension of L-phenylalanine **55** (20.0 g, 0.12 mmol) in methanol (250 mL), at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 24 hours, after which time the solution was evaporated at reduced pressure to give the L-phenylalanine methyl ester hydrochloride salt **301** (26.9 g, quant.).

Mp 156–162 °C (lit.,[221] 158–162 °C); $[\alpha]_D^{25}$ + 36.6 (*c.* 2 in EtOH) [lit.,[221] $[\alpha]_D^{20}$ + 32.0 (*c.* 1 in EtOH)]; **IR** (KBr)/cm^{-1}: ν_{max} 2852 (N–H), 1747 (C=O); **^{1}H NMR** (300 MHz; D_2O): δ_H 7.15–7.33 (5 H, m, C*H*, Ph), 4.31 (1 H, t, *J* 5.7 Hz, C*H*), 3.71 (3 H, s, CH_3–O), 3.16 (2 H, dABq, *J* 5.7 and 14.5 Hz, CH_2); **^{13}C NMR** (75 MHz; D_2O): δ_C 168.5 (*C*=O), 131.7 (*C*q, Ph), 127.4 (*C*H, Ph), 127.3 (*C*H, Ph), 126.1 (*C*H, Ph), 52.1 (*C*H), 51.6 (*C*H_3), 33.6 (*C*H_2); **LRMS** (+CI) *m/z* 214 (4%, M^+), 180 (100), 148 (25), 120 (81), 88 (27).

(2-Bromo-acetylamino)-phenyl-acetic acid methyl ester[222] 302



$C_{12}H_{14}NO_3Br$

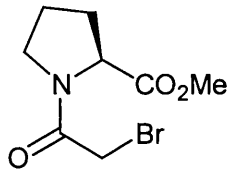
300.13 g.mol^{-1}

Bromoacetylchloride (2.8 mL, 33 mmol) was added, dropwise, to a solution of L-phenylalanine methyl ester hydrochloride **301** (5.0 g, 23 mmol) and triethylamine (6.2 mL, 44 mmol) in dichloromethane (100 mL) at 0 °C. The reaction mixture was stirred for 12 hours at room temperature, washed with water (2 x 50 mL) and dried over anhydrous magnesium sulfate. After filtration, the solvent was evaporated at reduced pressure to give the crude product. Purification by column chromatography

[silica, light petroleum (bp 40–60 °C)–ethyl acetate, 1:1] yielded the desired product **302** as a white solid (2.0 g, 78%).

Mp 77–78 °C (lit,[222] 80–82 °C); R_f [light petroleum (bp 40–60 °C)–ethyl acetate, 1:1] 0.85; $[\alpha]_D^{27}$ + 46.1 (*c.* 2 in $CHCl_3$) [lit.,[222] $[\alpha]_D^{20}$ + 57.3 (*c.* 1.4 in $CHCl_3$)]; **IR** (KBr)/cm^{-1}: ν_{max}: 3323 (N–H), 1731 (C=O), 1639 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.16–7.38 (5 H, m, C*H*, Ph), 7.03 (1 H, br, N*H*), 4.92 (1 H, td, *J* 6.05 and 7.6 Hz, C*H*), 4.06 (2 H, s, CH_2–Br), 3.88 (3 H, s, CH_3–O), 3.14–3.27 (2 H, m, CH_2–Ph); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 171.6 (*C*=O), 165.9 (*C*=O), 135.7 (*C*q, Ph), 129.6 (*C*H, Ph), 129.0 (*C*H, Ph), 127.7 (*C*H, Ph), 53.8 (*C*H), 52.8 (CH_3), 42.7 (CH_2), 38.2 (CH_2); **LRMS** (+FAB) *m/z* 300 (33%, M^+), 256 (100), 196 (58), 162 (26), 120 (35).

1-(2-Bromo-acetyl)-pyrrolidine-2-carboxylic acid methyl ester[223] 306



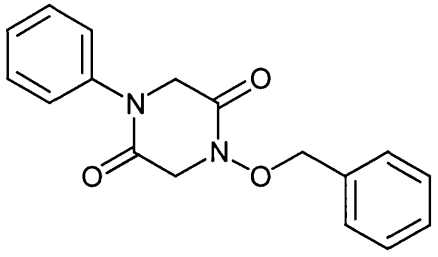
$C_8H_{12}NO_3Br$

250.09 $g.mol^{-1}$

Bromoacetylbromide (3.4 mL, 39 mmol) was added, dropwise, to a solution of L-proline methyl ester hydrochloride **305** (7.1 g, 42 mmol) and triethylamine (11.4 mL, 81 mmol) in dichloromethane (100 mL), at 0 °C. The reaction mixture was stirred for 12 hours at room temperature, washed with water (2 x 25 mL) and dried over anhydrous magnesium sulfate. After filtration, the solvent was evaporated at reduced pressure and the crude product was purified by column chromatography [silica, light petroleum (bp 40–60 °C)–ethyl acetate, 1:1] to yield **306** a colourless oil (5.0 g, 51%).

R_f [light petroleum (bp 40–60 °C)–ethyl acetate, 1:1] 0.45; $[\alpha]_D^{27}$ – 93.0 (*c.* 1 in MeOH) [lit.,[223] $[\alpha]_D^{22}$ – 96.7 (*c.* 1 in MeOH)]; **IR** (thin film)/cm^{-1}: ν_{max} 1732 (C=O), 1639 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 2 conformers in a ratio 5:1, 4.49 (1 H, dd, *J* 3.8 and 8.3 Hz, C*H*, *minor*), 4.30 (1 H, dd, *J* 3.9 and 8.3 Hz, C*H*, *major*), 4.01 (2 H, ABq, *J* 12.8 Hz, C*H*$_2$–Br, *major*), 3.90 (2 H, ABq, *J* 12.8 Hz, C*H*$_2$–Br, *minor*), 3.71 (3 H, s, C*H*$_3$–O, *minor*), 3.66 (3 H, s, C*H*$_3$–O, *major*), 3.55–3.63 (4 H, m, C*H*$_2$, *major and minor*), 1.93–2.05 (8 H, m, C*H*$_2$, *major and minor*); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 172.1 (O–*C*=O, *major*), 171.9 (O–*C*=O, *minor*), 165.2 (N–*C*=O, *minor*), 165.0 (N–*C*=O, *major*), 59.3 (*C*H, *minor*), 59.1 (*C*H, *major*), 52.8 (*C*H$_3$–O, *minor*), 52.2 (*C*H$_3$–O, *major*), 47.4 (*C*H$_2$–N, *minor*), 47.0 (*C*H$_2$–N, *major*), 41.8 (*C*H$_2$–Br, *minor*), 41.7 (*C*H$_2$–Br, *major*), 31.2 (*C*H$_2$, *minor*), 29.0 (*C*H$_2$, *major*), 24.8 (*C*H$_2$, *major*), 22.2 (*C*H$_2$, *minor*); **LRMS** (+CI) *m/z* 250 (16%, M$^+$), 206 (100), 146 (99), 70 (26).

1-Benzyloxy-4-phenyl-piperazine-2,5-dione <u>310</u>



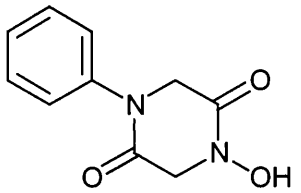
$C_{17}H_{16}N_2O_3$

296.32 g.mol^{-1}

[(2-Bromo-acetyl)-phenyl-amino]-acetic acid methyl ester **208** (318 mg, 1.11 mmol) was added to a stirred solution of *O*-benzylhydroxylamine **304** (449 mg, 2.77 mmol) in methanol (20 mL). The resulting mixture was refluxed for 5 days. After cooling the solid that precipitated gave 1-benzyloxy-4-phenyl-piperazine-2,5-dione **310** as white crystals (99 mg, 30%).

Mp 215–217 °C; **IR** (KBr)/cm^{-1}: ν_{max} 1650 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.30–7.50 (m, 10 H, C*H*, Ph), 5.02 (s, 2 H, CH_2), 4.37 (s, 2 H, CH_2), 4.34 (s, 2H, CH_2); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 162.6 (*C*=O), 161.3 (*C*=O), 139.8 (*C*q, Ph), 134.8 (*C*q, Ph), 129.3 (*C*H, Ph), 128.9 (*C*H, Ph), 128.6 (*C*H, Ph), 128.3 (*C*H, Ph), 127.0 (*C*H, Ph), 125.8 (*C*H, Ph), 72.5 (*C*H_2), 51.7 (*C*H_2), 51.6 (*C*H_2); **LRMS** (+EI) *m/z* 296 (3%, M$^+$), 91 (100); **HRMS** (+EI) Found: M$^+$, 296.11554; $C_{17}H_{16}N_2O_3$ requires *M*, 296.11548.

1-Hydroxy-4-phenyl-piperazine-2,5-dione 311



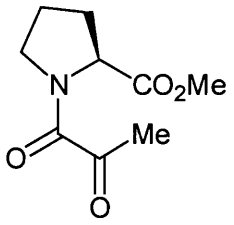
$C_{10}H_{10}N_2O_3$

206.20 g.mol^{-1}

1-Benzyloxy-4-phenyl-piperazine-2,5-dione **310** (80 mg, 0.27 mmol) was dissolved in a mixture of methanol (2.5 mL) and ethyl acetate (2.5 mL) and hydrogenated under an atmosphere of hydrogen in the presence of palladium black (8 mg) at room temperature for 48 hours. After the catalyst was removed by filtration, the filtrate was evaporated at reduced pressure to yield 1-hydroxy-4-phenyl-piperazine-2,5-dione **311** (50 mg, 89%).

Mp > 220 °C; **IR** (KBr)/cm^{-1}: ν_{max} 2361 (O–H), 1658 (C=O); **^{1}H NMR** (300 MHz; DMSO-d_6): δ_H 7.20–7.43 (m, 5 H, C*H*, Ph), 4.22 (s, 2 H, CH_2), 3.98 (s, 2 H, CH_2); **^{13}C NMR** (75 MHz; DMSO-d_6): δ_C 162.6 (*C*=O), 161.3 (*C*=O), 139.8 (*C*q, Ph), 129.3 (*C*H, Ph), 128.6 (*C*H, Ph), 127.0 (*C*H, Ph), 51.7 (*C*H_2), 51.6 (*C*H_2); **LRMS** (+EI) *m/z* 190 (100%, M$^+$ – OH), 106 (77), 77 (42); **HRMS** (+EI) Found: M$^+$, 206.06935; $C_{10}H_{10}N_2O_3$ requires *M*, 206.06914.

1-(2-Oxo-propionyl)-pyrrolidine-2-carboxylic acid methyl ester[224] 314



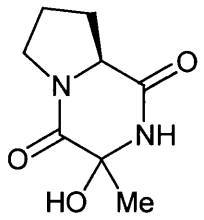
$C_9H_{13}NO_4$

199.18 g.mol^{-1}

Pyruvic acid (4.6 g, 52 mmol) and dicyclohexylcarbodiimide (10.8 g, 52.2 mmol) were added to a mixture of L-proline methyl ester hydrochloride **305** (7.2 g, 43 mmol) and triethylamine (7.3 mL, 52 mmol) in dichloromethane (250 mL) at 0 °C. After stirring at room temperature for 24 hours, the dicyclohexylurea by–product was removed by filtration and the solvent was evaporated at reduced pressure. The crude product was purified by column chromatography [silica, light petroleum (bp 40–60 °C)–ethyl acetate, 4:1] to yield **314** as a yellow oil (4 g, 48%).

R_f [light petroleum (bp 40–60 °C)–ethyl acetate, 1:1] 0.42; $[\alpha]_D^{27}$ – 70.9 (*c.* 1.1 in $CHCl_3$) [lit.,[224] $[\alpha]_D^{20}$ – 93.1 (*c.* 1.1 in $CHCl_3$)]; **IR** (thin film)/cm^{-1}: ν_{max} 1745 (C=O), 1716 (C=O), 1645 (C=O); **^{1}H NMR** (400 MHz; $CDCl_3$): δ_H 2 conformers in a ratio 3:2, 4.71 (1 H, dd, *J* 4.0 and 8.6 Hz, C*H*, *major*), 4.39 (1 H, dd, *J* 4.0 and 8.5 Hz, C*H*, *minor*), 3.63 (3 H, s, C*H*$_3$–O, *minor*), 3.61 (3 H, s, C*H*$_3$–O, *major*), 3.44–3.70 (4 H, m, C*H*$_2$, *major and minor*), 3.22 (3 H, s, C*H*$_3$–CO, *minor*), 2.30 (3 H, s, C*H*$_3$–CO, *major*), 1.67–2.10 (8 H, m, C*H*$_2$, *major and minor*); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 197.6 (*C*=O, *major*), 197.1 (*C*=O, *minor*), 172.3 (O–*C*=O, *major*), 171.4 (O–*C*=O, *minor*), 162.6 (N–*C*=O, *major*), 162.1 (N–*C*=O, *minor*), 59.4 (*C*H, *major*), 59.3 (*C*H, *minor*), 52.0 (*C*H$_3$–O, *major*), 51.8 (*C*H$_3$–O, *minor*), 47.6 (*C*H$_2$–N, *major*), 47.1 (*C*H$_2$–N, *minor*), 31.1 (*C*H$_2$, *major*), 28.2 (*C*H$_2$, *minor*), 26.4 (*C*H$_3$–CO, *major*), 25.9 (*C*H$_3$–CO, *minor*), 24.8 (*C*H$_2$, *minor*), 21.8 (*C*H$_2$, *major*); **LRMS** (+FAB) *m/z* 200 (58%, M$^+$), 133 (100).

3-Hydroxy-3-methyl-hexahydro-pyrrolo[1,2-a]pyrazine1,4-dione[225] 315



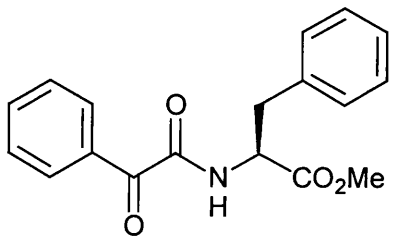
$C_8H_{12}N_2O_3$

184.19 g.mol^{-1}

Ammonia gas was bubbled through a solution of 1-(2-oxo-propionyl)-pyrrolidine-2-carboxylic acid methyl ester **314** (2.0 g, 10 mmol) in dimethoxyethane (100 mL) at 0 °C. The reaction mixture was stirred at room temperature, under an atmosphere of ammonia, for 12 hours. The precipitate formed was filtered and washed with diethyl ether to yield **315** as a white solid (4.0 g, 75%).

Mp 170 °C (partially melt) / 195 °C [lit.,[225] 185 (partially melt) / 200 °C]; **IR** (KBr)/cm^{-1}: ν_{max} 3221 (O–H), 3120 (N–H), 1660 (C=O); **^{1}H NMR** (300 MHz; DMSO-d$_6$): δ_H 8.53 (1 H, br, O*H*), 6.28 (1 H, br, N*H*), 4.13–4.21 (1 H, m, C*H*), 3.29–3.50 (2 H, m, CH_2), 1.72–2.17 (4 H, m, CH_2), 1.41 (3 H, s, CH_3); **^{13}C NMR** (75 MHz; DMSO-d$_6$): δ_C 167.2 (*C*=O), 167.1 (*C*=O), 81.1 (*C*q), 50.1 (*C*H), 45.3 (*C*H$_2$–N), 28.9 (*C*H$_2$), 27.3 (*C*H$_3$), 22.2 (*C*H$_2$); **LRMS** (+FAB) *m/z* 185 (100%, M$^+$), 167 (67), 154 (76), 137 (62), 70 (41).

2-(2-Oxo-2-phenyl-acetylamino)-3-phenyl-propionic methyl ester[226] 317



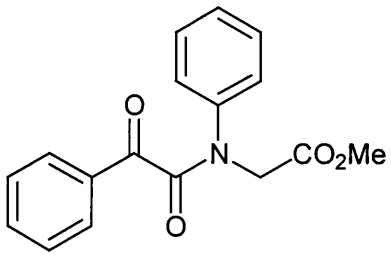
$C_{18}H_{17}NO_4$

311.33 g.mol^{-1}

Benzoylformic acid **316** (3.3 g, 22 mmol) and dicyclohexylcarbodiimide (4.6 g, 22 mmol) were added to a mixture of L-phenylalanine methyl ester hydrochloride **301** (4.0 g, 18 mmol) and triethylamine (3.1 mL, 22 mmol) in dichloromethane (40 mL), at 0 °C. After stirring at room temperature for 24 hours, the dicyclohexylurea by–product was removed by filtration and the solvent was evaporated at reduced pressure. The crude product was purified by column chromatography [silica, light petroleum (bp 40–60 °C)–ethyl acetate, 3:1] to yield **317** as a clear oil (2.3 g, 40%).

R_f [light petroleum (bp 40–60 °C)–ethyl acetate, 1:1] 0.81; $[\alpha]_D^{27}$ + 50.2 (*c.* 1 in $CHCl_3$), [lit.,[226] $[\alpha]_D^{20}$ + 57.3 (*c.* 1.4 in $CHCl_3$)]; **IR** (thin film)/cm^{-1}: ν_{max} 2952 (N–H), 1732 (C=O), 1651 (C=O), 1651 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 8.23–8.30 (2 H, m, C*H*, Ph), 7.15–7.68 (8 H, m, C*H*, Ph), 4.94–4.99 (1 H, m, C*H*), 3.73 (3 H, s, CH_3–O), 3.19 (2 H, dABq, *J* 5.6 and 13.9 Hz, CH_2); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 187.0 (*C*=O), 171.1 (*C*=O), 161.4 (*C*=O), 135.5 (*C*q, Ph), 134.4 (*C*H, Ph), 133.1 (*C*q, Ph), 131.1 (*C*H, Ph), 129.2 (*C*H, Ph), 128.7 (*C*H, Ph), 128.5 (*C*H, Ph), 127.3 (*C*H, Ph), 53.3 (*C*H), 52.5 (*C*H_3), 37.9 (*C*H_2); **LRMS** (+EI) *m/z* 311 (10%, M^+), 206 (12, M^+ – Ph – CO), 178 (21, M^+ – Ph – CO – CO), 105 (100), 91 (41), 77 (87), 51 (32); **HRMS** (+EI) Found: M^+, 311.11491; $C_{18}H_{17}NO_4$ requires *M*, 311.11575.

[(2-Oxo-2-phenyl-acetyl)-phenyl-amino]-acetic acid methyl ester <u>320</u>



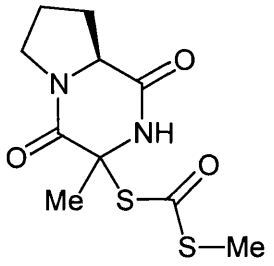
$C_{17}H_{15}NO_4$

297.28 g.mol^{-1}

Benzoyl formic acid **316** (1.3 g, 8.5 mmol) was treated with oxalyl chloride (1.4 mL, 16 mmol) in the presence of several drops of dimethylformamide in dry dichloromethane (15 mL) at room temperature for 2 hours. Organic solvent and excess oxalyl chloride were removed under reduced pressure to give the acid chloride **319**, which was dissolved in dichloromethane (15 mL) and added dropwise to a stirred solution of phenylamino-acetic acid methyl ester **207** (707 mg, 4.28 mmol) and triethylamine (4.1 mL, 30 mmol) in dichloromethane (30 mL) at 0 °C. When addition was complete, the resulting solution was allowed to warm to room temperature and stirred overnight. The organic solvent was removed under reduced pressure and the residue partitioned between water (50 mL) and dichloromethane (50 mL). The aqueous phase was extracted with dichloromethane (2 x 50 mL) and the combined organic extracts dried over anhydrous magnesium sulfate, filtered and the solvent removed under reduced pressure. Purification by column chromatography [silica, light petroleum (bp 40–60 °C)–ethyl acetate, 4:1] gave [(2-oxo-2-phenyl-acetyl)-phenyl-amino]-acetic acid methyl ester **320** as a pale yellow oil (1.10 g, 87%).

R_f [light petroleum (bp 40–60 °C)–ethyl acetate, 1:1] 0.42; **IR** (thin film)/cm^{-1}: ν_{max} 1747 (C=O), 1676 (C=O), 1654 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.96–7.98 (2 H, m, C*H*, Ph), 7.19–7.53 (8 H, m, C*H*, Ph), 4.56 (2 H, s, CH_2), 3.82 (3 H, s, CH_3); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 190.3 (*C*=O), 168.9 (*C*=O), 167.5 (*C*=O), 140.0 (*C*q, Ph), 134.4 (*C*H, Ph), 133.3 (*C*q, Ph), 129.7 (*C*H, Ph), 129.5 (*C*H, Ph), 128.7 (*C*H, Ph), 128.6 (*C*H, Ph), 127.8 (*C*H, Ph), 52.5 (CH_3), 50.5 (CH_2); **LRMS** (+EI) *m/z* 297 (6%, M^+), 192 (46, M^+ – Ph – CO), 164 (55, M^+ – Ph – CO – CO), 105 (100), 77 (100), 51 (42); **HRMS** (+EI) Found: M^+, 297.09937; $C_{17}H_{15}NO_4$ requires *M*, 297.10010.

Attempted synthesis of dithiocarbonic acid *S*-methyl ester *S*-(3-methyl-1,4-dioxo-octahydro-pyrrolo[1,2-a]pyrazin-3-yl) ester 329

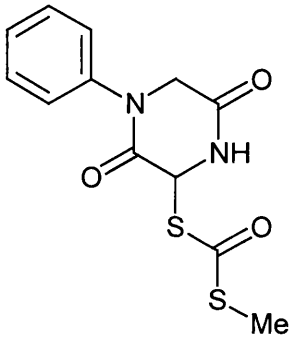


$C_{10}H_{14}N_2O_3S_2$

274.36 g.mol^{-1}

Sodium hydride (67 mg, 2.8 mmol) was added to a solution of **3**-hydroxy-3-methyl-hexahydro-pyrrolo[1,2-a]pyrazine1,4-dione **315** (500 mg, 2.71 mmol) in dimethylformamide (5 mL) at 0 °C. When hydrogen evolution had ceased, carbon disulfide (5.0 mL, 82 mmol) was added and the solution was stirred for 5 minutes prior to the addition of the methyl iodide (0.2 mL, 3.2 mmol) was added. After the reaction mixture was stirred for 12 hours at room temperature, all the volatiles were removed at reduced pressure. The crude product was subjected to column chromatography [silica, light petroleum (bp 40–60 °C)–ethyl acetate, 1:1] but no desired products were obtained but starting material **315** was recovered (110 mg).

Attempted synthesis of dithiocarbonic acid *S*-(3,6-dioxo-4-phenyl-piperazin-2-yl) ester *S*-methyl ester 332



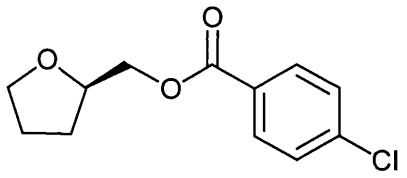
$C_{12}H_{12}N_2O_3S_2$

296.37 g.mol^{-1}

Sodium hydride (3.36 mg, 0.14 mmol) was added to a solution of 1-hydroxy-4-phenyl-piperazine-2,5-dione **311** (30.0 mg, 0.14 mmol) in dimethylformamide (1 mL) at 0 °C. When hydrogen evolution had ceased, carbon disulfide (258 μL, 4.28 mmol) was added and the solution was stirred for 5 minutes prior to the addition of the methyl iodide (10 μL, 0.2 mmol) was added. After the reaction mixture was stirred for 12 hours at room temperature, all the volatiles were removed at reduced pressure. The crude product was subjected to column chromatography [silica, light petroleum (bp 40–60 °C)–ethyl acetate, 1:1] but none of the desired products were isolated

4.1.5. Mechanistic studies

(*R*)-4-chlorobenzoic acid tetra-hydro-furan-2-ylmethyl ester 409



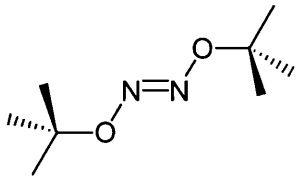
$C_{12}H_{13}ClO_3$

240.66 $g.mol^{-1}$

Para-chlorobenzoic acid (1.1 g, 7.3 mmol) was heated in thionyl chloride (20 mL) at reflux. After 2 hours, thionyl chloride was removed at reduced pressure at temperatures not exceeding 40 °C, to yield a semi-solid crude product. The crude acid chloride **408** was then dissolved in diethyl ether (5 mL) and a mixture of (*R*)-tetrahydrofurfuryl alcohol **407** (500 mg, 4.89 mmol) and triethylamine (1.0 mL, 7.3 mmol) in diethyl ether was then added dropwise at 0 °C. After the reaction mixture was stirred at room temperature for 4 hours, the triethylammonium chloride salt was filtered and the solvent removed at reduced pressure. The crude product so obtained was purified by column chromatography (silica, dichloromethane) to yield **409** as a transparent oil (800 mg, 68%).

R_f (dichloromethane) 0.25; $[\alpha]_D^{27}$ – 26.1 (*c.* 1 in $CHCl_3$); **IR** (thin film)/cm^{-1}: ν_{max} 1722 (C=O); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 7.89 (2 H, d, *J* 8.5 Hz, C*H*, Ph), 7.31 (2 H, d, *J* 8.5 Hz, C*H*, Ph), 4.25–4.30 (1 H, m, C*H*–O), 4.16–4.21 (2 H, m, CH–C*H*$_2$–O), 3.69–3.86 (2 H, m, CH_2–C*H*$_2$–O), 1.56–2.00 (4 H, m, CH_2); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 165.9 (*C*=O), 139.7 (*C*q, Ph), 131.4 (*C*H, Ph), 129.0 (*C*H, Ph), 76.9 (*C*H), 68.8 (*C*H_2), 67.4 (*C*H_2), 28.4 (*C*H_2), 26.1 (*C*H_2), one *C*q missing; **LRMS** (+FAB) *m/z* 241 (100%, M^+), 139 (100), 85 (52), 71 (62); **HRMS** (+FAB) Found: M^+, 240.05598; $C_{12}H_{13}ClO_3$ requires *M*, 240.05532.

Di-*tert*-butyl hyponitrite[195] 412



$C_8H_{18}N_2O_2$

174.24 g.mol^{-1}

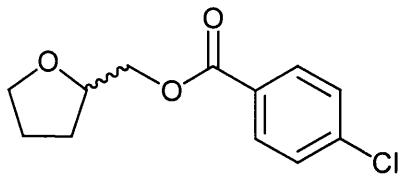
To a solution of zinc chloride (2.4 g, 20 mmol) in diethyl ether (16 mL) under nitrogen was added an excess of *tert*-butylbromide **411** (16.0 mL, 0.14 mol), under nitrogen. After cooling to 0 °C, sodium hyponitrite hydrate **410** (2.1 g, 20 mmol if anhydrous*) was then added in one portion. The reaction mixture was stirred for 1 hour and left at + 4 °C for 12 hours. The solid formed was filtered and washed with dry diethyl ether. The combined filtrates were washed with ice-cold water (50 mL) and dried over anhydrous magnesium sulfate. After filtration, the solvent was removed at reduced pressure at room temperature to give a yellow solid, which was washed with a small amount of cold methanol and dried at reduced pressure to yield a white solid (1.2 g, 34%).

Mp 45 °C (unstable) (lit.,[195] 82–83 °C); **^{1}H NMR** (300 MHz; $CDCl_3$): δ_H 1.37 (s, CH_3); **^{13}C NMR** (75 MHz; $CDCl_3$): δ_C 81.1 (*C*q), 27.7 (CH_3).

* The formula of the commercially-available salt is given as $Na_2N_2O_2.xH_2O$. The value of x is not specified but the material is believed to be close to anhydrous.

Typical procedure for the racemisation studies of (*R*)-4-chlorobenzoic acid tetra-hydro-furan-2-ylmethyl ester 409

The procedure used is based on that described by Roberts *et al.*[178]



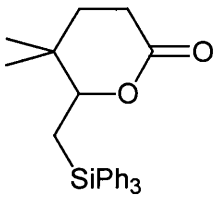
$C_{12}H_{13}ClO_3$

240.66 $g.mol^{-1}$

To a benzene solution (1 mL) of (*R*)-4-chlorobenzoic acid tetra-hydro-furan-2-ylmethyl ester **409** (99% ee) (250 mg, 1.03 mmol) under nitrogen was added di-*tert*-butyl hyponitrite (18 mg, 0.1 mmol, 10 mol%) and the thiol catalyst solution (5 mol% for catalyst containing 2 thiol functions, 10 mol% for catalyst containing 1 thiol function) in benzene (1 mL). The reaction mixture was then heated at 60 °C for 3 hours. To determine the enantiomeric excess of the product, a homogeneous sample was taken and analysed using the Chiralcel-OD column [eluent: 0.05% isopropanol in heptane; t_R 15 (*S*) and 18 (*R*) min].

Representative procedure for thiol-catalysed hydrosilylation of 5,5-dimethyl-6-methylene-tetrahydro-pyran-2-one 418

The procedure used is based on that described by Roberts *et al.*[176]



$C_{26}H_{25}O_2Si$

400.58 g.mol^{-1}

5,5-Dimethyl-6-methylene-tetrahydro-pyran-2-one **418** (140 mg, 1.00 mmol), triphenylsilane (281 mg, 1.10 mmol) and the thiol-catalyst (2.5 mol% for catalyst containing 2 thiol functions, 5 mol% for catalyst containing 1 thiol function) were added under nitrogen to a 5 mL flask. 1,4-Dioxane (2 mL) was used as a solvent and after dissolution of all the reagents, di-*tert*-butyl hyponitrite (8.7 mg, 50 µmol, 5 mol%) was added. After the reaction mixture was heated at 60 °C for 3 hours, the solvent was removed at reduced pressure and the crude product **419** analysed by ^{1}H NMR to determine the yields of conversion (70–99%).

5,5-dimethyl-6-methylene-tetrahydro-pyran-2-one:[176] **^{1}H NMR** (400 MHz; $CDCl_3$): δ_H 4.72 (1 H, d, *J* 1.7 Hz, vinyl *H*), 4.42 (1 H, d, *J* 1.7 Hz, vinyl *H*), 2.70 (2 H, t, *J* 7.1 Hz, C*H*$_2$CO), 1.74 (2 H, t, *J* 7.1 Hz, C*H*$_2$C(CH$_3$)$_2$), 1.27 (6 H, s, C*H*$_3$).

5,5-Dimethyl-6-[(triphenylsilanyl)-methyl]-tetrahydro-pyran-2-one:[176] **^{1}H NMR** (400 MHz; $CDCl_3$): δ_H 7.67 (6 H, m, C*H*, Ph), 7.45 (9 H, m, C*H*, Ph), 4.19 (1 H, dd, *J* 2.1 and 11.5 Hz, CH–O), 2.45 (2 H, m, C*H*$_2$CO), 1.86 (1 H, dd, *J* 11.5 and 15.0 Hz, SiC*H*), 1.65 (3 H, m, C*H*$_2$C(CH$_3$)$_2$ and SiC*H*), 1.06 (3 H, s, C*H*$_3$), 1.03 (3 H, s, C*H*$_3$).

Copper(I) triflate-benzene complex[227] 428

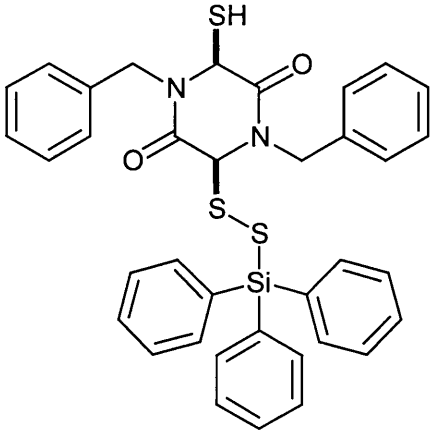
$CuOTf(C_6H_6)_{0.5}$

$C_4H_3CuF_3O_3S$

290.73 g.mol^{-1}

Cu_2O **427** (1.0 g, 7.0 mmol), benzene (40 mL) and trifluoromethanesulfonic anhydride (2.3 g, 8.1 mmol) were added under nitrogen into a 50 mL two-necks flask. The reaction mixture was heated under reflux for 6 hours until the red Cu_2O had dissolved and afforded a clear solution. The hot reaction mixture was filtered and cooled to room temperature under nitrogen. After crystallisation from the reaction mixture, the desired complex was filtered, washed with fresh benzene (2 x 5 mL) and dried at reduced pressure to yield white crystals (2.6 g, 65%). The copper triflate-benzene complex **428** was used immediately due to its instability.

Attempted synthesis of 1,4-dibenzyl-3-mercapto-6-triphenylsilanyldisulfanyl-piperazine-2,5-dione 431

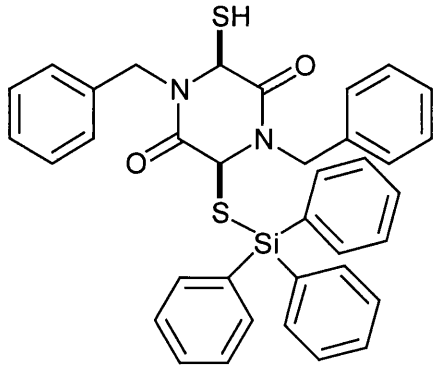


$C_{36}H_{32}N_2O_2S_3Si$

648.94 $g.mol^{-1}$

To a benzene solution (1 mL) of 1,4-dibenzyl-piperazine-2,5-dione **214** (20 mg, 50 μmol) under an atmosphere of nitrogen was added di-*tert*-butyl hyponitrite (9.0 mg, 50 μmol) followed by a solution of triphenylsilane thiol (16 mg, 50 μmol) in benzene (1 mL). The reaction mixture was then heated at 60 °C for 3 hours and after cooling to room temperature, TLC analysis showed the formation of a product. The solvent was then removed at reduced pressure and the crude product was subjected to a column chromatography [silica, light petroleum (bp 40–60 °C)–ethyl acetate, 1:1] but in contact with silica the crude product decomposed and the desired product **431** was not isolated.

Attempted synthesis of 1,4-dibenzyl-3-mercapto-6-triphenylsilanylsulfanyl-piperazine-2,5-dione 432



$C_{36}H_{32}N_2O_2S_2Si$

616.87 $g.mol^{-1}$

To a benzene solution (1 mL) of 1,4-dibenzyl-piperazine-2,5-dione **214** (20 mg, 50 μmol) under an atmosphere of nitrogen was added di-*tert*-butyl hyponitrite (9 mg, 50 μmol) followed by a solution of triphenylsilane (14 mg, 50 μmol) in benzene (1 mL). The reaction mixture was then heated at 60 °C for 3 hours and cooled to room temperature. The solvent was then removed at reduced pressure and the crude product was subjected to column chromatography [silica, light petroleum (bp 40–60 °C)–ethyl acetate, 1:1] but only starting material **214** was recovered.

4.2. Biological experiments

4.2.1. General experimental

Biological assays were performed on supercoiled pBR322 DNA in the absence of oxygen. The final product was stored in aliquots at 0.5 μg/1 μL in Tris-HCl (tris(hydroxymethyl)aminomethane hydrochloride) (10 mM, pH 7.4) at –22 °C. Incubation of plasmid DNA with various agents was conducted in Microfuges tubes (Eppendorf). Components were added to a total volume of 80 μL and contained 2 μg of plasmid DNA. The components were mixed by a short burst in the Microfuge and after incubation at 37 °C for 60 minutes, EDTA (200 μM) solution was added (20%, v/v) to inhibit further reaction, and the samples loaded onto 1% agarose (Type I; sigma) gels. Electrophoresis was conducted in a mini-submarine apparatus in Plexiglas equipped with two gates and one comb. The resulting gel was exposed to ultraviolet light and then photographed.

4.2.2. Experimental procedures

Tris-HCl (1 M, pH 7.4) solution

To a solution of Tris-base (12.1 g) in doubly distilled water (50 mL) was added 1 M aqueous hydrochloric acid to pH 7.4. Doubly distilled water was then added to give a 100 mL solution of Tris-HCl (10 mM, pH 7.4).

50X TAE (Tris-acetate-EDTA) buffer solution

To a liter container was added Tris-Base (242 g), glacial acetic acid (57.1 mL) and 0.5M aqueous EDTA (pH 8) (100 mL). Doubly distilled water was then added to make a 1 L solution, which was stored at room temperature.

1X TAE (Tris-acetate-EDTA) buffer solution

50x TAE buffer solution (20 mL) was added to doubly distilled water (980 mL). The resulting solution was stored at room temperature.

Preparation of an agarose gel for electrophoresis

To agarose (1 g) was added 1X TAE buffer solution (100 mL). The resulting solution was mixed, heated in a microwave over 1 minute and after swirling to mix, heated until the agarose was boiling gently. After cooling until hand-hot (50 °C), the stain ethidium bromide (5 μL) was added and the resulting solution swirled to mix. This mixture was poured into the casting tray and left for 20 minutes to set. After the gel had set, the casting gates and the comb were removed. The gel so formed and the reservoir were covered with 1X TAE buffer to 0.5-1 cm above the level of the gel.

6X Gel loading buffer

Glycerol (30 mL), bromophenol blue (2.5 mL) and xylene cyanol (2.5 mL) were added to doubly distilled water (70 mL). The mixture was kept in aliquots and stored at + 4 °C. The 6X loading buffer is thus dye allowing the progress of the electrophoresis to be monitored visibely. In a 1% agarose gel, bromophenol blue co-migrates with 300 to 400 bp DNA and xylene cyanol co-migrates with 3 to 4 kb DNA fragments.

1 Kb Plus DNA Ladder

The 1 Kb Plus DNA Ladder is commonly used for sizing linear double-stranded DNA fragments from 100 bp to 12 kb. The ladder contains a total of twenty bands of DNA fragments and was loaded in one well for each experiment.

General procedure

All the sample mixtures, which contained Plasmid DNA (0.5 μg/1 μL) (4 μL), were prepared in a glove box in an atmosphere of nitrogen. Tris-HCl (1 M) was added in 8 μL aliquots to give a final concentration of Tris-HCl 100 mM. $CuCl_2$ (2.4 mM) and $FeCl_3$ (2.4 mM) solutions were added in 10 μL aliquots to give concentrations of $CuCl_2$ 300 μM and $FeCl_3$ 300 μM respectively. Similarly, EDTA (0.32 mM) solution was added in 5 μL aliquots until the concentration of EDTA was 200 μM and glutathione (GSH) (1.6 mM) solution was added in 10 μL aliquots to give a concentration of GSH of 200 μM. *N,N'*-dimethyl-3,6-epidithio-2,5-diketopiperazine **70** (3.3 mg), due to its insolubility, was firstly dissolved in the minimum amount of DMSO (1 mL), then 10 μL of this solution was diluted with doubly distilled water (150 μL) to obtain 1 mM as the desired concentration of *N,N'*-dimethyl-3,6-epidithio-2,5-diketopiperazine **70**. This solution was added in 16 μL aliquots to give a concentration of 200 μM. The sample of DMSO was made using DMSO (10 μL) diluted with doubly distilled water (150 μL) and was used in 16 μL aliquots. Gliotoxin **1** was dissolved in absolute ethanol to give a 1 mM solution, which was added in 16 μL aliquots to give a final concentration of 200 μM. After addition of all the above products, doubly distilled water was added to make 80 μL of final solution.

The samples were then incubated at 37 °C for 2 hours. After incubation, an aliquot (10 μL) from each sample was mixed with 3 μL of EDTA (200 μM) and 2 μL of 6X gel loading buffer. 6 μL of each mixture were then loaded into the wells in the gel. 1 Kb plus DNA Ladder (2 μL) was also loaded into one well in each row. Electrophoresis was then performed at 100V and after 1 hour, the stained gel revealed the DNA fragmnents under ultraviolet light and a photograph was taken.

Chapter 5

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