A new orthogonal protecting group strategy for lanthionine-containing peptides

Marianne Françoise Andrée Groussier

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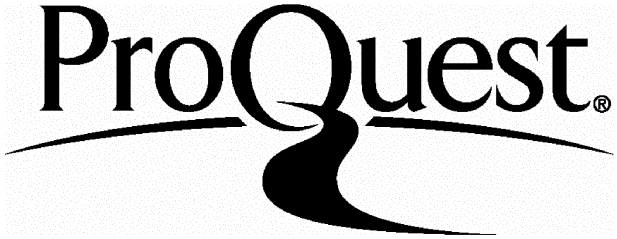

To Roger

To my parents for giving me their love of Britain and the English language

To both my grandfathers for giving me their love of science

"Persévérons (dit Socrates): ou bien nous trouverons ce que nous cherchons, ou bien nous serons moins sûrs de savoir ce que nous ne savons point; de toute manière, nous n'aurons pas perdu notre peine."

Platon, Théétète.

(Let us persevere (says Socrates): either we will find what we are looking for, or we will be less sure of what we do not know; either way, we will not have suffered in vain.)

Declaration: I, Marianne Groussier, hereby state that the following is entirely my own work and has not been submitted for any other degree or examination.

Marianne Groussier

August 2000

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Merci à tous.

Abstract

The synthesis of lanthionine residues bearing photolabile protecting groups and the use of these compounds for the synthesis of lanthionine-containing peptides is described in this thesis. A new orthogonal approach has been developed towards bridged peptidomimetics such as sandostatin analogs.

Several synthetic pathways were followed to synthesise these lanthionine residues and several types of photolabile groups were also tried. The final route involves the making of (*R, R*) lanthionine residues bearing NV and NVOC groups from (*S*)-serine and (*R, R*)-cystine. The intermediates (*R*)-cysteine and (*R*)-3-iodoalanine were synthesised with the appropriate protecting groups. The iodoalanine intermediates were obtained in high yields as mixtures of rotamers. They were subsequently used to synthesise the final lanthionine residues following a high-yielding and fast route. The formation of small amounts of diastereoisomers was observed. These were easily separated from the final compounds.

Photolabile cleavages studies of NV and NVOC groups were carried out and optimised in solution before SPPS. Finally, several attempts at synthesising a sandostatin analog were carried out on a resin, incorporating the lanthionine residues into cyclic octapeptides. Difficulties were encountered during photolabile cleavages on a polystyrene support. However, efficient photolabile cleavage was finally obtained on Tentagel resin.

Other sections of this thesis describe the large family of lanthionine-containing peptide lantibiotics and synthetic routes leading to lanthionine residues as well as the different strategies elaborated for the cyclisation of small peptides.

Abbreviations

Al: allyl

Ala: alanine

Alloc: allyloxycarbonyl

APCI: atmospheric pressure chemical ionisation

Arg: arginine

Asn: asparagine

Asp: aspartic acid

ATP: adenosine 5'-triphosphate

AviCys: 2-aminovinylcysteine

B: base

BAL: backbone amide linker

Bn: benzyl

Boc: *tert*-butyloxycarbonyl

BOP: benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate

Bom: benzyloxymethyl

Bzl: benzyl

Cbz: benzyloxycarbonyl

ClZ: 2-chlorobenzyloxycarbonyl

COSY: correlated spectroscopy

Cys: cysteine

DBF: dibenzofulvene

DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene

DCM: dichloromethane

Dde: 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidine)ethyl

Ddz: α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl

DEAD: diethyl azodicarboxylate

Dha: 2,3-didehydroalanine

Dhb: 2,3-didehydrobutyrine

DIC: diisopropylcarbodiimide

DIEA: diisopropylethylamine

DMAD: dimethyl azodicarboxylate

Dmb: 2,4-dimethoxybenzyl

DMF: *N,N*-dimethylformamide

DMSO: dimethyl sulfoxide

EI: electron impact

eq.: equivalent

ESP: electrospray

FAB: fast atom bombardment

FLECl: 1-(9-fluorenyl)ethyl chloroformate

FLEOC: 1-(9-fluorenyl)ethyloxycarbonyl

Fm: 9-fluorenylmethyl

Fmoc: 9-fluorenylmethyloxycarbonyl

FmocCl: 9-fluorenylmethyl chloroformate

Glu: glutamic acid

Gln: glutamine

Gly: glycine

His: histidine

HATU: *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HBTU: *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

hCT: human calcitonin

HMPA: hexamethylphosphoramide

HOAt: 1-hydroxy-7-azabenzotriazole

HOBt: 1-hydroxybenzotriazole

HPLC: high-performance liquid chromatography

HyAsp: 3-hydroxyaspartic acid

Ile: isoleucine

I.R.: infrared

Lan: *meso*-lanthionine

LC-MS: liquid chromatography-mass spectroscopy

Leu: leucine

Lys: lysine

LysN-Ala: lysinoalanine

MBHA: 4-methylbenzhydrylamine

Me: methyl

MeLan: 3-methyllanthionine

mesyl: methanesulfonyl

Met: methionine

Ms: methanesulfonyl

NBS: *N*-bromosuccinimide

NMM: *N*-methylmorpholine

n.m.r.: nuclear magnetic resonance

NV: 6-nitroveratryl or 4,5-dimethoxy-2-nitrobenzyl

NVOC: 6-nitroveratryloxycarbonyl or 4,5-dimethoxy-2-nitrobenzyloxycarbonyl

NVOCCl: 6-nitroveratryl chloroformate or 4,5-dimethoxy-2-nitrobenzyl chloroformate

OxRes: oxime resin

p: *para*

Pac: phenacyl

PAC: *p*-alkoxybenzyl alcohol

PAL: 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid

PCOR: peptide cyclisation on an oxime resin

PEG: polyethylene glycol

Phe: phenyalanine

Phenoc: 4-methoxyphenacyloxycarbonyl

Pro: proline

PS: polystyrene

PyAOP: 7-azabenzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate

PyBOP: benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate

SAR: structure-activity relationship

Ser: serine

SPPS: solid-phase peptide synthesis

tBu: *tert*-butyl

tert: tertiary

TEA: triethylamine

TFA: trifluoroacetic acid

th.: theoretical

THF: tetrahydrofuran

Thr: threonine

t.l.c.: thin layer chromatography

TMS: trimethylsilyl

TMSCl: trimethylsilyl chloride

tosyl: toluenesulfonyl

trityl: triphenylmethyl

Trt: triphenylmethyl

Trp: tryptophan

Tyr: tyrosine

U.V.: ultraviolet

Val: valine

VT: variable temperature

Xaa: amino acid

Z: benzyloxycarbonyl

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1. Lanthionine and lantibiotics

1. Background

1. 1. Lanthionine

Lanthionine is an unusual amino acid, a monosulfide analogue of cystine first isolated from sodium carbonate-treated wool in 1941,[1] then found in human hair, chicken feathers and lactalbumin shortly aftertwards[2,3] (**Figure 1**). (*S, R*) or (D, L) Lanthionine is the natural isomer but other diastereoisomers such as (*R, R*) lanthionine have been synthesised.

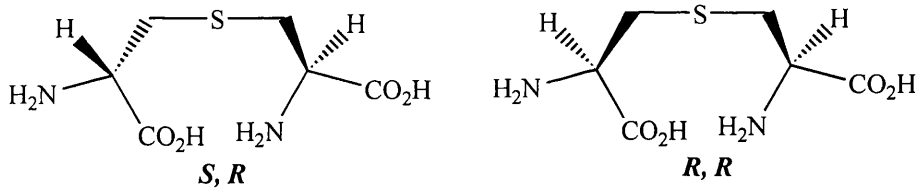


Figure 1: (*S, R*) Lanthionine and (*R, R*) lanthionine.

*Lan*thionine is quite abundant naturally, present in bacterial cell walls[4] as well as in a large family of peptides exhibiting anti*biotic* properties, the *lantibiotics*.

When lanthionine residues are incorporated into peptides, they give rigidity and constraints to their backbone and form monosulfide bridges that make lanthionine-containing peptides more stable towards enzymatic degradation.[5] These bridges are also less prone to reduction than the disulfide bridges (present in cystine-containing peptides).

Such interesting properties have led to extensive studies of lantibiotics in recent years and to the developments of several pathways for the synthesis of lanthionine-containing peptides, compounds of prime interest as peptidomimetics.[6]

1. 2. Characteristics of lantibiotics

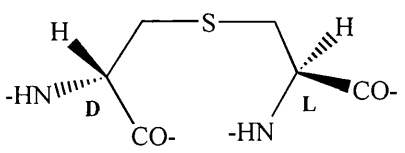
The first lantibiotic was discovered in 1928,[7,8] as a substance limiting lactic fermentation. This particular substance has since then been acknowledged as nisin,[9] and today, about thirty other lantibiotics have also been identified and their structure determined. Among the most studied ones and in chronological order of discovery are subtilin, ancovenin, epidermin, gallidermin, Pep5, cinnamycin, duramycin, mersacidin. They are all peptides produced by certain Gram-positive bacteria which inhibit the growth of other Gram-positive bacteria and they all contain monosulfide bridges. The main characteristics of some lantibiotics are summarised in **Table 1**: nisin Z varies in one residue from nisin A (His 27 replaced by Asn), duramycin C presents also small variations in residues from duramycin.

Lantibiotic	Origin and molecular mass (Da)		Lan	Melan	Dha	Dhb	Others
Type A							
Nisin A	*Lactococcus lactis*	3353	1	4	2	1	0
Nisin Z	*Lactococcus lactis*	3330	1	4	2	1	0
Subtilin	*Bacillus subtilis*	3317	1	4	2	1	0
Epidermin	*Staphylococcus epidermidis*	2164	2	1	0	1	AviCys
Gallidermin	*Staphylococcus gallinarum*	2164	2	1	0	1	AviCys
Pep5	*Staphylococcus epidermidis*	3488	2	1	0	2	Oxobutyryl
Epilancin K7	*Staphylococcus epidermidis*	3032	2	1	2	2	Hydroxypropionyl
Type B							
Cinnamycin	*Streptomyces cinnamoneus*	2042	4	2	0	0	HyAsp, LysN-Ala
Duramycin	*Streptomyces cinnamoneus*	2014	4	2	0	0	HyAsp, LysN-Ala
Duramycin C	*Streptomyces griseoluteus*	2008	4	2	0	0	HyAsp, LysN-Ala

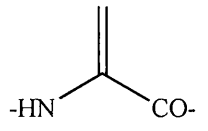
Table 1: Characteristics of some lantibiotics.[10]

Lantibiotics are polycyclic peptides of 19 to 34 amino acids. They are fairly small peptides (< 4 kDa). All lantibiotics contain rings made of *meso*-lanthionine (Lan), 3-methyllanthionine (MeLan), 2-aminovinylcysteine (AviCys) or lysinoalanine (LysN-Ala)

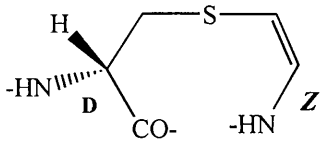
and other unusual amino acids such as 2,3-didehydroalanine (Dha), 2,3-didehydrobutyrine (Dhb) and 3-hydroxyaspartic acid (HyAsp).[11] (**Figure 2**).



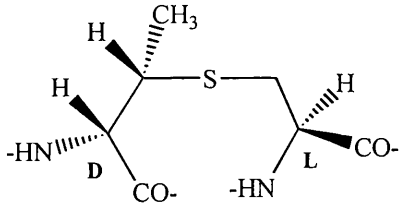
(*2S, 6R*)-lanthionine, *meso*-lanthionine



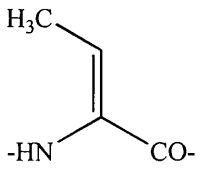
2,3-didehydroalanine



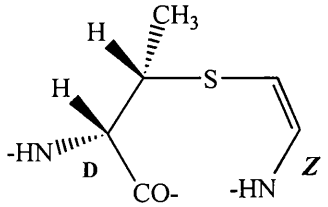
S-[(*Z*)-2-aminovinyl]-D-cysteine



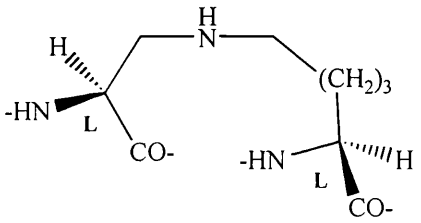
(*2S,3S 6R*)-3-methyllanthionine



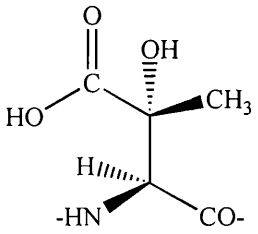
(*Z*)-2,3-didehydrobutyrine



S-[(*Z*)-2-aminovinyl]-3-methyl-D-cysteine



(*2S, 8S*)-lysinoalanine



erythro-3-hydroxy-L-aspartic acid

Figure 2: Unusual amino acids in lantibiotics[11, 12]

Studies of conformations and structural features of lantibiotics led Jung[13] to classify them into two types, type A and type B (**Table 1**). Lantibiotics of type A are strongly cationic peptides of relatively large molecular mass (over 2100 Da). They have a close similarity in the number and position of the monosulfide bridges and the types of unusual amino acids found in them. Type-A lantibiotics are screw-shaped peptides which act mainly by forming pores in the cytoplasmic membrane of the target cell.[14]

Lantibiotics of type B are weakly cationic peptides of smaller molecular mass. They are also structurally related and the presence of side-chain-to-tail bridges in their structure make them more globular than lantibiotics of type A. Some type-B lantibiotics are inhibitors of enzymes such as angiotensin-converting enzyme and phospholipase A and may consequently have therapeutic uses.[12] Nisin and Pep 5 are typical lantibiotics of type A whereas duramycin and cinnamycin represent type B (**Figure 3**).

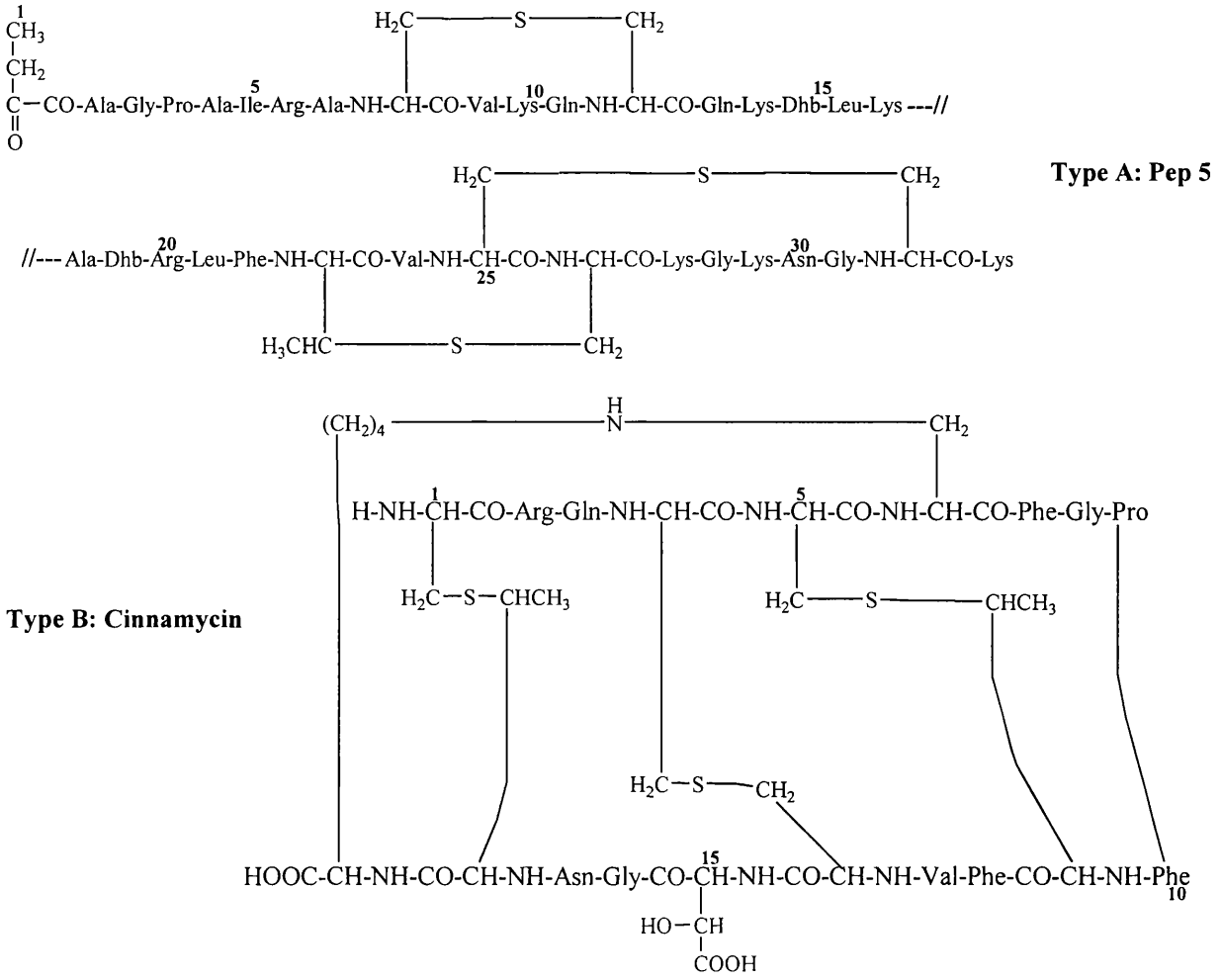


Figure 3: Type A and type B lantibiotics.[14]

Nisin was first produced in a large scale in the fifties[15,16,17] and has been used since then as a natural food preservative. It also exhibits interesting antibacterial activities such as antimalarial potency but its physical properties such as poor solubility have so far prevented further developments as a drug.[18]

It is a fairly large peptide of 34 amino acids presenting 5 monosulfide bridges, A, B, C, D, E (**Figure 4**): A from a lanthionine residue, B, C, D and E from four 3-methyllanthionine residues. Three other unusual amino acids are incorporated into the molecule (two dehydroalanine residues and one dehydrobutyrine residue).

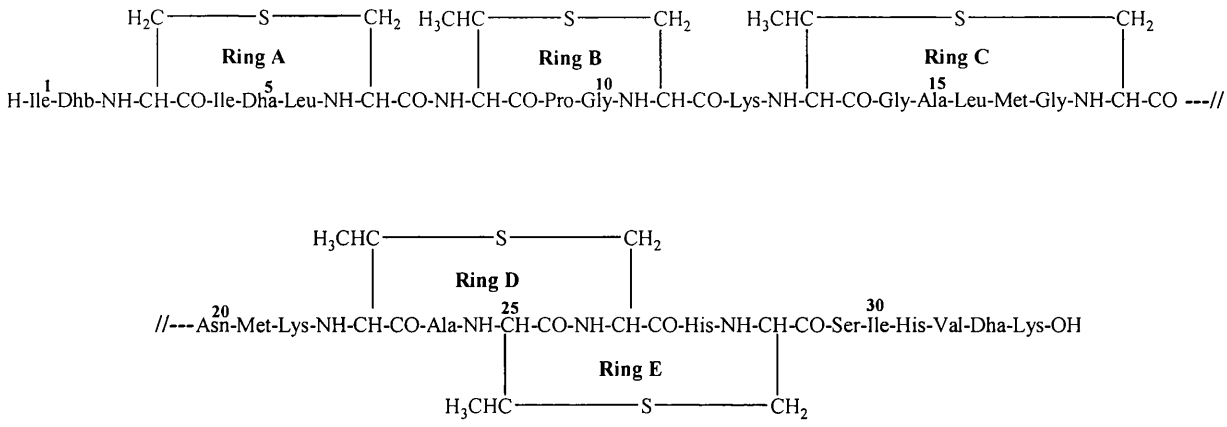


Figure 4: Nisin

The structure and conformation of nisin were first revealed by Gross *et al.* in 1971.[19,20] Most of its sequence could be determined by Edman degradation. However, new methods had to be developed to determine the presence of some unusual amino acids such as 2,3-didehydroalanine.[21]

For example, spontaneous oxidative deamination of the resulting *N*-terminal Dha residue occurs, leading to the formation of a pyruvyl group, which blocks the sequence determination. With mild acid hydrolysis, a peptide amide and pyruvyllysine are obtained (**Figure 5**).

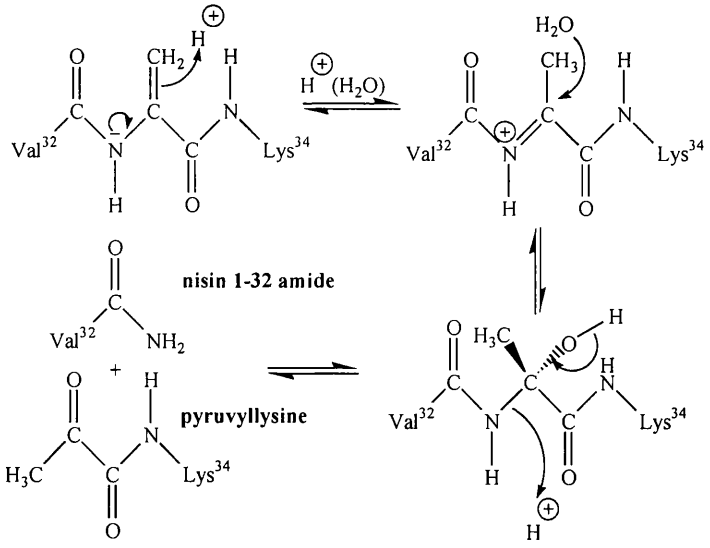


Figure 5: Acid hydrolysis of nisin at Dha 33 gives a peptide amide and pyruvyllysine.[13]

New determination methods of Dha and Dhb were carried out using addition of benzylthiol and mercaptoethylamine. Also, oxidative cleavage or desulfurization with Raney nickel was performed on lanthionine and methyllanthionine bridges to give alanine and aminobutyric acid residues.[13] The correct structure of nisin was perfectly determined with these thorough methods, as later confirmed by n.m.r. analyses.

Subtilin has structural features and antibacterial properties very close to the ones shown by nisin. Elucidation of its conformation therefore followed closely the work carried out on nisin.[22] Ancovenin was then studied by Shiba *et al.*.[18]

At about the same time, an extensive study of epidermin (**Figure 6**) and gallidermin was carried out by Jung *et al.*.[23,24] After having divided epidermin (made of 22 residues) into two large parts called P1 and P2 (fragment P1 residues 1 to 13, fragment P2 residues 14 to 22), they elucidated its sequence using mass spectrometry, n.m.r. spectroscopy, gas-phase sequencing and synthetic methods. Epidermin and gallidermin only differ from each other in position 6, occupied by Ile in epidermin and by Leu in gallidermin. They are both good therapeutic agents against acne. They can be used to replace the antibiotic erythromycin to treat juvenile acne without any side-effects.[13]

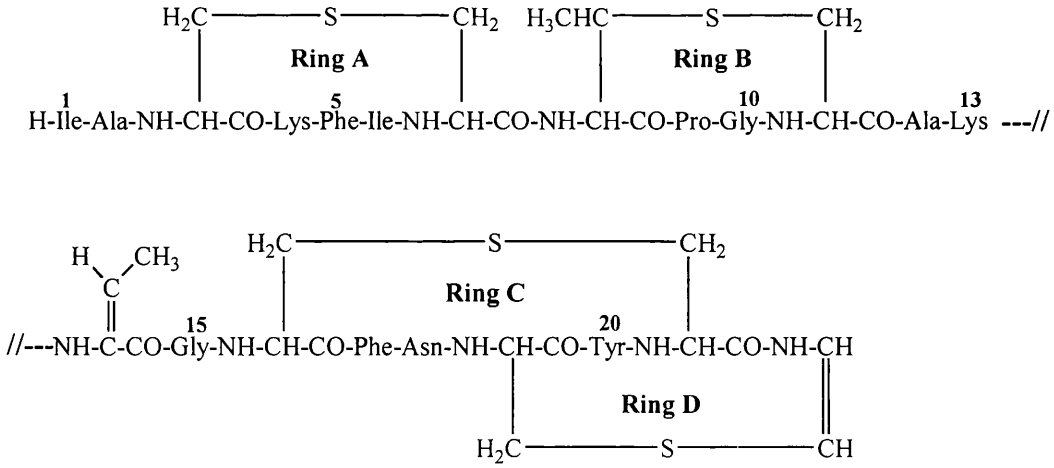


Figure 6: Epidermin

Today, conformations and structures of most of the known lantibiotics are determined or being studied. New methods are now available for this purpose, such as n.m.r. analyses[14] or automated sequential Edman degradation coupled to direct detection by electrospray-ionization mass spectrometry.[25]

1. 3. Biosynthesis of lantibiotics

A major breakthrough in the studies of lantibiotics was made by Jung *et al.*[13] when they elucidated some biological mechanisms which are involved in the natural making of lantibiotics. Lantibiotics are made from ribosomally synthesised precursor peptides,[26,27,28] whereas most other peptide antibiotics are formed from large multienzyme complexes.[29]

The "structural gene" for each lantibiotic codes a linear peptide, a lantibiotic prepeptide, of two distinct parts: a leader peptide and a prolantibiotic. The structural genes of the most common type-A lantibiotics have been identified.[11] The prolantibiotic contains only the 20 proteinogenic amino acids. It subsequently undergoes post-translational modifications as follows. Dehydrations at the serine and threonine residues create α and β unsaturated double bonds. To them are added stereospecifically thiol groups of neighbouring cysteine residues to form sulfide rings (**Figure 7**).

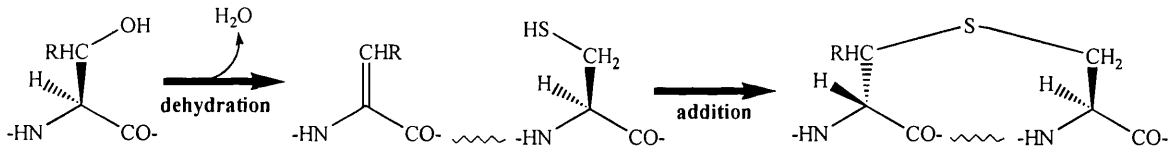


Figure 7: Dehydration and addition steps taking place during the biosynthesis of lantibiotics.[30] If R=H, L-serine is transformed into 2,3-didehydroalanine then *meso*-lanthionine is made, if R=CH_3, L-threonine is transformed into (*Z*)-2,3-didehydrobutyrine then (*2S, 3S, 6R*)-3-methyllanthionine is made.

These additions are followed by cleavage of the leader peptide by a signal peptidase and oxidative decarboxylation of some residues to give unusual amino acids. These chemical processes are carried out by modifying enzymes. These processes have been identified but their mechanisms are still to be discovered. For example, the stereospecificity of the lanthionine residues obtained remains unexplained.

Lantibiotics have very interesting properties for drug design because of the high number of unusual amino acids found in these polycyclic peptides. It is very likely that the monosulfide bridges are there to give local rigidity and protease stability to the structure and the unsaturated amino acids might increase local reactivity.[11]

So far, nisin remains the most attractive lantibiotic for its use in the food industry. Very recent work has focused on structure activity relationships studies of nisin, especially the importance of the bridges[31] or the fact that nisin is actually much more active when used in interaction with a membrane-anchored lipid, lipid II. Indeed, the high activity of this antibiotic appears to be closely related to the presence of lipid II, as studies of model membrane suggest. This would make nisin "a special case" among pore-forming antibiotics.[32]

It is still very challenging to understand all the processes involved in the synthesis and mode of action of lantibiotics. Therefore two routes are opened for future therapeutic uses of these peptides. Once the biosynthesis of the lantibiotics is fully understood, mutant producing strains may be developed, leading to new antibiotics. Alternatively, when direct and high yielding synthetic pathways are created, new lantibiotics can be invented.

1. 4. Synthetic lanthionine-containing peptides

Until now, nisin is the only lantibiotic to have been successfully synthesised by Shiba *et al.*[18,22,33] in an overall yield of 10 %. The synthesised nisin was identical in all aspects to the naturally occurring peptide. It was made by condensation of four main segments in solution. The whole synthesis took nearly ten years and remains a prodigious achievement that cannot be easily reproduced to synthesise more lantibiotics quickly and as cheaply as possible.

However, the synthesis of smaller lanthionine-containing peptides was met with wider success, especially for SAR studies of active compounds[6] and for conformational studies.[34] SAR studies of peptides and enzymes have been extensively developed in the past 20 years.[35,36] Most peptides are very flexible and adopt numerous conformations depending on their environment. Artificial constraints such as disulfide[37] or monosulfide bridges reduce the flexibility of the active peptides studied and in some cases increase the potency and specificity of the constrained analogs or peptidomimetics compared to the "free" species.

Lanthionine bridges have therefore been incorporated into small peptides for SAR studies. Goodman *et al* synthesised lanthionine-bridged opioid enkephalin[38,39,40,41] as well as lanthionine-somatostatin analogs.[42,43] Both these compounds were made again by Jarosinski[44] later on using new synthetic methods. Goodman *et al.* also worked on the synthesis of cyclolanthionine,[34,45] to determine its conformation and structure, comparing with cyclo-cystine, also used as constraints in the design of peptidomimetics.

Opioid enkephalins and their receptors are present in the mammalian brain and intestine. Extensive studies have been carried out to determine the receptor specificity and activity of these enkephalins. So far, three types of opiate receptors have been identified and the enkephalins appear to be selective to δ receptors. However, naturally occurring enkephalins such as [Leu^5] enkephalin[38] are too flexible for conformational studies, so more rigid enkephalin analogs had to be designed. Lanthionine-bridged opioid enkephalins were therefore synthesised (**Figure 8**).

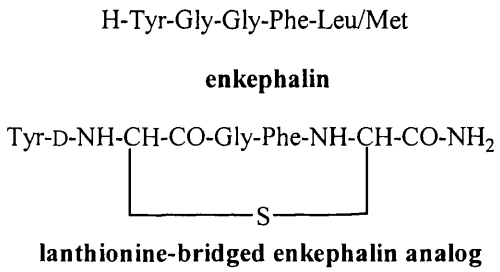


Figure 8: Design of a constrained enkephalin analog.[44]

An increase in activity but no change in selectivity were observed when this lanthionine-bridged analog was tested on δ receptors.[38] However, further studies with β,β-dimethylated lanthionine enkephalin analogs[41] revealed that some of these molecules showed high potency and δ selectivity along with high activity.

The cyclic tetradecapeptide hormone somatostatin has recently been extensively studied[42,43] especially for its role in neural transmission. Several somatostatin analogs were synthesised and their activities studied, among them synthetic sandostatin and lanthionine-sandostatin analogs (**Figure 9**). Replacing the disulfide bridge with a monosulfide bridge led to increased selectivities of the analogs for somatostatin receptors.[43]

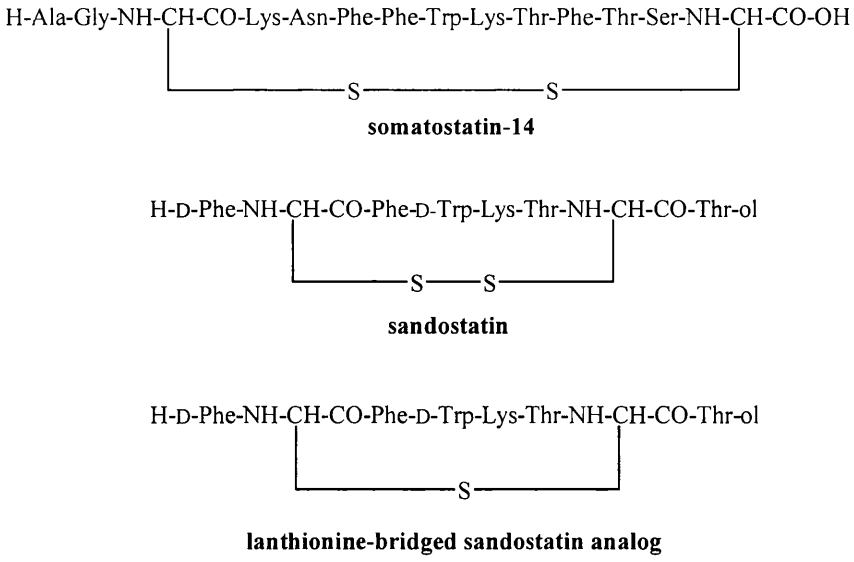


Figure 9: Design of constrained somatostatin and sandostatin analogs.[42]

Finally, cyclolanthionine analogs have been synthesised to study their conformation and draw comparisons with cyclo-cystine.[34,45] As both these constrained structures are incorporated into peptidomimetics, it is of prime interest to know their spatial arrangements. The crystal structure and conformation in solution of cyclolanthionine (**Figure 10**) as well as β,β-dimethylcyclolanthionine peptides were elucidated.

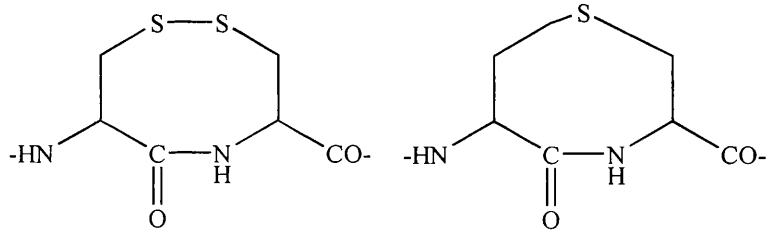


Figure 10: Cyclo-cystine and cyclolanthionine peptides.[34,45]

Conformation studies of the four stereoisomeric cyclolanthionine residues (**Figure 11**) showed that the amide bond in the ring is in a *cis* conformation and that there is nearly no deviation from the planarity in all four stereoisomers.[45]

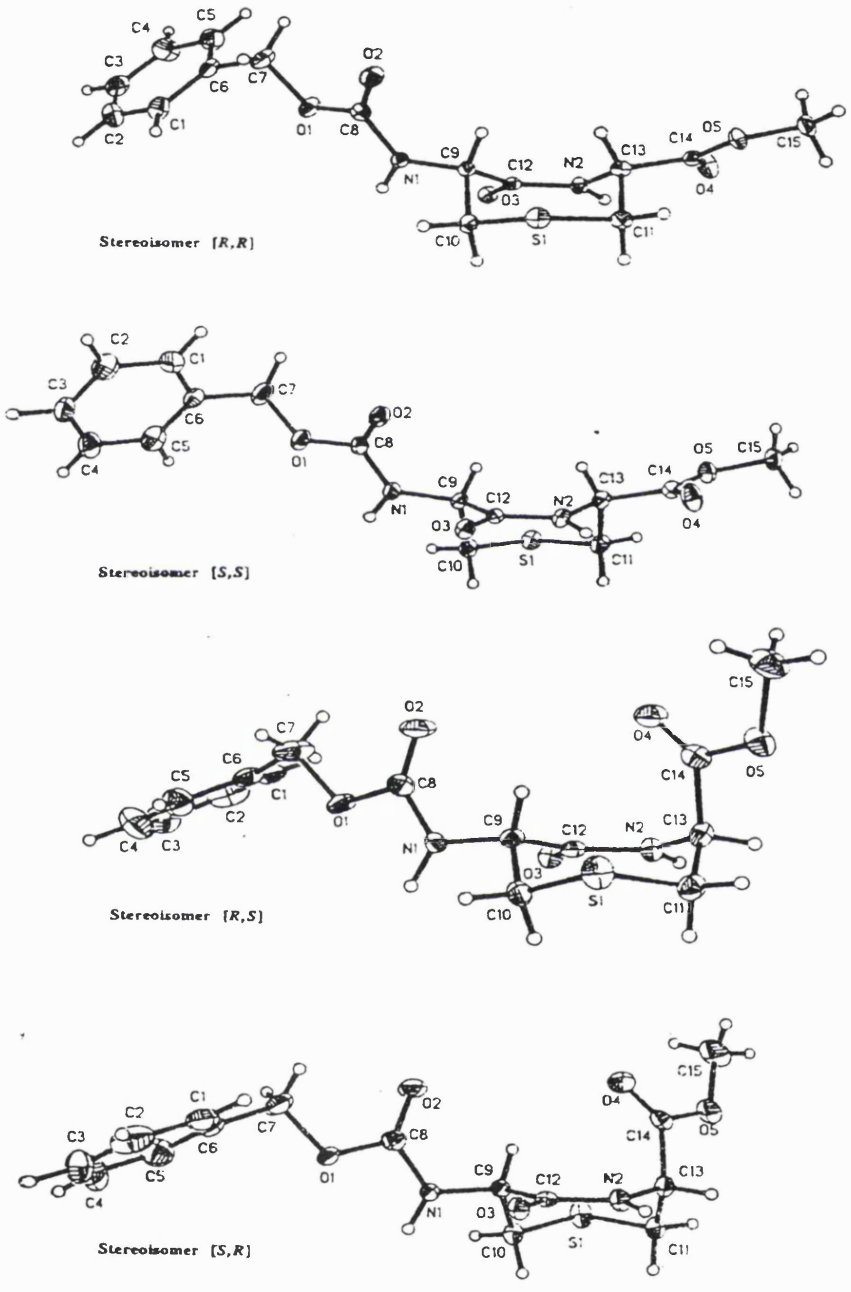


Figure 11: ORTEP view of the four stereoisomeric cyclolanthionine derivatives. The atoms are drawn with 30 % probability ellipsoids.[45]

2. Synthesis of lanthionine

2. 1. Desulfurisation of disulfides

One of the oldest methods for the preparation of lanthionine was developed by Gleason and Harpp.[46,47,48] Their work on small-ring sulfur compounds led them to use selective desulfurisation, i.e. desulfurisation of only one sulfur out of the two, to make monosulfide compounds, using tris(diethylamino)phosphine, $(Et_2N)_3P$. Carboxy groups have to be protected as they can react with $(Et_2N)_3P$, giving amides. This reaction was then extended to a wide range of disulfides and the first lanthionine derivative was synthesised with a high yield (96 %) (**Figure 12**).

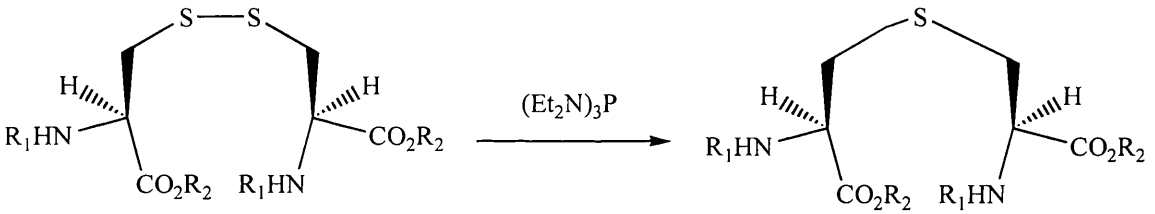


Figure 12: Synthesis of lanthionine by desulfurisation of cystine.[48] R_1 = $COCF_3$ or $CO_2CH_2C_6H_5$ and R_2 = CH_3 or C_2H_5

This is a stereospecific desulfurisation, an inversion of configuration taking place at one of the carbon atoms α to the disulfide bond (**Figure 13**).

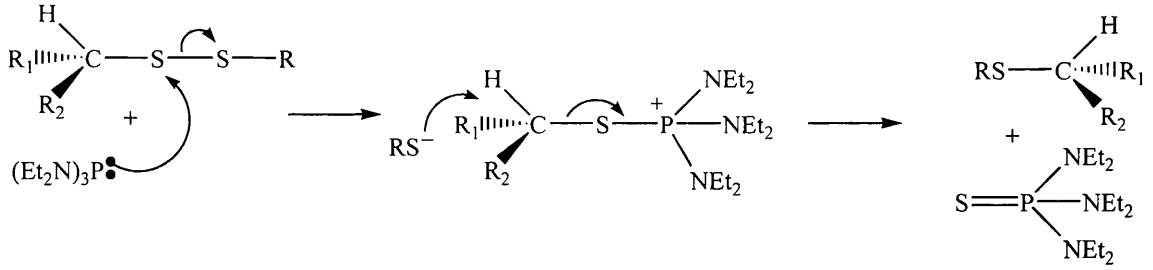


Figure 13: Mechanism of desulfurisation

The major drawback of this method is the synthesis of numerous by-products with unsymmetrical substituted disulfides. Indeed, with these reagents, three products can be expected[48] (**Figure 14**).

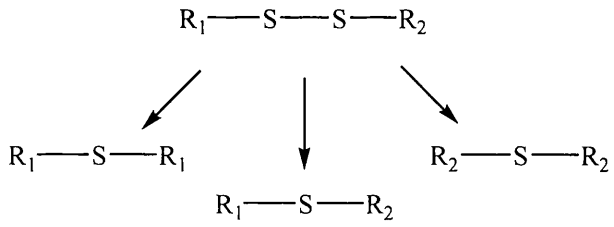


Figure 14: Lanthionine residues made from desulfurisation of unsymmetrically substituted cystine residues.[48]

Unsymmetrical lanthionine residues have been synthesised with this method, purifying the final product by medium pressure liquid chromatography,[49] but the yields were fairly low (58 %). An interesting way to overcome the problem is to link both the carboxy functions of cystine together with ethylene glycol temporarily, so that only one compound is made from desulfurisation of this cyclic cystine.[50] Unsymmetrical lanthionine residues were obtained with quantitative yields, without racemization, nor dimerization. The most famous illustration of this method is the synthesis of each ring of nisin by Shiba *et al.*[18,22,33] As each ring is synthesised like a cyclic cystine, only one main lanthionine residue was obtained after each desulfurisation, in a reasonable yield (**Figure 15**).

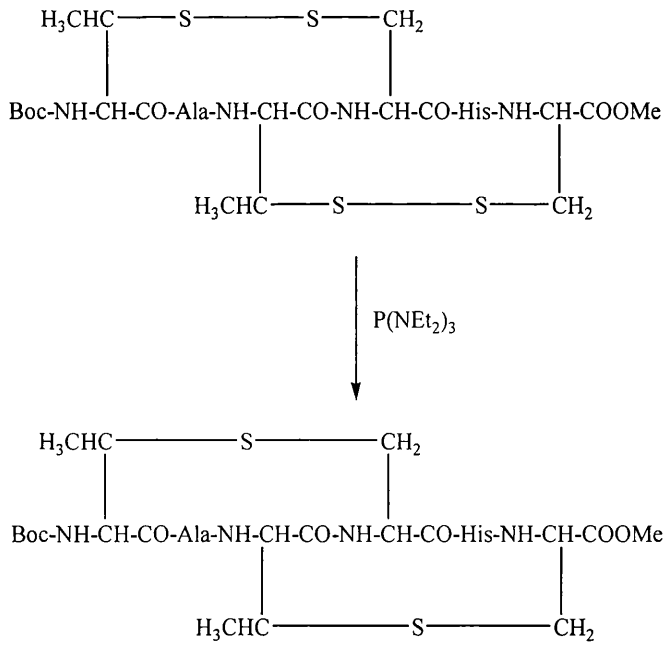


Figure 15: Synthesis of ring D and ring E of nisin.[18]

The four big segments synthesised this way (ring A, ring B, ring C, ring D + ring E) were then assembled in solution to make the final nisin.

Desulfurisation is a fast and efficient method and avoids racemisation, but it can lead to several by-products and is not easily compatible with base-labile protecting groups. Therefore, new synthetic pathways were explored to make lanthionine, from cysteine and serine residues. Three main strategies exist, depending partly on the protecting groups used, with dehydroalanine, serine β-lactone or halogenoalanine as β-alanyl cation equivalent (**Figure 16**).

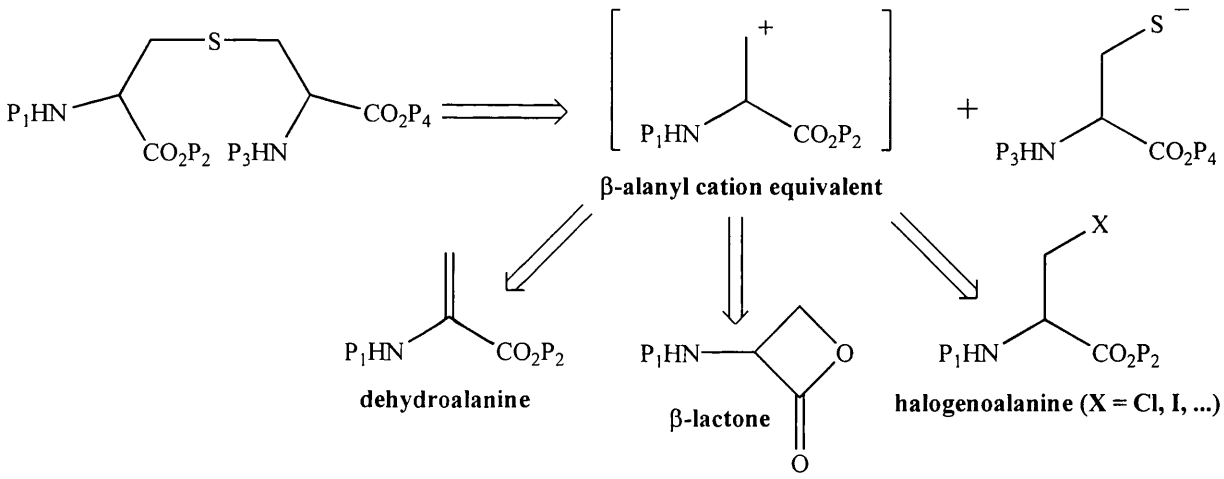


Figure 16: Retrosynthetic pathways to synthesise lanthionine from cysteine and serine residues. P_1, P_2, P_3 and P_4 are protecting groups.

2. 2. Dehydroalanine as β-alanyl cation equivalent

Residues of protected lanthionine were successfully synthesised[51] by Michael addition of a cysteine residue on a dehydroalanine residue, with an excellent yield of 72%, using cesium carbonate as a catalyst (**Figure 17**).

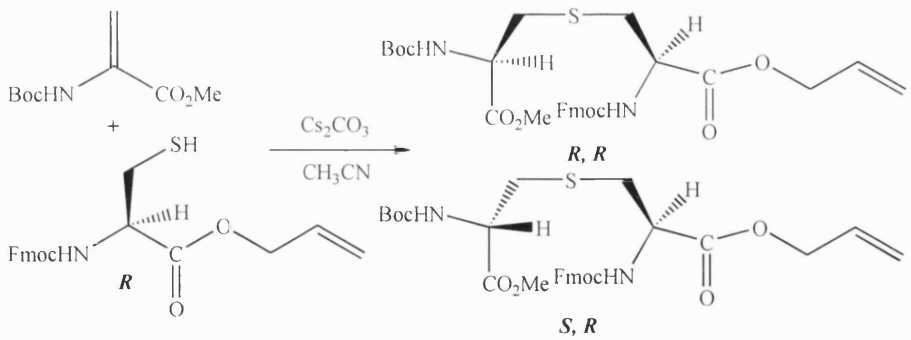


Figure 17: Synthesis of lanthionine residues using a dehydroalanine intermediate.[51]

Although this method is high-yielding, it is not stereospecific: however, the two diastereoisomers could easily be separated afterwards by HPLC. *tert*-Butyloxycarbonyldehydroalanine methyl ester was synthesised by action of carbodiimides on *tert*-butylserine methyl ester with CuCl as a catalyst[52] (several other routes to dehydroalanine residues have been published[53,54,55,56,57]).

This pathway gave very good results in peptide synthesis, mimicking the biosynthesis of lanthionine rings in lantibiotics. Lanthionine-bridged enkephalin analogs were synthesised with this method on a solid support by the attack of a cysteine anion on a dehydroalanine residue (**Figure 18**).[38]

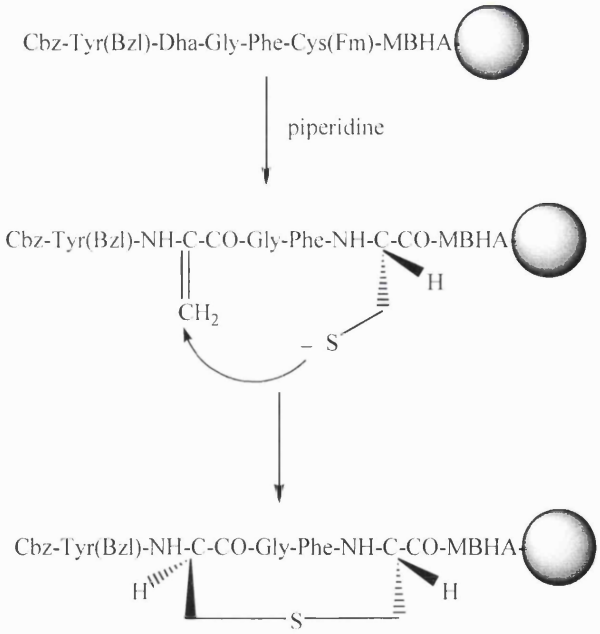


Figure 18: Synthesis of an enkephalin analog on solid-support *via* a dehydroalanine intermediate.[38]

The lanthionine ring obtained is (*S, R*), which is the favoured lanthionine conformation in lantibiotics.[44] It appears that there is no problem of diastereoisomers when the natural pathway for lanthionine-containing peptides is followed.

2. 3. Serine β-lactone as β-alanyl cation equivalent

This pathway has given lanthionine residues in high yields and excellent stereoselectivities, from the attack of a serine β-lactone by a cysteine residue, using cesium carbonate as a catalyst. Vederas *et al.* developed a methodology using dimethylazodicarboxylate and triphenylphosphine as catalysts to transform *N*-protected serine residues into serine β-lactones in THF.[58] Improvements of this Mitsunobu reaction,[59] carrying out the experiment in acetonitrile for example, led to yields of 70-80 %.[60,61,62] Goodman *et al.*[5] made an efficient synthesis of lanthionine residues from serine β-lactone, using this methodology (**Figure 19**).

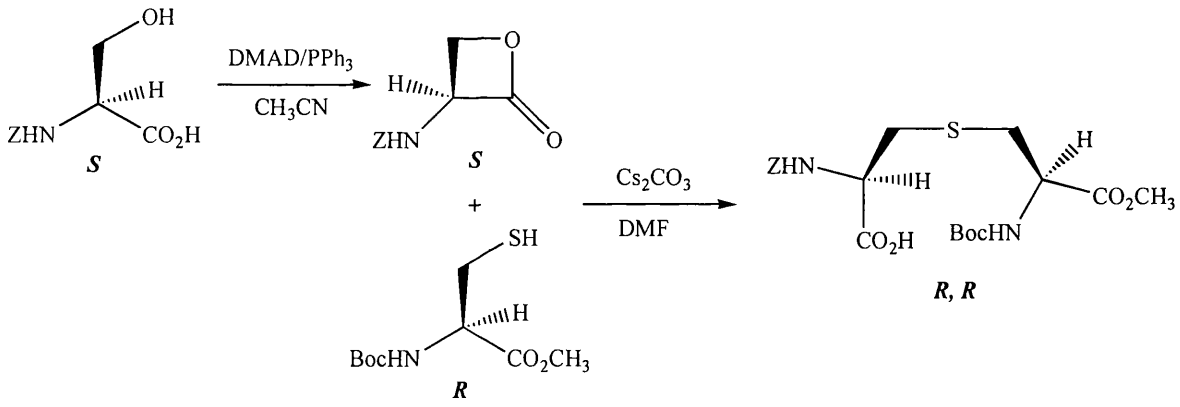


Figure 19: Synthesis of serine β-lactone and lanthionine residues

The advantage of this method is that the opening of the β-lactones leads to stereochemically pure compounds, however, the yield of the formation of lanthionine is fairly low (50 %) as there is a competing reaction taking place, in where a thioester is formed.[5] Cesium acts as a counteranion for the thiolate and the salt therefore formed attacks the β-lactone following two pathways, a or b (**Figure 20**).

When β,β-dimethylcysteine analogs are used, β,β-dimethyl lanthionine residues[5,6] are made without by-products with an excellent yield of 92 % (**Figure 20**, when R = H, both pathways a (*O*-acyl fission) and b (*O*-alkyl fission) occur, when R = CH_3, pathway a does not occur). Indeed, the bulky alkyl groups prevent the *O*-acyl fission.[5] Efficient synthetic pathways have been studied to make β,β-dimethylcysteine analogs.[63,64]

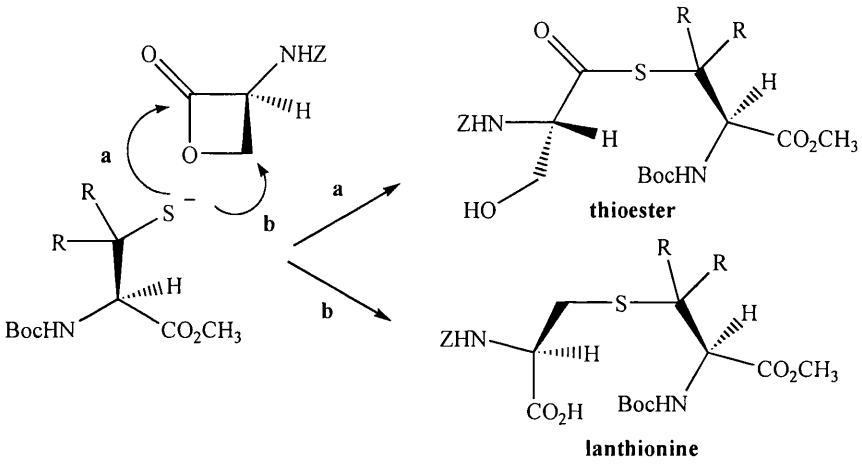


Figure 20: Competitive reactions when a thiolate residue attacks a serine β-lactone.

Another way to influence the outcome of the reaction has been reported recently.[65] Prenylated cysteine residues were synthesised *via* the opening of β-lactones after attack of prenyl thiolates using sodium hydride or triethylamine as basic reagents (**Figure 21**).

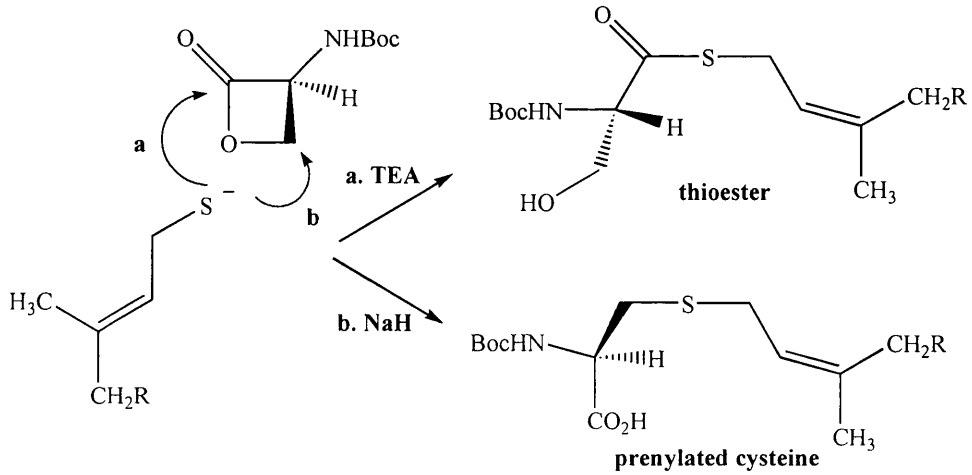


Figure 21: Synthesis of prenylated cysteine from serine β-lactone, R = $C_{10}H_{17}$ or $C_{15}H_{25}$.[65]

These bases were the determinant factors for the major products obtained as sodium hydride led mainly to prenylated cysteine derivatives, whereas triethylamine led mainly to thioester derivatives.

Finally, the nature of the protecting groups on the residues are very important. Indeed, when Bradley *et al.*[51] attempted the synthesis of lanthionine residues with serine β-lactone and *N*-Fmoc-cysteine allyl ester, no reaction took place.

This synthetic pathway has been successfully followed to synthesise lanthionine residues that were later used for the synthesis of lanthionine-bridged opioid enkephalin,[41] lanthionine-bridged sandostatin[39] and cyclolanthionine analogs.[34,45]

2. 4. Halogenoalanine residues and other β-alanyl cation equivalents

The most successful approach to lanthionine residues was discovered by Dugave and Ménez[66] in 1997 and involves the use of an iodoalanine residue as β-alanyl cation equivalent. This work derives from thorough searches of the best β-alanyl cation equivalent available.

Chloroalanine was added to a cysteine thiolate made from cystine treated with ammonia and sodium then potassium hydroxide in the first lanthionine synthesis by du Vigneaud *et al.*.[67,68] More work followed on this method,[69] however, a serious drawback is the racemisation of lanthionine residues in the presence of the strong base used in this synthesis. Aziridine has also been used as a β-alanyl cation equivalent.[70] All these methods led to difficult purification, low yields and problems of racemisation and recent attempts at improving them failed.[5,50]

In the meantime, aziridine[71] and iodoalanine residues[72,73] derived from *O*-tosylserine[74] were investigated as β-alanyl cation equivalent for the synthesis of organozinc reagents and syntheses using organocuprates, but no further work was carried out on lanthionine synthesis until Dugave and Ménez studied the synthesis of functionalised amino acids using *N*-trityl serine.[75]

The bulky *N*-trityl group had previously shown its potential as a protecting group for serine[76] in Mitsunobu reactions.[77] It prevents α-proton abstraction by non-

nucleophilic bases and α-ester saponification. Dugave and Ménez reported the synthesis of *N*-trityl iodoalanine residues from *O*-mesyl-*N*-tritylserine residues using sodium iodide in acetone. Iodoalanine residues were obtained as mixtures of rotamers (other *N*-tritylated amino acids have been reported as rotamers[78]). When treated in the same way, *O*-mesyl-*N*-tritylthreonine residues gave mostly aziridine. When heated, *O*-mesyl-*N*-tritylserine residues gave aziridines. Nucleophilic substitution of the iodine by a selection of malonate-related nucleophiles gave either aziridines or triesters.

Shortly afterwards, these iodoalanine residues were used to synthesise lanthionine residues[66] in high yields (**Figure 22**).

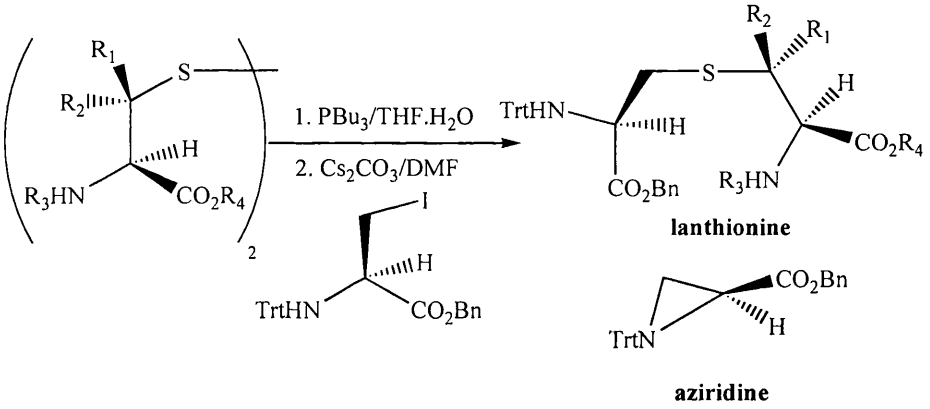


Figure 22: Synthesis of lanthionine residues from cystine and iodoalanine residues.[66] R_1, R_2, R_3 and R_4 are different groups (see Table 2).

A small amount of aziridine was also formed during the reaction. When lanthionine residues were made (R_1 = R_2 = H), no racemisation was detected but a mixture of rotamers was obtained. In these cases, the enantiomeric purity was determined (> 98 %) by desulfurisation of the lanthionine residues with Raney Nickel, giving alanine residues, followed by derivatisation of these alanine residues with a chiral reagent, then separation by HPLC of the diastereoisomers obtained. Several different protecting groups were tried on the lanthionine residues (**Table 2**).

Disulfide configurations, R_1, R_2	R_3	R_4	Aziridine (%)	Lanthionine (%)
2R, H, H	Fmoc	tBu	8	88
2R, H, H	Boc	Me	17	79
2R, 3R, H, CH_3	Boc	Me	7	83
2S, CH_3, CH_3	Boc	Me	13	81
2R, H, H	Fmoc	H	35	41

Table 2: Different types of lanthionine residues synthesised from iodoalanine residues.[66]

This method is fast, with high yields and low racemisation. This is the best method published so far to synthesise lanthionine residues quickly and efficiently.

Neither chloroalanine nor iodoalanine residues were used as intermediates to synthesise lanthionine-containing peptides on a solid support, but bromoalanine residues, made from serine residues, were used by Jarosinski *et al.*[44] to synthesise enkephalin analogs (**Figure 23**) and sandostatin analogs.

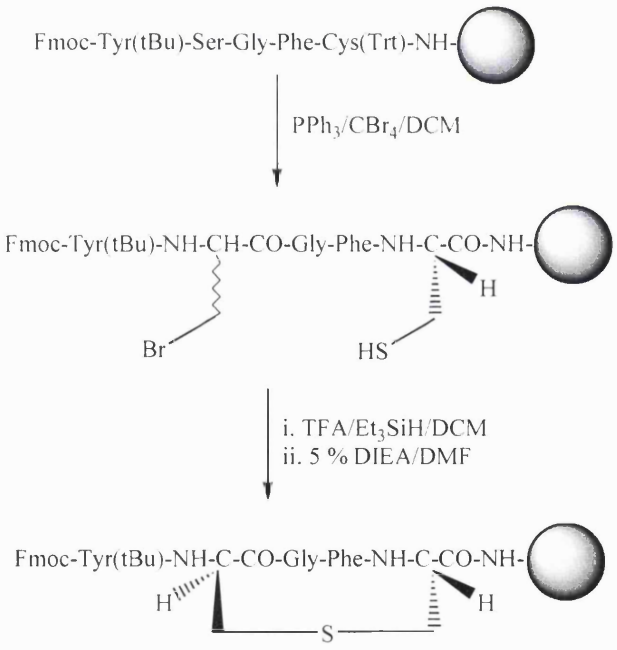


Figure 23: Synthesis of an enkephalin analog on a solid-support *via* a bromoalanine intermediate.[44]

In the presence of base, cysteine residues attack bromoalanine residues to form lanthionine bridges. It is believed that the attack proceeds *via* dehydroalanine formation. Indeed, only one lanthionine diastereoisomer (*S, R*) is obtained with this method, either

from (*R*) or (*S*) bromoalanine and (*R*) cysteine; this is a similar result to that obtained by the synthesis of enkephalin analogs from dehydroalanine residues (see section **2. 2.**).[38]

2. Orthogonal protecting group strategy for the cyclisation of small peptides

1. Cyclisation of small peptides

1. 1. Principles of cyclisation

The cyclisation of peptides containing proteinogenic amino acids, *via* lactam, disulfide or monosulfide bridges can be quite difficult for small peptides of six or fewer residues. The ease of peptide cyclisation will depend on the sequence, whether the cyclisation step involves sterically hindered amino acids or whether the linear peptide folds readily.[79]

Until recently, cyclic peptides used to be synthesised as linear precursors in solution or on solid support and then cyclised in solution.[80] However, there are several synthetic problems associated with cyclisation in solution, particularly intermolecular side-reactions (polymerisations) and the lack of solubility of the peptides in the chosen solvents.

Several methods for on-resin cyclisation of small peptides have therefore been developed.

1. 2. Solid-phase peptide synthesis

SPPS involves synthesising a peptide on a solid support or resin (**Figure 24**).[81] The peptide is usually linked to the resin by a linker, by its *C*-terminus (synthesising peptides from the *C*-terminus reduces the risk of racemisation[82]).

Each amino acid is added in turn and in order of the peptide sequence and coupled to the growing peptide by means of coupling reagents. Usually its amine function is protected by an N^{α}-protecting group and any side-chain of the amino acid likely to react

with carboxy or amine functions is protected by side-chain protecting groups, which are cleaved using different conditions to the N^{α}-protecting groups. In between addition of amino acids, capping of the resin ensures that any peptide with failed sequences will not grow on the resin, alongside the desired peptide.

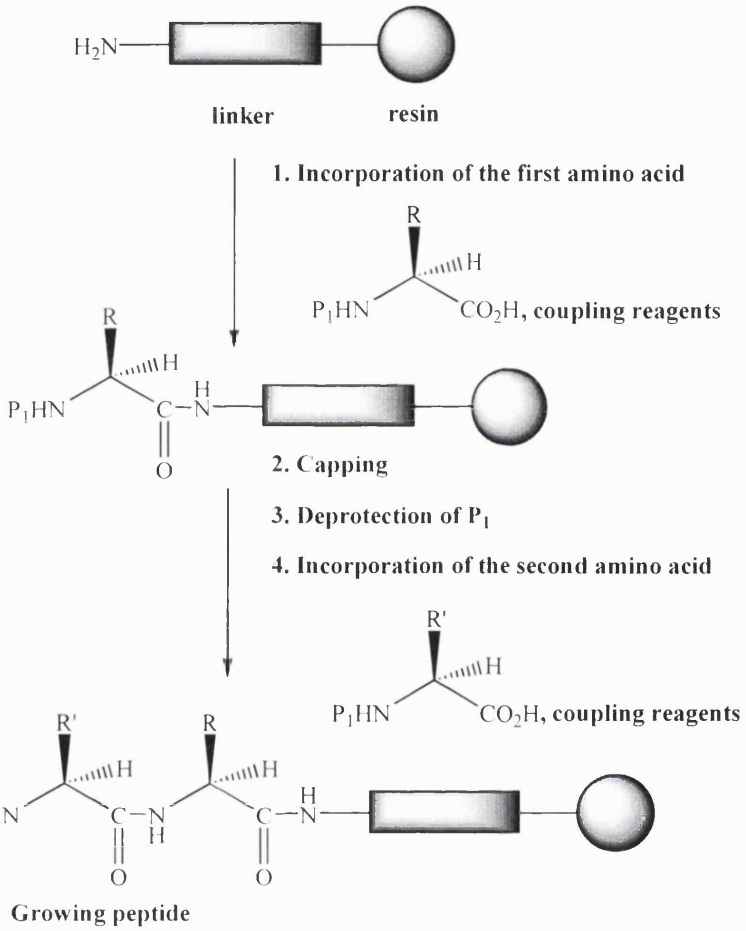
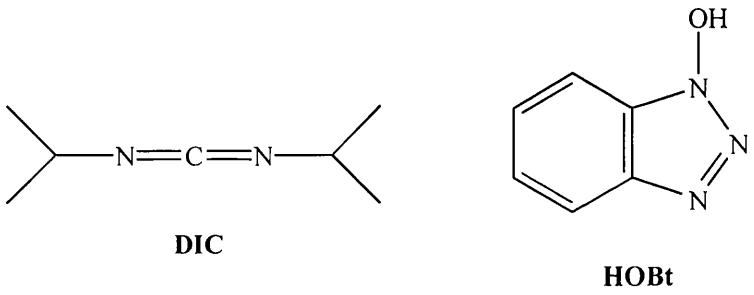


Figure 24: Principles of SPPS.[83] Growing of a peptide chain on a solid support (P_1 is a N^{α}-protecting group, R and R' are the different side-chains of the amino acids incorporated, they are protected by side-chain protecting groups if necessary.

There are numerous advantages to SPPS compared to solution peptide synthesis. Among them is the possibility of using a huge excess of soluble reagents that can be washed off the resin.

Carbodiimides are the most common types of coupling reagents used in peptide synthesis.[82,83] Several types exist depending on their solubility and the solubility of the by-product urea formed after formation of the peptide bond. DIC (**Figure 25**) is most commonly used in SPPS as its urea by-product is soluble in DMF/dioxane and is easily washed off the solid support.

Other coupling methods involves the use of activated esters either synthesised beforehand such as pentafluorophenyl esters[82,83] or synthesised *in situ*. The most common coupling reagent used for the *in situ* synthesis of an activated ester is HOBt (**Figure 25**).[82] Very often, HOBt is used alongside DIC, for a more efficient and clean coupling step.[82]



DIC **HOBt**

Figure 25: DIC and HOBt. Two of the most common coupling reagents, often used together for efficient coupling steps.

Resins used for SPPS must be totally inert to the reaction conditions and have good physical properties compatible with the solvents needed.[84,85,86] Polystyrene was originally used as the resin when Merrifield first developed SPPS.[87] Since then, other polymers such as polyacrylamide or polyethylene glycol-polystyrene (or Tentagel) have been used. PEG-PS is particularly good as this support permits complete solvation of the reactive sites.[80]

The range of resins available for SPPS today is very large as is the number of linkers used in conjunction with these resins. The role and nature of the linkers, particularly those used for the cyclisation of peptides, are discussed below. Both resins and linkers are very important tools for a successful SPPS.

Another requirement of SPPS is to have N^{α}-protecting groups that are easily removed by a method "orthogonal" to the method for the removal of the side-chain protecting groups. This means that if the N^{α}-protecting groups (such as Boc) are cleaved by mild acid (such as TFA), then the side-chains should be protected by groups which are not cleaved by mild acids (for example benzyl groups cleaved by very strong acids like HF), so that no side-reaction occurs where side-chain protecting groups would be cleaved at the same time as N^{α}-protecting groups.

Two methodologies have been developed in SPPS, using either Fmoc or Boc groups as N^{α}-protecting groups. The Boc methodology was developed first and usually involves the use of benzyl protecting groups for side-chain protection. The most recent and convenient methodology involves the use of Fmoc groups as N^{α}-protecting groups (cleaved by bases such as piperidine) and tBu/Boc groups as side-chain protecting groups (**Figure 26**).

An important advantage of the Fmoc methodology is that the Fmoc groups are U.V. detectable and the integration of amino acids on the growing peptides can be monitored.

Usually, to reduce the number of steps involved in SPPS (which increases the overall yield), the peptide is cleaved from the resin using similar reagents to those used for cleaving the side-chain protecting groups, so that the free peptide is obtained in one step.

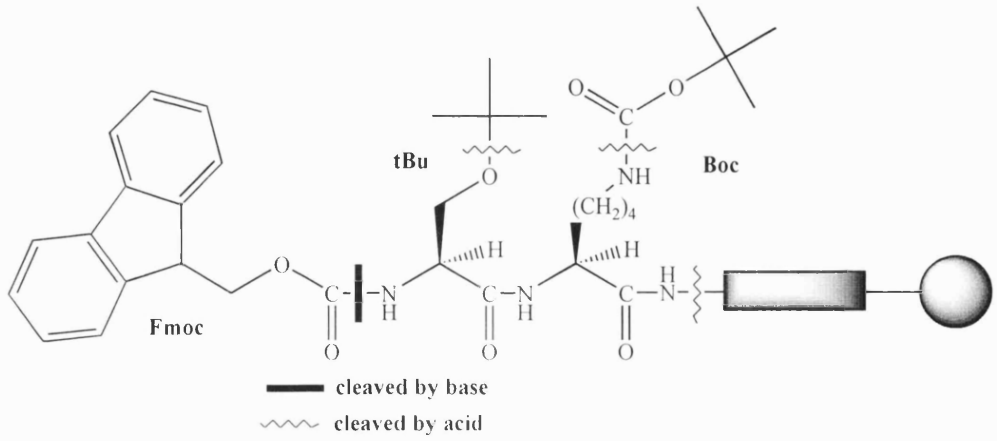


Figure 26: Example of SPPS of a small dipeptide built with the Fmoc methodology.

Again, a large selection of protecting groups and coupling reagents has been developed in the past 20 years and details of those specifically used for the cyclisation of small peptides are described below.

2. Three dimensional orthogonal protecting group strategy

2. 1. On-resin cyclisation methods in SPPS

An important advantage of on-resin cyclisation is the reduced risk of intermolecular reactions as there is a large distance between growing chains on the solid support. This is called the "pseudodilution" effect.[88] To increase the distance between growing chains, it is often better to have a resin with a low to medium loading.[80]

Several strategies have been developed to cyclise small peptides, with different types of anchoring of the peptide to the resin (*C*-terminal anchoring, side-chain anchoring or backbone anchoring *via* a linker) and cyclisation occurring either from side-chain to side-chain, from side-chain to terminus or in a head-to-tail fashion, forming either homodetic rings (peptide bond) or heterodetic rings (among them disulfide or monosulfide bridges).[80]

A convenient way to classify all these methods is to look at the tools (resins or protecting groups) used to perform these cyclisations. This way, two approaches can be identified: PCOR and peptide cyclisation with the use of several types of linkers to anchor the peptide to the resin, this second approach relying much more on a three dimensional orthogonal protecting group strategy.[79,80]

2. 2. The PCOR method

Kaiser[89] first developed polystyrene-bound oxime esters as supports in SPPS (only Boc methodology is compatible with oxime resins as the secondary amines used in Fmoc methodology to cleave the Fmoc groups can also lead to the cleavage of the growing peptide from the oxime resin).

Once the linear peptide has been synthesised on the resin, TFA is used to cleave Boc/tBu protecting groups, followed by DIEA to neutralise any remaining TFA.[90] Then,

cleavage of the peptide from the resin and cyclisation take place simultaneously, often catalysed by acetic acid.[79]

PCOR has been extensively used by Ösapay and Goodman to synthesise lanthionine-containing peptides.[34,39,40,42,43,45] For example, cyclolanthionine residues were synthesised on an oxime resin in high yields (**Figure 27**).

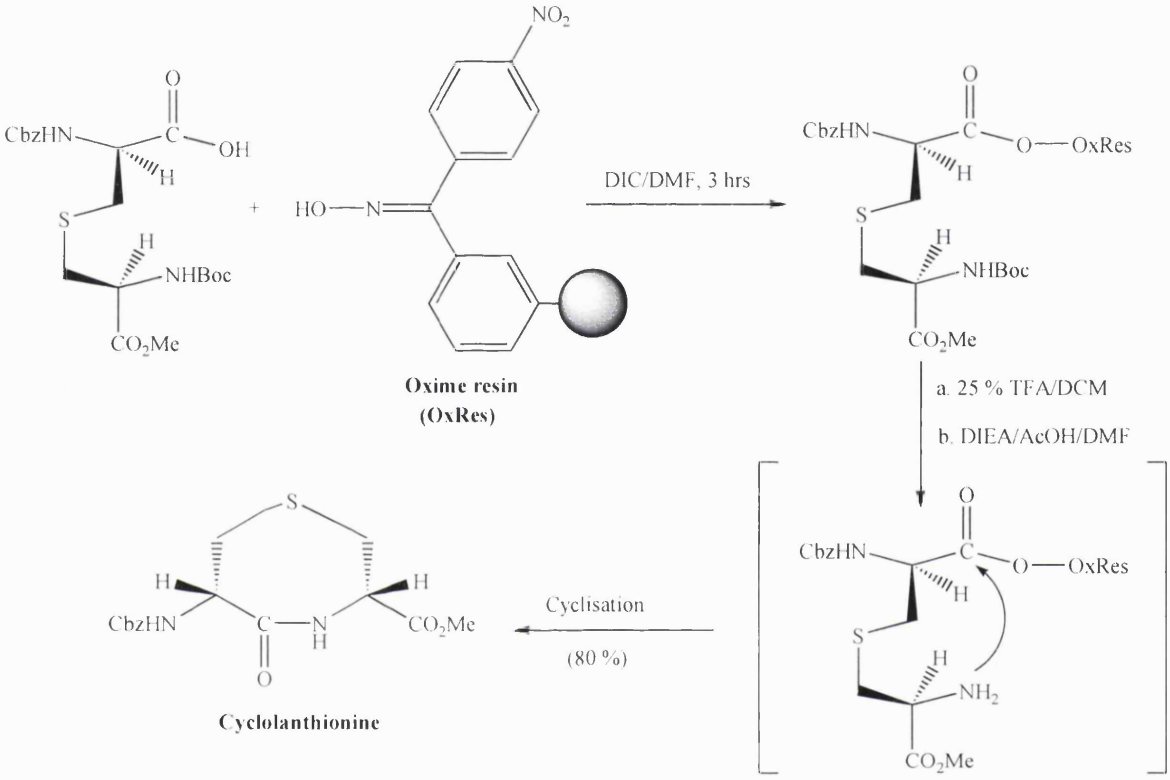


Figure 27: PCOR method used to synthesise the four stereoisomeric cyclolanthionine residues.[45] Synthesis of (*S, S*) cyclolanthionine.

Enkephalin analogs and sandostatin analogs were synthesised in a similar way in good yields. This method has also been used for the synthesis of depsipeptides.[91]

This is a quick and efficient method and cyclisation often proceeds in high yields. However, there are two major limitations of PCOR. Firstly it can only be used with Boc methodology. Secondly the peptide is cleaved from the resin when the cyclisation step occurs, so there is no possibility of extending the peptide on the resin after cyclisation. Therefore, polycyclic peptides cannot be synthesised using PCOR. Finally,

cyclodimerisation when cyclic tetrapeptides were synthesised has also been reported as a drawback of PCOR.[92]

In an attempt to overcome this drawback, Taylor *et al.*[93] combined the advantages of PCOR with a three dimensional orthogonal protecting group strategy. Three dimensional orthogonal protecting group strategy involves the use of a third type of protecting group, in addition to the N^{α}-protecting groups and side-chain protecting groups.

With Boc methodology, this third type of protecting group must not be cleaved by mild or strong acid and the method to cleave it must not affect Boc/tBu groups or Cbz/Bzl groups. Small multicyclic polypeptides were synthesised *via* anchoring of the side-chain of glutamic acid or aspartic acid to a *p*-nitrobenzophenone oxime resin,[94,95] with side-chain-to-tail cyclisation. Phenacyl groups were used as the third orthogonal protecting groups (removed by Zn/AcOH).

Subsequently, lactam-bridged hCT analogs were synthesised with a four dimensional orthogonal strategy (Boc/Bzl/Fmoc/Al) (**Figure 28**).[93]

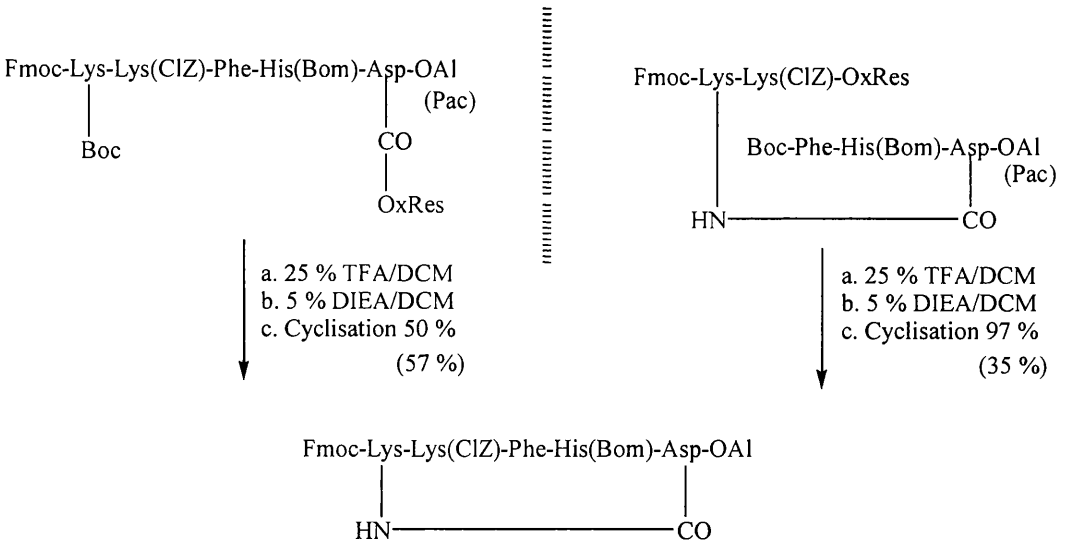


Figure 28: Use of PCOR method and four dimensional orthogonal strategy (Boc/Bzl/Fmoc/Al). Side-chain-to-side-chain cyclisation (left) and backbone cyclisation (right) are compared.[93] ClZ and Bom are protecting groups.

This method proceeds well as the Fmoc group is cleaved after removal of the peptide from the resin. The model cyclopentapeptide is obtained with much higher yields when allyl protecting-groups (Al) are used rather than phenacyl groups (Pac).

To limit the problem of dimerisation encountered with PCOR, another type of linker was used with a thioester spacer[96] to synthesise cyclic penta-, hexa- and hepta peptides. An improvement in terms of purity was noticed but the yields for these cases were low.

PCOR method is an efficient method to build small cyclic peptides with Boc methodology. Occasionally, a three dimensional orthogonal protecting group strategy can be used along this method. However, the use of a limited number of protecting groups and the necessary cleavage of the peptide from the resin during cyclisation give PCOR a fairly limited scope.

2. 3. Three dimensional orthogonal protecting group strategy: types of third protection available and examples

Cyclisation of small peptides *via* a three dimensional orthogonal protecting groups strategy proceeds well when it is carried out in a limited number of simple steps with high yields. The way the peptide is linked to the resin is important. Two main methods exist.

The peptide can be linked to the resin *via* its *C*-terminus (**Figure 29**).

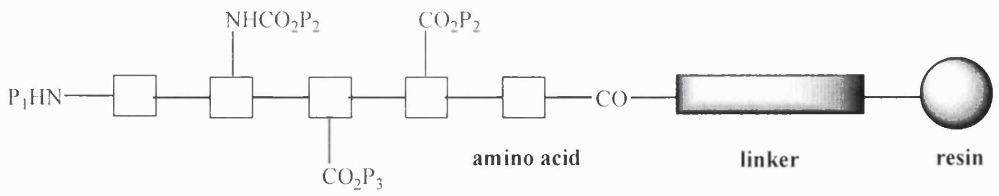


Figure 29: Peptide attached to the linker + resin *via* its *C*-terminus. P_1, P_2 and P_3 are protecting groups.

Three possibilities for cyclisation then become available, depending on the place and nature of the three types of protecting groups (P_1, P_2 and P_3) on the peptide. If P_1 is removed selectively and the peptide cleaved from the resin, then a head-to-tail cyclisation takes place. Alternatively, an *N*-terminus-to-side-chain cyclisation is carried out when P_1 and P_3 are cleaved selectively. Finally, the selective removal of groups P_2 leads to a side-chain-to-side-chain cyclisation.

The second method commonly used is to anchor the peptide to the resin *via* one of its side-chains (**Figure 30**).

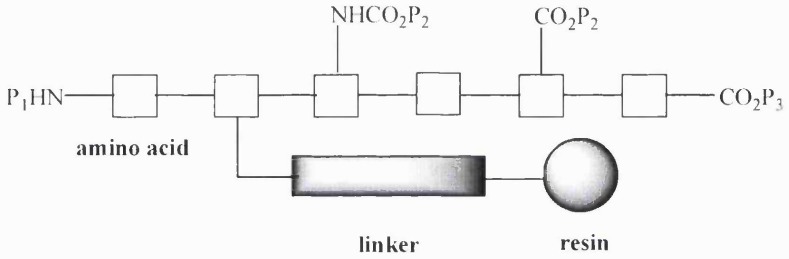


Figure 30: Peptide attached to the linker + resin *via* a side-chain. P_1, P_2 and P_3 are protecting groups.

Again several possibilities are available for cyclisation. For example, head-to-tail cyclisation occurs when P_1 and P_3 are cleaved whereas *N*-terminus-to-side-chain cyclisation is carried out when P_1 is cleaved and the peptide is removed from the resin. Aspartic acid, glutamic acid or lysine are usually used for backbone anchoring. Linkage *via* the backbone amide (using a special BAL linker[97]) has also been used.

The most common linkers[80] used to link the peptide to the resin using a three dimensional orthogonal protecting group strategy are MBHA, PAL and PAC (**Figure 31**).

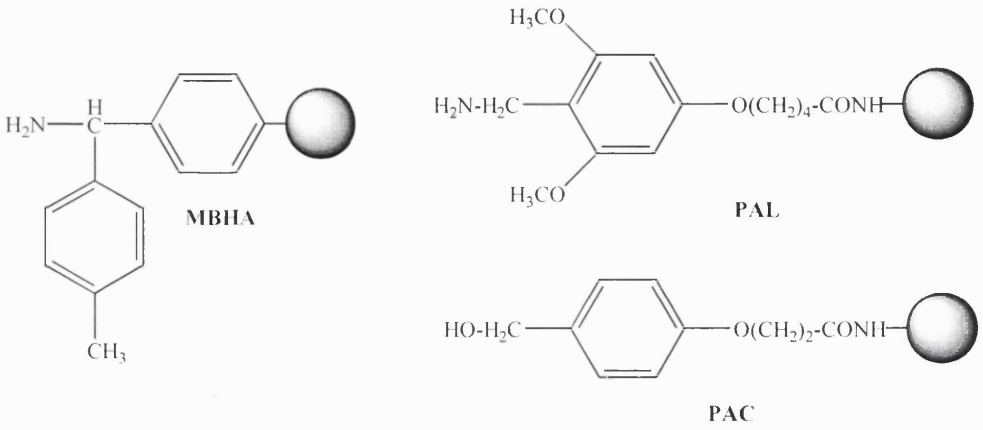


Figure 31: Types of linkers used for orthogonal protecting group strategy.[80]

The choice of third protecting groups is also very important. They must be compatible with all the reagents used during SPPS and removed under conditions where neither N^{α}-protecting groups nor side-chain protecting groups are affected.

Sometimes, both Boc and Fmoc methodologies are combined so that Boc, Fmoc and Bzl are used together. However, other protecting groups such as allyl, Dmb or Dde are used for the third orthogonal protecting group (**Figure 32**).[80]

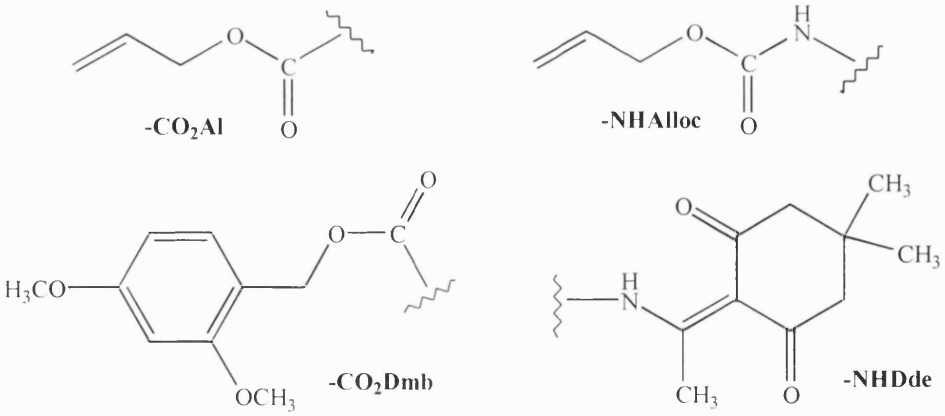


Figure 32: Third type of protecting group used in SPPS.[80]

A combination of both Boc and Fmoc methodologies were used in the earliest examples of three dimensional orthogonal protecting group strategy. Human growth hormone-releasing factor analogs were synthesised *via* side-chain-to-side-chain cyclisation.[98] The peptide was built with the Boc methodology with only two side-chains,

one of aspartic acid and one of lysine selectively protected with Fm and Fmoc groups. Orthogonal cleavage of these groups using base liberated the carboxy and amine groups necessary for side-chain-to-side-chain on-resin cyclisation.

In a similar manner, Fmoc/Bzl/Boc strategy was used[99] to synthesise side-chain-to-side-chain cyclic opioid dermorphin and β-casomorphin analogs. [100]

Fmoc/tBu/Al strategy is one of the most common three dimensional orthogonal protecting group strategies. The use of allyl groups in orthogonal protecting group strategy has been recently developed. Allyl protecting groups are cleaved with palladium tetrakistriphenylphosphine, $Pd(PPh_3)_4$[101,102] as a catalyst and scavengers such as silyl amines are used in the reaction. Different methods[103,104] have been developed to adapt the cleavage of allyl ester and Alloc to SPPS. Albericio *et al.* also recently found a way to cleave allyl group in neutral conditions, using phenyltrihydrosilane as a scavenger for the allyl group.[105]

A cyclic decapeptide was synthesised using an Fmoc/tBu/Al strategy.[106] Four methods were tried to build the peptide, starting at different points of the ring, anchoring either glutamic acid residues or aspartic acid residues and with the side-chain linked to a PEG-PS resin *via* a PAC or PAL linker (**Figure 33**). For example, aspartic acid protected by an Fmoc and allyl group was anchored on a resin + PAL linker *via* its β carboxy function, then a linear peptide was grown from this residue. Finally cyclisation was carried out. This method (c) gave the best yield and purity. This shows the importance of the nature of the linker used and of the place where the cyclisation takes place in the sequence of the chosen peptide.

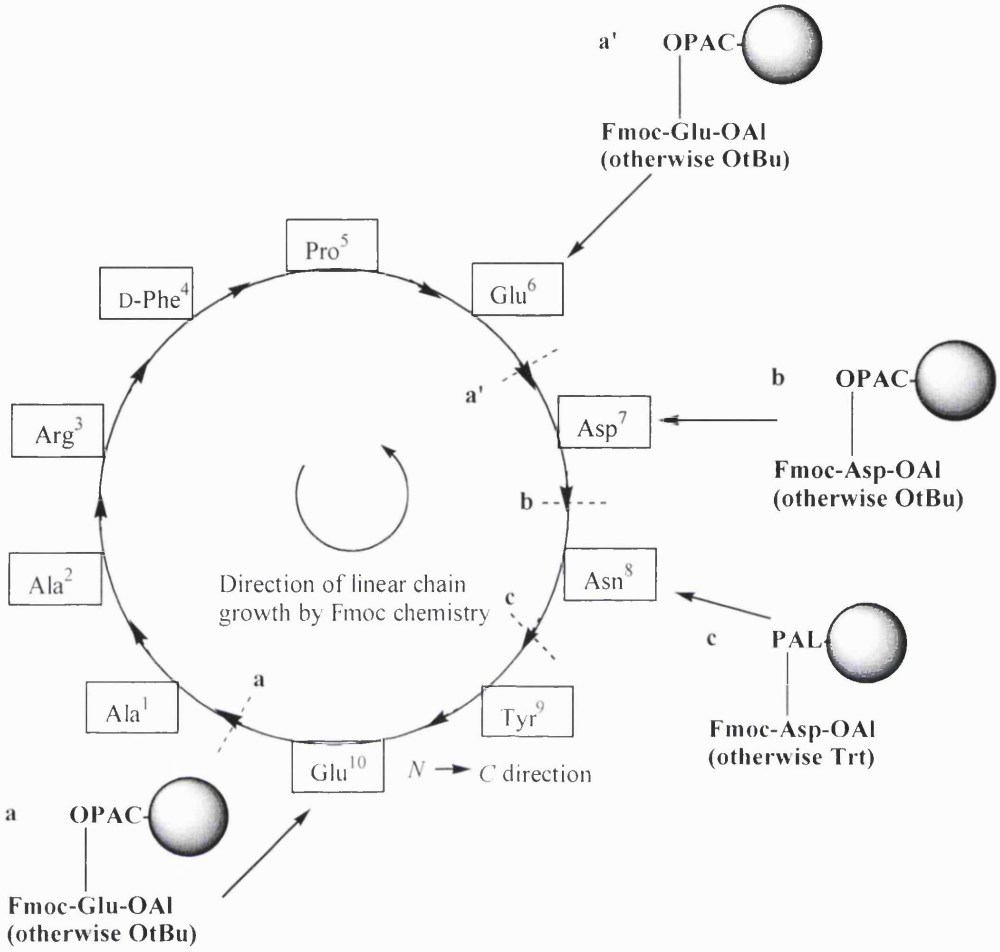


Figure 33: Four methods to build a decapeptide.[106] The perpendicular dashed lines indicate the final bond needed to achieve cyclisation in each case, a, a', b or c.

The same orthogonal strategy was used to anchor a Lys residue to a carbonate resin *via* its side-chain, its carboxy function being protected by an allyl ester.[107] This head-to tail cyclisation proceeded well and an hexapeptide was synthesised this way in a good yield (61 % cleavage yield, 71 % purity by HPLC).

Allyl protecting group used in a three dimensional orthogonal protecting group strategy is a convenient third protection and its use has led to efficient synthesis of cyclic peptides. Among others, it has been used to build cyclic peptides libraries,[108] synthesis of novel growth hormone releasing factor analogs[109] and synthesis of peptides with tripodal

side-chain bridges.[110] Moreover, the method has recently given very promising results for the synthesis of sterically crowded peptides such as bacitracin A[111] and oscillamide Y.[112]

Finally, synthesis of an analog of nodularin[113] comparing solution-phase and solid-phase methods has shown that even though the cyclisation step is carried out with a lower yield on the resin, SPPS is overall a better method in terms of practicality of the construction of the peptide.

Several other protecting groups have occasionally been used as third protection for a three dimensional orthogonal protecting group strategy. McMurray *et al.* reported the successful synthesis of head-to-tail cyclic decapeptides using Dmb esters to protect glutamic acid.[114,115] These esters are easily cleaved by 1 % TFA in dichloromethane. Optimisation of the reaction conditions to limit racemisation and oligomerisation was carried out.[115]

Dde protecting-groups were used in a four dimensional protecting-group strategy (Fmoc/tBu/Al/Dde) to synthesise a branched cyclic peptide (**Figure 34**). Dde was removed by 1.5 % hydrazine in DMF.

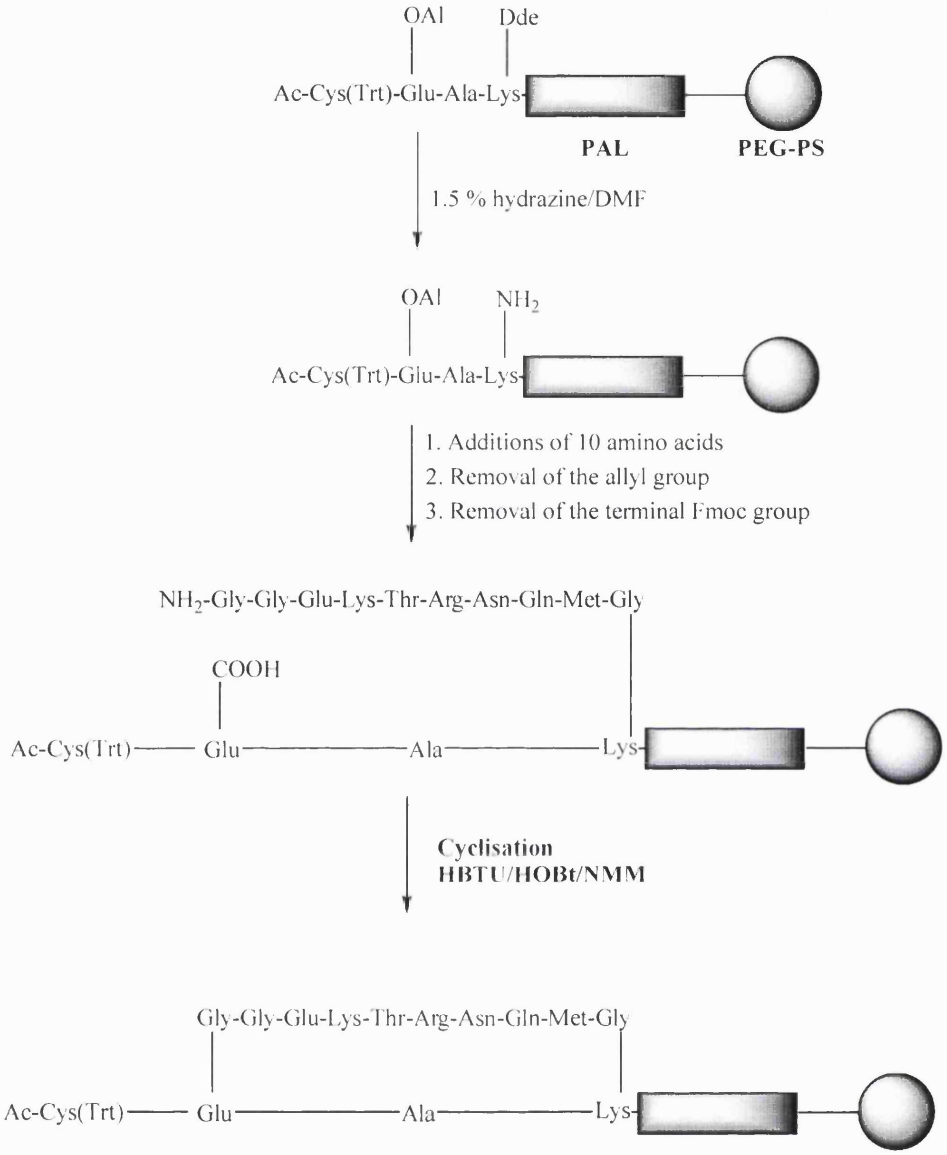


Figure 34: Synthesis of a decapeptide *via* a head-to-tail cyclisation using Fmoc/tBu/Al/Dde.[116] HBTU and HOBt are cyclisation reagents (see 2. 4.).

2. 4. Cyclisation reagents

Cyclisation reagents are coupling reagents used to promote the formation of the peptide bond when the linear peptide is cyclised. They are key elements to a successful cyclisation. They are usually derived from HOBt but are even more efficient in controlling by-reactions.

Several cyclisation reagents have been used (**Figure 35**).[117]

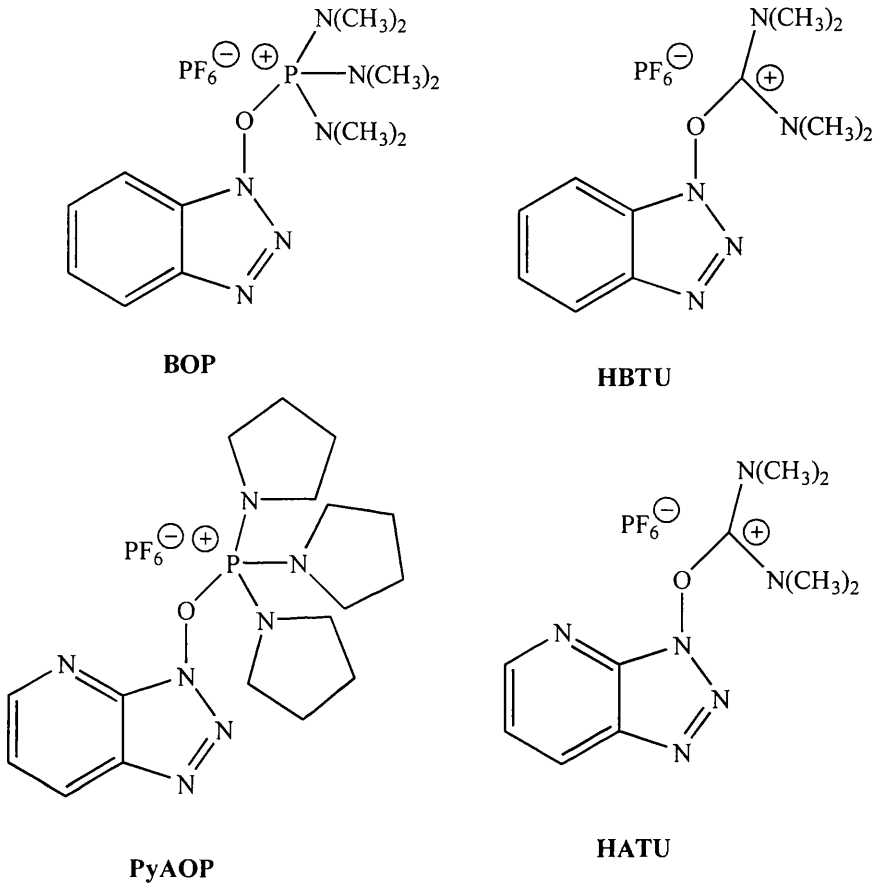


Figure 35: Cyclisation reagents.[117]

BOP is a good cyclisation reagent, especially when it is used alongside HOBt but the formation of a highly carcinogenic by-product (HMPA) during cyclisation led to the development of other reagents.

HBTU is a very efficient cyclisation reagent but racemisation during cyclisation still occurs in its presence as well as side-reactions like the formation of guanidinium by-products.[79,118]

HATU[119] and PyAOP[120] are better cyclisation reagents because the risk of racemisation is reduced when they are used. In addition, no guanidinium by-product is formed during cyclisation with PyAOP.

There is no rule for the choice of cyclisation reagents and optimisation for each particular case is necessary.

Three dimensional orthogonal protecting group strategy is a very good method for the synthesis of cyclic peptides. It is still in its infancy and each successful cyclisation depends largely on optimisations of the choice of resins, linkers, coupling reagents, site of cyclisation on the peptide and protecting groups. Allyl protecting groups in particular have been used very efficiently with this strategy.

However, so far, the quest for a "perfect" third type of protecting group, i.e. a protecting group removed by mild and neutral conditions by methods having no effect whatsoever on the other groups of the peptide or reagents employed in the synthesis is not over.

3. Photolabile protecting groups

3. 1. Introduction

Photolabile protecting groups have attractive properties: they contain chromophores which are sensitive to light, but they are stable to most of the reagents commonly used in chemistry.[121,122] A good photolabile group must be cleaved in high yields and have a short-lived excited state,[122] indeed the longer the excited state, the more likely it is that side-reactions will occur.

Photolabile protecting groups have been mainly designed to protect amine functions, carboxy functions, carbonyl functions and hydroxy functions. They have been used in nucleotide chemistry[123] and carbohydrate chemistry[124,125] but so far less use has been made of photolabile protecting groups in peptide synthesis.[126,127]

However, photolabile linkers have been synthesised,[128] for convergent SPPS in particular.[129] In convergent SPPS, fragments of large peptides or peptides with difficult sequences are built individually using SPPS and then the fragments are assembled in solution. It is extremely important to be able to cleave the fragments from their solid support without removing any of their side-chain protecting groups as the fragments are assembled in solution afterwards and any missing side-chain protecting group would lead to by-products. Photolabile linkers are therefore a good solution, as long as the light used to cleave them has a wavelength compatible with peptides (tryptophan for example is affected by light of wavelength of 320 nm and less[121]).

Among the photolabile protecting groups studied, three overall types are good candidates to protect amino acids, either to protect their amine function, or their carboxy function or both. They are 3,5-dimethoxybenzyl derivatives, phenacyl derivatives and nitrobenzyl derivatives.[121,122]

3. 2. 3,5-Dimethoxybenzyloxycarbonyl derivatives

As the photochemical reactivity of benzyl groups was reported,[130] work followed to study the photochemical reactivity of benzyl derivatives.[131,132] It was observed that methoxy groups at the *meta* position enhances the photochemical reactivity of benzyl acetates,[133] Chamberlin therefore developed a new photolabile group to protect amines of amino acids, the 3,5-dimethoxybenzyloxycarbonyl group (**Figure 36**).[134] This group is stable under the usual conditions of peptide synthesis.[121]

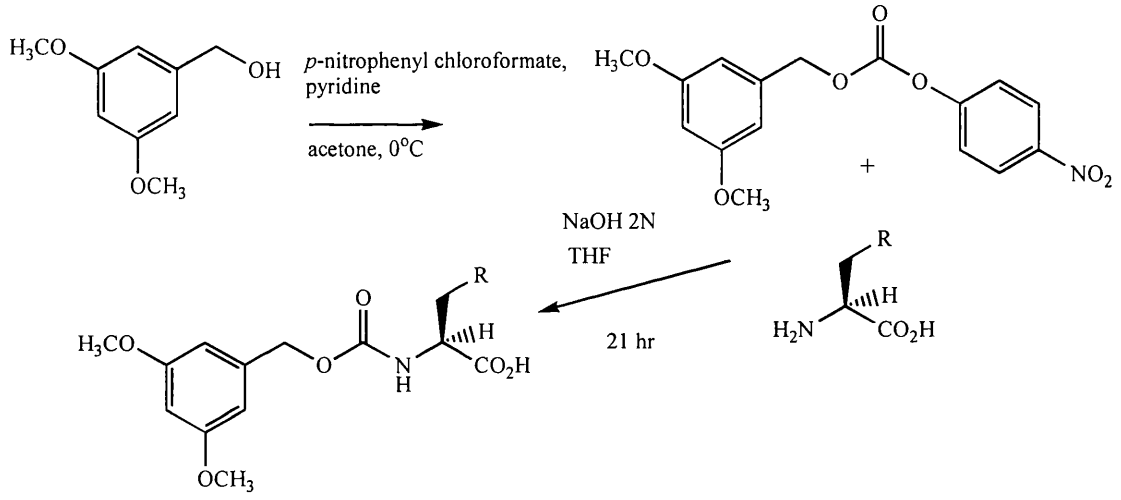


Figure 36: Use of 3,5-dimethoxybenzyloxycarbonyl group as *N*-protection.[132]

Shortly afterwards, Birr *et al.* developed the same protecting group using phosgene to make 3,5-dimethoxybenzyl chloroformate from the benzyl alcohol derivative. The chloroformate is then used in one step to protect the amine function of several amino acids that are then used in SPPS.[135]

This photosensitive group is cleaved by photosolvolysis.[136] This means that the photolabile cleavage proceeds well[134,135] (mercury high-pressure lamp, 90 min, yields above 60 % except for lysine 42 %[121]) in the presence of water.

A derivative of 3,5-dimethoxybenzyloxycarbonyl group was also made by Birr *et al.* The Ddz group is both photo- and acid labile.[137] This group is six times more sensitive to photochemical cleavage than 3,5-dimethoxybenzyloxycarbonyl and no water is needed for the cleavage.[122] Ddz groups absorb light around 280 nm and have been successfully

used as photolabile protecting groups for amino acids.[137] These groups were also used for acid labile *N*-protection in SPPS of a decapeptide.[138]

3. 3. Phenacyl derivatives

4-Methoxyphenacyl and α-methylphenacyl have both been developed as good photolabile protecting groups for the carboxy function of amino acids.[139] The interaction of the electrons between the carbonyl and the phenyl ring accounts for the low-lying excited states of this group. The protection is easily carried out using 4-methoxyphenacyl bromide and TEA in DMF (**Figure 37**). Photolabile cleavages are fairly long (4 to 17 hr) but proceed in high yields in ethanol or dioxane, except for tryptophan (yield 33%). This group is compatible with all the SPPS protecting groups and reagents with the exception of the trityl group which is affected by the photochemical cleavage.[122]

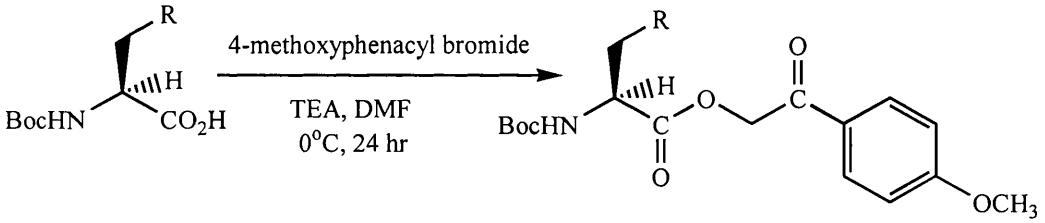


Figure 37: Use of 4-methoxyphenacyl group as carboxy protecting group.[139]

The Phenoc group has also been used to protect the amine function of some amino acids.[140] It is cleaved at 350 nm in quite good yields but the synthesis of the protected amino acids is quite complicated, proceeding *via* an oxime carbonate. The chloroformate intermediate is not stable enough to be used for this purpose.

4-Methoxyphenacyl and α-methylphenacyl groups are more photosensitive than the phenacyl group which has also been used as a protecting group for carboxy functions.[122,141] Phenacyl group has also been removed by sodium thiophenoxide[142] and selenophenol.[143] Very recently, 4-hydroxyphenacyl has been developed as a new "phototrigger" for ATP[144] or excitatory amino acids and peptides.[145]

Phenacyl resins have been used extensively in SPPS[129,146] but side-reactions can be encountered such as diketopiperazine formation.[147,148] They have also slow cleavage kinetics.[149]

3. 4. 2-Nitrobenzyl and 2-nitrobenzyloxycarbonyl derivatives

2-Nitrobenzyl and 2-nitrobenzyloxycarbonyl groups have been used extensively as protecting groups for the carboxy function and the amine function respectively.[121,122] 2-Nitrobenzyloxycarbonyl groups were among the first photolabile groups to be identified.[150,151] They have successfully protected amino acids.[152] The presence of an aromatic nitro group *ortho* to a carbon-hydrogen bond makes these groups very good photocleavable groups. Photolabile cleavage proceeds in high yields *via* an intramolecular oxygen transfer from the nitro group to the methylene[122] (**Figure 38**).

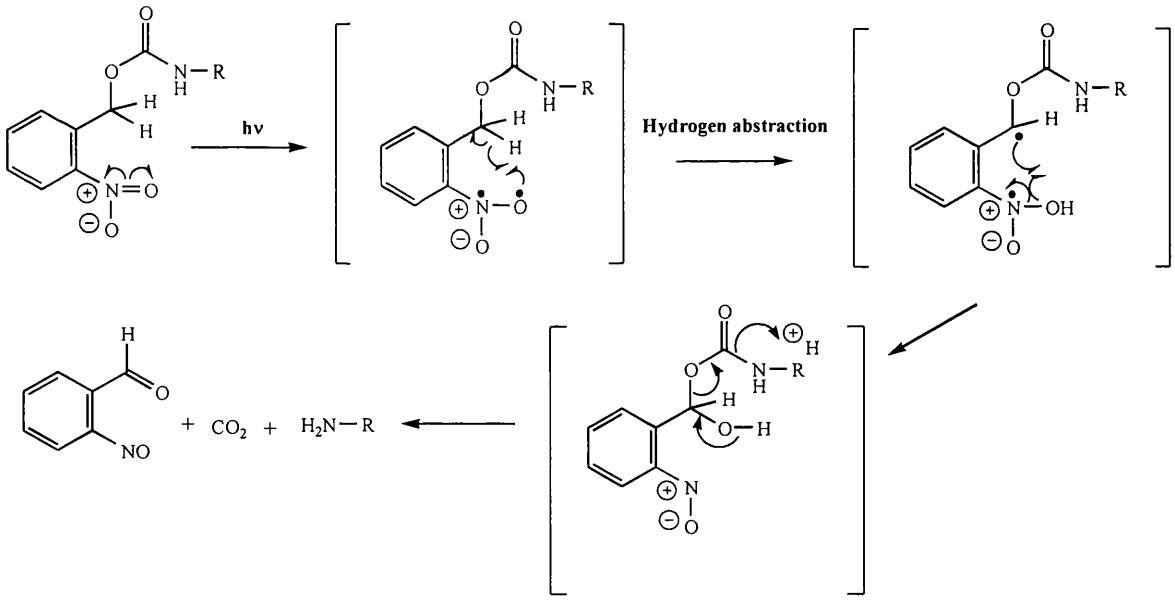


Figure 38: Mechanism of the photolabile cleavage of 2-nitrobenzyloxycarbonyl group.[122,153]

2-Nitrobenzyl groups are cleaved *via* a similar mechanism. 2-Nitrosobenzaldehyde is formed at the end of the reaction and further transformed into the dimer azobenzene-2,2'-dicarboxylic acid. This dark by-product acts as a light filter and can slow down the rate of the cleavage. If α-2-nitrophenyl-substituted 2-nitrobenzyl

groups replace 2-nitrobenzyl group, this problem is eliminated and the photolabile cleavage proceeds more efficiently.[122] The same is observed when α-2-nitrophenyl-substituted 2-nitrobenzyloxycarbonyl groups replace 2-nitrobenzyloxycarbonyl groups.[153]

6-Nitroveratryloxycarbonyl has also been used as a photolabile group. The photolabile cleavage of this group proceeds in a higher yield than for 2-nitrobenzyloxycarbonyl group and follows the same mechanism.[153] This group has been successfully used to protect amine functions of amino acids[153,154] or amino sugars.[155] The protection is easily made *via* the chloroformate (**Figure 39**).

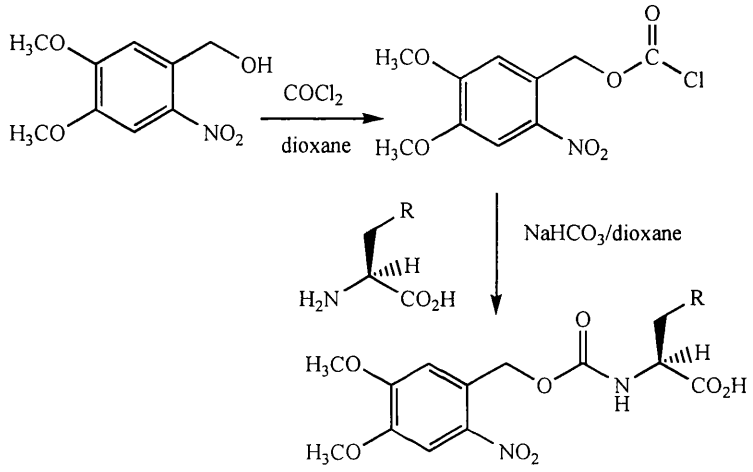


Figure 39: Synthesis of NVOC-amino acids.[154]

Photolabile cleavages proceeded in good yields when sulfuric acid was added during irradiation.[154] Indeed, if the liberated amine is not protonated, the aldehyde by-product formed during cleavage and derived from the NVOC groups can be attacked by this amine. Acidic conditions are therefore necessary to avoid these by-reactions. Most of the protecting groups and reagents used in SPPS are compatible with NVOC groups except the trityl group which is sensitive to irradiation.

Recently, NVOC groups and 6-nitroveratryl groups (protections for carboxy functions) have been used for chemical aminoacylation of transfer RNAs[156] and as photochemical protecting groups in DNA studies.[157] The photolabile cleavages were carried out in high yields. When both NVOC and 6-nitroveratryl groups were present, a

potassium acetate buffer was used during photolabile cleavage to keep the free amine protonated.[156]

Finally, linkers derived from 2-nitrobenzyl and 6-nitroveratryl have been used in SPPS. 2-Nitrobenzyl linkers have been synthesised and used with success but some drawbacks have also been reported, in particular these linkers are not fully compatible with Fmoc methodology as they are not completely stable to the piperidine used to cleave Fmoc groups.[129]

Recent work has been published on 6-nitroveratryl derived linkers[158,159] for combinatorial chemistry. Best photolabile cleavages (shorter half-lives of the linkers) were obtained in dioxane at 365 nm.[159] The same type of linkers has also been used in oligonucleotide chemistry.[160,161]

4. Aims of project

The aims of this project is to develop an efficient three dimensional protecting group strategy using photolabile groups to synthesise lanthionine-containing peptides with solid-phase methods.

The incorporation of monosulfide bridges into small peptides is a powerful tool in SAR studies. A quick and easy method to build lanthionine-containing peptides would be a great asset for such studies. Also, there is still today a need for synthetic techniques leading to portions of lantibiotics and ultimately new lantibiotics.

To achieve these targets, a solid-phase method enabling cyclisation and further elongation of the growing peptide on the resin is required. A side-chain-to-tail strategy was planned (**Figure 40**).

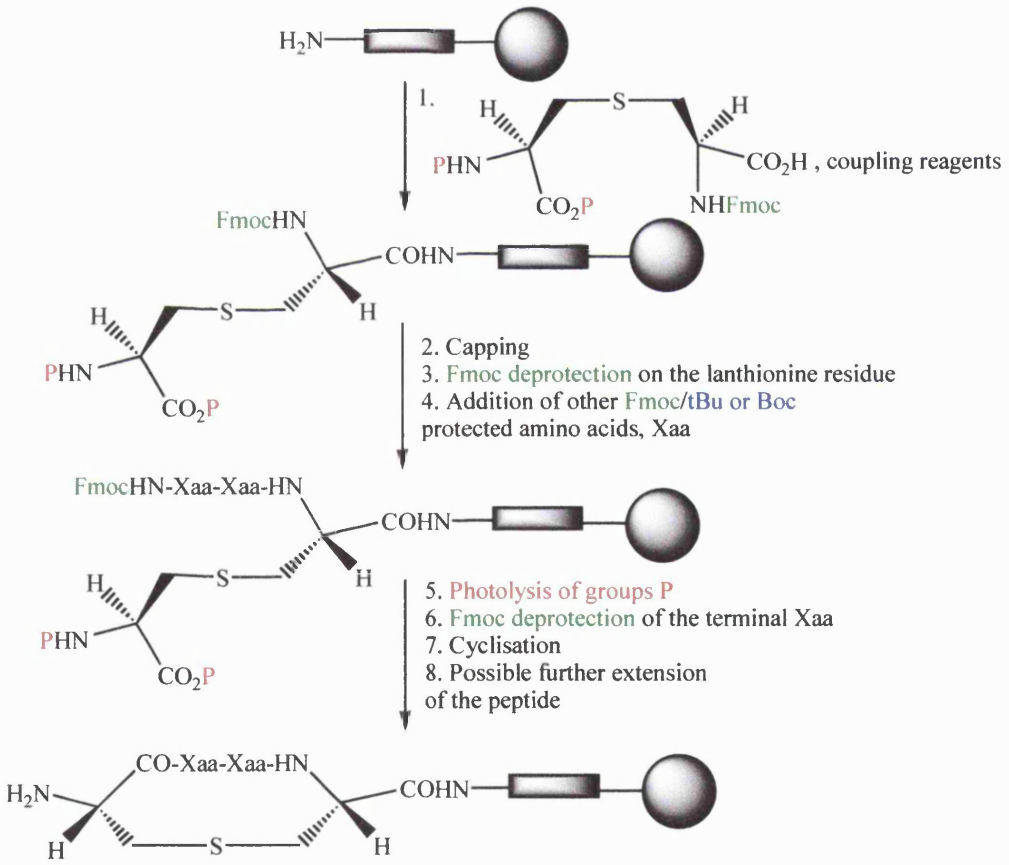


Figure 40: Three dimensional orthogonal protecting group strategy for the SPPS of lanthionine-containing peptides.

In this approach Fmoc groups were chosen for N^{α}-protection and tBu and Boc as side-chain protecting groups. This methodology can be adapted to both batch or continuous-flow peptide synthesis. The third type of protection must be orthogonal to both Fmoc and tBu/Boc; and therefore it must be resistant to both basic and acidic attack. Photolabile groups are an interesting option as they can be easily removed by light. Several types of photolabile protecting groups such as 3,5-dimethoxybenzyloxycarbonyl, 4-methoxyphenacyl and 6-nitroveratryl groups are compatible with SPPS. In addition, photolabile chemistry has already provided SPPS with promising linkers. This Fmoc/tBu or Boc/photolabile strategy would be a totally new three dimensional orthogonal protecting group strategy to build lanthionine-containing peptides. It would be a step towards polycyclic peptides and a synthetic approach for lantibiotics.

In order to develop this approach, synthetic pathways to novel lanthionine residues bearing photolabile groups had to be elaborated (**Figure 41**).

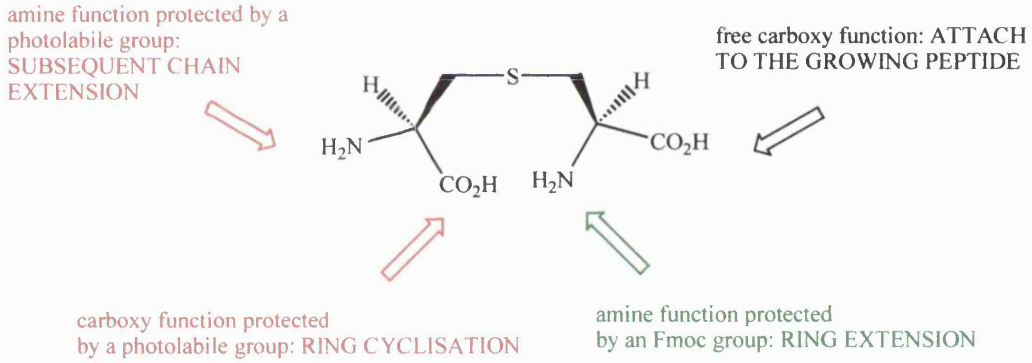


Figure 41: Target lanthionine residue.

It was decided to synthesise (*R, R*) lanthionine residues first, necessitating the use of the natural (*R*) cysteine and (*S*) serine residues which are cheap. Once the pathway is developed for one diastereoisomer, reproducing it to synthesise the other diastereoisomers should be straightforward.

Moreover, although the (*S, R*) diastereoisomer occurs naturally in lantibiotics, the (*R, R*) diastereoisomer has also been used, to synthesise sandostatin analogs in particular. So models of such analogs could be made from (*R, R*) lanthionine residues, following the Fmoc/Boc/photolabile strategy, to study the efficiency of this new strategy.

In chapter 3, the different strategies developed for the synthesis of lanthionine residues with photolabile protecting groups will be discussed.

In chapter 4, the photolabile tests carried out on these residues as well as the SPPS of a lanthionine-bridged sandostatin analog will be presented.

3. Synthesis of lanthionine residues with photolabile protecting groups

1. Towards lanthionine residues *via* serine β-lactone

Most of the existing methods for the synthesis of lanthionine residues use a cysteine residue and a β-alanyl cation equivalent (see **Chap. 1. Part 2.**). At the time this project started, serine β-lactones were the best β-alanyl cation equivalents available in terms of control of stereochemistry and synthetic accessibility.

The planned target lanthionine residue (see **Chap. 2. Part 4.**) bears two photolabile groups. 3,5-Dimethoxybenzyloxycarbonyl was chosen as the amine protecting group and 4-methoxyphenacyl was considered for the carboxy protecting group. The retrosynthetic pathway was therefore decided as starting from serine and cysteine to make lanthionine residues *via* serine β-lactones (**Figure 42**).

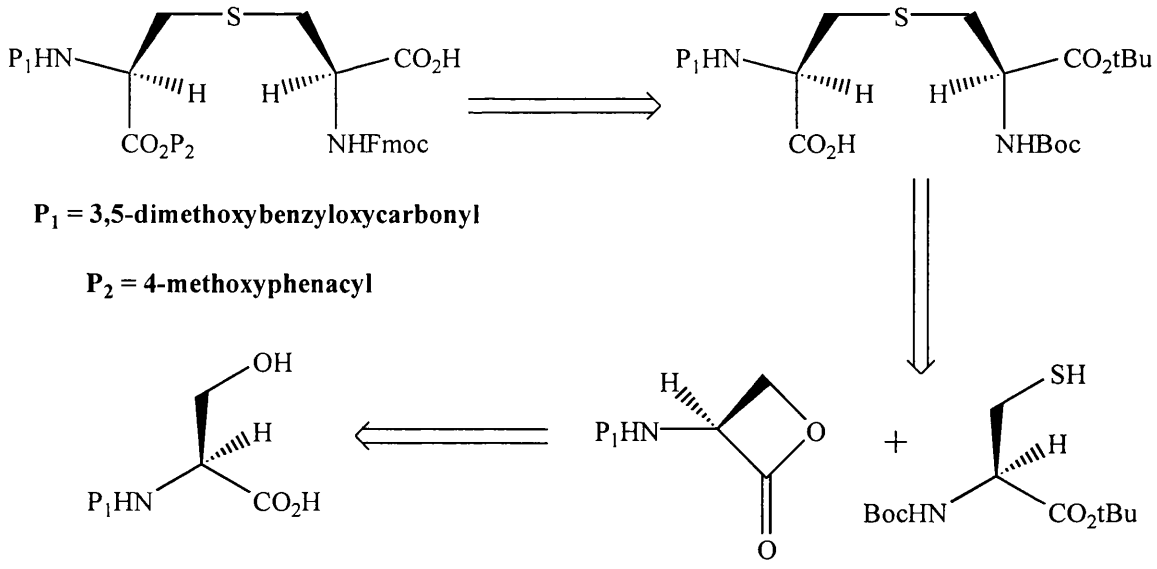


Figure 42: Retrosynthetic pathway to build the target lanthionine residue *via* serine β-lactones.

The photolabile groups were chosen for their adaptability to peptide synthesis (see **Chap. 2. Part 3.**) and the high yields reported for the cleavage steps. It was decided to

add the Fmoc group only at the end of the synthesis as when *N*-Fmoc-cysteine allyl ester residues were added to serine β-lactones, the ring opening reaction failed.[51]

1. 1. Synthesis of the cysteine moiety

Simple steps were chosen to synthesise Boc-Cys-OtBu. It was planned to start with the synthesis of the *tert*-butyl ester (**Figure 43**) as acidic conditions are required in this step; if a Boc group is already present on the molecule, the acidic conditions would cleave it.

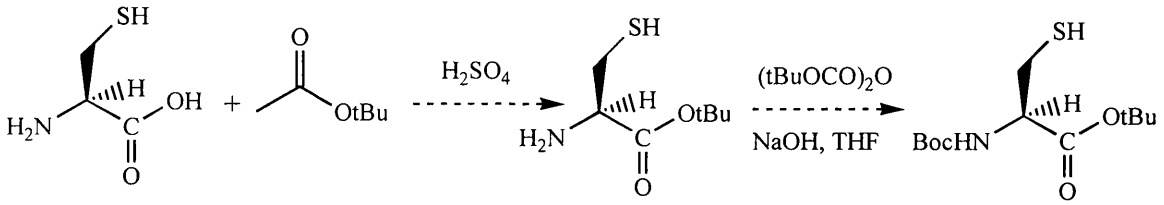


Figure 43: Planned route towards *N*-Boc-cysteine *tert*-butyl ester.

An efficient method to protect the carboxy function of amino acids with a *tert*-butyl group has been published[162] using *tert*-butyl acetate and perchloric acid. For a safer approach, sulfuric acid was first tried in this reaction.

A high-yielding protection strategy using di-*tert*-butyl dicarbonate in basic conditions[163] was planned for the protection of the amine function.

However, the first reaction did not go according to plan as the thiol function was also protected in the reaction. *N*-*tert*-Butyloxycarbonyl-*S*-*tert*-butylcysteine *tert*-butyl ester was eventually recovered (**Figure 44**).

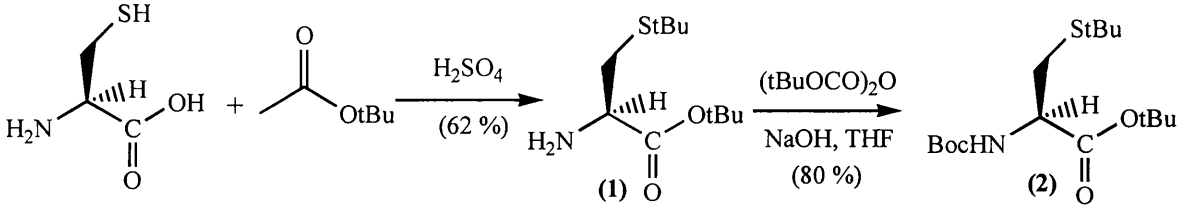


Figure 44: Synthesis of cysteine (2).

An easy way to overcome this difficulty is to use the commercially available *S*-*tert*-butylthiocysteine as starting material. As other parts of the synthetic pathway gave poor results (see below), the strategy was changed and these particular steps were not attempted.

1. 2. Synthesis of *N*-3,5-dimethoxybenzyloxycarbonylserine

It was decided to try the direct approach first to make *N*-3,5-dimethoxybenzyloxycarbonylserine *via* the chloroformate (**Figure 45**).[135]

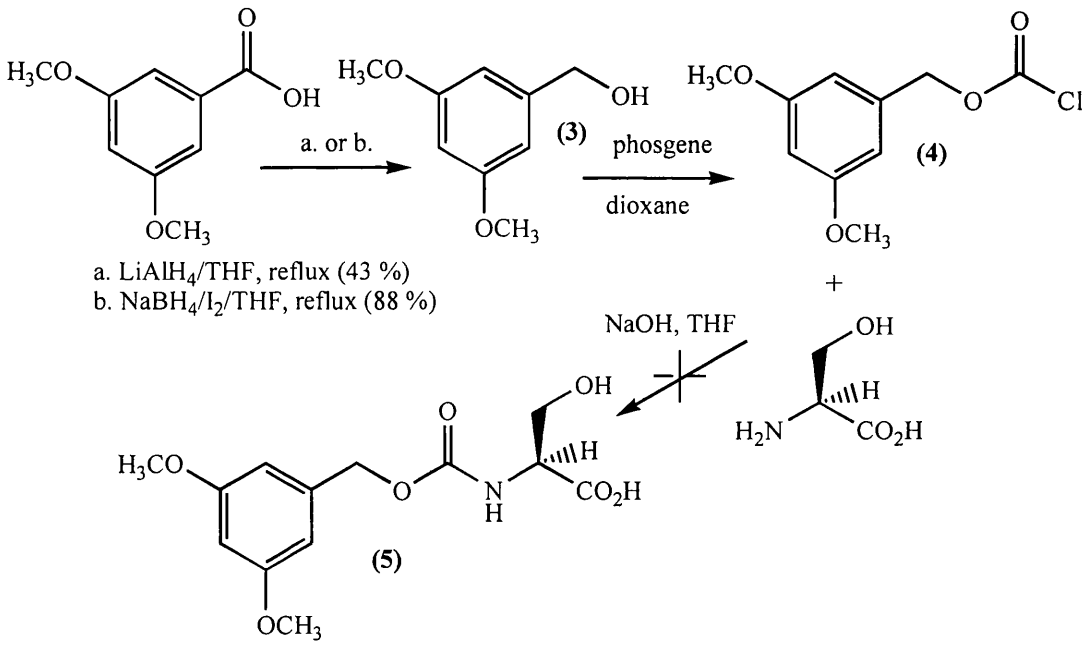


Figure 45: Towards *N*-3,5-dimethoxybenzyloxycarbonylserine *via* the chloroformate.

3,5-Dimethoxybenzyl alcohol **(3)** was easily synthesised from 3,5-dimethoxybenzoic acid using sodium borohydride and iodine[164]. The same reduction was much less successful with lithium aluminium hydride.

The chloroformate **(4)** was synthesised from the benzyl alcohol derivative using a solution of phosgene in toluene. Careful neutralisation of the excess phosgene had to be carried out before further work-up. As this chloroformate is fairly unstable, it was used straight away in the next step.

An attempted Schotten-Baumann reaction in basic conditions failed to give the *N*-protected serine even after another base (sodium hydrogen carbonate) and another solvent (dioxane) were tried. In all cases a mixture of 3,5-dimethoxybenzyl chloroformate and 3,5-dimethoxybenzyl alcohol was recovered. The lack of purification of the chloroformate might be partly responsible.

It was therefore decided to try another synthetic pathway originally developed by Chamberlin (**Figure 46**).[134] *N*-3,5-Dimethoxybenzyloxycarbonylserine is synthesised from a carbonate intermediate in basic conditions.

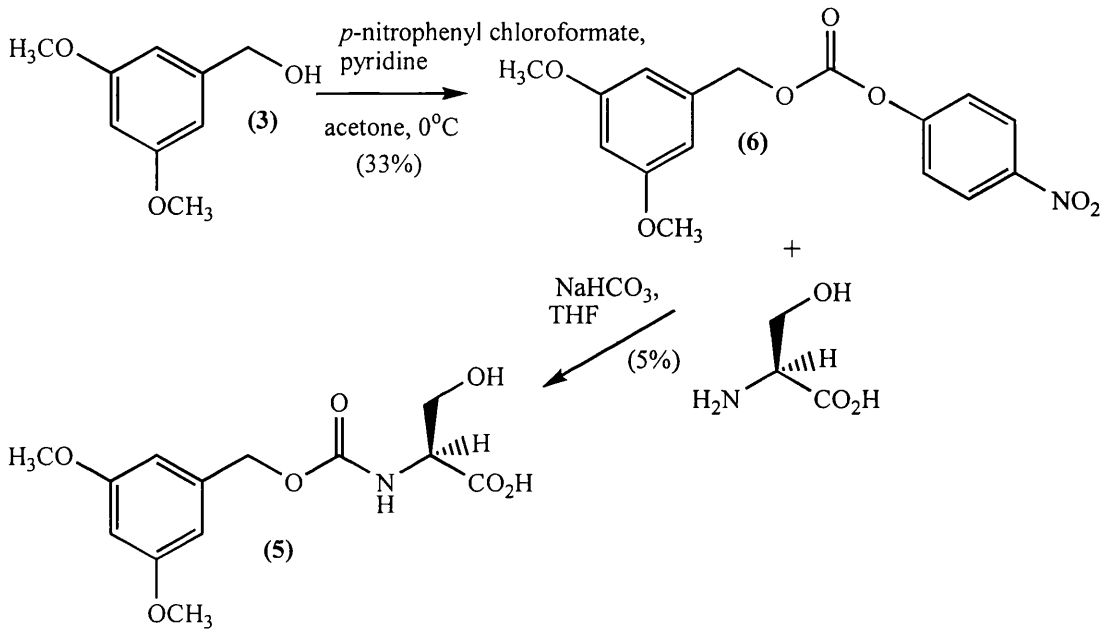


Figure 46: Towards *N*-3,5-dimethoxybenzyloxycarbonylserine *via* the carbonate.

The Schotten-Baumann reaction proceeded at a very low yield (5 %). Numerous attempts to improve the yield (trying sodium hydroxide as a base for example) were carried out to no avail. In particular, pH adjustments were made in the work-up to separate *p*-nitrophenol from the title compound.

1. 3. Synthesis of serine β-lactone residues

As difficulties were encountered in the synthesis of *N*-3,5-dimethoxybenzyloxycarbonylserine, *N*-benzyloxycarbonylserine was synthesised first to test the synthetic pathway leading to serine β-lactone residues. The methodology developed by Vederas *et al.*[62] to make serine β-lactone was followed (**Figure 47**), using DMAD or DEAD and triphenylphosphine.

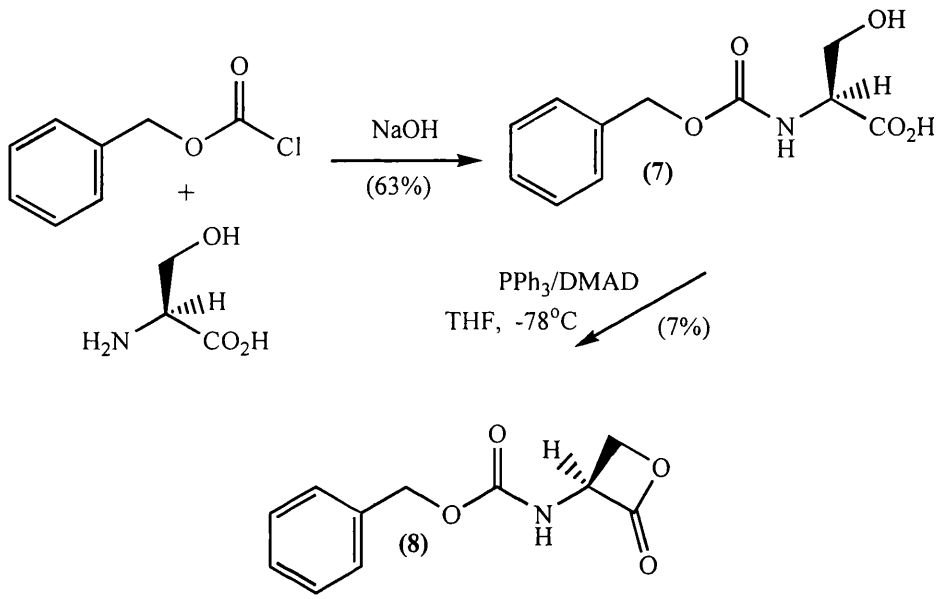


Figure 47: Synthesis of *N*-benzyloxycarbonylserine β-lactone.

N-Benzyloxycarbonylserine was synthesised from serine using benzyl chloroformate and base in good yield.[165]

The synthesis of serine β-lactone was reported to work well with DMAD and the crude product was easier to purify when DMAD rather than DEAD was used.[62] DEAD is available commercially whereas DMAD must be synthesised. It is a red liquid that decomposes quickly at room temperature and presents explosive hazards.

To synthesise the precursor of DMAD,[166] dimethylhydrazodicarboxylate, methods developed to make the precursor of DEAD were used.[167,168,169] The next step was adapted from the synthesis of methyl ethyl azodicarboxylate from its hydrazine precursor[170] (**Figure 48**).

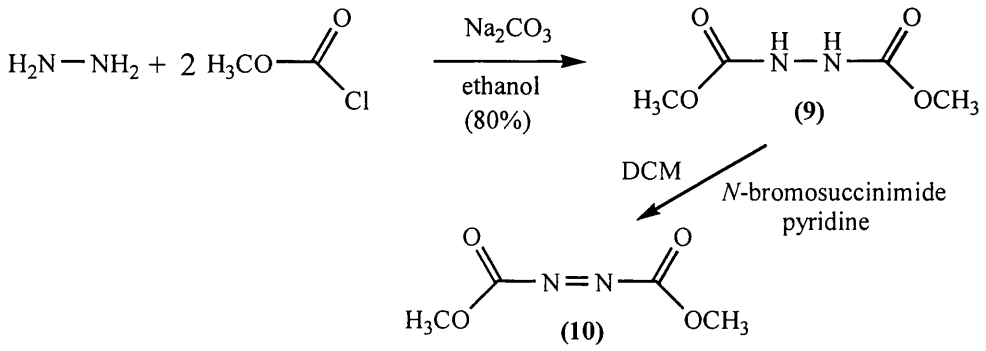


Figure 48: Synthesis of DMAD.

The synthesis of dimethyl hydrazodicarboxylate proceeded well. It had to be separated from its NaCl by-product by filtration in warm acetone and then used in the next step, alongside *N*-bromosuccinimide and pyridine, to make DMAD. As DMAD appeared pure by n.m.r. and is potentially explosive, no further purification was attempted. DMAD was used as quickly as possible in the synthesis of serine β-lactone, as it decomposes within a few days.

The synthesis of serine β-lactone proceeds in two steps. First DMAD is added to triphenylphosphine to make an adduct, then *N*-benzyloxycarbonylserine is added to this adduct and the Mitsunobu reaction takes place. The formation of the adduct must proceed at -78°C. If the adduct is not formed when the protected serine is added, no reaction will take place.

The purification of the crude product also presented difficulties. The by-product dimethyl hydrazodicarboxylate in particular tends to run at R_F values very close to the title compound. A careful flash column chromatography must be carried out for a proper purification of the product. When DEAD is used, it is impossible to separate the diethyl hydrazodicarboxylate by-product from the title compound.

The yield of the reaction was very low (7 %). It is believed that the key elements in this reaction are the purity of the DMAD used and very dry conditions. As the DMAD used in these attempts was still a bit wet with solvents, it is possible that this might lead to a limited formation of the intermediate adduct and a poor yield.

To synthesise lanthionine residues quickly and efficiently, easy high-yielding steps are required. As it was reported that lanthionine formation will not proceed with a

higher yield than 50 %, due to a competitive formation of thioester[5] (see **Chap. 1. Part 2.**) it is essential to achieve near quantitative yields in the rest of the synthesis. This first strategy had therefore to be reviewed.

2. Synthesis of lanthionine residues *via* iodoalanine

As the first strategy did not give satisfactory results, it appeared necessary to find an alternative to serine β-lactone and to change the photolabile *N*-protection. At that time in the project, a new method to synthesise lanthionine residues *via* iodoalanine residues was published by Dugave and Ménez.[66] The route seemed viable and proceeded in high yields. A new retrosynthetic pathway was therefore planned (**Figure 49**).

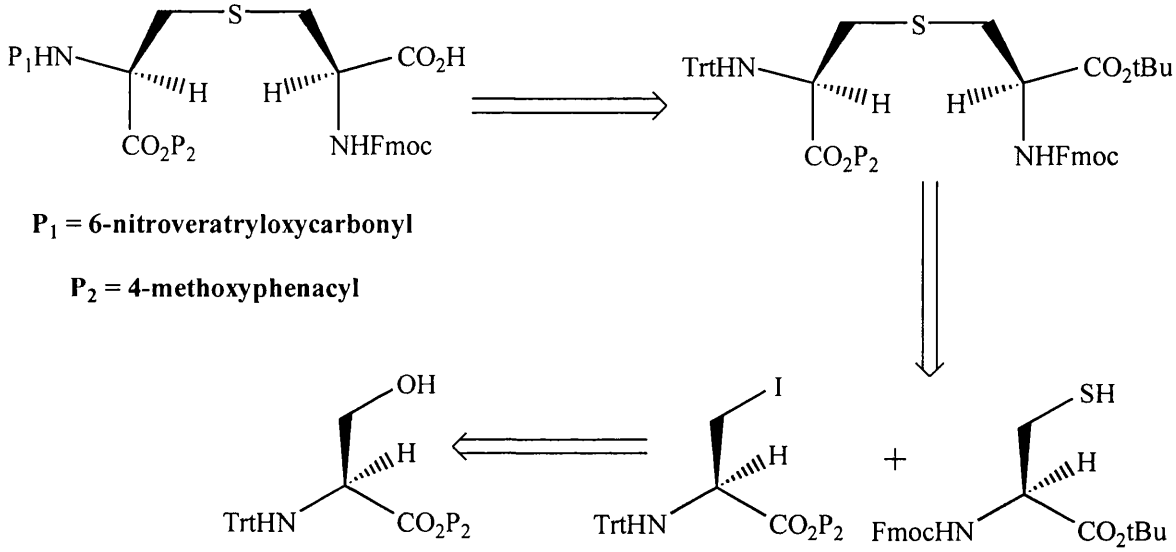


Figure 49: Retrosynthetic pathway to build the target lanthionine residue *via* iodoalanine.

6-Nitroveratryloxycarbonyl was chosen to replace 3,5-dimethoxybenzyloxy-carbonyl as the *N*-protection as it had also been used to protect amino acids and reasonable cleavage yields were reported.[154] 4-Methoxyphenacyl was retained as the photolabile carboxy protection.

The Fmoc group was introduced from the start on the cysteine residue as this protecting group was reported to be compatible with this pathway.[66]

A trityl group was used as *N*-protection on the serine and iodoalanine residues during the literature synthesis and was reported to be a good protecting group for this step. This bulky protecting group has been found to prevent α-ester saponification and α-proton abstraction by non nucleophilic bases.[75] It is easily removed by mild acid.

2. 1. Synthesis of the cysteine moiety

Starting from *S-tert*-butylthiocysteine, two protection steps were carried out. As noted previously (see **1. 1.**), it was necessary to initially protect the thiol function or it will be protected with a *tert*-butyl group in the first step. Three simple steps gave the desired cysteine residue (**Figure 50**).

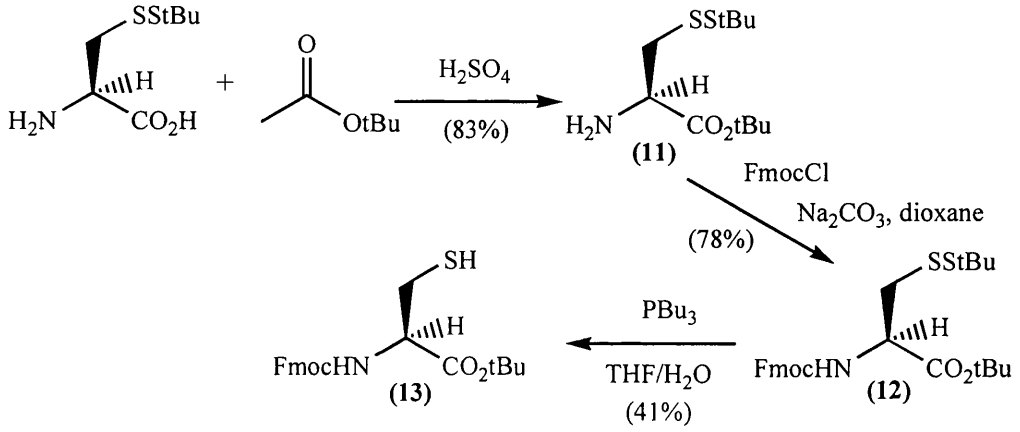


Figure 50: Synthesis of cysteine (13) from *S-tert*-butylthiocysteine.

The two first steps proceeded in high yields. The two protected intermediates were successfully purified by flash column chromatography.

The last step is the disulfide cleavage required to free the thiol function of the cysteine residue. In the original pathway towards lanthionine residues,[66] cystine was used to give a cysteine residue that was then used straightaway alongside iodoalanine to make lanthionine. The disulfide cleavage of the dimer was performed with tributylphosphine. It had been decided to use *S-tert*-butylthiocysteine instead of cystine as preliminary tests showed that cystine was not dissolved in a mixture of *tert*-butyl acetate and sulfuric acid. The cleavage of the thiol protecting group on *S-tert*-butylthiocysteine should lead to a similar cysteine residue than the one described in the original reference.[66]

Tributylphosphine was used for this cleavage, in wet THF. The main drawback of the reaction is the formation of *tert*-butyl thiol as a by-product, which presented problems in purification. Flash column chromatography was carried out but by-products resulted from the presence of *tert*-butyl thiol. The reaction proceeded to give a rather low yield (41 %).

It was consequently decided to avoid this problem by using cystine from the start of the pathway (**Figure 51**) (so there is no need of thiol protection and cleavage of the dimer will give the cysteine monomer without by-product).

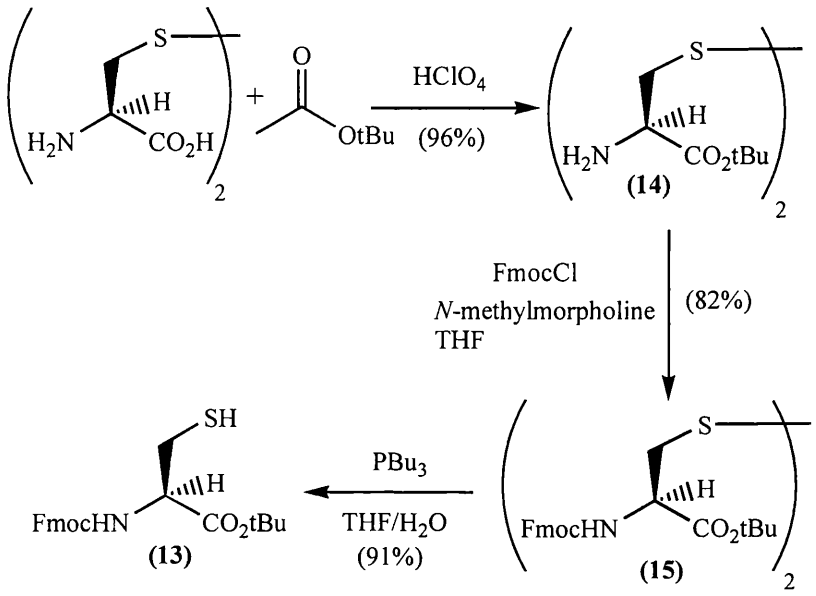


Figure 51: Synthesis of cysteine (13) from cystine.

Tests were carried out in acidic conditions to find an acid in which cystine dissolves well, however perchloric acid was the only suitable acid, as confirmed by reports in the literature.[171,172,173] The reaction proceeded in high yield, improving on the literature. The key element of the reaction is the increase of the pH in the work-up. It is important to reach at least pH 9-10 to recover most of the title compound. The next step must be carried out quickly afterwards as cystine bis-*tert*-butyl ester degrades, turning yellow.

A recent approach to the protection of cystine by an Fmoc group involves the use of Fmoc-*N*-hydroxysuccinimide ester and *N*-ethylmorpholine.[174] Using FmocCl and *N*-methylmorpholine, a similar method was carried out in high yield.

The main difficulty of the reaction is the purification of the title compound **(15)**. It is very hard to separate the by-product fluorenylmethyl alcohol from the title compound. Laborious flash column chromatography was successfully carried out (yield 82 %). An easier method, recrystallisation, has also given satisfactory results (yield 76 %), the title

compound being selectively recrystallised in dichloromethane: methanol (1: 4) at -20°C whereas fluorenylmethyl alcohol remains in solution.

The cleavage of the dimer cystine (**Figure 52**) worked with a very high yield once the right amount of water was added to the reaction mixture. Originally described as "wet THF",[66] the solvent system must actually consist of a large excess of water (at least 9:1, THF: water), as confirmed by methods described in the literature for the cleavage of disulfide in SPPS.[129,175] This might also explain why previous attempts with *S-tert*-butylthiocysteine were not very successful, probably lacking water in the reaction mixture.

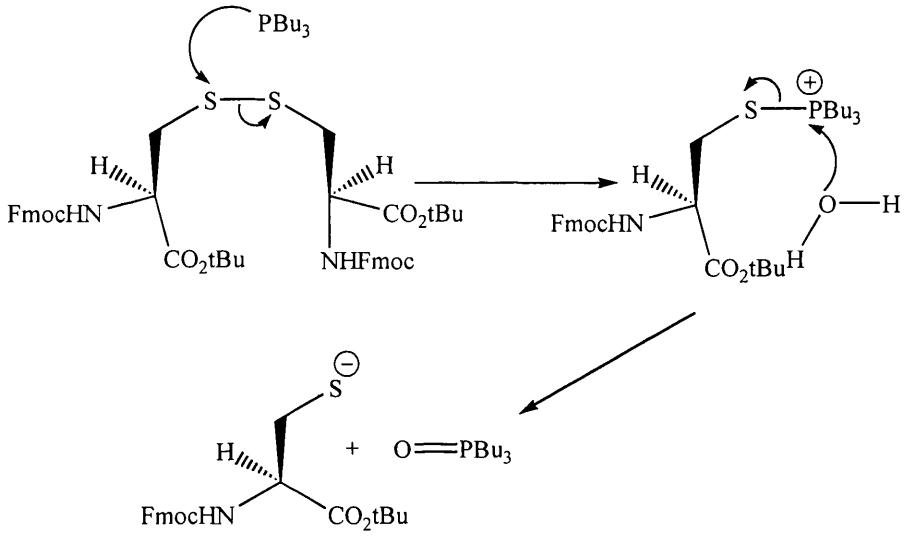


Figure 52: Mechanism of formation of cysteine residues from cystine residues using tributylphosphine.

Purification of the crude by flash column chromatography was carried out shortly after the reaction and gave the title compound in a very high yield (91 %)

2. 2. Synthesis of iodoalanine residues with 4-methoxyphenacyl as carboxy protecting group

Iodoalanine residues were synthesised from mesyl derivatives by Dugave and Ménez.[75] Protection of both the amine and the carboxy functions was necessary on the

serine residues, before synthesising the mesyl derivatives. The protection of the amine function was carried out first using a "one-pot" method developed by Theodoropoulos *et al.*[176] (**Figure 53**).

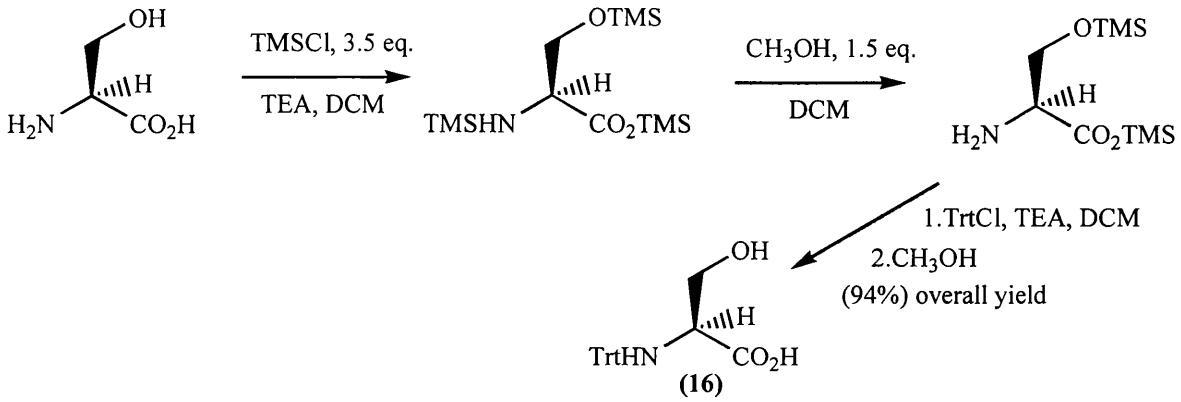


Figure 53: Synthesis of *N*-tritylserine.

The difficulty of protecting the amino group of serine with a trityl group is to have trityl chloride reacting only with this amine function and not with the alcohol function and the carboxy function also present in serine. An efficient way to overcome this problem is to first protect all three groups using trimethylsilylchloride, then to deprotect only the amine function with 1.5 eq. of methanol (silyl amino group being more easily cleaved by methanol than silyl ester or silyl ether) and finally to protect this amine with a trityl group. Fast and efficient deprotection of the silyl groups remaining on the molecule is carried out using an excess of triethylamine and methanol. This method gave *N*-trityl serine in a very high yield (94 %).

The diethylammonium salt of the title compound was also synthesised to check its purity by comparison of optical rotations ($[\alpha]_D$ -32.7°, 21°C, c 22.72 mg/ml in methanol; lit.[176] $[\alpha]_D$ -33°, 25°C, c 1 % in methanol).

4-Methoxyphenacyl bromide was added to a solution of *N*-tritylserine in DMF with triethylamine as a basic catalyst (**Figure 54**), following a known method.[139] Recrystallisation of the whitish crude product in ethanol gave the title compound as a white powder in a fairly good yield (55 %).

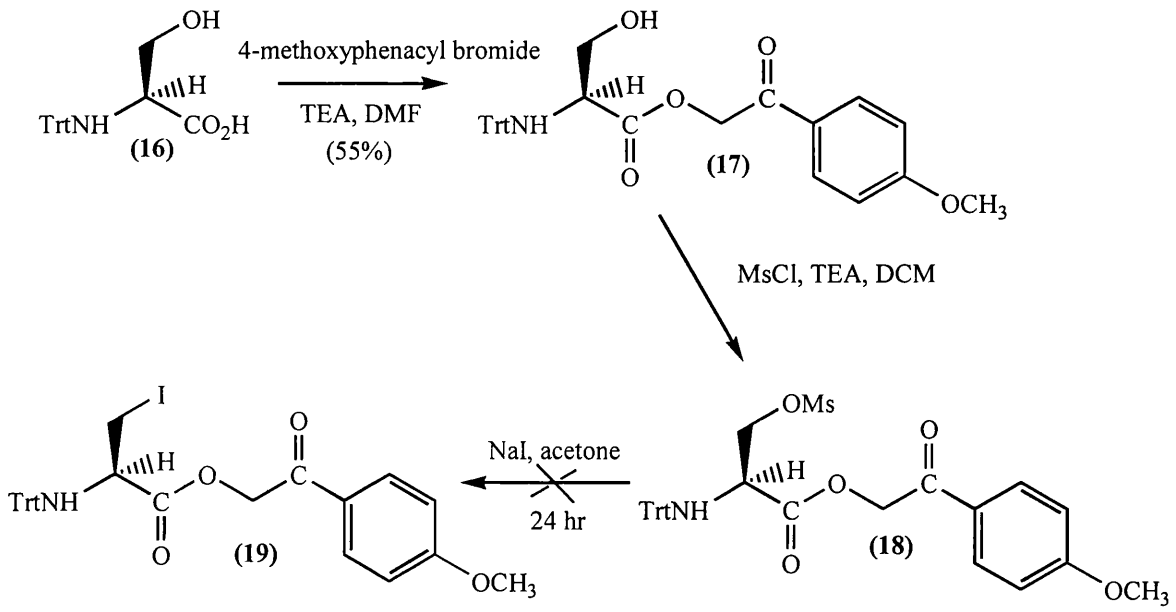


Figure 54: Towards iodoalanine (19).

The next step proceeded well to make the mesyl derivative following a common procedure.[177] As mesyl derivatives are usually fairly unstable, the next step was carried out without further purification. Later attempts at purifying the title compound by flash column chromatography led to the recovery of a small amount of aziridine by-product (**Figure 55**) alongside the title compound **(18)** (yield about 80 %).

A second method was used to protect the hydroxy function with a mesyl group. This was developed by Dugave and Ménez[75] and requires the use of THF instead of DCM and less TEA. In this case a large amount of aziridine by-product was recovered along with the title compound (yield 21 %) after purification by flash column chromatography.

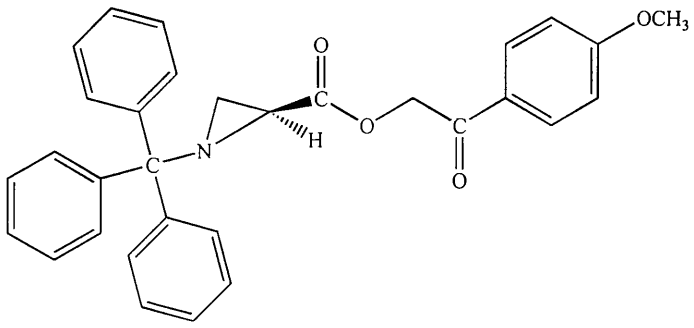


Figure 55: Aziridine by-product formed during mesylation of the hydroxy group of serine (17).

Most of the time, the mesyl derivative was therefore used in the next step without purification. Sodium iodide was added to the crude mesyl derivative in acetone.[75] Performed in light as well as in the dark, for 24 hr or 41 hr, the reaction did not work. The starting material **(18)** was recovered after extraction.

Another method was tried. Iodoalanine derivatives have been reported in the literature from serine derivatives using methyltriphenoxyphosphonium iodide (**Figure 56**).[73,178]

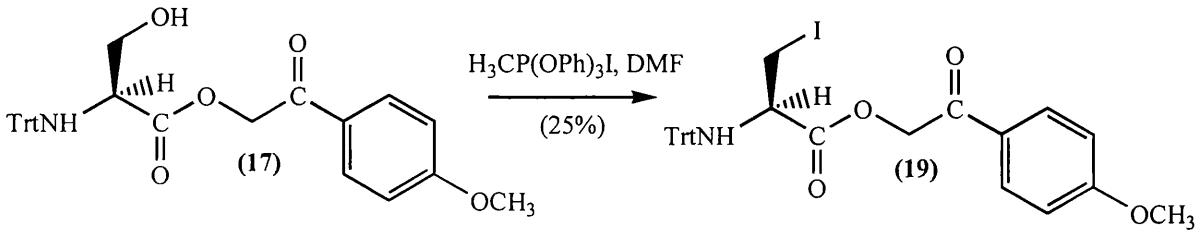


Figure 56: Synthesis of iodoalanine (19).

Methyltriphenoxyphosphonium iodide was obtained commercially but it degrades very quickly and was already 1/3 degraded when used in the reaction. The purification of this reagent requires the use of a glove-box in strictly dry conditions. As this was not available at the time, it was decided to use an excess of methyltriphenoxyphosphonium iodide in the reaction.

Purification was difficult and by-products derived from methyltriphenoxyphosphonium iodide were still mixed with the title compound after flash column chromatography. A low yield was recorded for this reaction (25 %). It is very probably due to the lack of purity of the reagent used in this reaction.

This pathway does not have high-yielding and efficient steps. The mesyl derivative is hard to obtain in a very pure and dry form and this accounts for the lack of success in synthesising the iodoalanine residue.

It was therefore decided to use the 6-nitroveratryl group as protection for the carboxy function of the serine and alanine residues. Recent work[156] had shown the advantage of cleaving this group and an NVOC group on the same molecule with the same wavelength, so this solution appeared like an interesting practical advantage for future SPPS work on the target lanthionine residues.

2. 3. Synthesis of iodoalanine residues with 6-nitroveratryl as carboxy protecting group

Starting from *N*-trityl serine **(16)**, a new synthetic pathway was planned, with 6-nitroveratryl as a carboxy protecting group (**Figure 57**).

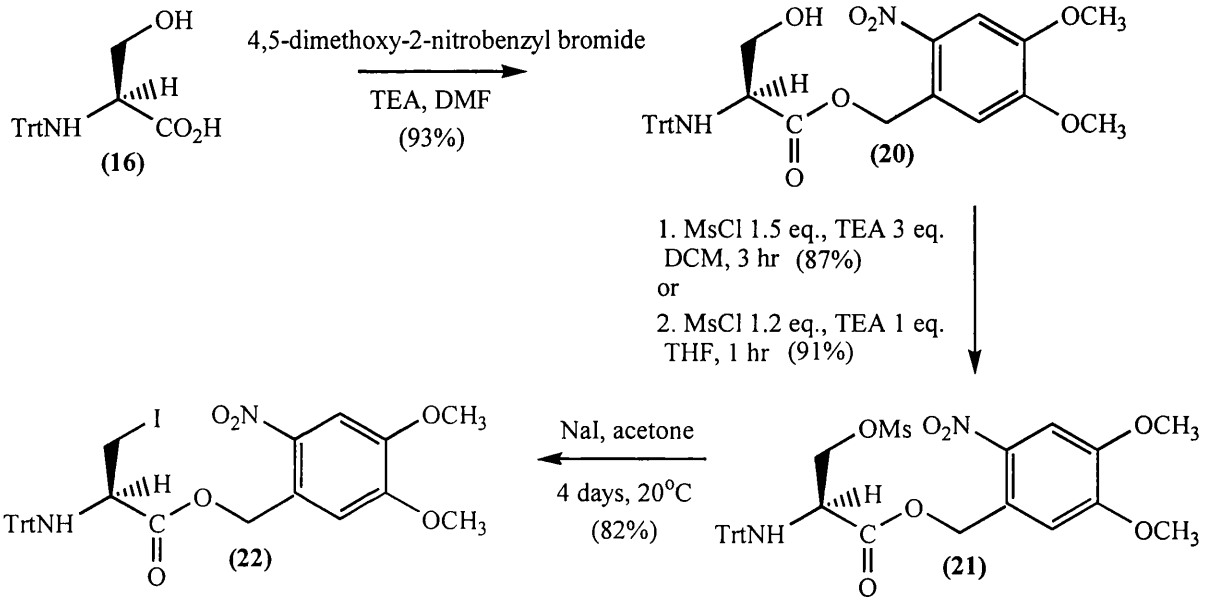


Figure 57: Synthesis of iodoalanine (22).

The same methodology was used for the 6-nitroveratryl ester **(20)** as for the 4-methoxyphenacyl ester **(17)**. The carboxy function was protected in a high yield without the need for purification.

Conversion to the mesyl derivative **(21)** using similar conditions proceeded in high yield (87 %). Separation of the by-product, methanesulfonic acid from the desired compound **(21)** was successfully achieved using flash column chromatography. Further improvement in the yield was observed (91 %) using the second method developed by Dugave and Ménez[75]. Similarly, the crude was purified successfully by flash column chromatography or recrystallisation.

The iodoalanine **(22)** was successfully synthesised from **(21)** using sodium iodide in acetone. A large excess of sodium iodide (11 eq.) is necessary. The reaction works best when both sodium iodide and the mesyl derivative **(21)** are separately totally dissolved in acetone before being mixed together. Also an improvement in yield is observed when the

mesyl derivative solution is added to a solution of sodium iodide and not the other way around.

If the reaction mixture is left for too long after completion and before any work-up takes place, then a large amount of the aziridine by-product **(23)** is formed. Finally, it seems that the "room" temperature plays a role in the completion of the reaction. After a quite low yield was obtained at 10°C, some tests were carried out. They showed that at 25°C, the reaction is completed in 24 hr and a small amount of aziridine by-product is also recovered. At 10°C, the reaction is nearly completed after 4 days but a small amount of mesyl derivative remains in the reaction mixture. Ideal conditions are therefore 4 days at 20°C. A high yield is obtained in these conditions, after purification by flash column chromatography (82 %).

2. 4. Synthesis of lanthionine residues

Following the published method,[66] cesium carbonate was added to a mixture of protected cysteine **(13)** and iodoalanine **(22)** dissolved in DMF (**Figure 58**).

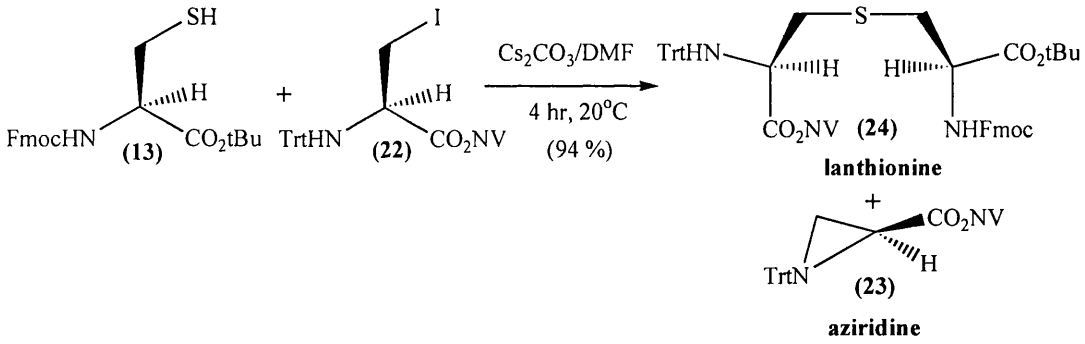


Figure 58: Synthesis of lanthionine from cysteine and iodoalanine residues.

Cesium carbonate has been found to be the best basic reagent for this nucleophilic substitution.[5] The order of addition in this reaction is important. If cesium carbonate is added to cysteine **(13)** on its own and in a small amount of solvent, partial cleavage of the Fmoc group takes place. Similarly, it is important not to concentrate the reaction mixture *in high vacuo* at the end of the reaction and to use a maximum of 1 equivalent of cesium

carbonate, otherwise partial cleavage of the Fmoc group is observed. It was established that adding cesium carbonate last and portionwise to the reaction mixture worked best.

Interestingly, there appears to be no formation of the elimination by-product dehydroalanine, even though a non-nucleophilic base is used in this reaction. This is due to the presence of the trityl group on the amine function, which has been shown to prevent α-proton abstraction.[75,77]

A small amount of aziridine by-product **(23)** is formed during the lanthionine synthesis, as previously reported.[66] Lanthionine is obtained with a higher yield when iodoalanine is used in slight excess (1.1 eq.) and if the reaction is carried out at 20°C. After thorough purification by flash column chromatography, lanthionine **(24)** is obtained in excellent yield (94 %).

A mixture of two inseparable lanthionine residues was obtained: this is discussed in section **2. 5.**

A 6-nitroveratryloxycarbonyl group was then required in place of the trityl group, and the *tert*-butyl group had to be removed to give the final lanthionine residue. It was initially decided to remove both the trityl and *tert*-butyl group in one step, then to selectively reprotect the amine but this alternative makes the work-up and the purification of the intermediates difficult as a free carboxy function is then present for several steps.

A synthetic pathway was therefore planned, with three steps giving the final lanthionine residue (**Figure 59**).

The cleavage of the trityl group with TFA to give **(25)** proceeded quantitatively. When only 4 eq. of acid are used, the very acid labile trityl group is removed but not the *tert*-butyl group which requires stronger acidic conditions to be cleaved. Neutralisation of the remaining TFA was carried out with NMM, so as not to remove the *tert*-butyl group by concentration of the remaining acid when the solvents are removed *in vacuo.* The NVOC protection was usually carried out straightaway, with no purification of the intermediate lanthionine **(25)**.

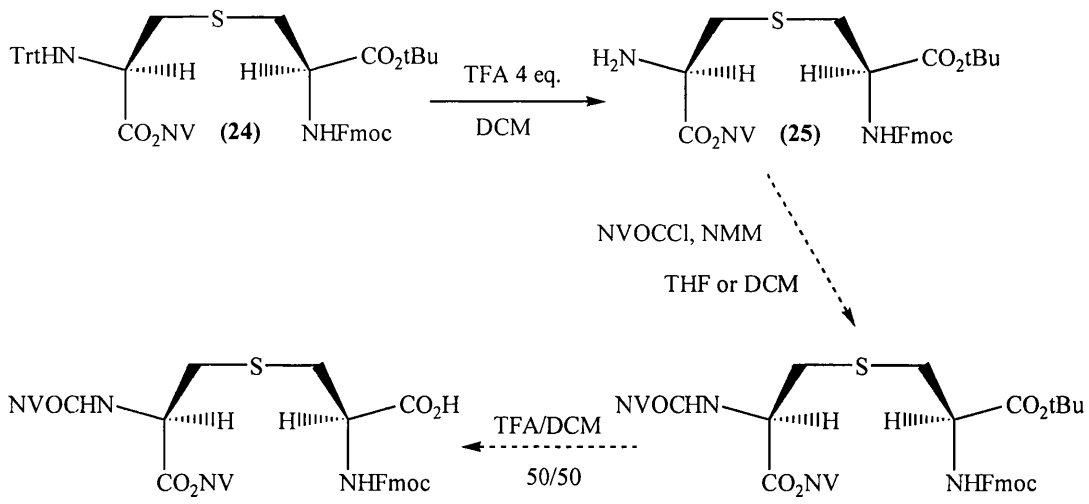


Figure 59: Synthetic pathway towards the final lanthionine residue.

The protection of an amine function by an NVOC group is usually carried out with Schotten-Baumann conditions in dioxane/sodium hydrogen carbonate solution but earlier attempts did not proceed well, as the starting material was not soluble in the basic solution. It was therefore decided to try organic conditions for this reaction. Conditions close to the ones developed to put the Fmoc group on the molecule were used, so as not to remove the Fmoc group in this basic step. As low yields were obtained in THF, the reaction was carried out in DCM, immediately after formation of the amine and neutralisation, without changing the solvent.

N.m.r. and mass spectroscopy analyses revealed that (*R*)-(4,5-dimethoxy-2-nitrobenzyl)-*N*-(4,5-dimethoxy-2-nitrobenzyloxycarbonyl)alanyl (*R*)-*tert*-butyl-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide was not made during this reaction. The amino group was successfully protected with NVOC, but the NV ester was unexpectedly hydrolysed in the process (**Figure 60**).

It seems that under these conditions the NV ester is very sensitive to base hydrolysis, being cleaved even more readily than the Fmoc group. Even though a minimal amount of base was used in the reaction, a slight excess appeared enough to trigger the cleavage of the NV group.

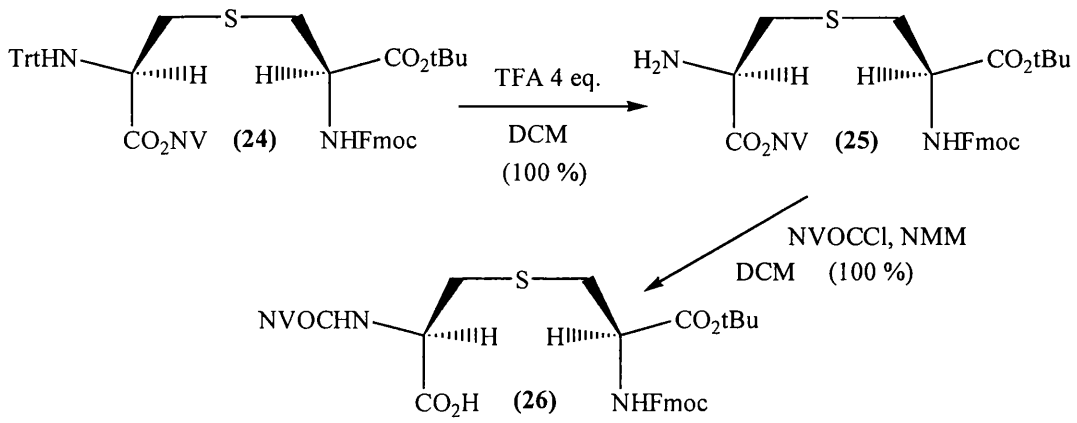


Figure 60: Synthesis of lanthionine (26).

In the initial formation of lanthionine, the presence of cesium carbonate with an NV ester was not a problem as the trityl group was present on the molecule; this has been shown to protect α-esters from saponification.[69,75] This was also confirmed by test reactions where lanthionine residues **(24)** were left with NMM in DCM or with NMM and NVOCCl in DCM. The residues bearing the Trt groups were not affected by the basic conditions and residues **(24)** were recovered intact.

Depending on the nature of the batches of lanthionine used, two types of lanthionine **(25)** have been recovered, with similar mass spectroscopy analyses but slightly different n.m.r. analyses. Again, their nature is discussed in section **2. 5.**

The Schotten-Baumann conditions described in the literature[154,155,157] were therefore tried again but this time the starting material was dissolved in dioxane[156] first before adding the aqueous solution of sodium hydrogen carbonate and the NVOCCl dissolved in dioxane.

During the preparation of the starting material lanthionine **(25)**, no neutralisation of the remaining TFA was carried out after the removal of the trityl group. Toluene was added several times to the reaction mixture and removal of the solvents *in vacuo* (dichloromethane and TFA first, then toluene) gave protected lanthionine **(25)** with a free amino group without removal of the *tert*-butyl group. Occasionally, lanthionine **(25)** was

purified by flash column chromatography, but most of the time the next step was carried out without further purification.

The amino group was successfully protected after 2 hr to give lanthionine **(27)** and in these conditions (**Figure 61**), neither hydrolysis of the NV ester, nor cleavage of the Fmoc group was observed.

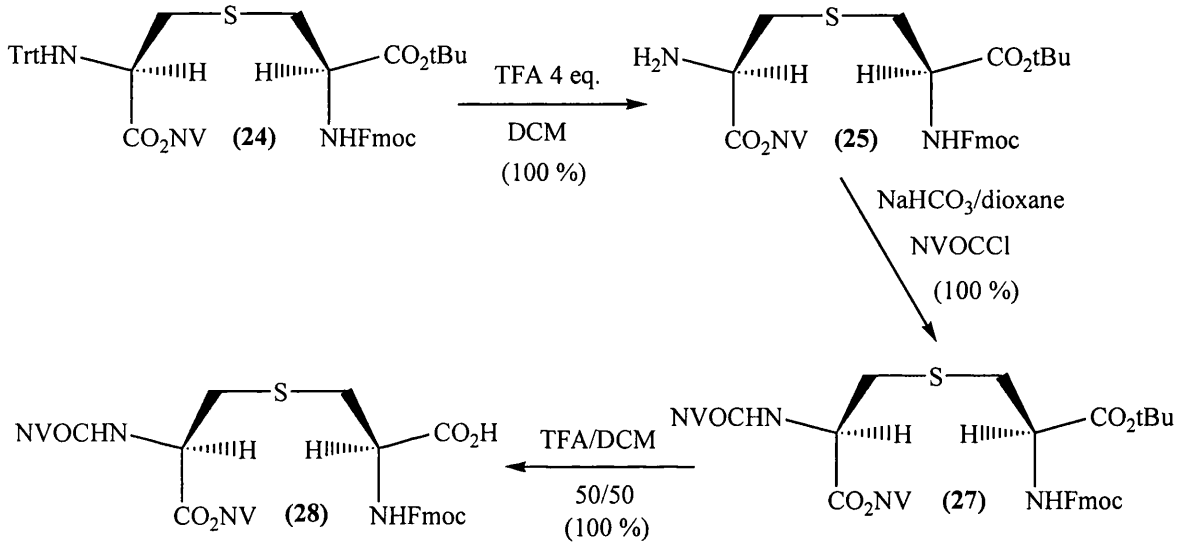


Figure 61: Synthesis of the final lanthionine residue (28).

In section **2. 5.** , the nature of the two types of fully protected lanthionine **(27)** recovered will be discussed.

Cleavage of the tBu group was easily carried out with a large excess of TFA in dichloromethane to give the desired lanthionine **(28)** in excellent yield. This material was now ready for SPPS.

2. 5. Iodoalanine and lanthionine residues: mixture of rotamers and/or diastereoisomers?

2. 5. 1. Iodoalanine residues

The synthesis of iodoalanine residues, from chloroalanine or *O*-tosylserine, with different protecting groups on the amine function, has been previously reported.[72,73] In this work, no evidence of rotamers was described.

Dugave and Ménez reported the formation of iodoalanine residues from *O*-mesyl serine and the presence of rotamers for such compounds.[75] This is accounted for by the presence of the very bulky trityl group on the amine, which has previously led to the reported formation of rotamers in homoserine residues.[78]

Iodoalanine **(22)** was also found to exist as inseparable rotamers. It is very clear from the n.m.r. spectrum (**Appendix 1** and **Appendix 2**) that two sets of signals are present for each set of protons on the molecule, with a major difference in chemical shift between some of them (3.58 ppm (1H, -C**H**-CH_2I, rotamer B) and 4.51 ppm (1H, -C**H**-CH_2I, rotamer A) for example). This effect would not be observed with enantiomers. These extra signals do not correspond to the aziridine by-product **(23)** which we have isolated and characterised (**Appendix 3**) nor to the dehydroalanine by-product (which would show distinct signals in the 6-7 ppm region). Iodoalanine **(19)** was also found to exist as rotamers by n.m.r. analysis.

Dugave and Ménez do not give a hypothesis for which bond suffers restricted rotation, justifying the presence of rotamers. It is speculated that the CH-CH_2 bond is probably most restricted in the molecule.

To confirm the presence of rotamers, VT experiments were carried out in DMSO, to temperatures of 100°C, in the hope of observing coalescence of some signals. This did not happen, presumably because the barrier of rotation of each rotamer is too high, even at very high temperatures. In addition, the formation of the aziridine by-product **(23)** starts at around 40°C and complicates further the experiment. These problems were also observed by Dugave and Ménez.[66]

Attempts to selectively recrystallise the iodoalanine rotamers were unsuccessful. Cleavage of the trityl group with TFA was then attempted, in the hope of isolating only one compound afterwards. Indeed, if the bulky trityl group is responsible for the formation of rotamers, removal of this group should give only one type of iodoalanine NV ester. Unfortunately, when the trityl group is removed from the molecule, protection against hydrolysis of the ester or formation of dehydroalanine is lost. The cleavage of the NV ester was clearly visible by n.m.r. Mass spectroscopy identified the title compound but other analyses were not good enough and a mixture of by-products was recovered.

A similar type of iodoalanine residue (with a trityl group as amine protecting group and an allyl group as carboxy protecting group) has been synthesised very recently, giving similar n.m.r. spectra to the ones obtained for iodoalanine **(19)** and **(22)**.[179] An elemental analysis of this iodoalanine residue (found: C, 60.1; H, 4.8; N, 2.7; I, 25.6. $C_{25}H_{24}NO_2I$ requires C, 60.4; H, 4.9; N, 2.8l; I, 25.5 for a mixture of rotamers 3: 1)[179] indicated that the additional peaks in the n.m.r. analyses were not due to an impurity, and therefore confirmed the existence of iodoalanine rotamers.

It seems that when the reaction is carried out at 20°C for 4 days (optimum conditions, see **2. 3.**) a larger proportion of one rotamer (called rotamer A) is formed than the other (called rotamer B). When the "room" temperature is higher, even more of rotamer A is formed, along rotamer B and some aziridine by-product. When the "room" temperature is lower, the main rotamer is rotamer B, with a minority of rotamer A and some remaining mesyl starting material. This shows that rotamer A has probably a lower ground state conformational energy but requires more energy to be formed than rotamer B. So rotamer A is the thermodynamic product whereas rotamer B is the kinetic product.

2. 5. 2. Lanthionine residues

All the lanthionine intermediates **(24)**, **(25)**, **(26)** and **(27)** (**Figure 62**) have been recovered as two sets of compounds having slightly different n.m.r. analyses but similar

mass spectroscopy. It is harder to identify two compounds for the final lanthionine **(28)** as this residue does not give very good n.m.r. spectra in $CDCl_3$. This lanthionine can only be identified with difficulty in deuterated DMSO (**Appendix 4** and **Appendix 5**).

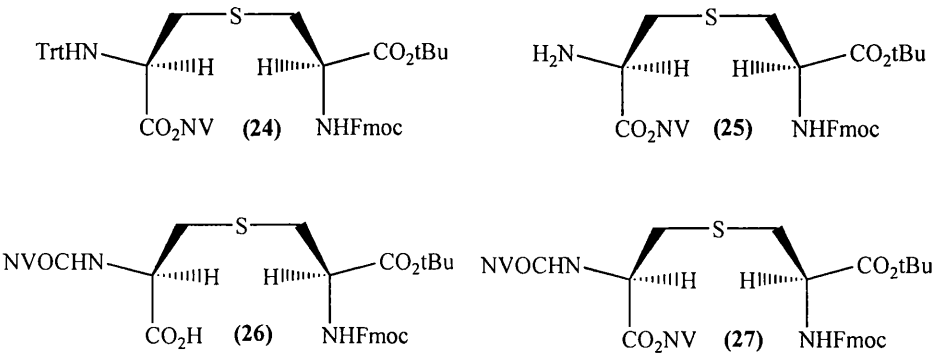


Figure 62: Lanthionine residues showing doubling of n.m.r. signals.

The two compounds recovered as lanthionine **(24)** are inseparable (**Appendix 6** and **Appendix 7**). By chance in the course of earlier attempts to synthesise these lanthionine residues, these two compounds were obtained separately (**Appendix 8** and **Appendix 9**) so comparison of the spectra made it easier to identify compound A and compound B in the spectrum of the mixture, however the separation of these residues is not usually possible.

Most of the time, lanthionine **(25)** was used straightaway without purification, but tests reactions were carried out on a small scale, with purification of these residues by flash column chromatography. Two compounds with different R_F (0.25 and 0.02, neat ethyl acetate) were recovered, with similar mass spectroscopy analyses but slightly different n.m.r. analyses (**Appendix 10** and **Appendix 11**). Careful chromatography also led to the separation of two lanthionines **(27)** (**Appendix 12**, **Appendix 13**, **Appendix 14** and **Appendix 15**), despite their very close R_F (0.85 and 0.9, neat ethyl acetate).

Dugave and Ménez synthesised similar compounds (**Figure 63**).[66]

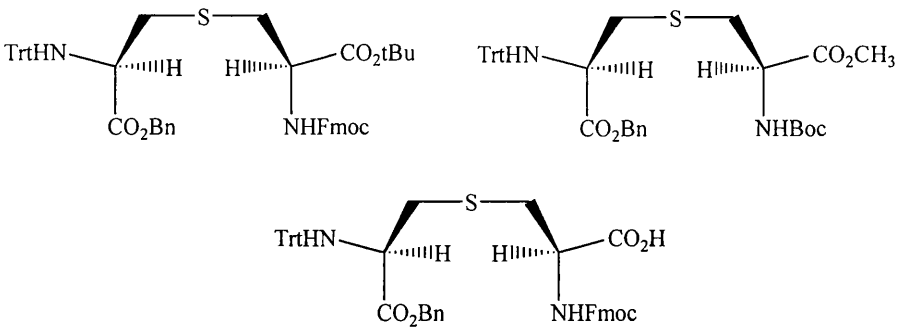


Figure 63: Some of the lanthionine residues synthesised by Dugave and Ménez.[66]

Doubling of some of the signals of the n.m.r. spectra of these compounds was also reported. This was attributed to the presence of rotamers, in line with the results observed for the iodoalanine residues.[75] These results were confirmed by a series of experiments (**Figure 64**) that led to the determination of the enantiomeric excesses of the lanthionine residues synthesised.

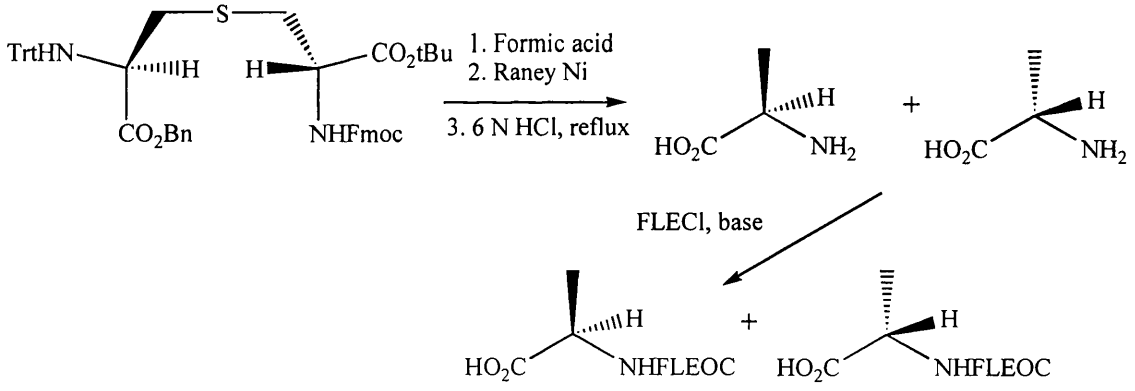


Figure 64: Conversion of lanthionine residues to derivatised alanine residues.[66]

The lanthionine residues were desulfurised with Raney nickel to give alanine residues. The chiral reagent FLECl was then used to derivatise these alanine residues. If lanthionine diastereoisomers had been formed during the coupling reaction, two alanine diastereoisomers would have been recovered and identified by HPLC. However, only one alanine chiral compound was identified by HPLC, proving that only a single lanthionine diastereoisomer was present. A total absence of racemisation during the coupling step

was therefore confirmed and the lanthionine residues obtained by Dugave and Ménez clearly identified as rotamers.

Lanthionines **(24)** to **(27)** have very similar n.m.r. spectra to the ones obtained by Dugave and Ménez and the method to synthesise them is also identical. It seems that these lanthionine residues could therefore also be rotamers. However, cleavage of the trityl group to make lanthionine **(25)** should eliminate most of the steric hindrance on the molecule and a unique lanthionine **(25)** should have been recovered. This is not the case as two lanthionines **(25)** are identified and separated by chromatography, as are later lanthionines **(27)**.

Some experiments were therefore carried out to prove the nature of these compounds. Time constraints precluded similar desulfurisation and derivatisation reactions.

VT experiments were carried out on lanthionine **(24)** in an attempt to identify the two compounds present as rotamers. No coalescence between peaks of compound A and peaks of compound B was observed. However, the two sets of signals of the methylene protons on the NV group became individually much sharper and seemed to coalesce, both in DMSO and toluene (**Appendix 16**). This experiment did not show whether compound A and compound B are rotamers or diastereoisomers but implies that each compound A and compound B are themselves actually a set of two rotamers. So lanthionine **(24)** might consist of two rotamers, each having one rotamer, so a mixture of four rotamers; or of two diastereoisomers, each having a rotamer. The presence of more than two rotamers appears quite possible, especially as most of the lanthionine residues synthesised by Dugave and Ménez have three rotamers. VT experiments were also attempted on lanthionines **(27)** and **(28)** but no coalescence was observed.

Separation of compound A and compound B of lanthionine **(24)** was attempted by reverse-phase HPLC, however, only one peak was observed. Similarly, lanthionine **(27)** gave only one peak by reverse-phase HPLC and lanthionine **(28)** gave two peaks so close to each other that no separation was possible. Normal-phase LC-MS of lanthionines **(24)** and **(27)** gave respectively two peaks each. No conclusive evidence regarding the nature of these lanthionine residues could be drawn from these results.

Recrystallisation of each type of lanthionine **(27)** was successfully carried out. However, the crystals obtained were too unstable for subsequent crystallographic analysis.

Attempts at synthesising cyclolanthionine from lanthionine **(24)** were carried out using DIEA and carbonyldiimidazole[66] (**Figure 65**).

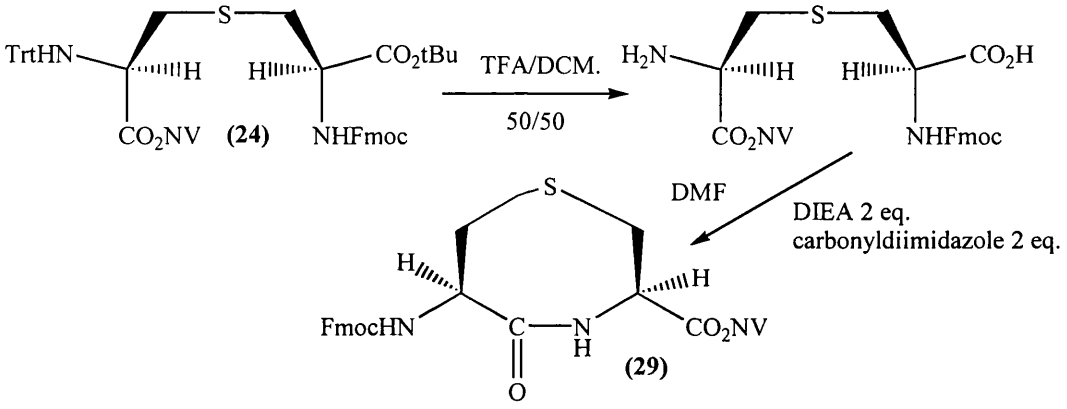


Figure 65: Synthesis of cyclolanthionine residues.

A batch of lanthionine **(24)** with a ratio of compound A: compound B, 3: 1 was used in the attempted reactions. It was necessary to recover the cyclolanthionine with a yield higher than 75 % to draw a conclusion about the nature of compounds A and B. If a mixture of cyclolanthionine diastereoisomers had been recovered (identified by n.m.r. by comparison with published data[34,45]) the nature of compounds A and B as diastereoisomers would have been proven. On the other hand, if only one type of cyclolanthionine residue had been identified by n.m.r., compounds A and B would have been shown to be rotamers.

Unfortunately, the reactions did not proceed with high enough yields to draw a conclusion to the experiment. A large number of by-products were recovered. The desired cyclic compound was identified by mass spectroscopy, alongside the starting material without its NV ester. In the second part of the experiment, it appears that the basic conditions are responsible for the saponification of the NV ester.

The key to whether rotamers or diastereoisomers are present lies in the coupling step to synthesise lanthionine **(24)**. Racemisation should not happen later in the synthetic pathway whereas it might take place during this nucleophilic substitution. It is hard to draw a correlation between the ratio of iodoalanine rotamers and the ratio of the two lanthionine residues obtained as both these sets of compounds are undergoing long work-up and purification by flash column chromatography before they are identified by n.m.r. However, it appears that iodoalanine rotamer A gives mainly lanthionine compound A and iodoalanine rotamer B mainly lanthionine compound B.

As mentioned previously (see **2. 4.**) these lanthionine compounds are obtained with a higher yield when iodoalanine is used in slight excess (1.1 eq.) and if the reaction is carried out at 20°C. Iodoalanine rotamer B was partially recovered when an excess of iodoalanine rotamers was used in the reaction, with a larger proportion of rotamer A than rotamer B in the starting mixture. It is possible that the conformation of rotamer A makes thiolate attack on this rotamer preferable.

A mechanistic explanation for the possible formation of diastereoisomers could be drawn from the previous observations (**Figure 66**).

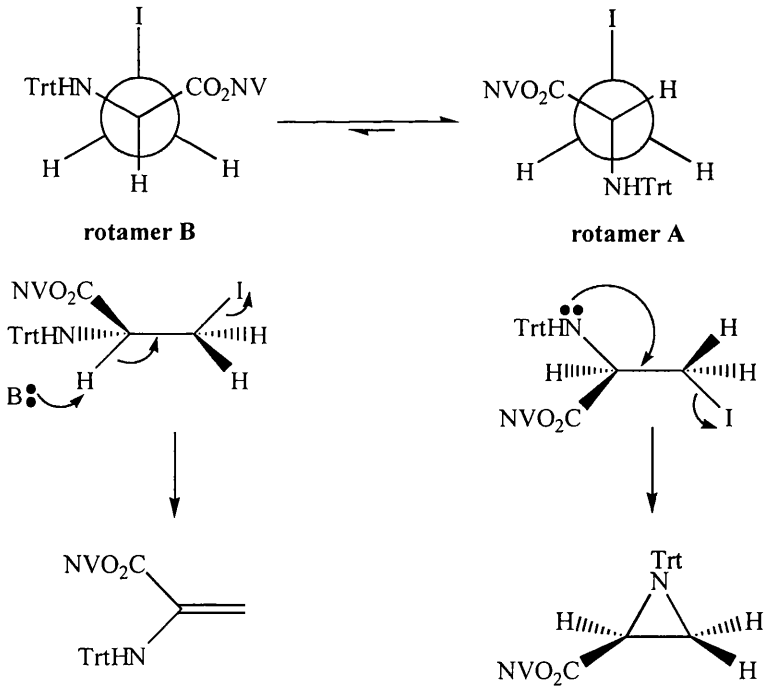


Figure 66: Possible mechanistic explanation for the presence of lanthionine diastereoisomers. B is the base cesium carbonate.

If the minor iodoalanine rotamer B has a conformation such that it can easily undergo elimination as a possible competing reaction, then a small amount of dehydroalanine might be formed in the reaction from rotamer B. This would then be attacked by the cysteine thiolate to give a racemic mixture of both lanthionine diastereoisomer A and lanthionine diastereoisomer B. The iodoalanine rotamer A on the other hand may have a conformation such that the formation of aziridine is easily carried out as a competing side-reaction, along with the synthesis of lanthionine diastereoisomer A

The representation of the most abundant iodoalanine rotamer A seems plausible as the two most bulky substituents NHTrt and I are the furthest apart in this conformation, so this is probably the most stable.

Lanthionines **(24)** are formed in a similar manner to the one used by Dugave and Ménez to synthesise lanthionine residues from iodoalanine residues. They reported the presence of rotamers for both iodoalanine and lanthionine residues and their n.m.r. spectra are very similar to the ones obtained for iodoalanine **(22)** and lanthionine **(24)**.

If there is little doubt that iodoalanine **(22)** consists of a mixture of rotamers, it is much harder to identify the nature of lanthionines **(24)** to **(28)**. With the facts presently available, it is difficult to know whether lanthionines **(24)** to **(28)** are mixtures of diastereoisomers or rotamers.

Further work is therefore needed on this particular point. Repeating the set of experiments done by Dugave and Ménez (desulfurisation then derivatisation of the alanine residues obtained) as well as molecular modelling studies will have to be considered.

At the time the final compound **(28)** was synthesised for SPPS, the nature of the residues (rotamers or diastereoisomers) was not known, and it was assumed that they were rotamers, as described in the literature.[66] Both types of compounds were therefore left mixed and used as such for SPPS. After SPPS, the excess of lanthionine **(28)** not used in the experiment can easily be recovered by a quick work-up and purification by reverse-phase column chromatography.

If it is proven that lanthionine **(24)** to **(28)** are diastereoisomers, the separation of these diastereoisomers can be easily carried out by flash column chromatography of lanthionine **(27)** before they are transformed into the final lanthionine **(28)**.

In summary, a quick, efficient and high-yielding pathway has been developed to synthesise lanthionine residues bearing photolabile groups. It is possible that a small amount of diastereoisomer is formed during the monosulfide formation. However, when optimum conditions are used for the synthesis of iodoalanine residues and lanthionine residues (respectively 4 days at 20°C and 4 hr at 20°C), lanthionine **(24)** is mainly recovered as compound A.

4. Solid-phase peptide synthesis with photolabile protecting groups

1. Photolabile tests

Before the final lanthionine **(28)** was incorporated into peptides, it was necessary to check that photolabile cleavage of the NV and NVOC groups would be feasible. Photolabile cleavages performed in solution may behave very differently when tried on a solid-support, however it was felt to be worth checking that photolabile cleavage was possible in solution before tackling solid-phase experiments.

Photolabile tests were carried out using two lamps, one broad range U.V. lamp, **lamp 1** and one longwave U.V. lamp emitting at 365 nm, **lamp 2**, either simultaneously or separately.

Preliminary tests were performed on lanthionine **(24)** in methanol, for a total of 9 hr 30 using **lamp 1** (**Figure 67**).

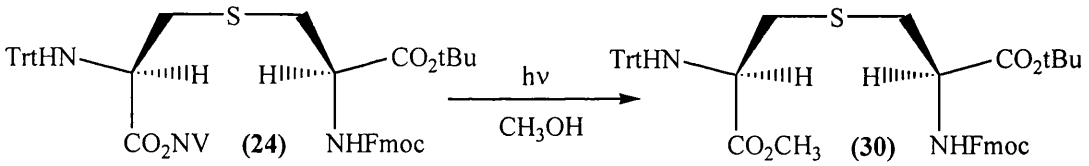


Figure 67: Photolabile cleavage performed on lanthionine (24).

Methanol was chosen as several photolabile cleavages of NVOC protecting groups and NVOC-type linkers have been successfully carried out in methanol[154,159] or ethanol.[155] Several fractions were recovered after purification of the crude mixture by flash column chromatography. The main fraction was identified as (*R*)-methyl-*N*-tri-phenylmethylalanyl (*R*)-*tert*-butyl-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(30)** (12 %). This shows that the photolabile cleavage of the NV ester was partially successful, but that methanol is an unsuitable solvent. As the trityl group is well-known for its lack

of stability during NV and NVOC cleavage,[121] no further tests were carried out on lanthionine **(24)**.

Simultaneously, other experiments were carried out on lanthionine **(28)**. Methanol was tried first as solvent, for 3 hr, using **lamp 1** with no success. As lanthionine **(28)** was only partially soluble in methanol, an acetonitrile: methanol mixture was subsequently used, as acetonitrile has been used in a number of photochemical experiments involving NVOC protecting groups and NVOC-type linkers.[157,160,161] The photochemical reaction was set up again for 5 hr 30, at the end of which HPLC analyses and mass spectroscopy revealed that partial photolabile cleavage had occurred but a large amount of starting material was still present.

A further attempt, using both lamps simultaneously was tried for 7 hr 30 in acetonitrile and methanol. Mass spectroscopy revealed partial photolabile cleavage of both NV ester and NVOC group. However, a characteristic by-product (**Figure 68**) was also identified by mass spectroscopy.

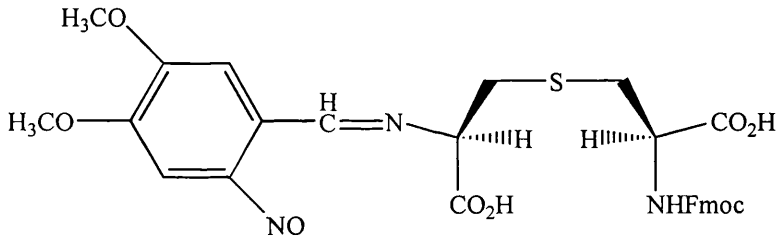


Figure 68: By-product formed during photolabile cleavage of lanthionine (28).

During photolabile cleavage of the NVOC groups, benzaldehyde by-products are formed that can then be attacked by the liberated amines to give this by-product.[156] It is therefore important to work in slightly acidic conditions to keep these amines protonated. Sulphuric acid[154] or a buffer of potassium acetate/acetic acid (pH = 4.5)[156] have been used for this purpose. A mixture of acetonitrile and potassium acetate solution was therefore used as solvent for photolabile cleavage, using **lamp 1** first for 6 hr (**Figure 69**).

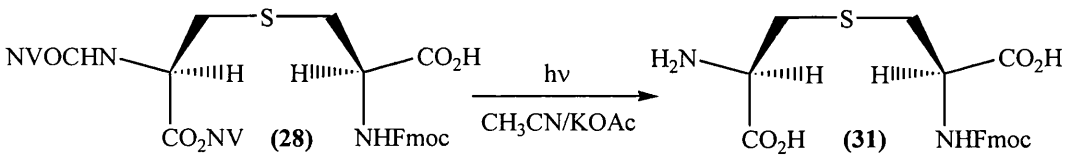


Figure 69: Photolabile cleavage performed on lanthionine (28) in acetonitrile/KOAc.

Keeping the same solvent system, the experiment was carried out again using **lamp 2** for 12 hr, with samples analysed every 2 hr. The lamp was changed to test the photolabile cleavage at a wavelength (365 nm) compatible with peptide synthesis[121] After 4 hr and 8 hr, mass spectroscopy revealed the presence of the product **(31)** in addition to the starting material **(28)**. After 12 hr, no starting material was detected in the reaction mixture, but it was also very hard to identify any other compound present.

A final set of experiments were tried in dioxane and potassium acetate solution. Dioxane has been reported to be a very good solvent for photolabile cleavage of NVOC linkers (the photolysis half lives of such linkers are the shortest when performed in this solvent[159]). This would also be a good solvent for the solid support during peptide synthesis. HPLC analysis revealed that no starting material remained in the reaction mixture after 5 hr 30.

After several tests, the best conditions for the photolabile cleavage of NV and NVOC groups were identified. The quickest and more complete cleavages were observed in dioxane and a solution of potassium acetate with **lamp 2**. In addition, sparging of the solvents 30 min prior to experiment and bubbling of nitrogen during the cleavage also help to reduce the risk of by-reactions with oxygen.

2. Stability of NV ester to SPPS conditions

The difficulties encountered with saponification of the NV ester in basic conditions during the synthesis of the final lanthionine residues prompted additional experiments before SPPS. During SPPS, removal of the Fmoc group is performed usually using 20 % piperidine in DMF prior to addition of the next amino acid. The next amino acid is then added to the solid support, after preliminary activation with DIC and HOBt. If the NV ester was cleaved under the basic conditions used to remove the Fmoc group, the addition of an activated amino acid alongside an excess of DIC and HOBt might lead to the activation of the liberated carboxy group on the lanthionine residue and possible peptide by-products (**Figure 70**).

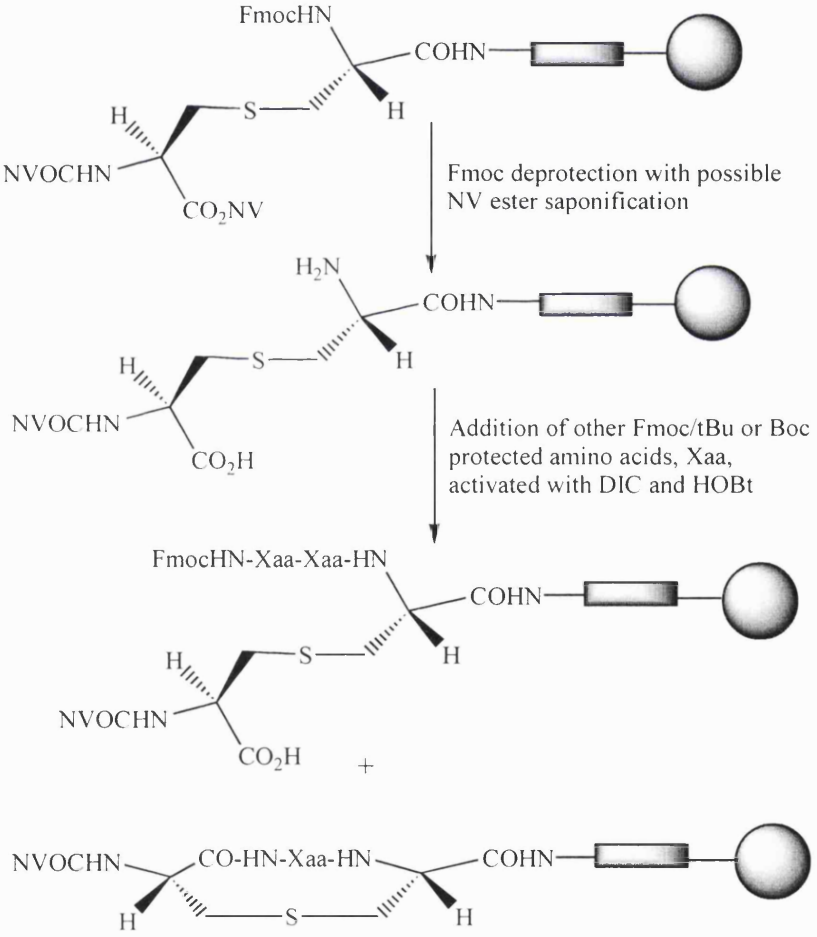


Figure 70: Possible SPPS side-reactions if NV ester saponification occurs during Fmoc cleavage.

Lanthionine **(28)** was dissolved in 20 % piperidine in DMF and the solution left to stand for 20 min, which are the usual reaction conditions for a SPPS Fmoc deprotection. The dibenzofulvene-piperidine adduct formed during the reaction is extracted with a phosphate buffer[180] (pH between 5.5 and 6). After work-up, the crude product was analysed by n.m.r. Very characteristic peaks belonging to the dibenzofulvene and 6-nitroveratryl alcohol by-products were identified, which shows that both Fmoc and NV groups were removed by 20 % piperidine.

DBU has also been used for cleavage of the Fmoc group.[180,181,182] Both 2 % DBU, 2 % piperidine in DMF and 5 % DBU in DMF were tried. These basic systems have been used previously to minimise side-reactions in SPPS. However, when lanthionine **(28)** was dissolved in these systems, cleavage of both the Fmoc and NV groups was observed.

Although there was no certainty that the NV ester would be cleaved during Fmoc cleavage when performing the reaction on a solid-support, in view of these results, it was decided to plan the coupling steps to minimise the risk of side-reactions if cleavage did take place.

DIC and HOBt are the most common coupling reagents used in SPPS. However, if DIC and HOBt are present during coupling, there is a substantial risk of also activating the carboxy group liberated from a potential NV ester saponification, leading to side-reactions (**Figure 70**).

It was therefore decided to use pentafluorophenyl esters of *N*-Fmoc protected amino acids,[183,184] after the lanthionine residue had been incorporated into the growing peptide (**Figure 71**).

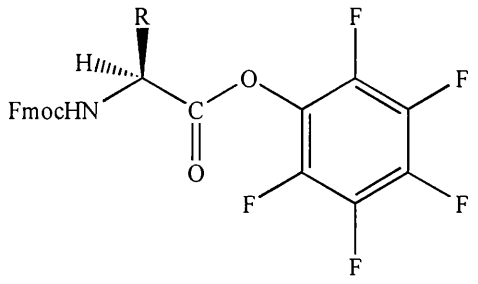


Figure 71: Pentafluorophenyl ester of an Fmoc protected amino acid.

3. SPPS of a sandostatin analog

3. 1. Batchwise synthesis

It was decided to synthesise a sandostatin analog (**Figure 72**), previously made using PCOR methods[42,43] or on-resin attack of a bromoalanine residue by a cysteine residue.[44] N.m.r analyses, mass spectroscopy data and HPLC techniques are therefore available for comparison of these methodologies and the one presently developed.

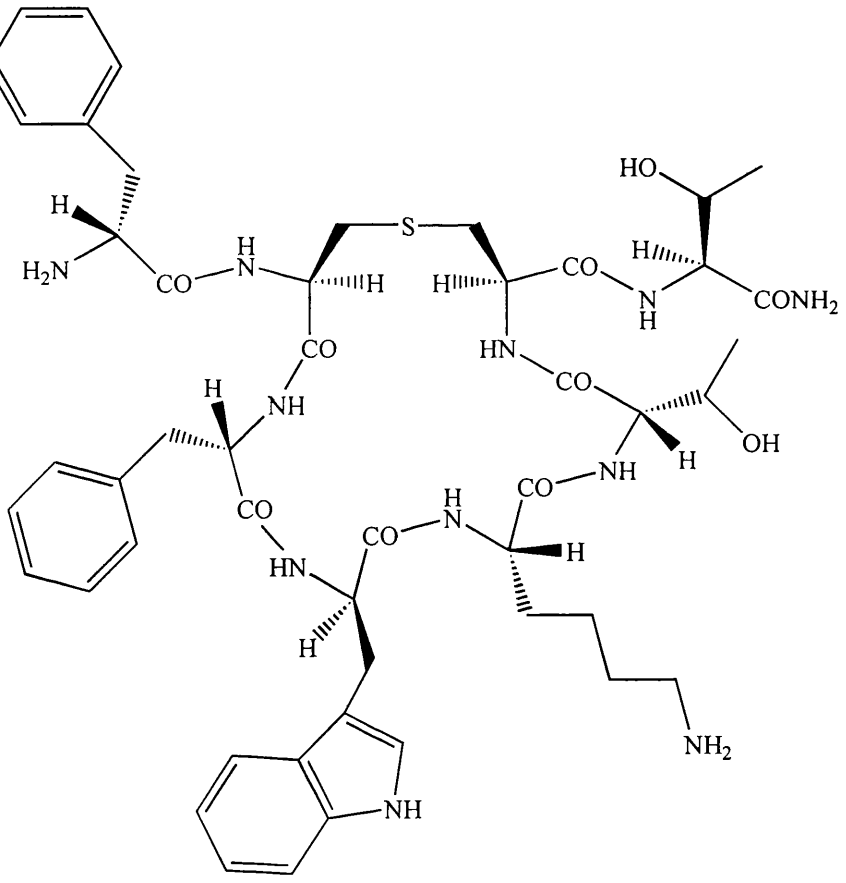


Figure 72: Target sandostatin analog.

The target peptide is a peptide amide. Several amide resins are available for its synthesis. The Rink amide MBHA resin (**Figure 73**) was chosen as it is cleaved by fairly strong acidic conditions (about 80 to 90 % TFA) and has shown good qualities (in particular reduction of side-reactions) for the synthesis of peptide amides.[185,186,187] It has also been used previously for the synthesis of sandostatin analogs.[44] This resin was

preferred to milder acid-labile resins such as the Sieber resin[188] because slightly acidic conditions are planned for the photolabile cleavage and any risk of cleavage of the peptide from the resin at this stage must be avoided.

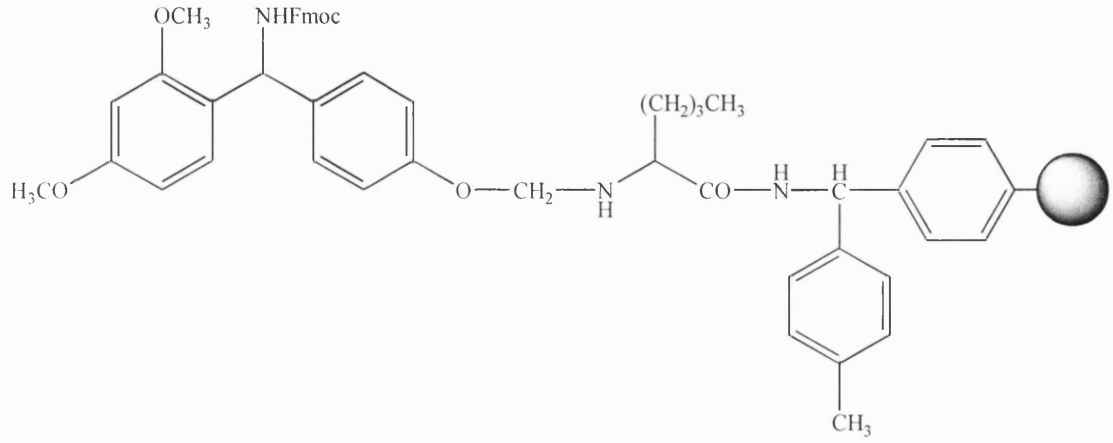


Figure 73: Rink amide MBHA resin.

The chosen resin is polystyrene-based. It was anticipated that this should not be a problem for photolabile cleavages as several photolabile experiments with nitrobenzyl linkers had been successfully carried out on this solid support.[189,190,191]

It was decided to use the common HOBt/DIC coupling reagents to incorporate the first Thr residue and lanthionine **(28)** and to use pentafluorophenyl esters of Fmoc amino acids and HOBt to incorporate the remaining residues.

HATU/HOAt/DIEA were chosen as cyclisation reagents as it is one of the most efficient system of coupling reagents used for the cyclisation of small peptides, its use reducing considerably the risk of racemisation and side-reaction.[192]

The first part of the pathway involves the synthesis of the peptide up to the photolabile cleavage (**Figure 74**).

The SPPS of this sandostatin analog was performed in a Merrifield bubbler, batchwise. Incorporation of the first Thr residue proceeded well (as confirmed by Kaiser test[193,194]). Incorporation of the lanthionine residue also proceeded very well. After 1 hr, the Kaiser test was pale yellow, revealing the absence of any remaining amine. Incorporation of the second Thr residue as well as Lys, Trp and Phe was carried out easily using the pentafluorophenyl esters of these amino acids and HOBt for 30 to 45

min. Triple couplings were only necessary for Lys and Phe for which a pale green coloration of the Kaiser test revealed some remaining amine after the second coupling.

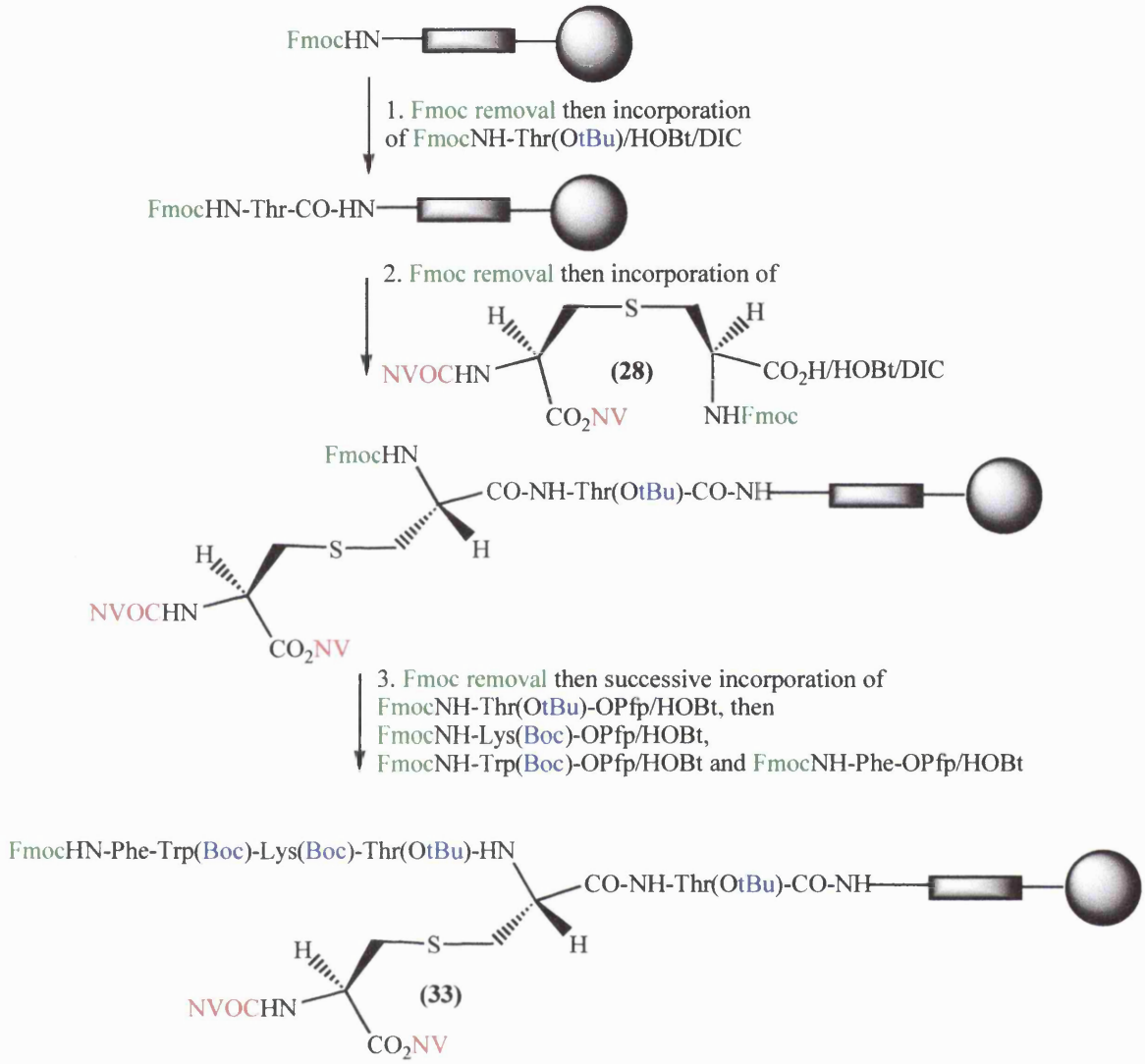


Figure 74: First part of the synthesis of a sandostatin analog on solid-support.

A portion of the resin was removed at this stage and the peptide cleaved from this portion was analysed. Mass spectroscopy analysis revealed that linear peptide **(33)** had been properly synthesised.

The photolabile cleavage was carried out for 10 hr after which the Kaiser test appeared red burgundy, indicating at least partial cleavage of the NVOC group. It was

therefore decided to proceed with the cyclisation step for half of the resin, keeping the other half under argon. Cyclisation was attempted with HATU/HOAt/DIEA. A small attenuation of colour was observed on the Kaiser test. Incorporation of the last residue and cleavage of the peptide from the resin using a common acidic mixture (TFA: water: thioanisole, 96: 2: 2)[44] were therefore carried out (**Figure 75**).

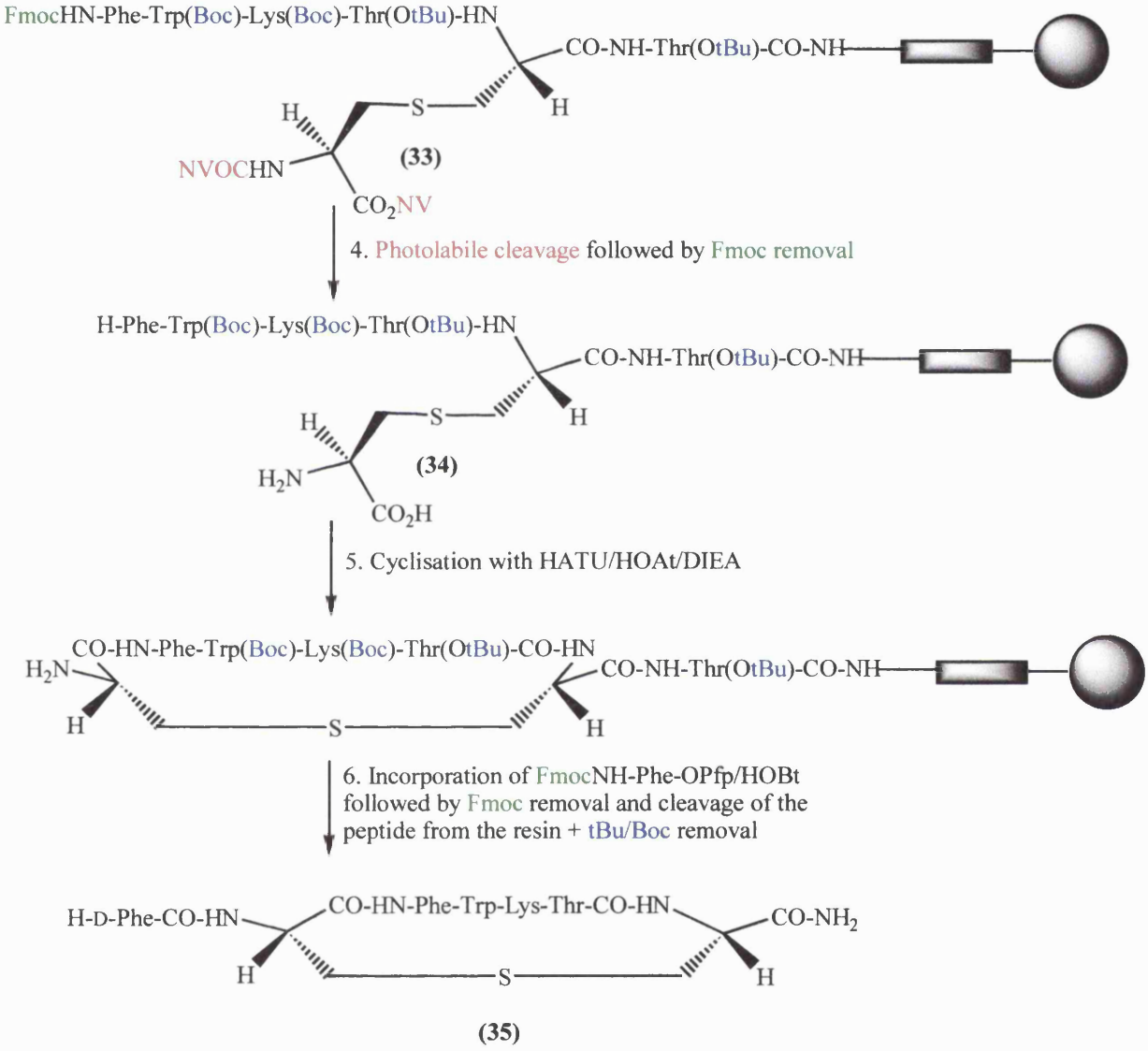
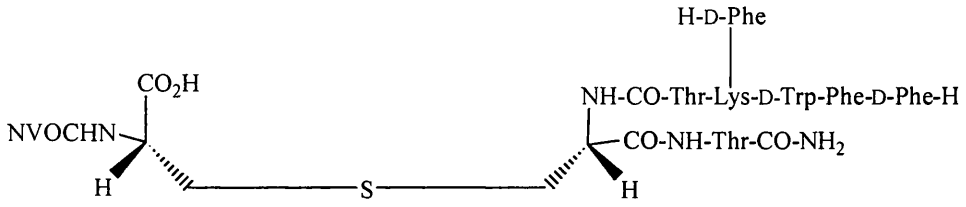


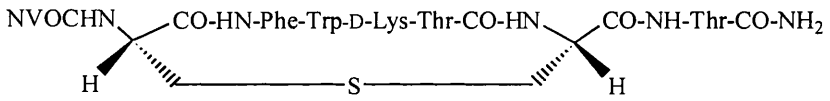
Figure 75: Photolabile cleavage leading to a sandostatin analog.

Subsequent mass spectroscopy of the peptide after the photolabile cleavage revealed that the photolabile cleavage was not complete. Peptide **(33)** was still identified after 10 hr.

The target peptide **(35)** was not eventually recovered. Two peptide by-products **(36)** and **(37)** (**Figure 76**) were identified in the final crude product obtained. This implies that the photolabile cleavage of the NV ester is quickly carried out whereas the NVOC group is cleaved much more slowly.



(36)



(37)

Figure 76: By-products formed during the first attempt at synthesising sandostatin analog (35).

Not only did this result show that the photolabile cleavage of the NVOC group was not complete in 10 hr in the chosen conditions but also that the cyclisation step was not complete either. Products with a free amine resulting from photolabile cleavage of the NVOC group must have been present in the mixture obtained from the resin as the Kaiser test was not yellow after 10 hr, however such products were not recovered or identified at this stage.

The second half of the resin was therefore used for further photolabile cleavage. After 30 hr and a slight change in the Kaiser test, cyclisation and incorporation of D-Phe were tried in a similar manner as before.

The crude mixture obtained from the cleavage of the resin did not give any positive result by mass spectroscopy nor HPLC. It is very likely that the resin was kept

for so long (two weeks) before being submitted to further photolabile cleavage that degradation of the peptide had occurred.

Peptide **(33)** was therefore resynthesised as before, and subjected to a much longer photolabile cleavage. A U.V. monitoring of this cleavage was carried out, *via* disappearance of a characteristic peak at 250 nm (NVOC/NV). 70 hr were necessary for completion of the reaction. The Kaiser test only changed colour after 50 hr. No NVOC group was detected in the photolabile filtrate and on the resin after 70 hr. Mass spectroscopy clearly revealed the disappearance of the starting material **(33)** and the formation of peptides **(38)** (**Figure 77**) and **(34)**.

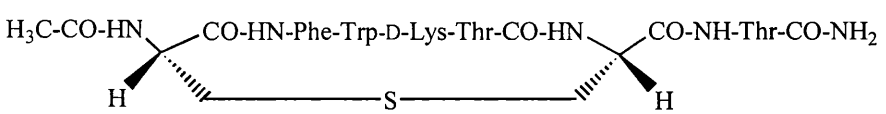
(38)

Figure 77: Peptide (38) formed during the second attempt at synthesising sandostatin analog (35).

Cyclisation was carried out using PyAOP/HOAt/DIEA. This system of cyclisation reagents has been reported to be as efficient as HATU/HOAt/DIEA with the added advantage of eliminating any possibility of guanidinium by-product formation.[118,120]

Incorporation of the final amino acid and acid treatment gave a crude product which was purified by HPLC. Two by-products **(39)** and **(40)** were identified by mass spectrometry (**Figure 78**).

(39)



(40)

Figure 78: Final by-products recovered from the second attempt at synthesising sandostatin analog (35).

These by-products show that an increase in the amount of potassium acetate present in the photolabile filtrate finally led to the capping of the free amine function on the lanthionine residue. The peptide could therefore not be further extended.

During this peptide synthesis, the Fmoc cleavage following the photolabile cleavage was very hard to perform. Four attempts were necessary in order to obtain a proper Kaiser test (purple) after 20 min of treatment with 20 % piperidine in DMF. Tests showed that the pH on the resin was very acidic before the first attempt at Fmoc cleavage. These observations confirm the presence of an acidic medium where capping of the free amine by acetate is highly likely to be carried out.

In this second attempt, the photolabile cleavage went to completion but in the very long time of 70 hr! This is too long a time for such reactions and for SPPS. Numerous side-reactions and spoiling of the peptide and resin can happen in such a long time. A build-up in the concentration of potassium acetate in the photolabile filtrate led to capping of the free amine on the lanthionine residue and the formation of by-products **(39)** and **(40)**. It was also very difficult to follow the progress of the photolabile cleavage by Kaiser test, as the potentially free amine appeared to have been capped by potassium acetate. Finally, the resin changed colour drastically after photolabile cleavage, turning from pale yellow to very dark brown beads. This might have resulted in some shielding effect of the U.V. light by the resin, considerably slowing down the progress of the photolabile cleavage.

Tentagel resins (polyethyleneglycol-polystyrene graft copolymer, **Figure 79**) have also been used as solid supports for successful photolabile cleavages of NVOC linkers[159] and dithiane-protected benzoin photolabile safety catch linkers.[149] In the latter, both polystyrene and Tentagel resins were used and the resin matrix did not seem to influence the photochemical reaction,[149] whereas these two resins have very different chemical reaction profiles.[195] It is possible that Tentagel resins would give much better results for the synthesis of this sandostatin analog, especially potentially increasing the speed of the photolabile cleavage by reducing any potential shielding effect. This resin also swells well in water. This could be an advantage when the small amount of aqueous

potassium acetate solution is used. Finally, it might be better to choose a resin with a lower loading as the rather high loading of the chosen resin (0.54 mmol/g) might have made the photolabile cleavage harder.

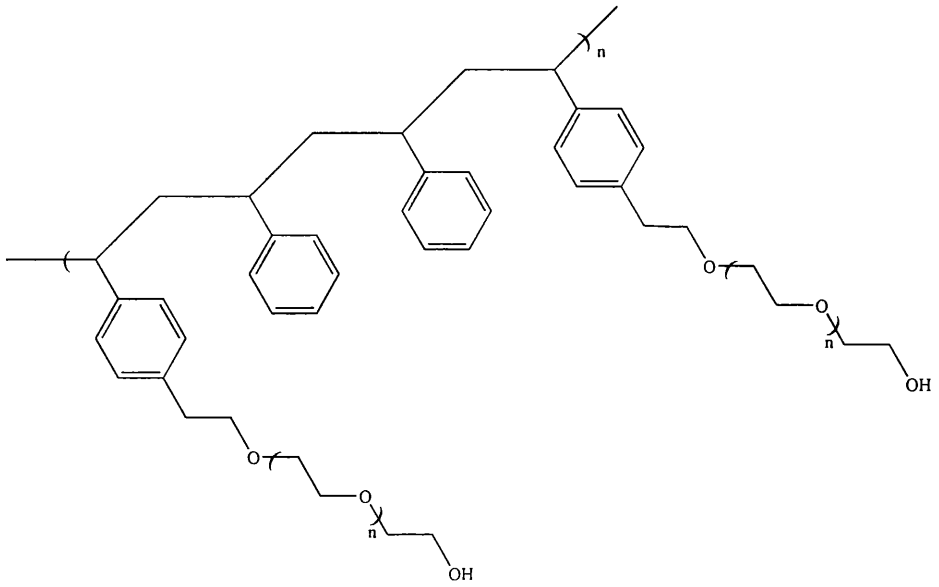


Figure 79: Tentagel resin.

3. 2. Tests on Tentagel resin

NovaSyn TGR® resin was chosen. This is a Tentagel-based support with a similar MBHA Rink amide linker and a lower loading than the previous resin. A small amount of resin was used for a photolabile test. Lanthionine **(28)** was incorporated directly on the solid support and the sample was submitted to 10 hr of photolabile cleavage. Quantitation of the substitution level showed a very good incorporation of the lanthionine residue (substitution level found 0.174 mmol/g, loading of the resin 0.21 mmol/g). After 10 hr, the peptide was cleaved from the resin and analysed by mass spectrometry. The presence of lanthionine **(41)** was clearly identified (**Figure 80**).

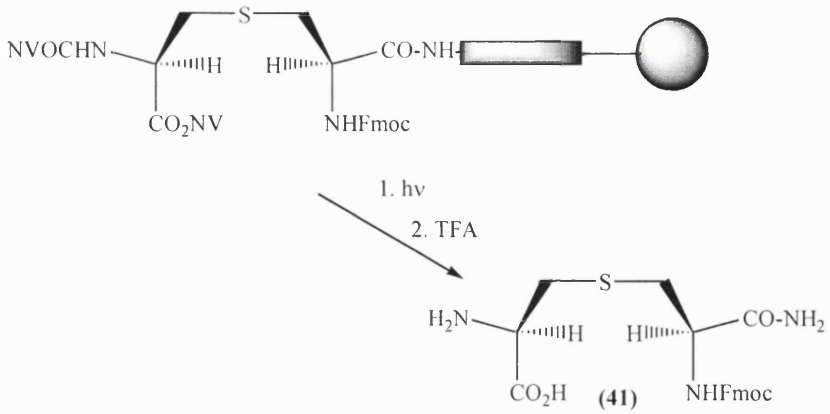


Figure 80: Synthesis of lanthionine (41).

This shows that the photolabile cleavage proceeds much faster and better on Tentagel resin. No dark brown coloration of the resin was observed this time. No partially cleaved lanthionine nor starting material **(28)** was identified by mass spectroscopy. Clearly changing the solid-support and lowering the loading has drastically improved the photolabile cleavage of the NV and NVOC groups in these conditions (365 nm, dioxane, KOAc solution, 10 hr). Moreover, calculation of the substitution level in this experiment proved that lanthionine **(28)** was incorporated on the solid support in satisfactory yield.

3. 3. Continuous-flow SPPS on Tentagel resin

Peptide **(33)** was easily resynthesised by continuous flow SPPS on a Millipore 9050 PepSynthesizer™ on NovaSyn TGR® resin. U.V. monitoring confirmed the proper incorporation of every amino acid, including lanthionine **(28),** which was manually incorporated *via* an external syringe.

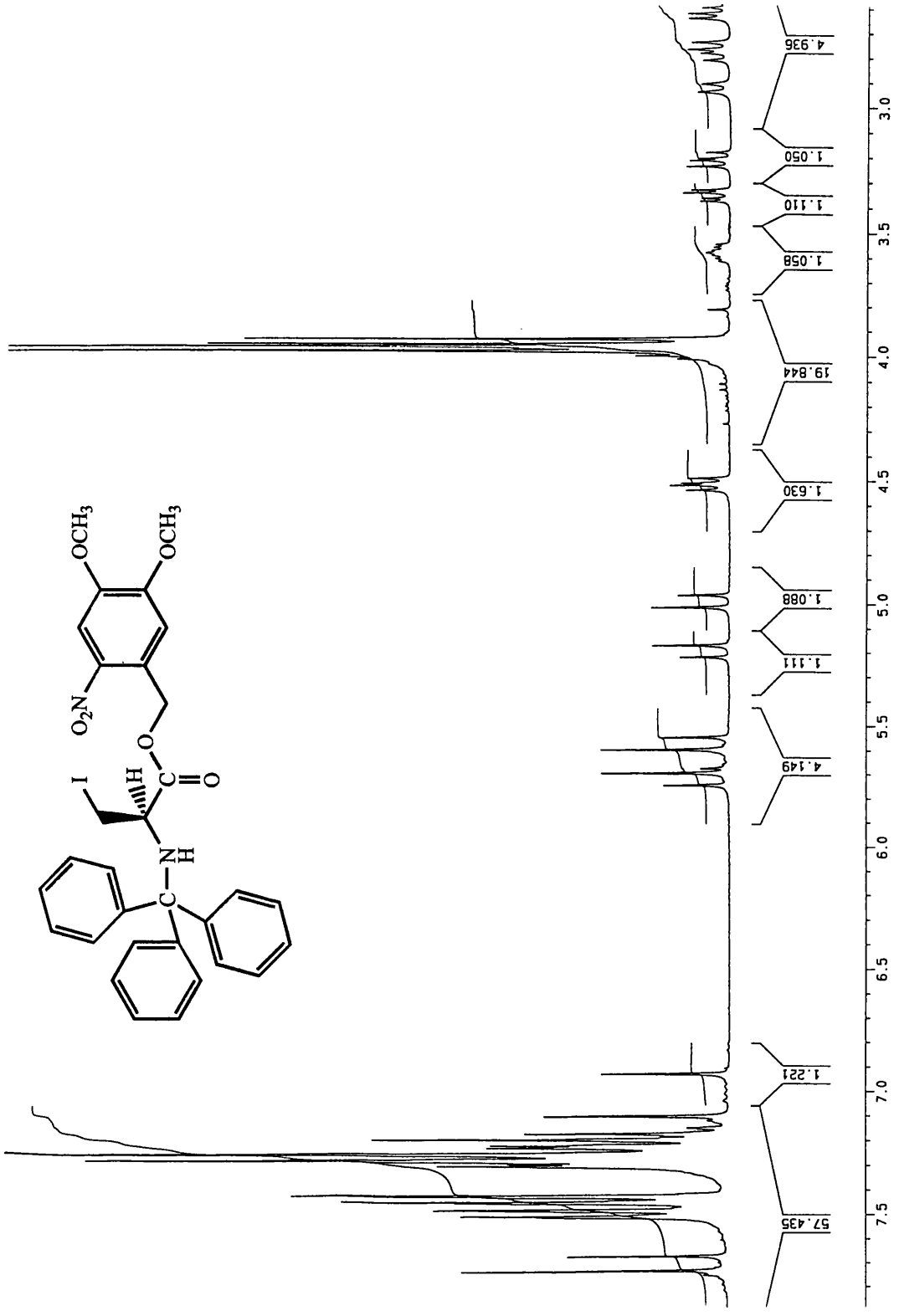
Photolabile cleavage was carried out for 30 hr in a similar manner as before, but with the possibility to thoroughly rinse the resin after photolabile cleavage as a sinter and tap were present at the bottom of the lamp used. After this time (considered maximum for reasonably efficient synthetic conditions), a small amount of peptide was cleaved from the resin and analysed by mass spectrometry. A large amount of peptide **(33)** was clearly identified, as well as a smaller amount of peptide **(34)**.

This synthesis proceeded much better than the two previous attempts but the photolabile cleavage is still not completed after 30 hr. As previous tests have shown the efficiency of the photolabile cleavage in solution and on this solid-support on lanthionine **(28)**, it is very likely that the limited success encountered here is due to the sequence of the target peptide itself. Indeed, the two aromatic residues present in the ring (Trp and Phe) may seriously restrict the efficiency of the photolabile cleavage (by shielding) and the cyclisation.

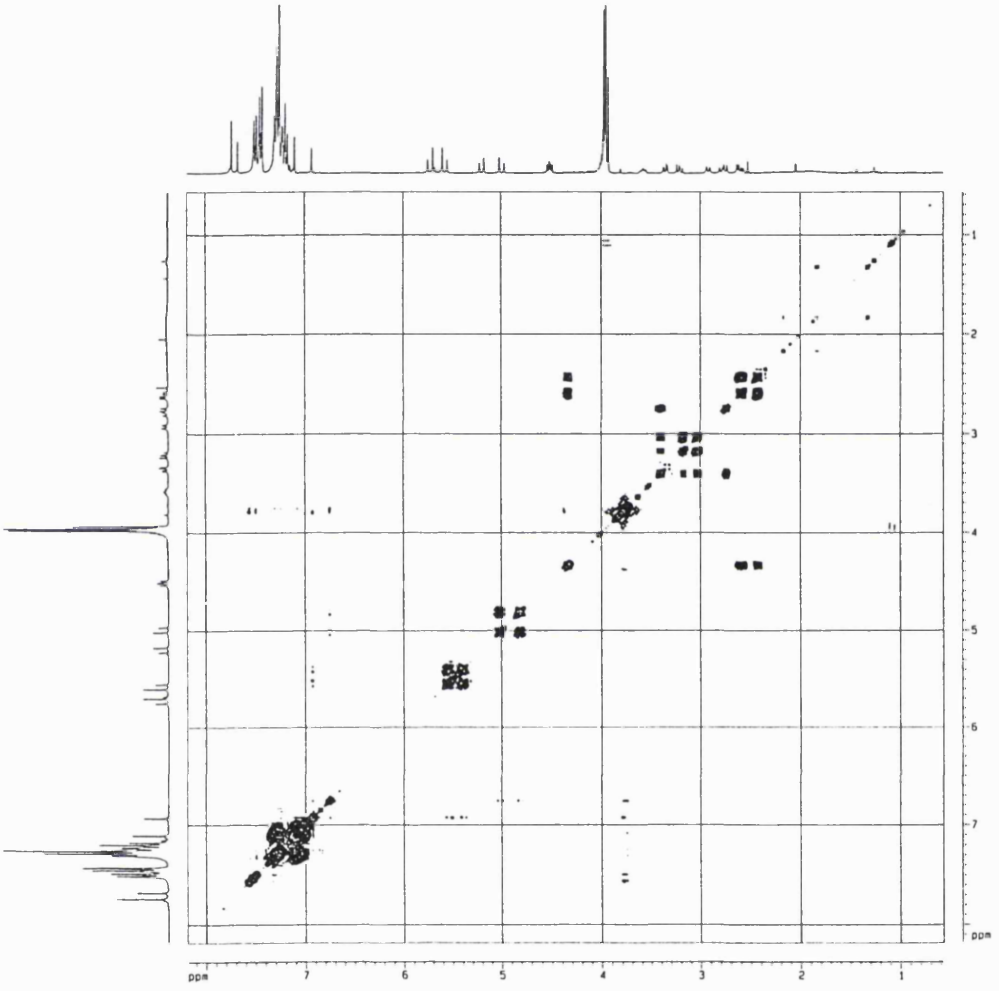
4. Future work

Incorporating lanthionine **(28)** in a much simpler peptide than sandostatin analog **(35)** (for example with only two small residues such as Ala in the ring) should be attempted to improve the efficiency of the photolabile cleavage.

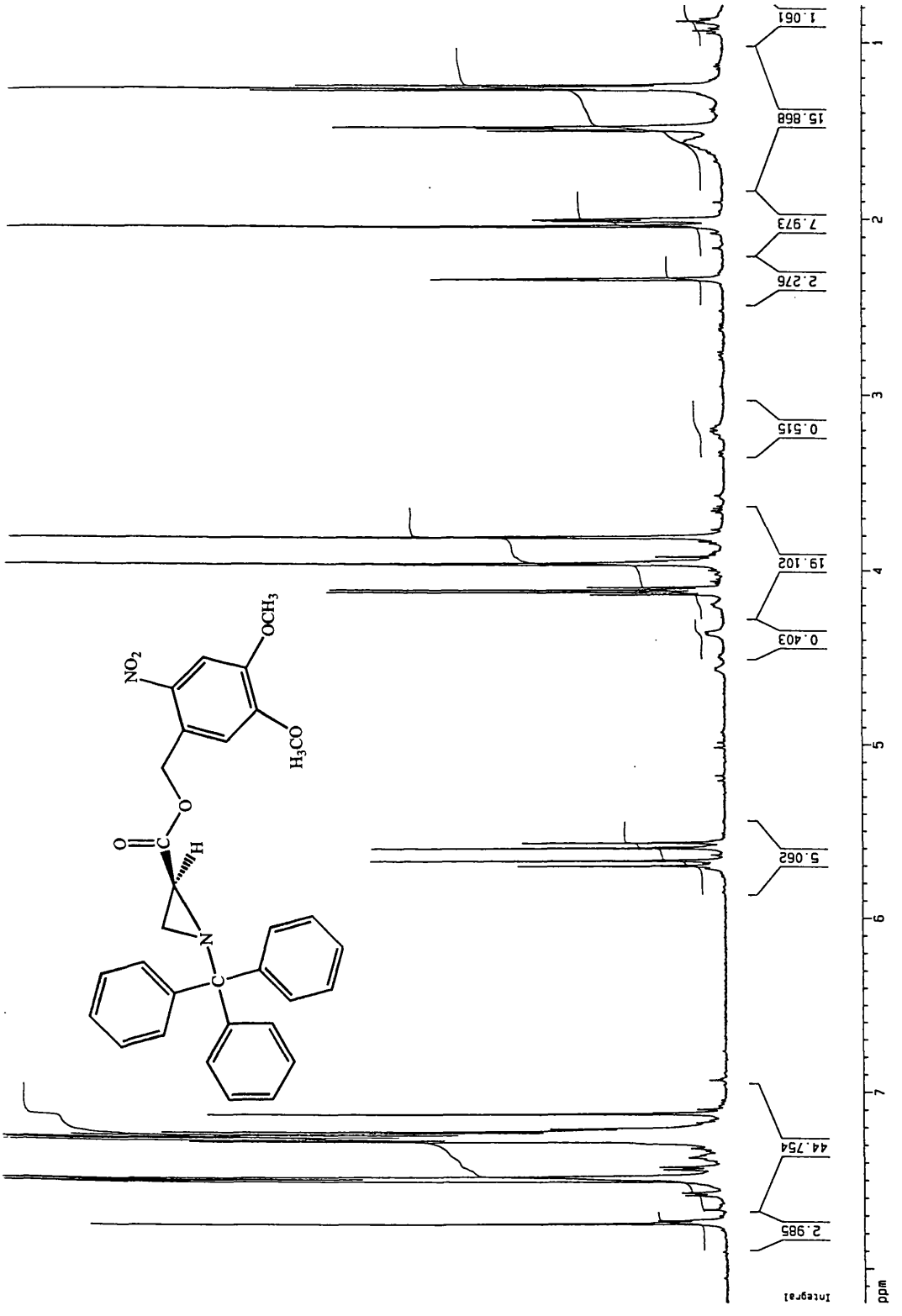
If a better photolabile cleavage is possible in these conditions, a four dimensional orthogonal protecting group strategy could be developed to ultimately synthesise bicyclic systems such as rings D and E of nisin. A fourth type of protecting group, orthogonal to Fmoc/Boc/photolabile could be found among the orthogonal protecting groups already developed in SPPS such as Al or Dde. As only a few small residues are present in these rings (e.g. Ala and His), it is hoped that the photolabile cleavage would proceed smoothly.



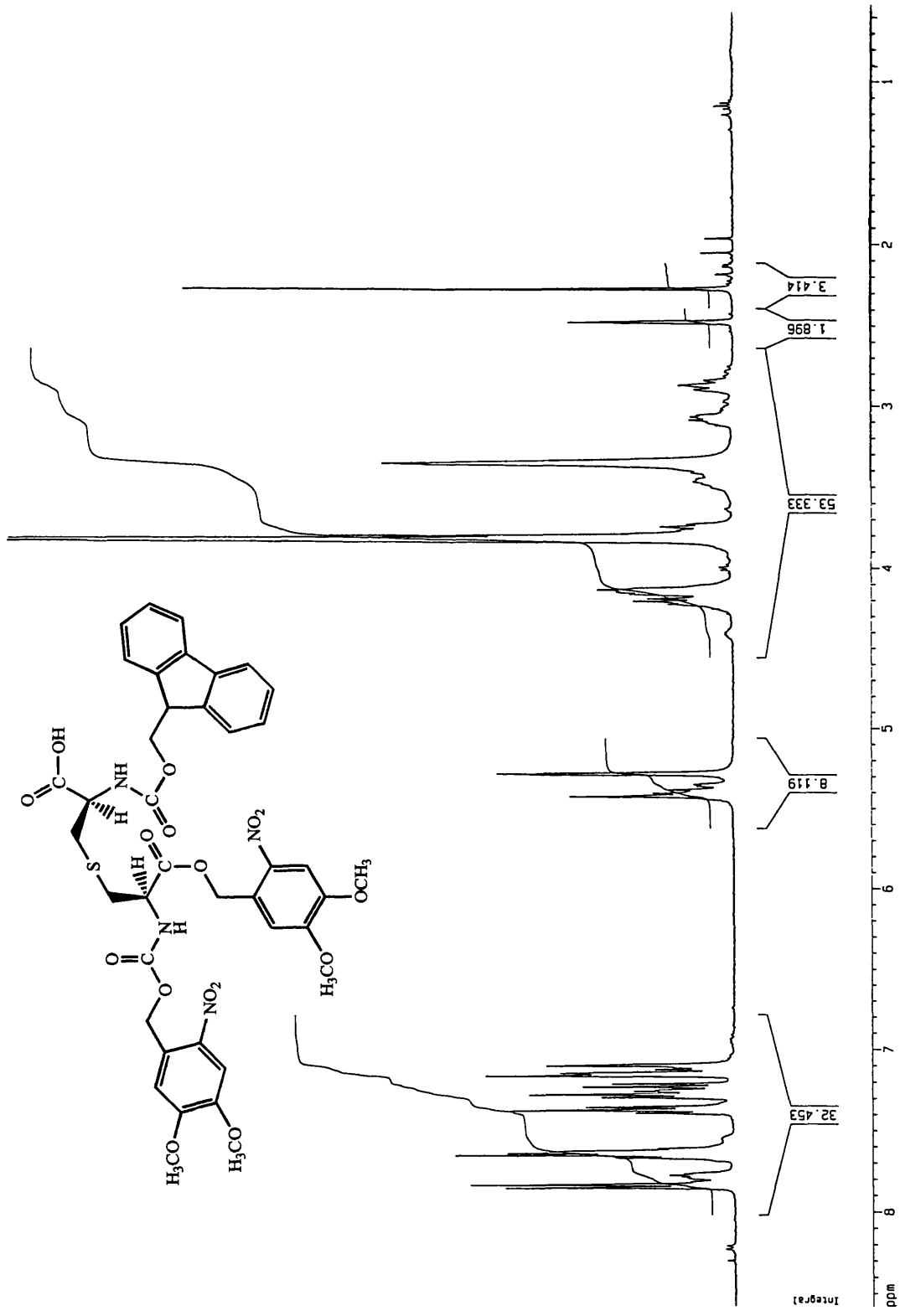
Appendix 1: ^{1}H n.m.r. spectrum of iodoalanine (22).



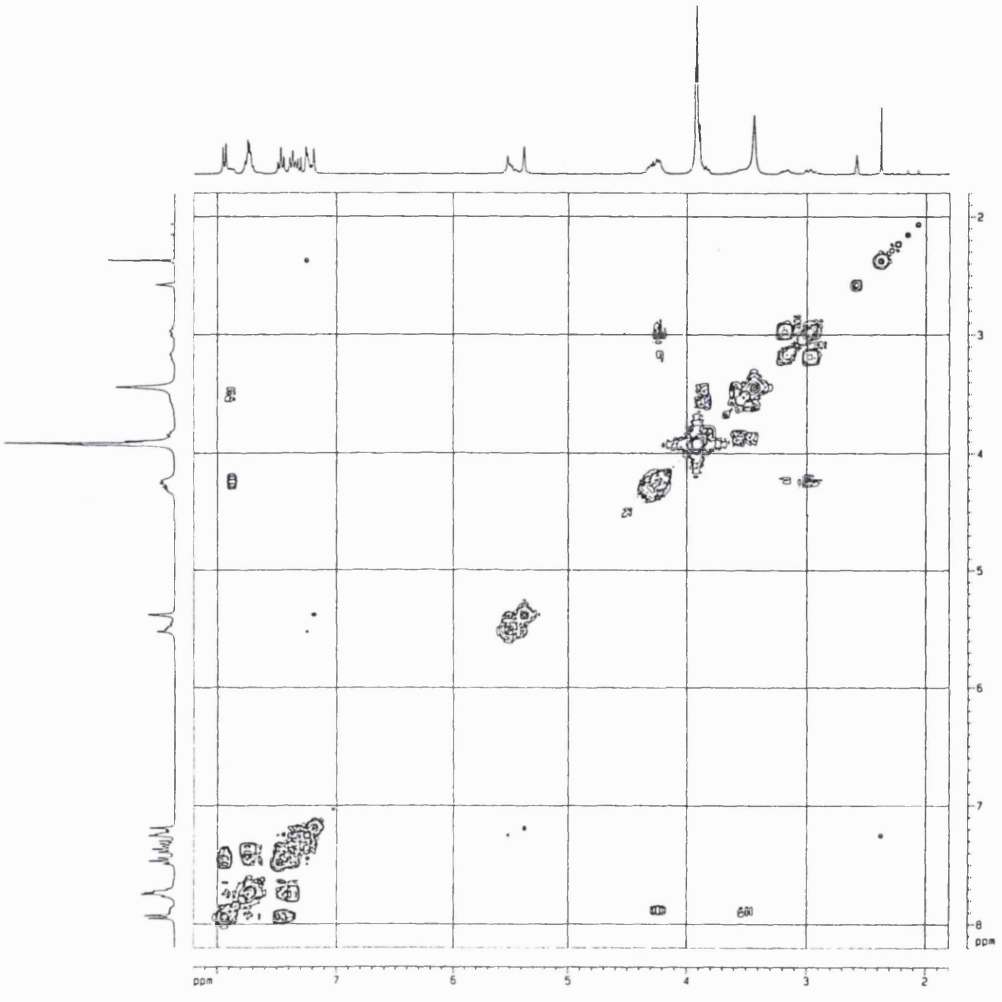
Appendix 2: COSY of iodoalanine (22).



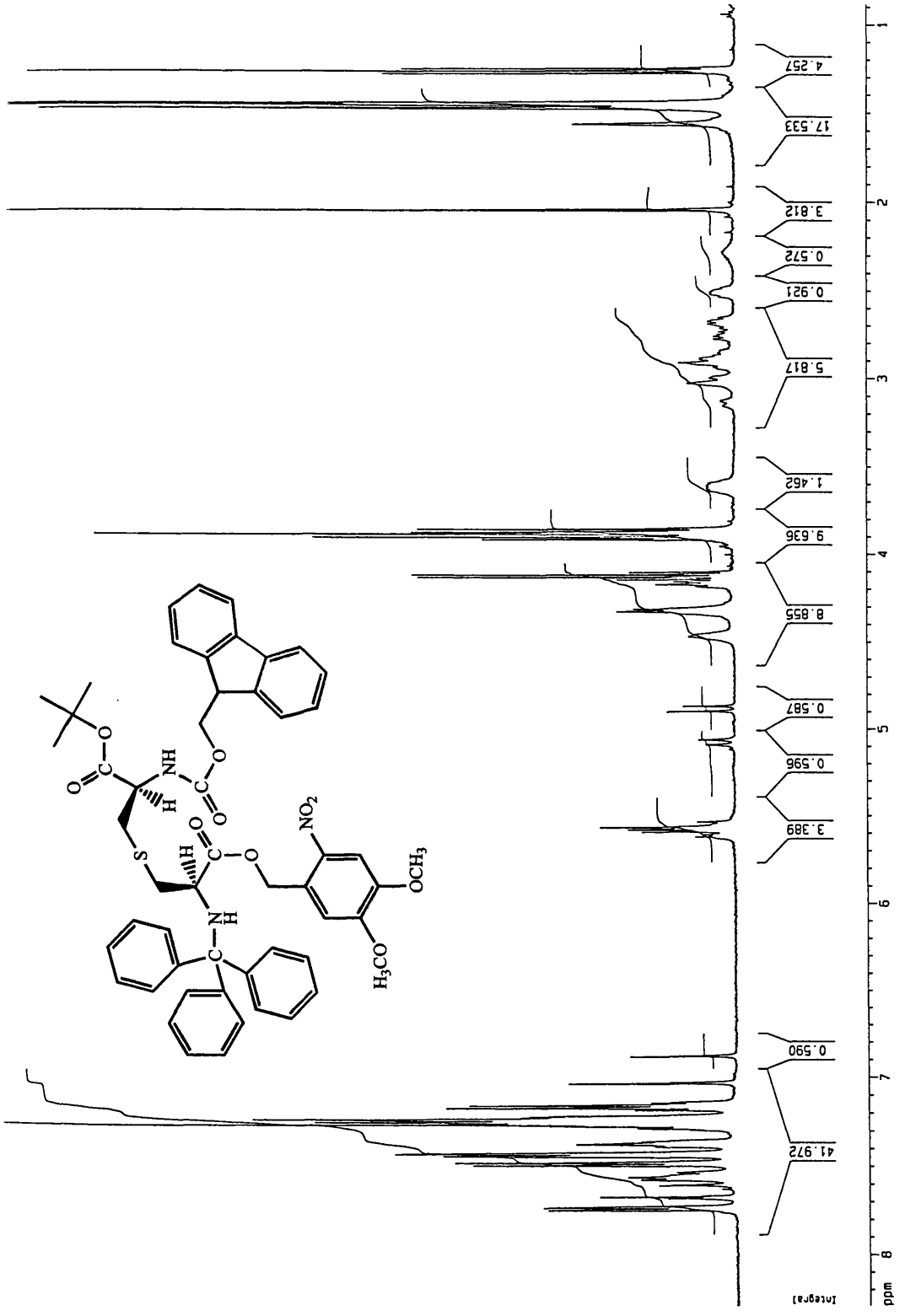
Appendix 3: ^{1}H n.m.r. spectrum of aziridine (23).



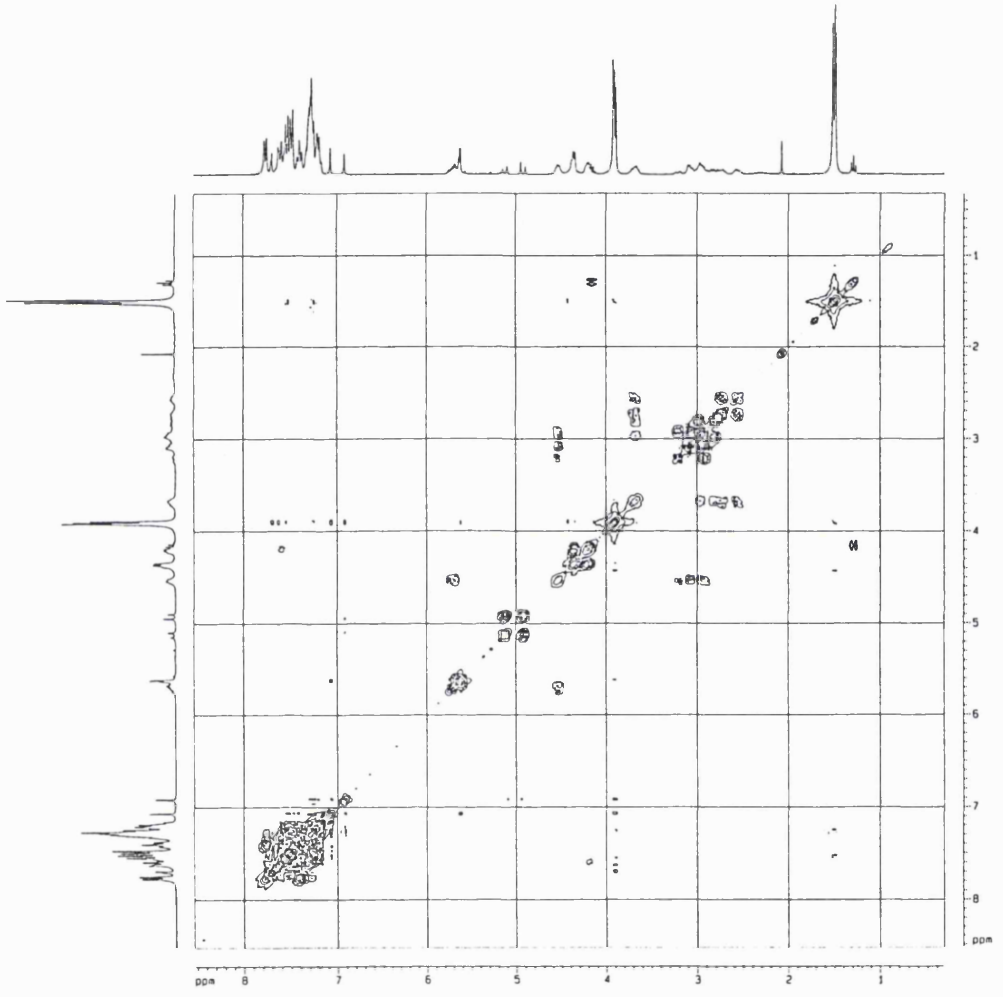
Appendix 4: ^{1}H n.m.r. spectrum of lanthionine (28).



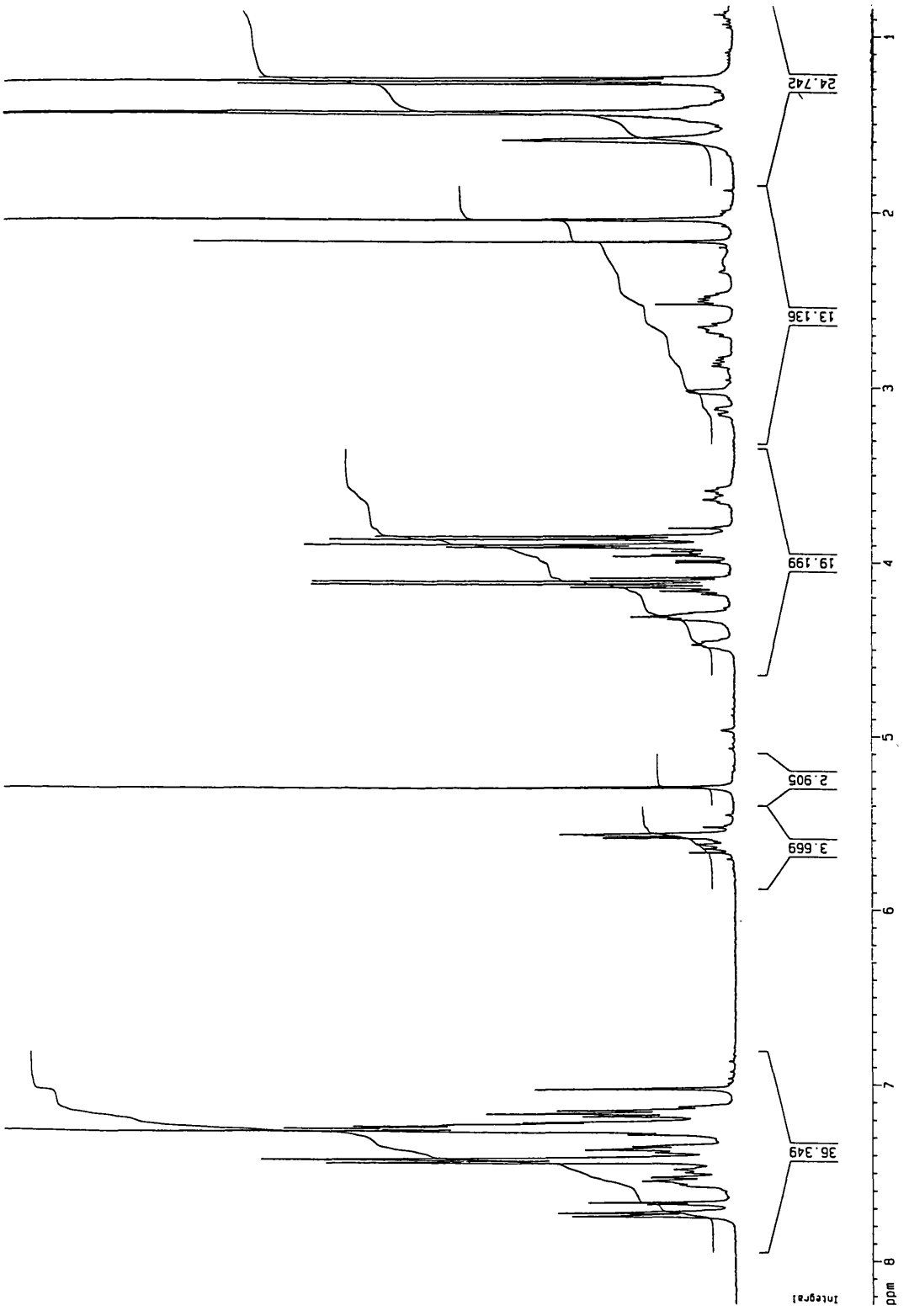
Appendix 5: COSY of lanthionine (28).



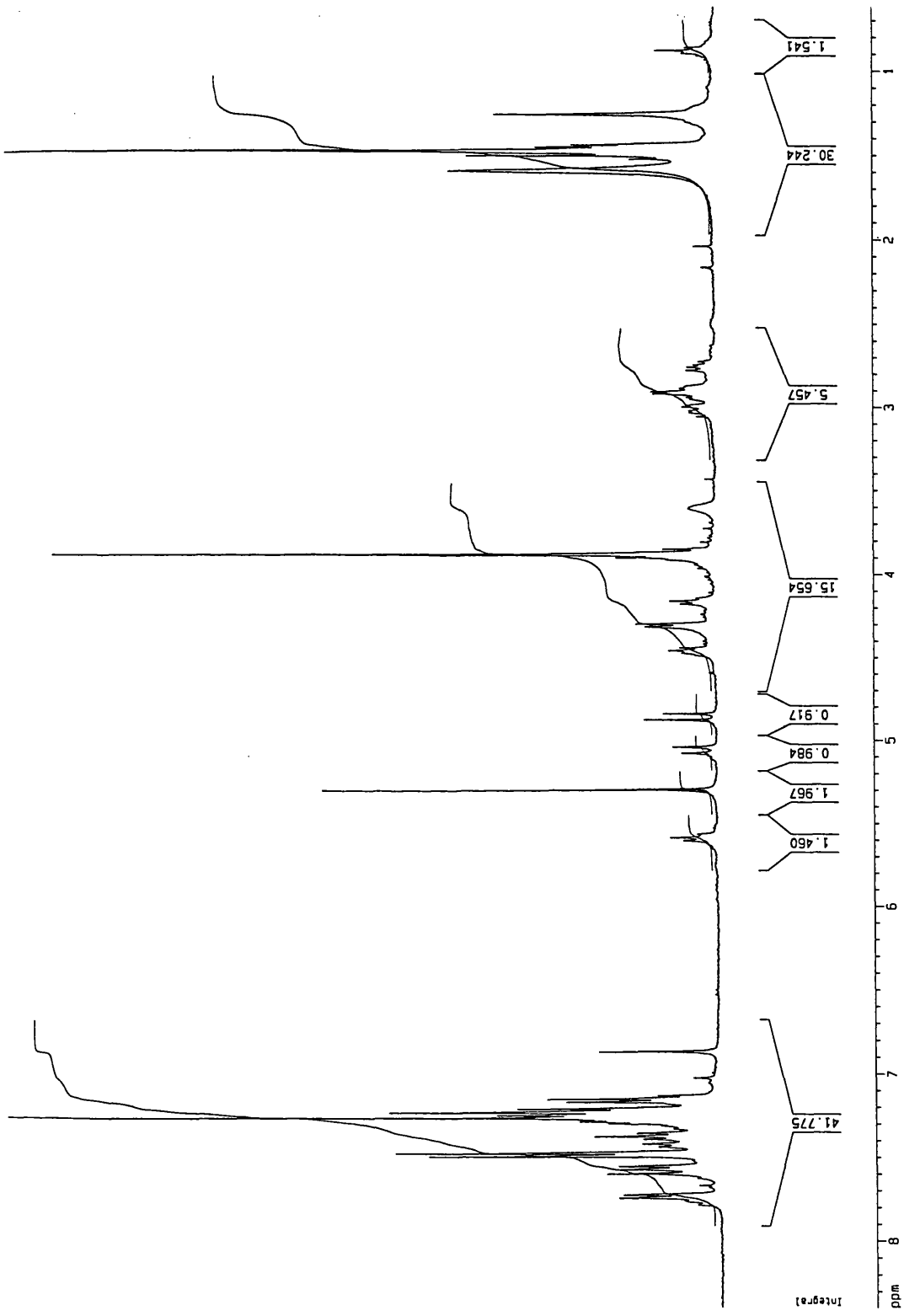
Appendix 6: ^{1}H n.m.r. spectrum of lanthionine (24): mixture of compounds A and B.



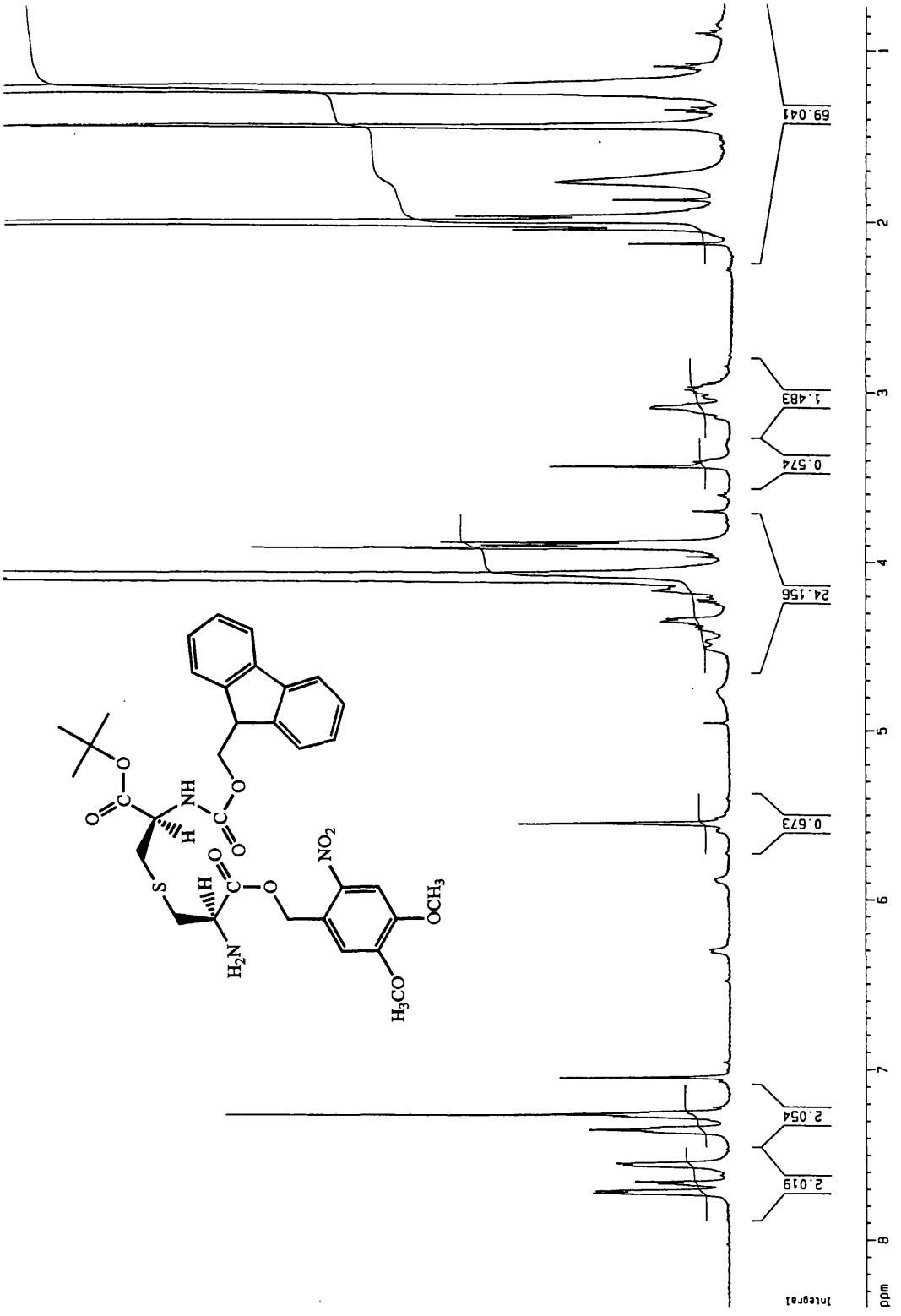
Appendix 7: COSY of lanthionine (24): mixture of compounds A and B.



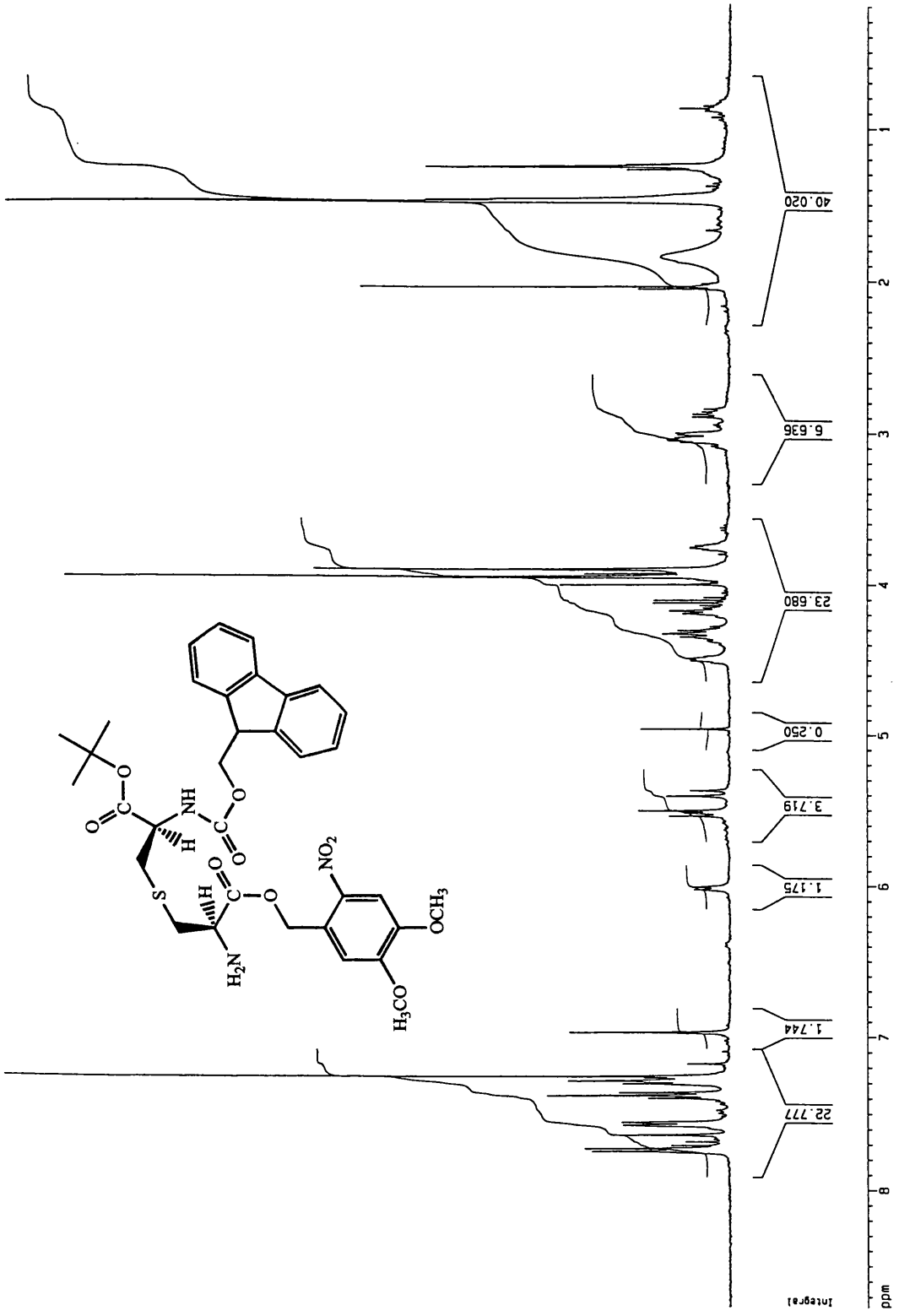
Appendix 8: ^{1}H n.m.r. spectrum of lanthionine (24): compound A.



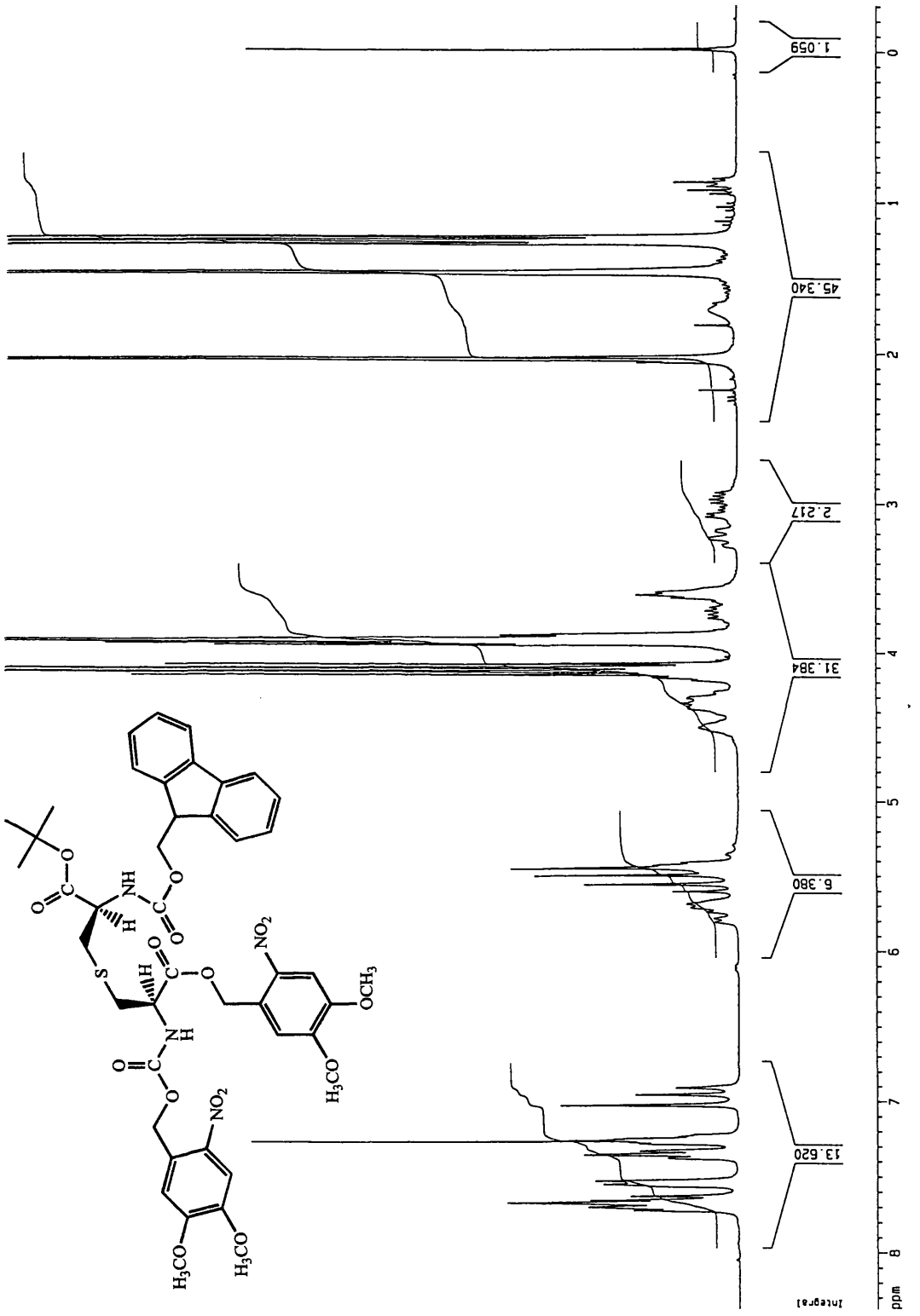
Appendix 9: ^{1}H n.m.r. spectrum of lanthionine (24): compound B.



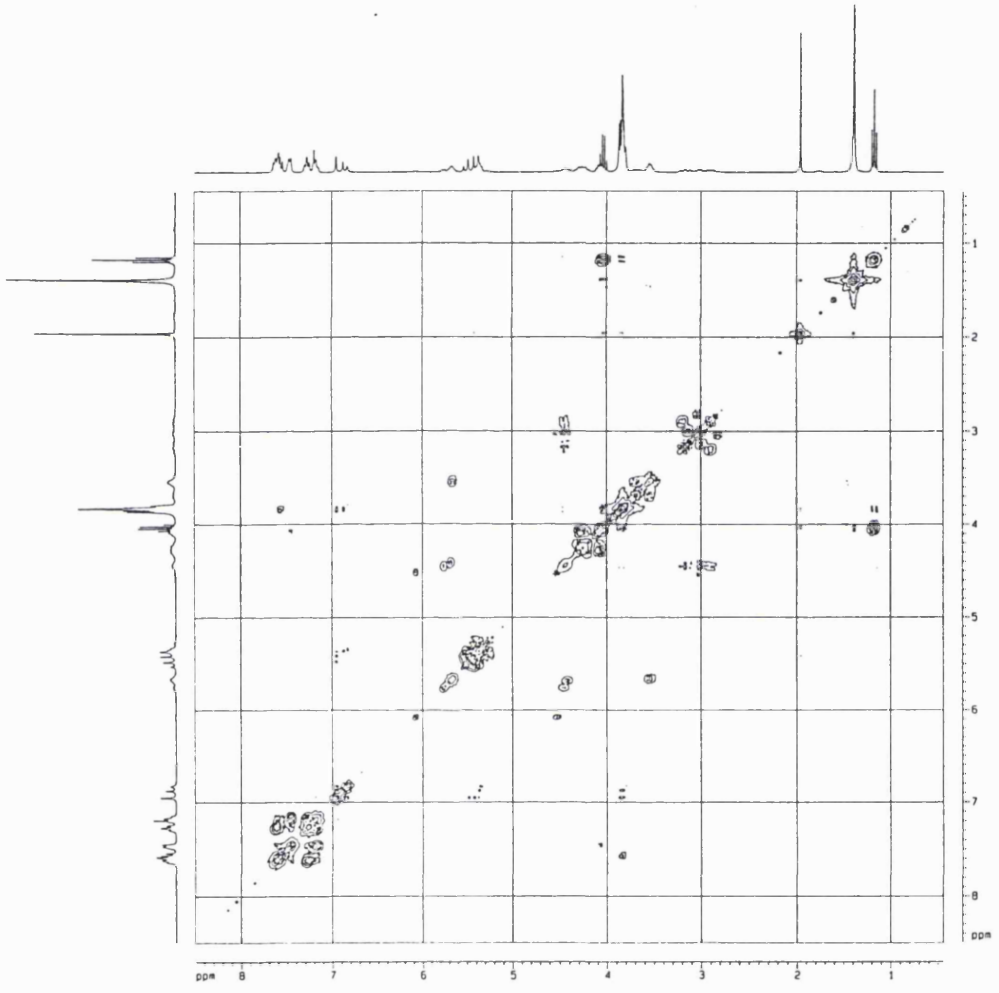
Appendix 10: ^{1}H n.m.r. spectrum of lanthionine (25): compound A.



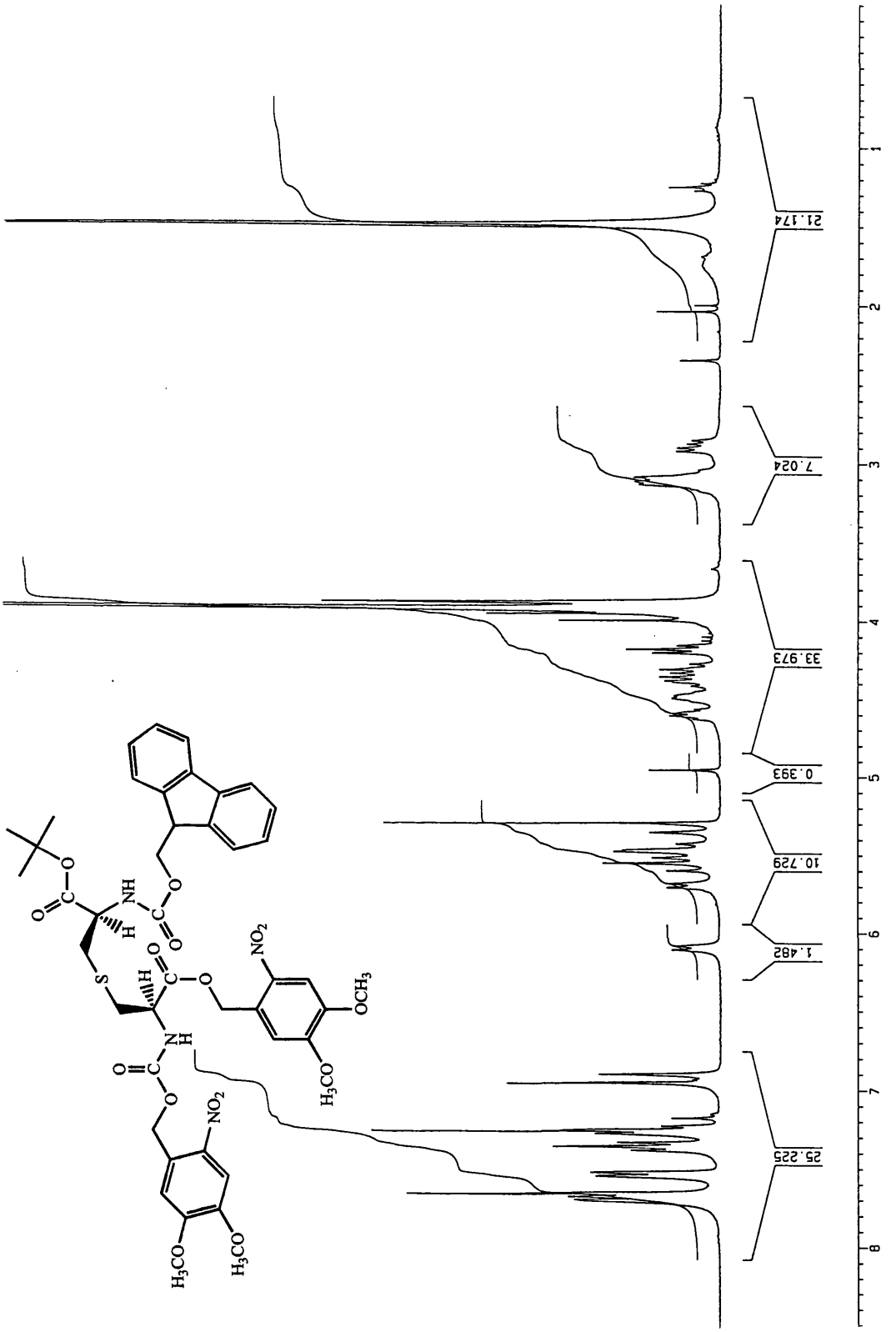
Appendix 11: ^{1}H n.m.r. spectrum of lanthionine (25): compound B.



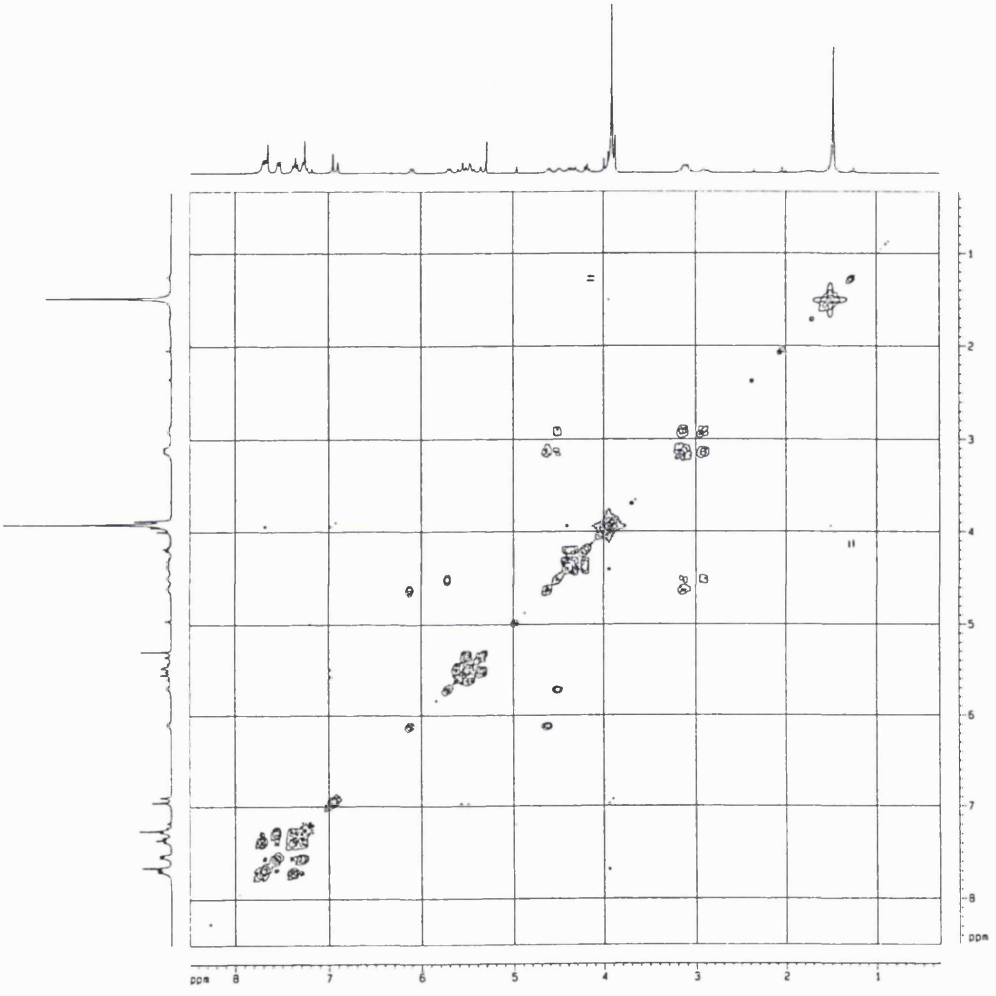
Appendix 12: ^{1}H n.m.r. spectrum of lanthionine (27): compound A.



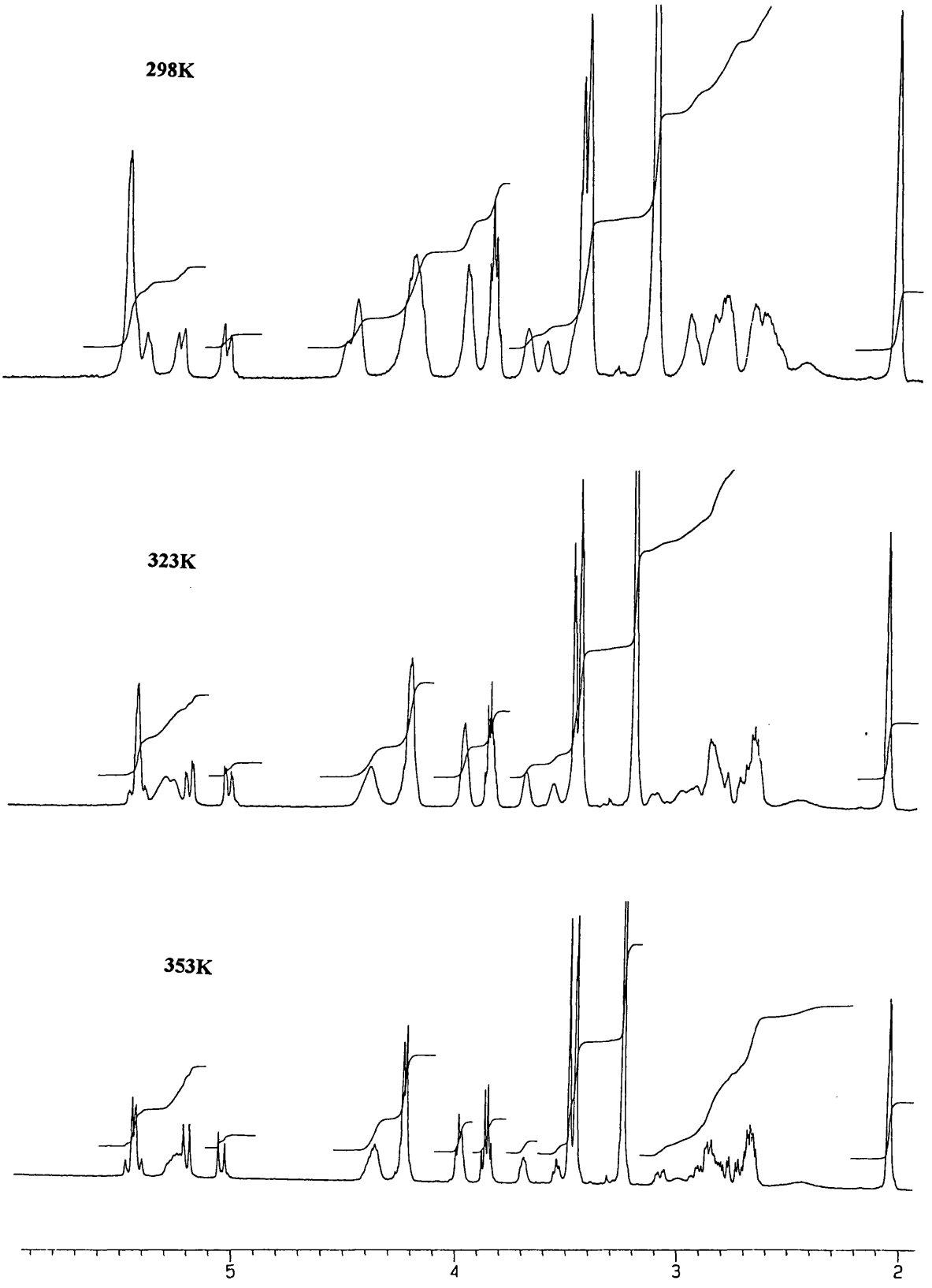
Appendix 13: COSY of lanthionine (27): compound A.



Appendix 14: ^{1}H n.m.r. spectrum of lanthionine (27): compound B.



Appendix 15: COSY of lanthionine (27): compound B.



Appendix 16: VT experiments on lanthionine (24) in toluene at 298K, 323K and 353K.

5. Experimental

Unless specified, all reagents were purchased from commercial suppliers and were used without further purification. For SPPS, all amino acids and resins were purchased from Novabiochem, except Thr(OtBu)-OPfp that was purchased from Bachem. Ether refers to diethyl ether. Brine refers to a saturated aqueous solution of sodium chloride. Ethanol refers to ethanol 96 v/v unless otherwise stated. Petroleum ether refers to petroleum ether 40-60 °C.

Optical rotations were measured on an Optical Activity polAAR 2000 polarimeter.

U.V. spectra were recorded on a computer driven UV Shimadzu 2041 PC spectrometer.

I.R. spectra were recorded on a Perkin-Elmer 1600 series FT-IR, a Nicolet FT-IR 94 and a computer driven FT-IR Shimadzu 8700 spectrometers. They are usually in solution in chloroform unless otherwise stated.

^{1}H n.m.r. and ^{13}C n.m.r. spectra were recorded on a Varian VXR 400, a Bruker AC 300, a Bruker AMX 300, a Bruker AMX 400 and a Bruker AVANCE 500 instruments. The chemical shift data for each signal is given in units of δ relative to tetramethylsilane (TMS) where δ (TMS) = 0. For ^{1}H n.m.r. spectra, the multiplicity of the signals is indicated as : s - singlet, d - doublet, t - triplet, m - multiplet, dd - doublet of doublets, dt - doublet of triplets, etc... For lanthionine residues, the notation -S-C**H**$_2$-CH-tBuFmoc was adopted to designate the half of the molecule bearing the tBu and the Fmoc group, for example.

EI^+, ESP^+, $APCI^+$ and $APCI^-$ mass spectra were recorded using a Micromass Quattro LC. FAB mass spectra (nominal and high resolution) were recorded using a VG ZAB SE mass spectrometer.

CHN analysis was carried out on a Perkin Elmer 2400 Elemental Analyser and S analysis was carried out on a Perkin Elmer 240 Elemental Analyser.

Melting points were determined using a Gallenkamp instrument and are uncorrected.

Normal-phase t.l.c. was carried out on pre-coated 0.25 mm thick Merck 60 F_{254} silica plates. Visualisation was by absorption of U.V. light, or by spraying with basic permanganate solution, or with ninhydrin solution (to reveal the amines). Normal-phase flash chromatography was carried out using silica gel 60 purchased from BDH; the column diameter is given in cm.

Reverse-phase t.l.c. was carried out on Merck RP-F_{254} plates. Visualisation was by absorption of U.V. light. Reverse-phase chromatography was carried out using silanized $silica_{60}$ gel purchased from BDH; the column diameter is given in cm.

THF was dried by distillation from a suspension of THF with sodium and benzophenone. Wet THF refers to commercial THF used without distillation. Dioxane was dried over anhydrous molecular sieves (4 Å) and used immediately afterwards and for SPPS, dioxane was distilled over calcium hydride. DCM was dried by distillation over calcium hydride or phosphorus pentoxide. DMF was dried by distillation over calcium hydride under reduced pressure. Methanol was dried by distillation over magnesium turnings. Acetic anhydride was dried by distillation over calcium hydride. Triethylamine and piperidine were dried by distillation over calcium hydride. Diisopropylethylamine was distilled over potassium hydroxide after previous drying over potassium hydroxide overnight.

Photolabile tests were carried out using two lamps, one broad range U.V. lamp from Applied Photophysics with outer condenser around the reaction vessel and lamp fitting inside the vessel, **lamp 1** (**Figure 81**), one Blak-Ray longwave U.V. lamp emitting at 365 nm, Model B 100 AP from UVP, **lamp 2**. Photolabile cleavages during solid phase peptide synthesis were carried out in the vessel of **lamp 1**, without **lamp 1** inside but with **lamp 2** used as a projector on the reaction vessel. **Lamp 3** was also used. It is similar to **lamp 1** with a sinter and a tap at the bottom of the reaction vessel.

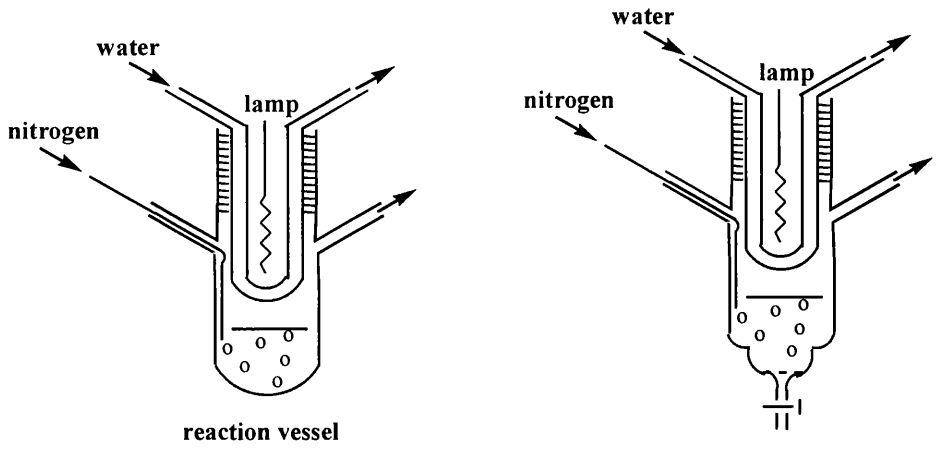


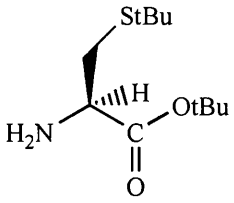
Figure 81: Lamp 1 and lamp 3.

Analytical reverse-phase HPLC was carried out using a Waters 600E quaternary gradient system pump, Rheodyne 7125 valve injector, Gilson 115 variable-wavelength U.V. detector and Hewlett-Packard HP3396A integrator with a Vydac^{TM} column, 4.6 mm x 25 cm. Preparative reverse-phase HPLC was carried out using a Waters 600E quaternary gradient system pump, Rheodyne 7125 valve injector, Gilson Holochrome variable-wavelength U.V. detector and Waters 745B Data Module with a Vydac^{TM} column, 21.4 mm x 25 cm. LC-MS reverse-phase and LC-MS normal phase were carried out using a Hewlett-Packard HP1100 for HPLC and Micromass Quattro LC for mass spectrometry. Solvents used were of HPLC grade; for reverse-phase : A : H_2O (0.1 % TFA) ; B : CH_3CN (0.1 % TFA), for normal phase : A' : hexane; B' : ethyl acetate. Retention times are given in min.

The names of all new compounds are indicated in italics. Literature compound names are given in normal text.

1. Synthesis of protected cysteine and cystine residues

1. 1. (*R*)-*S*-*tert*-Butylcysteine *tert*-butyl ester (1)



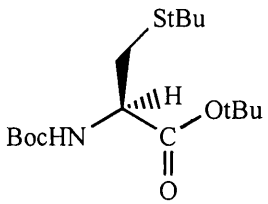
tert-Butyl acetate (75 ml, 557 mmol) and sulphuric acid (98 %, 0.6 ml, 5.5 mmol) were added to (*R*)-cysteine (605 mg, 5 mmol). A precipitate was formed immediately and persisted for 15 min. The reaction mixture was stirred at room temperature for 70 hr. The reaction mixture was cooled to 0°C and sodium hydroxide solution (2M) was then added to the mixture to adjust the pH to 11. The product was extracted with ethyl acetate (2 x 100 ml), the organic layer was dried over sodium sulphate and the solvent was removed *in vacuo* to give an oil. Compound **(1)** was recovered (720 mg, 62 %).

R_F: 0.35 (methanol: chloroform, 2: 1), visualisation by spraying with ninhydrin solution

^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 1.30 (9H, s, -SC(CH_3)$_3$) 1.45 (9H, s, -COOC(CH_3)$_3$) 2.73 (1H, dd, -C**H**$_2$, *J* 7.5, 0.8 Hz) 2.87 (1H, dd, -C**H**$_2$, *J* 3.7, 0.8 Hz) 3.51 (1H, m , -C**H**-)

mass spectrum (FAB) : m/z 178 (M^+ - tBu + 2H) 100 %, 234 (M^+ + H) 82 %

1. 2. (*R*)-*N*-*tert*-Butyloxycarbonyl-*S*-*tert*-butylcysteine *tert*-butyl ester (2)



(*R*)-*S*-*tert*-Butylcysteine *tert*-butyl ester **(1)** (720 mg, 3.1 mmol) was dissolved in a solution of sodium hydroxide (1M, 4.5 ml, 4.5 mmol, 1.4 eq.) and THF (3 ml). Di-*tert*-butyl dicarbonate (887 mg, 4.1 mmol, 1.3 eq.) was then added portionwise and the

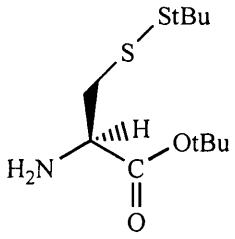
reaction mixture was stirred overnight at room temperature. The THF was removed *in vacuo* and the mixture dissolved in distilled water (50 ml). Extraction of the product was carried out with dichloromethane (3 x 50 ml). The organic layer was dried over sodium sulphate and the solvents removed *in vacuo* to give an oil. Purification was carried out by flash column chromatography (3 cm, hexane: ethyl acetate, 85: 15) (823 mg, 80 %).

R_F: 0.42 (hexane: ethyl acetate, 85: 15), visualisation by spraying with basic permanganate solution

^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 1.28 (9H, s, -SC(CH_3)$_3$) 1.41 (9H, s, -NH-COOC(CH_3)$_3$) 1.45 (9H, s, -COOC(CH_3)$_3$) 2.90 (1H, dd, -S-CH_2-, *J* 12.6, 4.5 Hz) 2.96 (1H, dd, -S-CH_2-, *J* 12.6, 4.5 Hz) 4.42 (1H, m , -C**H**-) 5.28-5.30 (1H, broad s, -N**H**-).

mass spectrum (FAB): m/z 166 (M^+ - 3tBu + 4H) 86 %, 222 (M^+ - 2tBu + 3H) 100 %, 278 (M^+- tBu + 2H) 16 %, 334 (M^+ + H) 11 %, 356 (M^+ + Na) 9 %

1. 3. (*R*)-*S*-*tert*-Butylthiocysteine *tert*-butyl ester (11)



(*R*)-*S*-*tert*-Butylthiocysteine hydrate (1 g, 4.8 mmol) was suspended in *tert*-butyl acetate (72 ml) and concentrated sulphuric acid (0.4 ml, 1.1 eq.) was added to this suspension, forming white clusters. Stirring of the reaction mixture for 15 min led to a clearer solution which was left at room temperature for 4 days. The clear reaction mixture was then cooled to 0°C and extracted with pre-cooled HCl (0.5 M, 4 x 15 ml). The aqueous solution was neutralised immediately to pH 7 with solid sodium hydrogen carbonate in a beaker. An ether layer (100 ml) was kept on top of the aqueous layer during neutralisation. The aqueous layer was then extracted with ether (1 x 100 ml, then 4 x 50 ml). The organic layer was washed with brine and dried over sodium sulphate. The solvent was removed *in vacuo* to give a clear oil. Purification was carried out by flash

column chromatography (5 cm, hexane then gradient up to neat ethyl acetate). The title compound was obtained as white crystals (1.05 g, 83 %).

R_F : 0. 40 (chloroform: methanol, 2: 1), visualisation by spraying with ninhydrin solution

^{1}H n.m.r. : δ ($CDCl_3$, 400 MHz) 1.32 (9H, s, -SC(C**H**$_3$)$_3$) 1.45 (9H, s, -COOC(C**H**$_3$)$_3$) 2.90 (1H, dd, -C**H**$_2$, *J* 13.2, 7.2 Hz)) 3.09 (1H, dd, -C**H**$_2$, *J* 13.2, 4.3 Hz) 3.72 (1H, dd, -C**H**-, *J* 4.3, 7.2 Hz) 5.43 (2H, broad s, -N**H**$_2$)

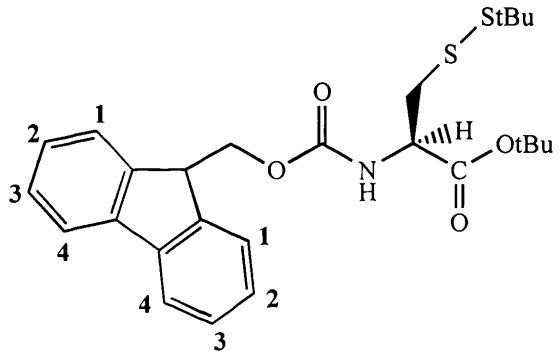
^{13}C n.m.r. : δ ($CDCl_3$, 100 MHz) 27.9, 29.7, 29.8, 44.8, 48.2, 54.0, 82.1, 175.8

mass spectrum (FAB) : m/z 210 (M^+ - tBu + 2H) 100 %, 266 (M^+ + H) 60 %

Found: (M^+ + H), 266.1248. $C_{11}H_{23}NO_2S_2$ requires 266.1232 (FAB)

Melting point: 53-57°C

1. 4. (*R*)-*S*-*tert*-Butylthio-*N*-(9-fluorenylmethyloxycarbonyl)cysteine *tert*-butyl ester (12)



(*R*)-*S*-*tert*-Butylthiocysteine *tert*-butyl ester **(11)** (454 mg, 1.71 mmol) was dissolved in a mixture of aqueous sodium carbonate solution (10 %, w/v, 7 ml) and dioxane (4 ml). This mixture was cooled to 0°C and FmocCl (580 mg, 1.3 eq.) dissolved in dioxane (4 ml) was added dropwise over 5 min. The reaction was stirred for 1hr. The reaction mixture was poured into distilled water (50 ml) and ether (50 ml). Solid citric acid was then added to the basic mixture (pH > 8) at 0°C to adjust the pH to 7. Extraction of the organic product was carried out with ether (5 x 50 ml). After washing with a small amount of water (5 ml), the ether layer was dried over sodium sulphate. Removal of the solvents *in vacuo* gave the title compound **(12)** as an oil (800 mg). Purification was

carried out by flash column chromatography (5 cm, hexane: ethyl acetate, 5: 1) and gave the title compound as a clear pale yellow oil (651 mg, 1.34 mmol, 78 %).

The reaction was also carried out in THF in the same proportions. The yields obtained were similar.

R_F : 0.45 (hexane: ethyl acetate, 4: 1), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

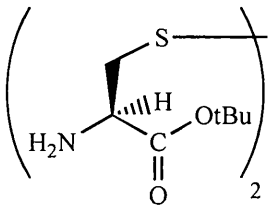
^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 1.31 (9H, s, -SC(C**H**$_3$)$_3$) 1.49 (9H, s, -COOC(C**H**$_3$)$_3$) 3.13 (1H, dd, -C**H**$_2$S, *J* 13.4, 5.3 Hz) 3.25 (1H, dd, -C**H**$_2$S, *J* 13.4, 4.2 Hz) 4.23 (1H, t, -C**H**CH$_2$OCO-, *J* 7.1 Hz) 4.35 (2H, m, -C**H**$_2$OCO) 4.57 (1H, m, -C**H**CH$_2$S-) 5.66 (1H, d, -N**H**-, *J* 7.4 Hz) 7.30 (2H, t, Ar**H 2**, *J* 6.9 Hz) 7.38 (2H, t, Ar**H 3**, *J* 7.6 Hz) 7.61 (2H, d, Ar**H 1**, *J* 7.5 Hz) 7.75 (2H, d, Ar**H 4**, *J* 7.5 Hz)

^{13}C n.m.r. : δ ($CDCl_3$, 100 MHz) 28.0, 29.8, 43.3, 47.1, 48.2, 54.4, 67.2, 83.0, 119.9, 125.2, 127.05, 127.7, 141.2, 143.8, 155.5, 169.3

mass spectrum (FAB): m/z 375 (M^+ - 2 tBu + 2H) 71 %, 432 (M^+ -tBu + 2H) 100 %, 488 (M^+ + H) 8 %, 510 (M^+ + Na) 10 %

Found: (M^+ + Na), 510.1749. $C_{26}H_{33}NO_4S_2Na$ requires 510.1764 (FAB)

1. 5. (*R, R*)-Cystine bis-*tert*-butyl ester (14)



(*R, R*)-Cystine (3.11 g, 13.0 mmol) was suspended in perchloric acid (70-72 %, 4.7 ml, 4.3 eq.) and *tert*-butyl acetate (75 ml) was added to the slurry. After 30 min, a clear solution was observed. After 2 hr, a white precipitate appeared in the solution. The reaction mixture was stirred for 3 days at room temperature.

The mixture was cooled to 0°C then basified with an aqueous sodium hydroxide solution (3 M, about 300 ml) until pH = 9-10. A first extraction was carried out with ether (3 x

100 ml) then ethyl acetate (3 x 100 ml). The aqueous solution was readjusted to pH 10 by cautious addition of aqueous sodium hydroxide (3M). A second extraction was then carried out with ether (2 x 100 ml) then ethyl acetate (2 x 100 ml). The combined organic layers were dried over sodium sulphate and removal of the solvents *in vacuo* gave a thick oil which was dried *in high vacuo*. A thick pale yellow slurry was recovered as the title compound (4.41 g, 96 %). This compound is fairly unstable, it turns yellow after a few days as it starts degrading. It was therefore used as quickly as possible in the next step.

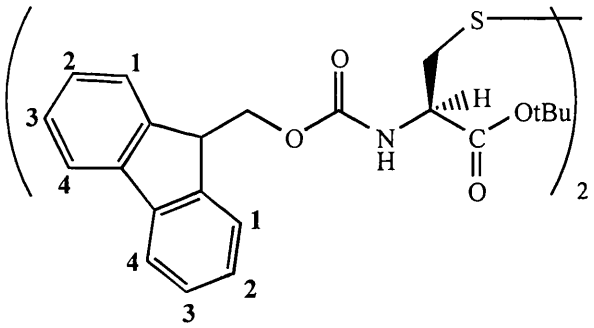
^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 1.42 (18H, m, -COOC(C**H**$_3$)$_3$) 2.85 (2H, dd, -C**H**$_2$S-, *J* 13.4, 7.8 Hz) 3.10 (2H, dd, -C**H**$_2$S-, *J* 13.4, 4.5 Hz) 3.66 (2H, dd, -C**H**CH$_2$S-, *J* 7.8, 4.4 Hz)

^{13}C n.m.r.: δ ($CDCl_3$, 100 MHz) 28.0, 43.9, 54.2, 81.9, 172.7

mass spectrum (APCI$^+$): m/z 297 (M^+ - tBu + 2H) 40 %, 353 (M^+ + H) 100 %

Found: (M^+ + H), 353.1569. $C_{14}H_{29}N_2O_4S_2$ requires 353.1563 (FAB)

1. 6. (*R, R*)-*N, N'*-bis-(9-Fluorenylmethyloxycarbonyl)cystine bis-*tert*-butyl ester (15)



(*R, R*)-Cystine bis-*tert*-butyl ester **(14)** (4.19 g, 11.9 mmol) was dissolved in dry THF (15 ml). *N*-Methylmorpholine (2.7 ml, 2.06 eq.) was added dropwise to the solution at 0°C. FmocCl (6.34 g, 2.06 eq.) dissolved in dry THF (15 ml) was added to the mixture over 5 min at 0°C. The reaction mixture was then stirred for 4 hr at room temperature. After concentration of the reaction mixture *in vacuo*, the residue was dissolved in ethyl acetate (60 ml) and an aqueous solution of potassium hydrogen sulphate (5 %, w/v, 60

ml). The organic layer was washed with an aqueous solution of potassium hydrogen sulphate (5 %, w/v, 3 x 30 ml) and water (3 x 30 ml). The aqueous layers were gathered and extracted with ethyl acetate (2 x 50 ml). The combined organic layers were dried over sodium sulphate and the solvents removed *in vacuo* to give a pale yellow solid. Recrystallisation in dichloromethane: methanol (1: 4) at -20°C gave the title compound as a white solid (7.21 g, 76 %).

Purification could also be carried out by flash column chromatography. For instance for 486 mg of starting material **(14)**, flash column chromatography (5 cm, hexane: ethyl acetate, 9: 1, gradient) gave the title compound **(15)** as a white solid (906 mg, 82 %).

R_F: 0.17 (hexane: ethyl acetate, 4: 1), visualisation by U.V. light and by spraying with basic permanganate solution

$[\alpha]_D$ -5.5° (15°C, c 41.09 mg/ml, $CHCl_3$) (lit.[174] $[\alpha]_D$ -6° (20°C, c 2 g/100ml, $CHCl_3$))

Found: C, 65.9; H, 6.0; N, 3.4; S, 8.0. $C_{44}H_{48}N_2O_8S_2$ requires C, 66.3; H, 6.1; N, 3.5; S, 8.0

ν_{max} ($CHCl_3$) 3427 (CONH), 3014 (CH), 1713 (C=O), 1507 (aromatic)

^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 1.49 (18H, s, -COOC(C**H**$_3$)$_3$) 3.21 (4H, m, -C**H**$_2$S-) 4.20 (2H, m, - C**H**CH$_2$OCO-) 4.37 (4H, m, -C**H**$_2$OCO) 4.59 (2H, m, -C**H**CH$_2$S-) 5.76 (2H, d, -N**H**-, *J* 5.7 Hz) 7.28 (4H, m, Ar**H 2**) 7.38 (4H, m, Ar**H 3**) 7.59 (4H, d, Ar**H 1**, *J* 7.4 Hz) 7.75 (4H, d, Ar**H 4**, *J* 7.4 Hz)

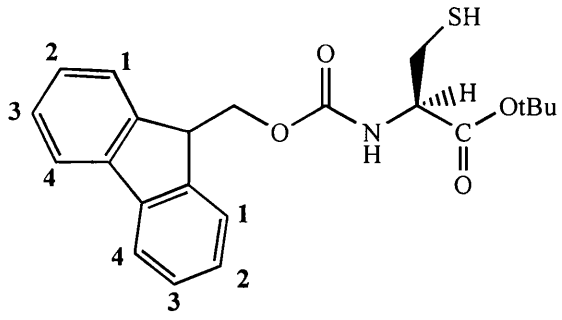
^{13}C n.m.r.: δ ($CDCl_3$, 100 MHz) 28.0, 41.9, 47.1, 54.1, 67.2, 83.1, 120.0, 125.2, 127.1, 127.7, 141.3, 143.7, 155.7, 169.3

mass spectrum (APCI$^+$): m/z 685 (M^+ - 2tBu + 3H) 25 %, 741 (M^+ - tBu + 2H) 24 %, 797 (M^+ + H) 100 %

Found: (M^+ + Na), 819.2750. $C_{44}H_{48}N_2O_8S_2Na$ requires 819.2780 (FAB)

Melting point: 141-144°C

1. 7. (*R*)-*N*-(9-Fluorenylmethyloxycarbonyl)cysteine *tert*-butyl ester (13)



1. 7. 1. From cysteine residues

(*R*)-*S*-*tert*-Butylthio-*N*-(9-fluorenylmethyloxycarbonyl)cysteine *tert*-butyl ester **(12)** (650 mg, 1.33 mmol) was dissolved in wet THF (10 ml) and tributylphosphine (0.35 ml, 1.33 mmol, 1 eq.) was added to the solution which was stirred at room temperature for 21 hr.

The reaction mixture was poured into ethyl acetate (50 ml) and the organic layer was washed with a citric acid solution (10 %, w/v, 40 ml) and brine (10 ml). After slow evaporation of the solvents in the fume cupboard a thick crude oil was obtained. Purification was carried out by flash column chromatography (5 cm, hexane: ethyl acetate, 5: 1). The title compound was recovered as a clear thick oil (218 mg, 41 %).

1. 7. 2. From cystine residues

(*R*, *R*)-*N,N'*-bis-(9-Fluorenylmethyloxycarbonyl)cystine bis-*tert*-butyl ester **(15)** (2.07 g, 2.6 mmol) was dissolved in THF (15 ml) and treated with tributylphosphine (0.64 ml, 2.6 mmol, 1 eq.). After 30 min, water (4 ml, about 80 eq.) was added to the solution. The reaction mixture was stirred for 4 hr at room temperature.

After removal of the solvents *in vacuo*, the residue was dissolved in ethyl acetate (200 ml) and the organic layer was washed with an aqueous solution of citric acid (10 %, w/v, 3 x 50 ml), with brine (2 x 20 ml) and with water (3 x 20 ml). The combined aqueous layers were extracted once with ethyl acetate (50 ml). Drying of the combined organic

layers over sodium sulphate followed by removal of the solvents *in vacuo* gave a pale yellow oil.

Purification by flash column chromatography (5 cm, hexane: ethyl acetate, 3: 1, gradient) gave the title compound as a clear oil which was dried on the freeze drier giving a white solid (1.89 g, 91 %).

R_F : 0.32 (hexane: ethyl acetate, 4: 1), visualisation by U.V. light and by spraying with basic permanganate solution

$[\alpha]_D$ +11.0° (26°C, c 101.75 mg/ml, $CHCl_3$)

Found: C, 66.1; H, 6.4; N, 3.4; S, 8.1. $C_{22}H_{25}NO_4S$ requires C, 66.1; H, 6.3; N, 3.5; S, 8.0

ν_{max} ($CHCl_3$) 3425 (CONH), 2983 (CH stretching), 1721 (C=O), 1505 (aromatic)

^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 1.48 (9H, s, -COOC(C**H$_3$**)$_3$) 2.97 (2H, m, -C**H$_2$**SH) 4.23 (1H, t, - C**H**CH$_2$OCO-, *J* 7.0 Hz) 4.39 (2H, m, -C**H$_2$**OCO) 4.53 (1H, m, -C**H**CH$_2$S-) 5.69 (1H, d, -N**H**-, *J* 6.8 Hz) 7.30 (2H, t, Ar**H 2**, *J* 7.4 Hz) 7.39 (2H, t, Ar**H 3**, *J* 7.4 Hz) 7.59 (2H, d, Ar**H 1**, *J* 7.2 Hz) 7.75 (2H, d, Ar**H 4**, *J* 7.5 Hz)

^{13}C n.m.r.: δ ($CDCl_3$, 75.47 MHz) 28.4, 47.6, 53.9, 55.9, 67.5, 83.5, 120.4, 125.5, 127.5, 128.2, 141.7, 144.1, 156.1, 169.4

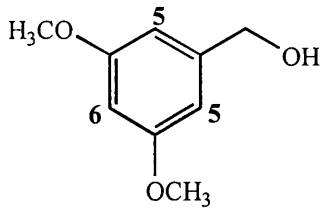
mass spectrum (EI^+) : m/z 344 (M^+ - tBu + 2H), 100 % 399 (M^+) 74 %

Found: (M^+ + H), 400.1583. $C_{22}H_{26}NO_4S$ requires 400.1572 (FAB)

Melting point: 63-67°C

2. Steps towards (*S*)-*N*-3,5-dimethoxybenzyloxycarbonylserine

2. 1. 3,5-Dimethoxybenzylalcohol (3)



2. 1. 1. Reduction with lithium aluminium hydride

Lithium aluminium hydride (270 mg, 7.1 mmol, 2 eq.) was suspended in dry THF (10 ml) under nitrogen. 3,5-Dimethoxybenzoic acid (647 mg, 3.55 mmol) dissolved in dry THF (10 ml) was added to the suspension and the reaction mixture was heated under reflux for 18 hr under nitrogen. Quenching of the cooled mixture was carried out by the dropwise addition of an aqueous sodium hydroxide solution (2M, 5 ml) followed by distilled water (4 ml) at 0°C. After removal of THF *in vacuo*, the mixture was extracted with ethyl acetate (2 x 50 ml). The organic layer was dried with sodium sulphate, followed by removal of the solvents *in vacuo*. The crude oil was recrystallised from ether: hexane (1: 1) at 0°C to give white crystals (92 mg, 43 %).

2. 1. 2. Reduction with sodium borohydride and iodine

3,5-Dimethoxybenzoic acid (1 g, 5.5 mmol) was dissolved in dry THF (40 ml) and sodium borohydride (522 mg, 13.7 mmol, 2.5 eq.) was added to the solution. Iodine (1.4 g, 5.5 mmol, 1 eq.) dissolved in dry THF (10 ml) was added to the mixture under nitrogen. The reaction mixture was heated under reflux overnight under nitrogen. It turned white from colourless after 30 min. Quenching of the cooled mixture was carried out with drops of methanol (10 ml) at 0°C. After removal of the solvents *in vacuo*, the crude residue was dissolved in ethyl acetate (50 ml) and water (50 ml). The mixture was extracted with ethyl acetate (2 x 50 ml). Drying of the organic layer with sodium sulphate

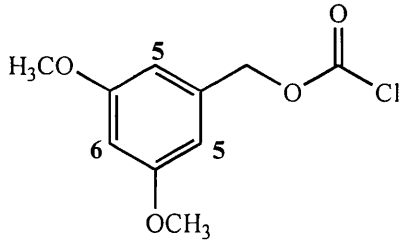
was followed by removal of the solvents *in vacuo*. The crude oil was dissolved in a minimum of ether and hexane at 0°C to give white crystals (810 mg, 88 %).

R_F: 0.56 (ethyl acetate + drops of formic acid), visualisation by spraying with basic permanganate solution

^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 3.78 (6H, s, -OC**H$_3$**) 4.62 (2H, s, ArC**H$_2$**OH) 6.35 (1H, t, Ar **H 6**, *J* 4.5 Hz) 6.50 (2H, d, Ar**H 5**, *J* 2.3 Hz)

mass spectrum (EI^+): m/z 139 $(C_6H_3(OCH_3)_2)^+$ + 2H) 65 %, 151 $(C_6H_3(OCH_3)_2CH_2^+)$ 37 %, 168 (M^+) 100 %

2. 2. 3,5-Dimethoxybenzyl chloroformate (4)



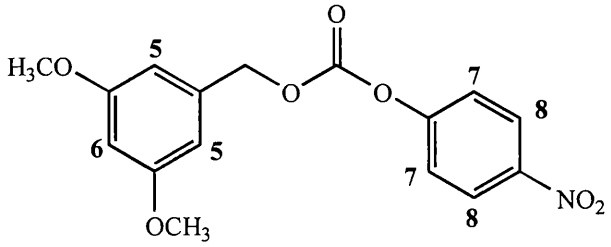
A solution of phosgene in toluene (6 ml, 1.93 M, 11.6 mmol, 3.1 eq.) was added to a solution of 3,5-dimethoxybenzyl alcohol **(3)** (623 mg, 3.7 mmol) dissolved in dry dioxane (20 ml) under argon. The reaction mixture was stirred under argon for 48 hr at room temperature. The excess of phosgene was removed from the reaction mixture by a flow of nitrogen and neutralised in a solution of concentrated ammonia, forming ammonium chloride and white fumes of hydrochloric acid. When the white fumes had disappeared, the solvents were removed *in vacuo* and the white solid obtained was used immediately for the next step without further purification as it is unstable.

R_F: 0.61 (dichloromethane), visualisation by spraying with basic permanganate solution

^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 3.78 (6H, s, -OC**H$_3$**) 5.20 (2H, s, ArC**H$_2$**O-) 6.45 (1H, m, Ar**H 6**) 6.50 (2H, d, Ar**H 5**, *J* 2.2 Hz)

No other analyses could be carried out as the product is unstable.

2. 3. 3,5-Dimethoxybenzyl-*p*-nitrophenyl carbonate (6)



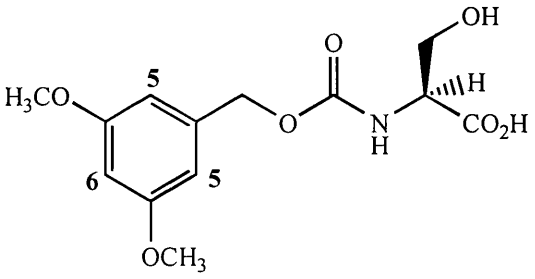
3,5-Dimethoxybenzyl alcohol **(3)** (280 mg, 1.7 mmol) was dissolved in acetone (5 ml) and pyridine (1.3 ml, 1.5 mmol, 0.9 eq.) at 0°C. *p*-Nitrophenyl chloroformate (304 mg, 1.5 mmol, 0.9 eq.) was added portionwise to the cooled mixture which was then stirred for 90 min. The reaction mixture was then poured into distilled water (7 ml), which led to the formation of a pale yellow precipitate which was collected by filtration and washed with distilled water. The crude product (330 mg, 33 %) was used immediately in the next step. ^{1}H n.m.r. analysis showed that 50 mol % of *p*-nitrophenol was mixed with the title compound.

R_F: 0.85 (methanol: chloroform, 1: 1), visualisation by spraying with basic permanganate solution

^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 3.81 (6H, s, -OC**H$_3$**) 5.23 (2H, s, ArC**H$_2$**O-) 6.47 (1H, t, Ar**H 6**, *J* 2.3 Hz) 6.57 (2H, d, Ar**H 5**, *J* 2.2 Hz) 7.39 (2H, dt, Ar**H 7**, *J* 9.4, 2.2 Hz) 7.50 (2H, dt, Ar**H 7** from *p*-nitrophenol, *J* 9.1, 2.3 Hz) 8.28 (2H, dt, Ar**H 8**, *J* 9.4, 2.2 Hz) 8.35 (2H, dt, Ar**H 8** from *p*-nitrophenol, *J* 9.1, 2.3 Hz).

mass spectrum (EI^+): m/z 151 ($C_6H_3(OCH_3)_2CH_2^+$), 100 % 333 (M^+) 29 %

2. 4. (*S*)-*N*-3,5-Dimethoxybenzyloxycarbonylserine (5)



2. 4. 1. Attempted Schotten-Baumann reaction using chloroformate (4)

Crude 3,5-dimethoxybenzyl chloroformate **(4)** was used immediately without purification. As an excess of reagents is not a problem in this reaction (excess of serine goes into the aqueous layer), amounts of reagents were calculated assuming the starting material was pure.

3,5-Dimethoxybenzyl chloroformate **(4)** (th. 3.7 mmol, 1.5 eq.) and (*S*)-serine (260 mg, 2.5 mmol) were dissolved in a mixture of THF (10 ml) and an aqueous sodium hydroxide solution (4 M, 7 ml). The reaction was carried out at 0°C and stirred overnight at room temperature. After removal of the organic solvent *in vacuo*, the aqueous mixture was adjusted to pH 1 with concentrated hydrochloric acid. The reaction mixture was then extracted with ethyl acetate (2 x 200 ml), followed by drying of the organic layer over sodium sulphate and removal of the solvents *in vacuo*.

The reaction was also carried out dissolving 3,5-dimethoxybenzyl chloroformate **(4)** (th. 0.54 mmol, 1.1 eq.) and serine (50 mg, 0.48 mmol) in a mixture of dioxane (4 ml) and an aqueous sodium hydrogen carbonate solution (80 mg in 2 ml distilled water, 0.95 mmol, 2 eq.). The work-up in this case was similar as that described above.

In all cases, t.l.c. (chloroform: methanol, 1: 2) revealed no disappearance of serine from the reaction mixture and ^{1}H n.m.r. spectra in $CDCl_3$ showed that a mixture of 3,5-dimethoxybenzyl chloroformate **(4)** and 3,5-dimethoxybenzyl alcohol **(3)** was recovered after extraction.

2. 4. 2. Schotten-Baumann reaction using carbonate (6)

(*S*)-Serine (80 mg, 0.75 mmol) was dissolved in aqueous sodium hydrogen carbonate (120 mg in 2 ml, 1.4 mmol, 1.5 eq.) and added to 3,5-dimethoxybenzyl-*p*-nitrophenyl carbonate **(6)** (315 mg, 0.9 mmol, 1.25 eq.) in THF (10 ml) at 0°C. The reaction mixture was stirred at room temperature overnight. After removal of THF *in vacuo* a white precipitate in a yellow syrup was recovered. The white precipitate was dissolved in aqueous sodium hydrogen carbonate (100 ml, 1 M). The aqueous mixture

was then cooled to 0°C and the pH (measured with a pH-meter) was adjusted with hydrochloric acid (1M) from 8.2 to 7. At this pH, *p*-nitrophenol (yellow syrup) was extracted from the mixture with ether (8 x 50 ml) until the aqueous layer became colourless. The aqueous mixture was then cooled to 0°C and the pH (measured with a pH-meter) was adjusted with hydrochloric acid (1M) from 8.6 to 1.5. The organic product was then extracted with ether (6 x 50 ml). After washing with brine and drying of the organic layer over sodium sulphate, the organic solvents were removed *in vacuo*. A very small amount of white solid **(5)** was recovered (yield smaller than 5 %), sufficient enough for analysis.

The reaction was also carried out adding serine (80 mg, 0.75 mmol) dissolved in aqueous sodium hydroxide (2 M, 2 ml) to 3,5-dimethoxybenzyl-*p*-nitrophenyl carbonate **(6)** (315 mg, 0.9 mmol, 1.25 eq.) dissolved in THF (4 ml) at 0°C. The work-up in this case was similar as above. No product was recovered from the organic layer in this case.

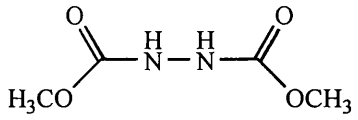
R_F: 0.53 (methanol: chloroform, 1: 1), visualisation by spraying with basic permanganate solution

^{1}H n.m.r.: δ (CD_3OD, 400 MHz) 3.75 (6H, s, -OC**H**$_3$) 3.86 (2H, m, -CH-C**H**$_2$-OH) 4.27 (1H, m, -C**H**-CH_2-OH) 5.03 (2H, s, ArC**H**$_2$O-) 6.39 (1H, t, Ar**H** **6**, *J* 2.2 Hz) 6.52 (2H, d, Ar**H** **5**, *J* 2.1 Hz)

mass spectrum (FAB): m/z 151 ($C_6H_3(OCH_3)_2CH_2^+$) 100 %, 168 ($C_6H_3(OCH_3)_2CH_2OH$) 15 %, 301 ($M^+ + 2H$) 17 %, 323 ($M^+ + Na + H$) 13 %

3. Synthesis of serine β-lactone residues

3. 1. Dimethyl hydrazodicarboxylate (9)[167]



In a three-necked flask equipped with two dropping funnels and a thermometer, hydrazine hydrate (5 ml, 0.1 mol) was added to ethanol (50 ml). The flask was then cooled to 10°C and methyl chloroformate (16 ml, 0.2 mol, 2 eq.) was added dropwise with stirring, keeping the temperature below 20°C. After one-half of the methyl chloroformate had been added to the mixture, an aqueous solution of sodium hydrogen carbonate (10.6 g in 50 ml, 0.1 mol, 1 eq.) was added dropwise simultaneously with the remaining methylchloroformate. The reaction was kept all the time below 20°C. After stirring of the reaction for 1 hr, water and ethanol were removed *in vacuo* to give a white solid, which was a mixture of sodium chloride and the title product. Separation of the two compounds was carried out by pouring the mixture into warm acetone, followed by filtration. The title compound was recovered from the filtrate after removal of the solvents *in vacuo* as a fine white powder (11.4 g, 0.08 mol, 80 %).

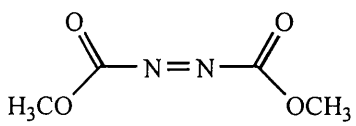
R_F: 0.66 (hexane: ethyl acetate, 2: 1), visualisation by absorption of U.V. light

^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 3.74 (6H, s, -C**H**$_3$) 6.60 (2H, broad s, N**H**-)

mass spectrum (EI^+): m/z 59 ($COOCH_3^+$) 100 %, 116 (M^+ - OCH_3 – H) 87 %, 148 (M^+) 20 %

Melting point: 122-125°C (lit.[167] 129.5-130.5°C)

3. 2. Dimethyl azodicarboxylate (10)[166]



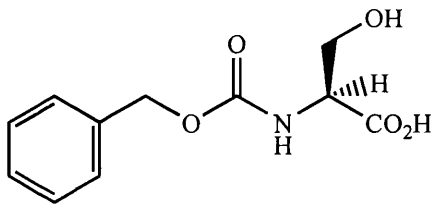
Dimethyl hydrazodicarboxylate **(9)** (7g, 47 mmol) was added to a solution of *N*-bromosuccinimide (previously recrystallised from boiling water, 12g, 67 mmol, 1.4 eq.) and pyridine (5.5 ml, 68 mmol, 1.4 eq.) in dichloromethane (250 ml). The mixture was left to stand for 30 min with occasional shaking. The reaction mixture was washed with distilled water (3 x 200 ml) and brine (20 ml), followed by drying of the organic layer over sodium sulphate. Removal of the solvents *in vacuo* (using teflon sleeves on the joints to avoid explosive hazards as the title compound is friction explosive) gave a red clear oil, (9 g, 62 mmol, some remaining dichloromethane). As the title compound is highly unstable, presenting explosive and flammable hazards, no further removal of dichloromethane nor purification was attempted, and the product was kept in the freezer to avoid further decomposition (bubbling at room temperature).

R_F: 0.41 (hexane: ethyl acetate; 2: 1), visualisation by absorption of U.V. light

^{1}H n.m.r.: δ ($CDCl_3$, 300 MHz) 4.10 (lit.[166] 4.07) (6H, s, -C**H**$_3$)

No further analyses could be carried out due to the high instability of the title compound.

3. 3. (*S*)-*N*-Benzyloxycarbonylserine (7)[165]

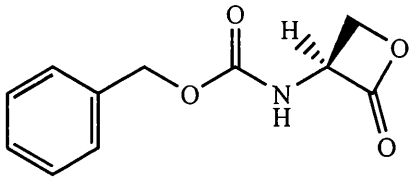


Benzyl chloroformate (7 ml, 49 mmol, 1 eq.) was added dropwise to a solution of (*S*)-serine (5g, 48 mmol) and aqueous sodium hydroxide (4 M, 30 ml) at 0°C. The reaction mixture was stirred for 90 min, then acidified to pH 2 with concentrated hydrochloric acid. Extraction with ethyl acetate (100 ml), followed by washing with brine (2 x 20 ml), drying of the organic layer over sodium sulphate and removal of the organic solvents *in vacuo* gave the title compound as a paste (7.40 g, 65 %).

R_F: 0.48 (hexane: ethyl acetate, 1: 2), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

^{1}H n.m.r.: δ (CD_3OD, 400 MHz) 4.58 (2H, m, -C**H**$_2$OH) 4.91 (2H, s, ArC**H**$_2$O-) 5.13 (1H, s, -C**H**-) 7.29-7.37 (5H, m, Ar**H**)

3. 4. (*S*)-*N*-Benzyloxycarbonylserine β-lactone (8)[62]



Triphenylphosphine (previously dried *in high vacuo*, 1.8 g, 6.9 mmol) was dissolved in dry THF (40 ml) under argon. The flask was then cooled to -78 °C. DMAD **(10)** (1 g, 6.9 mmol, 1 eq.) dissolved in dry THF (5 ml) was added to the clear colourless solution. As soon as a pale orange slurry had formed in the flask, (*S*)-*N*-benzyloxycarbonylserine **(7)** (1.64 g, 6.9 mmol, 1 eq.) dissolved in dry THF (8 ml) was added dropwise to the mixture. The reaction mixture was stirred under argon at -78°C for 30 min, then at room temperature for 3 hr. The solvents were removed *in vacuo* and the yellow thick crude oil obtained was purified by flash column chromatography (7 cm, hexane: ethyl acetate; 4: 1 then hexane: ethyl acetate; 3: 2, then pure ethyl acetate). The title compound **(8)** was recovered as a white solid (100 mg, 7 %).

The reaction was also carried out using triphenylphosphine (previously dried *in high vacuo*, 1.8 g, 6.9 mmol), DEAD (1.1 ml, 6.9 mmol, 1 eq.) and (*S*)-*N*-benzyloxycarbonylserine **(7)** (1.64 g, 6.9 mmol, 1 eq.) in the same conditions. The title product was visualised on the t.l.c. plate but could not be separated from diethyl hydrazodicarboxylate, even after purification of the reaction mixture by flash column chromatography (7 cm, hexane: ethyl acetate; 4: 1 then hexane: ethyl acetate; 3: 2, then pure ethyl acetate).

R_F: 0.13 (hexane: ethyl acetate; 2: 1), visualisation by spraying with basic permanganate solution

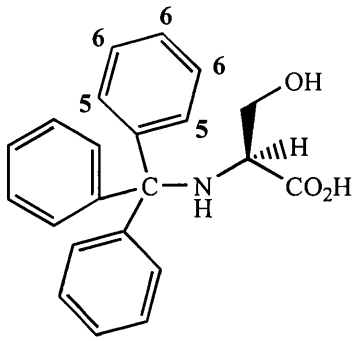
^{1}H n.m.r.: δ (CD_3OD, 400 MHz) 4.37 (lit[62] 4.4) (1H, ABX system, -C**H**$_2$OCO-, J_{AB} 4.8, J_{AX} 4.8 Hz) 4.43 (lit[62] 4.4) (1H, ABX system, -C**H**$_2$OCO-, J_{AB} 4.8, J_{BX} 6.6 Hz) 5.11 (lit[62]

5.12) (2H, s, ArC**H**$_2$O-) 5.14 (lit[62] 5.0-5.1) (1H, ABX system, -NHC**H**CO-, J_{AX} 4.8, J_{BX} 6.6 Hz) 7.28-7.38 (lit[62] 7.3-7.4) (5H, m, Ar**H**)

mass spectrum (FAB): m/z 91 ($C_6H_5CH_2^+$) 100 %, 137 ($C_6H_5CH_2CO_2H$ + H) 10 %, 222 (M^+ + H) 13 %, 244 (M^+ + Na) 11 %

4. Synthesis of iodoalanine (19) *via* protected serine residues

4. 1. (*S*)-*N*-Triphenylmethylserine (16)[176]



(*S*)-Serine (4.2 g, 40 mmol) was suspended in dichloromethane (70 ml) under nitrogen. Trimethylsilyl chloride (19.51 ml, 140 mmol, 3.5 eq.) was added to the suspension and the mixture was refluxed for 20 min. After having cooled the reaction mixture to room temperature, triethylamine (19.6 ml, 140 mmol, 3.5 eq.) in dichloromethane (40 ml) was added, forming a white slurry and white fumes of hydrochloric acid. The mixture was heated under reflux for 45 min then gradually cooled to 0°C. At this temperature, the mixture was treated dropwise with anhydrous methanol (2.5 ml, 60 mmol, 1.5 eq.) in dichloromethane (10 ml) then allowed to attain room temperature. Triethylamine (5.6 ml, 40 mmol, 1 eq.) was added to the mixture followed by triphenylmethyl chloride (11.25 g, 40 mmol, 1 eq.) portionwise. A white slurry in a pale brown solution was observed. The reaction mixture was then stirred for 18 hr at room temperature. An excess of triethylamine (10 ml) and methanol (100 ml) was added to the reaction mixture until the white slurry disappeared and a bright yellow solution was formed. The solvents were then evaporated *in vacuo* to give a pale yellow paste. This solid was partitioned between ethyl acetate and ether (200 ml, 1: 1) and a pre-cooled solution of citric acid (5 %, w/v, 200 ml). Extraction of the aqueous layer was carried out with ethyl acetate and ether (2 x 120 ml, 1: 1). The combined organic layers were washed with a solution of sodium hydroxide (1M, 2 x 80 ml) and water (2 x 40 ml). The combined aqueous layers were washed with ether (80 ml) and ethyl acetate (80 ml) and neutralised with glacial acetic acid at 0°C. The precipitated white solid was extracted with ethyl acetate (3 x 120 ml) and the combined organic layers were washed with water (2 x

40 ml) and dried over sodium sulphate. Evaporation of the solvent *in vacuo* and washing of the crude compound with hexane gave a fine white powder as the title compound (13.0 g, 94 %).

R_F: 0.62 (chloroform: methanol, 2: 3), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

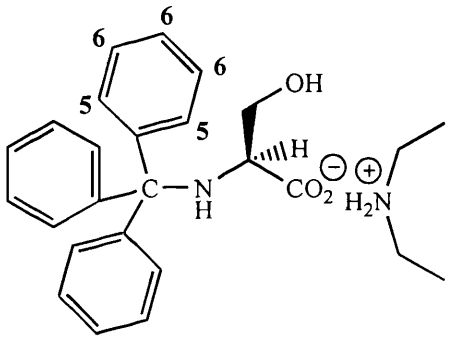
^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 2.77 (1H, dd, -C**H**$_2$OH, *J* 10.9, 5.0 Hz) 3.49 (1H, m, -C**H**-) 3.71 (1H, dd, -C**H**$_2$OH , *J* 10.9, 2.6 Hz) 7.19-7.29 (9H, m, Ar**H 6**) 7.40-7.42 (6H, m, Ar**H 5**)

^{13}C n.m.r.: δ ($CDCl_3$, 100 MHz) 58.6, 62.7, 71.9, 127.2, 128.3, 128.5, 144.4, 175.1

mass spectrum (FAB): m/z 243 (Trt^+) 100 %, 370 (M^+ + Na) 5 %

Found: (M^+ + Na), 370.1419. $C_{22}H_{21}NO_3Na$ requires 370.1430 (FAB)

Melting point: 150-154°C



N-Triphenylmethylserine (340 mg, 0.98 mmol) was dissolved in ethyl acetate (5 ml, the few remaining white clusters that did not dissolve were removed by filtration). An excess of diethylamine (0.3 ml, 3 eq.) was added to the clear solution. After removal of the solvent *in vacuo* and washing of the slurry obtained with ether, the diethylammonium salt of the title compound was obtained as a white powder.

$[\alpha]_D$ -32.7° (21°C, c 22.72 mg/ml, methanol) (lit.[176] $[\alpha]_D$ -33° (25°C, c 1%, methanol))

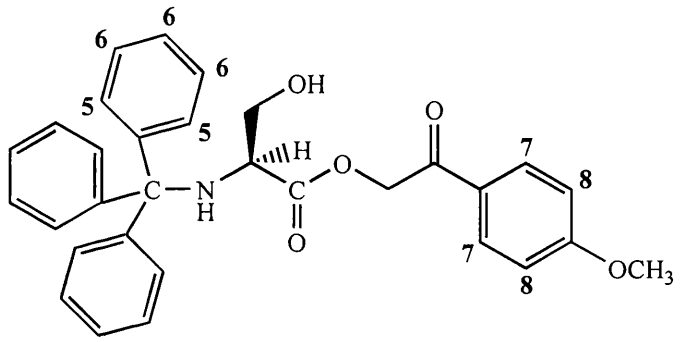
ν_{max} ($CHCl_3$) 3663 (OH), 1632 (CO_2^-), 1595 (aromatic)

^{1}H n.m.r.: δ ($CDCl_3$, 300 MHz) 1.24 (6H, t, $HN(CH_2C\mathbf{H_3})_2$, *J* 7.3 Hz) 2.76-2.84 (5H, m, $HN(C\mathbf{H_2}CH_3)_2$ + -C**H**$_2$OH) 3.24 (1H, dd, -C**H**CH_2OH, *J* 6.6, 3.0 Hz) 3.25 (1H, dd, -C**H**$_2$OH , *J* 22.6, 3.0 Hz) 7.16-7.27 (9H, m, Ar**H 6**) 7.46-7.48 (6H, m, Ar**H 5**)

^{13}C n.m.r.: δ ($CDCl_3$, 100 MHz) 12.2, 42.8, 59.5, 65.2, 72.3, 127.4, 128.7, 129.9, 147.3, 180.2

mass spectrum (APCI$^-$): m/z 346 (M^-) 100 %

4. 2. (S)-N-*Triphenylmethylserine 4-methoxyphenacyl ester* (17)



(*S*)-*N*-Triphenylmethylserine **(16)** (5g, 14.4 mmol) was dissolved in DMF (20 ml) under nitrogen. Triethylamine (2 ml, 14.4 mmol, 1 eq.) was added to the yellow solution and a white slurry was gradually formed. 4-Methoxyphenacyl bromide (3.3 g, 14.4 mmol, 1 eq.) dissolved in DMF (10ml) was added to this slurry and the reaction mixture was left in the fridge under nitrogen for 40 hr.

The reaction mixture was poured into ice water (200 ml) and a whitish precipitate was immediately formed. This precipitate was recovered by filtration and then suspended in petroleum ether and stirred for at least 5 min. After filtration, the crude solid was dried on the freeze drier then recrystallised from ethanol to give the pure title compound as a white powder (3.9 g, 55 %).

R_F: 0.55 (hexane: ethyl acetate, 1: 1), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

$[\alpha]_D$ -8.3° (26°C, c 53.15 mg/ml, $CHCl_3$)

ν_{max} ($CHCl_3$) 3462 (OH), 3018 (CH), 2983 (OCH_3), 1737 (C=O ester), 1679 (C=O ketone), 1597 (aromatic)

^1H n.m.r.: δ ($CDCl_3$, 300 MHz) 3.64 (1H, m, -C**H**$_2$OH) 3.72 (1H, m, -C**H**$_2$OH) 3.84 (3H, s, -OC**H**$_3$) 4.07 (1H, m, -C**H**-) 4.77 (1H, d, -CO_2C**H**$_2$CO-, *J* 16.4 Hz) 5.00 (1H, d, -

CO_2CH_2CO-, *J* 16.4 Hz) 6.89 (2H, d, Ar**H 8**, *J* 8.9 Hz) 7.16-7.28 (9H, m, Ar**H 6**) 7.52 (6H, d, Ar**H 5**, *J* 7.4 Hz) 7.77 (2H, d, Ar**H 7**, *J* 8.9 Hz)

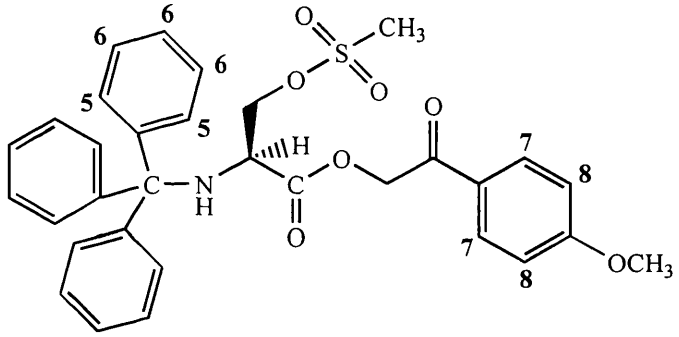
^{13}C n.m.r.: δ ($CDCl_3$, 100 MHz) 55.5, 58.5, 65.7, 66.2, 70.9, 114.1, 126.3, 126.5, 127.9, 128.8, 130.3, 145.7, 164.4, 173.6, 191.6

mass spectrum (FAB): m/z 243 (Trt^+) 100 %, 518 (M^+ + Na) 10 %

Found: (M^+ + Na), 518.1943. $C_{31}H_{29}NO_5Na$ requires 518.1957 (FAB)

Melting point: 120-121°C

4. 3. (S)-O-*Methanesulfonyl*-N-*triphenylmethylserine 4-methoxyphenacyl ester* (18)



(*S*)-*N*-Triphenylmethylserine 4-methoxyphenacyl ester **(17)** (500 mg, 1 mmol) was dissolved in dry dichloromethane (2 ml). Triethylamine (0.42 ml, 3 mmol, 3 eq.) was added to the solution at 0°C. Methanesulfonyl chloride (0.16 ml, 2 mmol, 2 eq.) was then added slowly to the reaction mixture at 0°C. The reaction was stirred for 2 hr at room temperature.

The reaction mixture was poured into a mixture of dichloromethane (10 ml) and saturated ammonium chloride solution (10 ml). After washing with saturated sodium hydrogen carbonate solution (10 ml), the organic layer was dried over sodium sulphate. After removal of the solvents *in vacuo*, the title compound was recovered as a pale yellow paste (579 mg, 1 mmol) which was used in the next step without further purification.

In some cases, purification was carried out by flash column chromatography (for 500 mg of starting material **(17)**, 5 cm, hexane: ethyl acetate, 2: 1), the title compound **(18)** was recovered as a pale yellow paste (470 mg, 80 %).

(*S*)-*N*-Triphenylmethylserine 4-methoxyphenacyl ester **(17)** (241.5 mg, 0.49 mmol) was dissolved in dry THF (4 ml). Triethylamine (0.08 ml, 0.59 mmol, 1.2 eq.) was added to the solution at 0°C. Methanesulfonyl chloride (0.06 ml, 0.73 mmol, 1.5 eq.) was added slowly to the reaction mixture at 0°C. The reaction was stirred for 4 hr at room temperature.

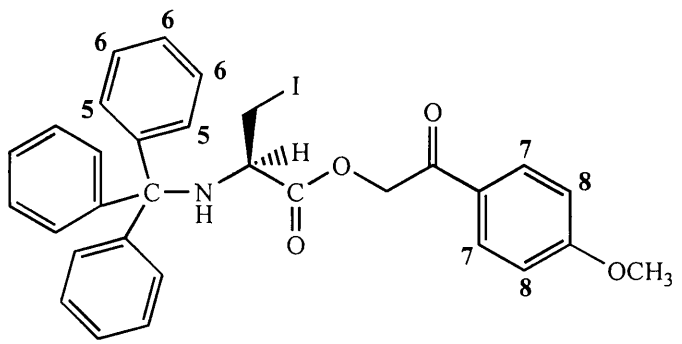
After removal of the solvents *in vacuo*, the residue was dissolved in ether and ethyl acetate (100 ml, 1: 1) and ice cold water (30 ml). The organic layer was washed twice with ice cold water (2 x 30 ml) and with brine (20 ml). The organic layer was dried over sodium sulphate, followed by removal of the solvents *in vacuo* to give a yellow oil. Purification was carried out by flash column chromatography (2.5 cm, DCM then 1 % methanol in DCM). The title compound was recovered as a pale yellow paste (60 mg, 21 %).

R_F: 0.55 (hexane: ethyl acetate, 1: 1), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 2.89 (1H, d, -N**H**-, *J* 10.1 Hz) 3.04 (3H, s, -$SO_2C\mathbf{H_3}$) 3.81-3.83 (3H, s, -$OC\mathbf{H_3}$ and 1H, m, -C**H**-) 4.29 (1H, dd, -$C\mathbf{H_2}$OH, *J* 10.0, 5.3 Hz) 4.45 (1H, dd, -$C\mathbf{H_2}$OH, *J* 10.0, 4.1 Hz) 4.65 (1H, d, -$CO_2C\mathbf{H_2}CO$-, *J* 16.1 Hz) 4.89 (1H, d, -$CO_2C\mathbf{H_2}CO$-, *J* 16.1 Hz) 6.88 (2H, d, Ar**H 8**, *J* 9.0 Hz) 7.14-7.26 (9H, m, Ar**H 6**) 7.47-7.49 (6H, d, Ar**H 5**, *J* 7.6 Hz) 7.72 (2H, d, Ar**H 7**, *J* 9.0 Hz)

mass spectrum (FAB): m/z 500 (aziridine, M^+ + Na) 100 %, 596 (M^+ + Na) 31 %

4. 4. (R)-N-*Triphenylmethyl-3-iodoalanine 4-methoxyphenacyl ester* (19)



4. 4. 1. Using sodium iodide

Crude (*S*)-*O*-methanesulfonyl-*N*-triphenylmethylserine 4-methoxyphenacyl ester **(18)** (724 mg, 1.26 mmol) was dissolved in acetone (12 ml) and sodium iodide (1.9 g, 10 eq.) was added to the solution. The reaction mixture was stirred under nitrogen for 41 hr in the dark.

The mixture was then poured into ether (50 ml) and sodium thiosulphate solution (10 %, w/v, 50 ml), followed by washing of the organic layer with sodium thiosulphate solution (10 %) until this layer lost its bright yellow colour. Drying of the organic layer over sodium sulphate was followed by removal of the solvents *in vacuo* to give a yellow oil (344 mg).

^{1}H n.m.r. spectra in $CDCl_3$ showed that the starting material **(18)** was recovered after extraction.

4. 4. 2. Using methyltriphenoxyphosphonium iodide

(*S*)-*N*-Triphenylmethylserine 4-methoxyphenacyl ester **(17)** (300 mg, 0.61 mmol) and crude methyltriphenoxyphosphonium iodide (1.51g, ^{1}H n.m.r. and ^{31}P n.m.r. spectra in $CDCl_3$ revealed one third by-products mixed with it, about 2 eq.) were dissolved in dry DMF (10 ml) under nitrogen. After stirring in the dark for 2 hr, the reaction mixture was poured into ether (40 ml). The organic layer was washed with distilled water (2 x 10 ml), sodium thiosulphate solution (1 M, 10 ml) then distilled water again (3 x 10 ml). The organic layer was dried over sodium sulphate and the solvents were removed *in vacuo* to give a clear oil. Purification by flash column chromatography (5 cm, hexane followed by hexane: ethyl acetate, 4: 1 followed by neat ethyl acetate) gave the title compound as an oil, however the purification was incomplete and some by-products derived from methyltriphenoxyphosphonium iodide were mixed with it (92 mg, 25 %).

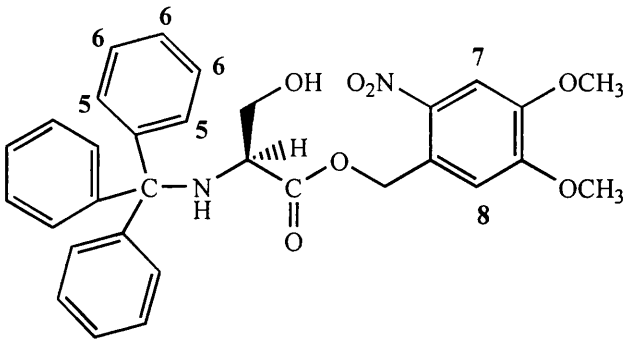
R_F: 0.74 (dichloromethane), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) among impurities were 2 rotamers A + B (3: 2): 2.60 (1H, dd, -C**H$_2$**I, *J* 13.0, 5.7 Hz, A) 2.78 (1H, m, -C**H$_2$**I, A) 2.84 (1H + 1H, d, -N**H**-, *J* 9.5 Hz, A + B) 3.20 (1H, dd, -C**H$_2$**I, *J* 9.9, 5.8 Hz, B) 3.34 (1H, dd, -C**H$_2$**I, *J* 9.9, 3.2 Hz, B) 3.62 (1H, m, -C**H**-, B) 3.82 (3H + 3H, s, -OC**H$_3$**, A + B) 4.50 (1H, dd, -C**H**-, *J* 9.3, 5.7 Hz, A) 4.77 (1H, d, -CO_2C**H$_2$**CO-, *J* 16.1 Hz, B) 4.93 (1H, d, -CO_2C**H$_2$**CO-, *J* 16.1 Hz, B) 5.25 (1H, d, -CO_2C**H$_2$**CO-, *J* 15.9 Hz, A) 5.35 (1H, d, -CO_2C**H$_2$**CO-, *J* 15.9 Hz, A) 6.60-6.90 (2H + 2H, m, Ar**H 8**, A + B) 7.15-7.27 (9H + 9H, m, Ar**H 6**, A + B) 7.44-7.50 (6H + 6H, m, Ar**H 5**, A + B) 7.76-7.83 (2H + 2H, m, Ar**H 7**, A + B)

mass spectrum (APCI$^+$): m/z 497 (M^+ - $C_6H_4OCH_3$ + H) 44 %, 628 (M^+ + Na) traces

5. Synthesis of iodoalanine (22) *via* protected serine residues

5. 1. (S)-N-*Triphenylmethylserine 4,5-dimethoxy-2-nitrobenzyl ester* (20)



(*S*)-*N*-Triphenylmethylserine **(16)** (2.1 g, 6.0 mmol) was dissolved in DMF (12 ml) under nitrogen. Triethylamine (0.85 ml, 6.1 mmol, 1 eq.) was added to the solution. 4,5-Dimethoxy-2-nitrobenzyl bromide (1.9 g, 6.9 mmol, 1.1 eq.) dissolved in DMF (3 x 5 ml) was added dropwise to the reaction mixture at 0°C. The reaction mixture was stirred under nitrogen for 24 hr.

After removal of the solvents *in high vacuo*, the residue was dissolved in water: ethyl acetate (100 ml, 1: 1) the aqueous layer was extracted with ethyl acetate (6 x 75 ml) and the combined organic layers were washed with water (6 x 40 ml). The organic layer was dried over sodium sulphate, followed by removal of the solvents *in vacuo* to give the title compound as a dark yellow solid (3.0 g, 93 %).

R_F: 0.85 (chloroform: methanol, 2: 3), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

$[\alpha]_D$ -17.9°(25°C, c 62.16 mg/ml, $CHCl_3$)

Found: C, 68.0; H, 5.5; N, 5.0. $C_{31}H_{30}N_2O_7$ requires C, 68.6; H, 5.6; N, 5.2

ν_{max} ($CHCl_3$) 3593 (OH), 3021 (CH stretching), 2940 (OCH_3), 1737 (CO ester), 1583 (aromatic), 1523 ($C\text{-}NO_2$)

^1H n.m.r.: δ ($CDCl_3$, 400 MHz) 3.54 (1H, dd, -C**H**$_2$OH, *J* 5.8, 10.7 Hz) 3.61 (1H, dd, -C**H**-, *J* 10.7, 3.9 Hz) 3.83 (1H, dd, -C**H**$_2$OH , *J* 5.8, 3.9 Hz) 3.89 (3H, s, -OC**H**$_3$) 3.91 (3H, s, -OC**H**$_3$) 4.92 (1H, d, -CO_2C**H**$_2$-, *J* 15.2 Hz) 5.24 (1H, d, -CO_2C**H**$_2$-, *J* 15.2 Hz) 6.84 (1H, s, Ar**H 8**) 7.16-7.29 (9H, m, Ar**H 6**) 7.46-7.48 (6H, m, Ar**H 5**) 7.65 (1H, s, Ar**H 7**)

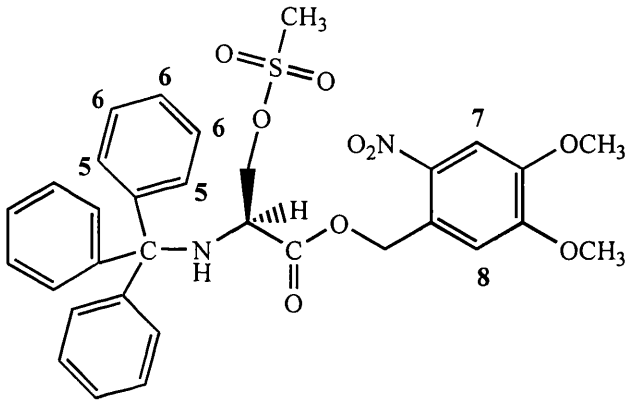
^{13}C n.m.r.: δ ($CDCl_3$, 100 MHz) 56.2, 56.5, 58.0, 63.7, 64.9, 70.9, 107.9, 109.8, 126.6, 127.8, 127.9, 128.6, 139.5, 145.4, 147.9, 153.5, 173.1

mass spectrum (FAB): m/z 243 (Trt^+) 100 %, 675 (M^+ + Cs) 29 %

Found: (M^+ + Cs), 675.1107. $C_{31}H_{30}N_2O_7Cs$ requires 675.1090 (FAB)

Melting point: 166-168°C

5. 2. (S)-O-*Methanesulfonyl*-N-*triphenylmethylserine 4,5-dimethoxy-2-nitro-benzyl ester* (21)



(*S*)-*N*-Triphenylmethylserine 4,5-dimethoxy-2-nitrobenzyl ester **(20)** (1.5 g, 2.8 mmol) was dissolved in dichloromethane (10 ml). Triethylamine (1.16 ml, 8.3 mmol, 3 eq.) was added to the solution at 0°C. Methanesulfonyl chloride (0.43 ml, 5.5 mmol, 2 eq.) was added slowly to the reaction mixture at 0°C. The reaction was stirred for 2 hr 15 at room temperature.

The reaction mixture was poured into dichloromethane (40 ml) and washed with a saturated solution of ammonium chloride (20 ml), then a saturated solution of sodium hydrogen carbonate (2 x 30 ml) and the organic layer was dried over sodium sulphate. Removal of the solvents *in vacuo* gave a yellow oil which was purified by flash column chromatography (6 cm, hexane: ethyl acetate, 2: 1 gradient). The title compound was recovered as a pale yellow solid (1.5 g, 87 %).

(*S*)-*N*-Triphenylmethylserine 4,5-dimethoxy-2-nitrobenzyl ester **(20)** (1.6 g, 3.0 mmol) was dissolved in dry THF (18 ml). Triethylamine (0.45 ml, 3.2 mmol, 1.1 eq.) was

added to the solution at 0°C. Methanesulfonyl chloride (0.25 ml, 3.2 mmol, 1.1 eq.) was added slowly to the reaction mixture at 0°C. The reaction was stirred for 4 hr at room temperature.

After removal of the solvents *in vacuo*, the residue was dissolved in ether (50 ml), ethyl acetate (50 ml) and ice cold water (30 ml). The organic layer was washed twice with ice cold water (2 x 30 ml) and with brine (20 ml). The organic layer was dried over sodium sulphate, followed by removal of the solvents *in vacuo* to give a yellow oil.

After recrystallisation (DCM: methanol, 1:4, -20°C), the title compound was recovered as a light cream solid (1.7 g, 93 %).

Purification was also carried out by flash column chromatography. For instance for 1.5 g of starting material **(20)**, flash column chromatography (5 cm, hexane: ethyl acetate, 1: 1, gradient) gave the title compound **(21)** as a pale yellow solid (1.5 g, 87 %). This mesyl derivative degrades only slowly (in a matter of weeks) once it has been purified.

R_F: 0.17 (hexane: ethyl acetate, 2: 1), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

$[\alpha]_D$ -1.4° (26°C, c 22.25 mg/ml, $CHCl_3$)

Found: C, 61.4; H, 5.1; N, 4.5; S, 5.3. $C_{32}H_{32}N_2O_9S$ requires C, 61.9; H, 5.2; N, 4.5; S, 5.2

ν_{max} ($CHCl_3$) 3028 (CH stretching), 1740 (C=O ester), 1583 (aromatic), 1523 (C-NO_2)

^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 2.96 (3H, s, -SO_2CH_3) 3.74 (1H, m, -C**H**-) 3.93 (3H, s, -O**CH$_3$**) 3.94 (3H, s, -O**CH$_3$**) 4.25 (1H, dd, -**CH$_2$**SO_2-, *J* 10.2, 6.4 Hz) 4.45 (1H, dd, -**CH$_2$**SO_2-, *J* 10.2, 4.1 Hz) 4.88 (1H, d, -CO_2**CH$_2$**-, *J* 14.2 Hz) 5.14 (1H, d, -CO_2**CH$_2$**-, *J* 14.2 Hz) 6.86 (1H, s, Ar**H 8**) 7.16-7.28 (9H, m, Ar**H 6**) 7.47-7.50 (6H, m, Ar**H 5**) 7.66 (1H, s, Ar**H 7**)

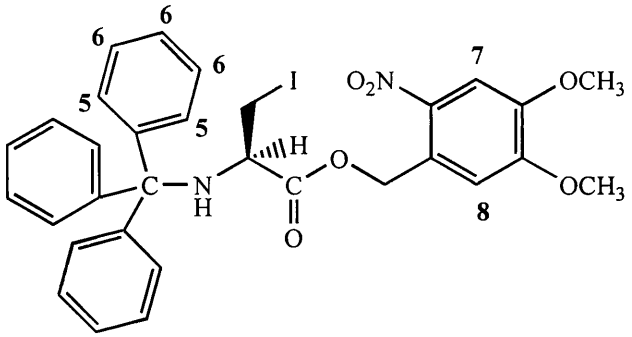
^{13}C n.m.r.: δ ($CDCl_3$, 75.47 MHz) 38.0, 56.3, 56.8, 57.1, 64.5, 71.25, 71.6, 108.4, 111.4, 126.2, 127.2, 128.5, 129.0, 140.4, 145.6, 148.8, 153.9, 171.8

mass spectrum (FAB): m/z 241 (Trt^+ - 2H) 100 %, 348 (M^+ - NV - mesyl + 2H) 28 %, 543 (M^+ - mesyl + H) 73 %, 621 (M^+ + H) 2 %, 643 (M^+ + Na) 3 %

Found: (M+ + Na), 643.1726. $C_{32}H_{32}N_2O_9SNa$ requires 643.1742 (FAB)

Melting point: 129-131°C (dec.)

5. 3. (R)-N-*Triphenylmethyl-3-iodoalanine 4,5-dimethoxy-2-nitrobenzyl ester* (22)



(*S*)-*O*-Methanesulfonyl-*N*-triphenylmethylserine 4,5-dimethoxy-2-nitrobenzyl ester **(21)** (1.0 g, 1.6 mmol) was dissolved in acetone (3 ml) then this solution was added dropwise slowly to a solution of sodium iodide (2.6 g, 17.3 mmol, 11 eq.) dissolved in acetone (5 ml). The reaction mixture was stirred under nitrogen at room temperature for 4 days.

Pouring of the reaction mixture into ether (50 ml) and ethyl acetate (50 ml) and an aqueous solution of sodium thiosulphate (10 %, w/v, 40 ml) was followed by washing of the organic layer with an aqueous solution of sodium thiosulphate (10 %, w/v, 2 x 40 ml) and with water (2 x 20 ml). The combined aqueous layers were extracted once with ether (25 ml) and ethyl acetate (25 ml). The combined organic layers were dried over sodium sulphate, followed by removal of the solvents *in vacuo* to give a pale yellow paste which was purified by flash column chromatography (3.5 cm, hexane: ethyl acetate, 1: 1, gradient). The title compound was recovered as a pale yellow solid (881 mg, 84 %).

R_F: 0.80 (hexane: ethyl acetate, 1: 2), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

$[\alpha]_D$ -6.1° (26°C, c 23.6 mg/ml, $CHCl_3$)

ν_{max} ($CHCl_3$) 3014 (CH stretching), 1737 (C=O ester), 1583 (aromatic), 1523 (C-NO_2)

^{1}H n.m.r.: δ ($CDCl_3$, 300 MHz) 2 rotamers A + B (7: 4): 2.61 (1H, dd, -C**H**$_2$I, *J* 13.0, 5.9 Hz, A) 2.77 (1H, dd, -C**H**$_2$I, *J* 13.0, 8.5 Hz, A) 2.92 (1H, d, -N**H**-, *J* 9.7 Hz, B, no visible signal for -N**H**-, A), 3.21 (1H, -C**H**$_2$I, *J* 9.8, 7.2 Hz, B) 3.35 (1H, -C**H**$_2$I, *J* 9.8, 3.3 Hz, B) 3.58 (1H, -C**H**CH$_2$I, m, B) 3.93-4.01 (6H + 6H, m, -OC**H**$_3$, A + B) 4.51 (1H, dd, -C**H**CH$_2$I, *J* 8.5, 5.9 Hz, A) 4.99 (1H, d, -C**H**$_2$OCO-, *J* 14.7 Hz, B) 5.19 (1H, d, -

CO_2CH_2-, *J* 14.7 Hz, B) 5.57 (1H, d, -CO_2CH_2-, *J* 15.2 Hz, A) 5.72 (1H, d, -CO_2CH_2-, *J* 15.2 Hz, A) 6.83 (1H, s, Ar**H 8**, B) 7.10 (1H, s, Ar**H 8**, A) 7.12-7.34 (9H + 9H, m, Ar**H 6**, A + B) 7.42-7.46 (6H, m, Ar**H 5**, A) 7.47-7.51 (6H, m, Ar**H 5**, B) 7.67 (1H, s, Ar**H 7**, B) 7.74 (1H, s, Ar**H 7**, A)

^{13}C n. m. r.: δ ($CDCl_3$, 75.47 MHz) 10.0, 56.8, 57.0, 57.1, 57.4, 64.2, 71.3, 71.5, 108.4, 108.6, 110.1, 111.4, 126.6, 127.0, 127.2, 127.3, 128.5, 128.5, 128.8, 129.0, 140.4, 145.8, 145.9, 148.7, 153.7, 170.5

mass spectrum ($APCI^+$): m/z 243 (Trt^+) 100 %, 547 (aziridine$^+$ + Na) 12 %, 675 (M^+ + Na) 10 %

Found: (M^+ + Na), 675.0968. $C_{31}H_{29}N_2O_6INa$ requires 675.0991 (FAB)

Melting point: 56-59°C

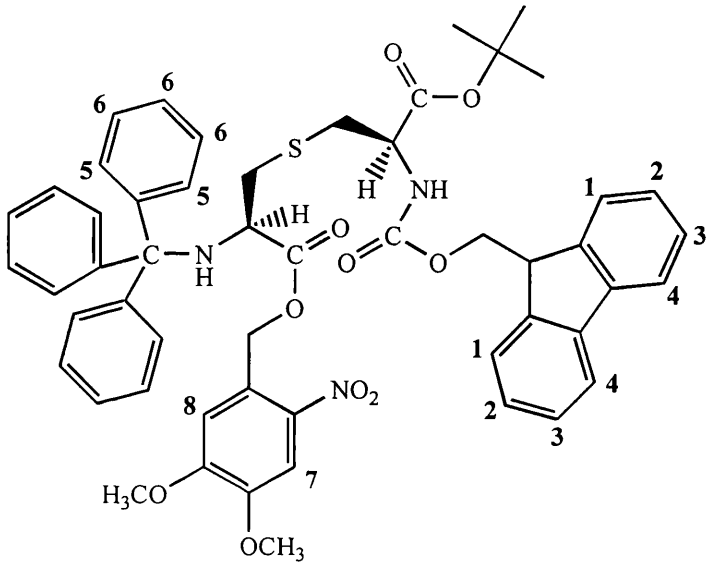
A test reaction was carried out in an attempt to determine whether the iodoalanine residues are mixtures of rotamers. (*R*)-*N*-Triphenylmethyl-3-iodoalanine 4,5-dimethoxy-2-nitrobenzyl ester **(22)** (54 mg, 0.08 mmol) was dissolved in DCM (2 ml). Trifluoroacetic acid (0.5 ml) was added to the solution and the reaction mixture was stirred for 4 hr. Removal of the solvents *in vacuo* gave a crude yellow product.

6-Nitroveratryl alcohol was clearly identified by ^1H n.m.r.

mass spectrum ($APCI^+$): m/z 411 ((*R*)-3-iodoalanine 4,5-dimethoxy-2-nitrobenzyl ester, M^+ + H) 6 %

6. Synthesis of lanthionine residues

6. 1. (R)-*(4,5-Dimethoxy-2-nitrobenzyl)*-N-*triphenylmethylalanyl* (R)-tert-*butyl*-N-*(9-fluorenylmethyloxycarbonyl)alanyl sulfide* (24)



A mixture of (*R*)-*N*-(9-fluorenylmethyloxycarbonyl)cysteine *tert*-butyl ester **(13)** (245 mg, 0.61 mmol) and (*R*)-*N*-triphenylmethyl-3-iodoalanine 4,5-dimethoxy-2-nitrobenzyl ester **(22)** (450 mg, 0.69 mmol, 1.1 eq.) in DMF (6 ml) was treated with cesium carbonate (200 mg, 0.61 mmol, 1 eq.) under nitrogen. The reaction was stirred for 4 hr at room temperature (20°C).

The reaction mixture was dissolved in ethyl acetate (100 ml) and an aqueous solution of citric acid (10 %, w/v, 20 ml). The aqueous layer was extracted twice with ethyl acetate (2 x 50 ml) and ether (2 x 60 ml). The combined ethyl acetate layers were washed with an aqueous solution of citric acid (10 %, w/v, 20 ml) and water (8 x 30 ml) then dried over sodium sulphate. The combined ether layers were washed with an aqueous solution of citric acid (10 %, w/v, 20 ml) and water (4 x 30 ml) then dried over sodium sulphate. Removal of the solvents *in vacuo* from the combined organic layers gave an oil which was then purified by flash column chromatography (3.5 cm, hexane: ethyl acetate, 4: 1, gradient). The title compound **(24)** was obtained as a pale yellow light solid (532 mg, 94 %, 0.57 mmol).

When the reaction was carried out with equimolar amounts of **(13)** and **(22)** or at a lower temperature the yield dropped significantly (for example 82 % for a mixture of **(13)**: **(22)**, 1: 1 at 20°C).

R_F: 0.65 (hexane: ethyl acetate, 1: 1), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

$[\alpha]_D$ +1.8° (12°C, c 21.47 mg/ml, $CHCl_3$)

ν_{max} ($CHCl_3$) 3028 (CH stretching), 1730 (C=O, broad), 1583 (aromatic), 1521 (C-NO_2)

^{1}H n.m.r.: δ ($CDCl_3$, 500 MHz) 2 diastereoisomers A + B (2: 1): 1.45 (9H + 9H, m, -C(C**H$_3$**)$_3$, A + B) 2.52 (1H, m, TrtNV-CH-C**H$_2$**-S-, A) 2.68 (1H, m, TrtNV-CH-C**H$_2$**-S-, A) 2.77 (1H, m, TrtNV-CH-C**H$_2$**-S-, B) 2.86 + 3.03 (1H, m, -S-C**H$_2$**-CH-tBuFmoc, A) 2.91 (2H, m, TrtNV-CH-C**H$_2$**-S- + -S-C**H$_2$**-CH-tBuFmoc, B) 3.00 (1H, m, -S-C**H$_2$**-CH-tBuFmoc, B) 3.03 + 3.14 (1H, m, -S-C**H$_2$**-CH-tBuFmoc, A) 3.61 (1H + 1H, m, TrtNV-C**H**-CH_2-S-, A + B) 3.87-3.91 (6H + 6H, m, -OC**H$_3$**, A + B) 4.17 (1H + 1H, t, -C**H**-CH_2-OCONH-, *J* 7.1 Hz, A + B) 4.32 (2H + 2H, m, -CH-C**H$_2$**-OCONH-, A + B) 4.47 (1H + 1H, m, tBuFmoc-C**H**-CH_2-S-, A + B) 4.88 (1H, d, -CO_2-C**H$_2$**-Ar, *J* 14.8 Hz, B) 5.07 (1H, d, -CO_2-C**H$_2$**-Ar, *J* 14.8 Hz, B) 5.56 ((2H, m, -CO_2-C**H$_2$**-Ar, A) + (1H + 1H, -N**H**-Trt, (A + B)) + (1H + 1H, -N**H**-CO_2-CH_2, (A + B))) 6.88 (1H, s, Ar**H 8**, B) 7.03 (1H, s, Ar**H 8**, A) 7.16 (2H + 2H, m, Ar**H 2**, A + B) 7.21-7.29 (9H + 9H, m, Ar**H 6**, A + B) 7.35 (2H + 2H, m, Ar**H 3**, A + B) 7.43 (6H, m, Ar**H 5**, A) 7.48 (6H, m, Ar**H 5**, B) 7.55 (2H + 2H, m, Ar**H 1**, A + B) 7.60 (1H, s, Ar**H 7**, B) 7.67 (1H, s, Ar**H 7**, A) 7.73 (2H + 2H, d, Ar**H 4**, *J* 7.4 Hz, A + B)

^{13}C n.m.r.: δ ($CDCl_3$, 75.47 MHz) 28.4, 36.3, 38.5, 44.8, 45.0, 47.5, 48.2, 48.9, 54.7, 54.8, 56.7, 56.8, 57.0, 57.1, 64.2, 64.3, 67.6, 67.7, 71.3, 71.7, 83.4, 83.5, 108.4, 108.6, 110.6, 110.8, 111.0, 120.4, 125.5, 126.9, 127.1, 127.5, 128.2, 128.4, 128.9, 129.1, 140.2, 141.7, 144.1, 144.2, 146.0, 148.5, 148.7, 153.8, 154.0, 156.1, 169.7, 169.9, 171.5, 173.4

mass spectrum (FAB): m/z 924 (M^+ + H) 81 %, 946 (M^+ + Na) 100 %

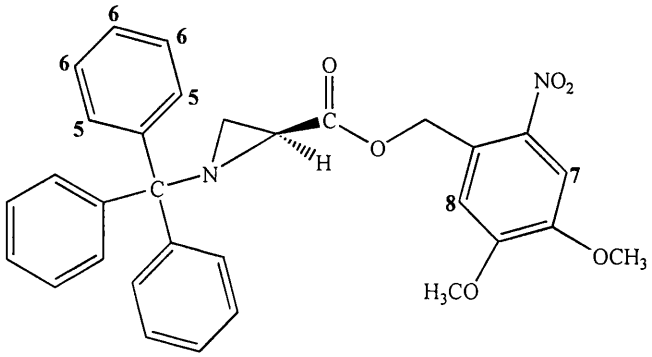
LC-MS reverse-phase: 21.20 min (60 % to 90 % B in 20 min, no TFA in the solvent system) ($APCI^+$): m/z 924 (M^+ + H) 1 %, 946 (M^+ + Na) 7 %

LC-MS normal-phase: 25.19 min + 26.52 min (25 % B') ($APCI^+$): m/z 925 (M^+ + H) 1 %, 946 (M^+ + Na) 1 % and 925 (M^+ + H) 1 %, 946 (M^+ + Na) 1 %

Found: (M^+ + Na), 946.3349. $C_{53}H_{53}N_3O_{10}SNa$ requires 946.3312 (FAB)

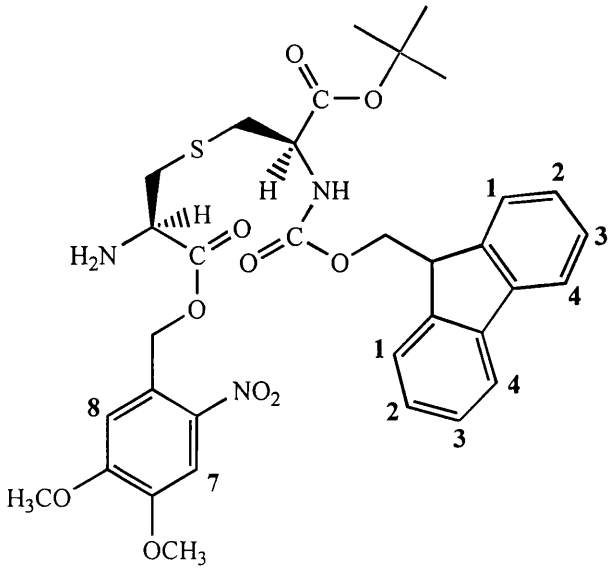
Melting point: 54-58°C

Aziridine **(23)** was recovered as a by-product of the reaction. It can also be produced during the formation of iodoalanine **(22)**.



^{1}H n.m.r.: δ ($CDCl_3$, 500 MHz) 1.48 (1H, m, CH-C**H**$_2$-N-) 2.00 (1H, dd, CH-C**H**$_2$-N-, *J* 6.0, 2.5 Hz) 2.34 (1H, m, -N-C**H**-CO_2-) 3.81 (3H, s, -OC**H**$_3$) 3.96 (3H, s, -OC**H**$_3$) 5.58 (1H, d, -CO_2-C**H**$_2$-Ar, *J* 15.1 Hz) 5.68 (1H, d, -CO_2-C**H**$_2$-Ar, *J* 15.1 Hz) 7.12 (1H, s, Ar**H 8**) 7.20-7.27 (9H, m, Ar**H 6**) 7.48 (6H, m, Ar**H 5**) 7.74 (1H, s, Ar**H 7**)

6. 2. (R)-*(4,5-Dimethoxy-2-nitrobenzyl)alanyl* (R)-tert-*butyl*-N-*(9-fluorenyl-methyloxycarbonyl)alanyl sulfide* (25)

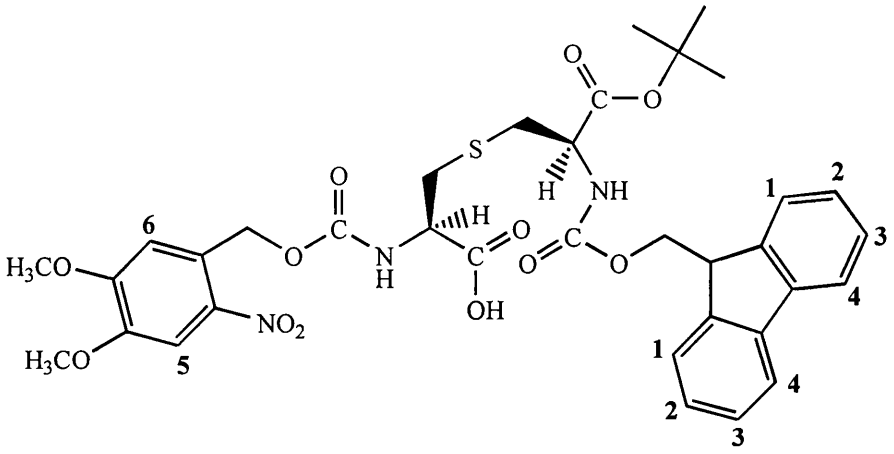


(*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-triphenylmethylalanyl (*R*)-*tert*-butyl-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(24)** (76 mg, 0.082 mmol) was dissolved in dry dichloromethane (4 ml). Trifluoroacetic acid (0.025 ml, 0.33 mmol, 4 eq.) was added to the solution and the reaction mixture was stirred for 4 hr 30 at room temperature.
After removal of the solvents *in vacuo*, the pale yellow oil obtained was purified by flash column chromatography (1.5 cm, hexane: ethyl acetate, 1: 2 then neat ethyl acetate, then methanol). Two types of residues **(25)**, compound A and compound B were obtained from the column, each as a pale yellow oil (A: B, 5: 1, 56 mg, 0.082 mmol, 100 %).
mass spectrum (FAB) (identical for both compounds): m/z 179 (DBF) 92 %, 196 (NV) 80 %, 682 (M^+) 70 %

Compound A: R_F: 0.02 (neat ethyl acetate), visualisation by absorption of U.V. light and by spraying with basic permanganate solution
^{1}H n.m.r.: δ ($CDCl_3$, 500 MHz) 1.44 (9H, m, -C(C**H**$_3$)$_3$) 1.77 (2H, broad s, -N**H**$_2$) 2.98 (2H, m, H_2NNV-CH-C**H**$_2$-) 3.10 (2H, m, -S-C**H**$_2$-CH-tBuFmoc) 3.42 (1H, m, H_2NNV-C**H**-CH_2-S-) 3.92 (6H, m, -OC**H**$_3$) 4.15 (1H, m, -C**H**-CH_2-OCONH-) 4.37 (2H, m, -CH-C**H**$_2$-OCONH-) 4.50 (1H, m, tBuFmoc-C**H**-CH_2-S-) 5.55 (2H, m, -CO_2-C**H**$_2$-Ar) 5.88 (1H, m, -OCON**H**-) 6.31 (1H, m, -OCON**H**-) 7.05 (1H, s, Ar**H 8**) 7.26 (2H, m, Ar**H 2**) 7.35 (2H, t, Ar**H 3**, *J* 7.1 Hz) 7.56 (2H, d, Ar**H 1**, *J* 6.5 Hz) 7.66 (1H, m, Ar**H 7**) 7.72 (2H, d, Ar**H 4**, *J* 5.8 Hz)

Compound B: R_F: 0.25 (neat ethyl acetate), visualisation by absorption of U.V. light and by spraying with basic permanganate solution
^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 1.48 (9H, m, -C(C**H**$_3$)$_3$) 1.85 (2H, broad s, -N**H**$_2$) 2.87 (1H, m, H_2NNV-CH-C**H**$_2$-) 3.03 (3H, m, H_2NNV-CH-C**H**$_2$-S- + -S-C**H**$_2$-CH-tBuFmoc) 3.76 (1H, m, H_2NNV-C**H**-CH_2-S-) 3.90 (3H, s, -OC**H**$_3$) 3.95 (3H, s, -OC**H**$_3$) 4.18 (1H, m, -C**H**-CH_2-OCONH-) 4.33 (2H, m, -CH-C**H**$_2$-OCONH-) 4.50 (1H, m, tBuFmoc-C**H**-CH_2-S-) 5.39 (1H, d, -CO_2-C**H**$_2$-Ar, *J* 14.4 Hz) 5.52 (1H, d, -CO_2-C**H**$_2$-Ar, *J* 14.4 Hz) 6.02 (1H, d, -OCON**H**-, *J* 7.4 Hz) 6.97 (1H, s, Ar**H 8**) 7.29 (2H, t, Ar**H 2**, *J* 7.6 Hz) 7.38 (2H, t, Ar**H 3**, *J* 7.4 Hz) 7.57 (2H, d, Ar**H 1**, *J* 7.6 Hz) 7.64 (1H, s, Ar**H 7**) 7.74 (2H, d, Ar**H 4**, *J* 7.4 Hz)

6. 3. (R)-N-*(4,5-Dimethoxy-2-nitrobenzyloxycarbonyl)alanyl* (R)-tert-*butyl*-N-*(9-fluorenylmethyloxycarbonyl)alanyl sulfide* (26)



(*R*)-(4,5-dimethoxy-2-nitrobenzyl)-*N*-triphenylmethylalanyl (*R*)-*tert*-butyl-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(24)** (102 mg, 0.11 mmol) was dissolved in dry dichloromethane (4 ml). Trifluoroacetic acid (0.033 ml, 0.44 mmol, 4 eq.) was added to the solution and the reaction mixture was stirred for 4 hr 30 at room temperature. Neutralisation was then carried out at 0 °C with *N*-methyl morpholine (0.024 ml, 0.22 mmol, 2 eq.). The solvents were removed *in vacuo* and the crude (*R*)-(4,5-dimethoxy-2-nitrobenzyl)alanyl (*R*)-*tert*-butyl-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(25)** obtained was dissolved in THF (0.5 ml). *N*-Methylmorpholine (0.024 ml, 0.22 mmol, 2 eq.) was added, followed by a solution of 4,5-dimethoxy-2-nitrobenzyl chloroformate (60 mg, 0.22 mmol, 2 eq.) in THF (0.5 ml). The reaction mixture was stirred for 2 hr at room temperature.

Removal of the solvents *in vacuo* followed by purification by flash column chromatography (1.5 cm, hexane: ethyl acetate, 4: 1) gave the title compound as a yellow paste (31 mg, 39 %).

(*R*)-(4,5-dimethoxy-2-nitrobenzyl)-*N*-triphenylmethylalanyl (*R*)-*tert*-butyl-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(24)** (50 mg, 0.054 mmol) was dissolved in dry dichloromethane (4 ml). Trifluoroacetic acid (0.017 ml, 0.22 mmol, 4 eq.) was added to the solution and the reaction mixture was stirred for 4 hr 30 at room temperature. Neutralisation was then carried out at 0 °C with *N*-methyl morpholine (0.03 ml, 0.22

mmol, 2 eq.). A further addition of *N*-methylmorpholine (2 eq., 0.11mmol, 0.015 ml) was followed by an addition of 4,5-dimethoxy-2-nitrobenzyl chloroformate (2.4 eq., 0.13 mmol, 37 mg) in dichloromethane (2 ml). The reaction mixture was stirred overnight under nitrogen at room temperature.

The crude mixture was dissolved in dichloromethane (50 ml) and washed twice with an aqueous solution of citric acid (5%, w/v, 2 x 10 ml) then water (10 ml). The organic layer was dried over sodium sulphate and the solvents were removed *in vacuo* to give a dark yellow oil.

Purification by flash column chromatography (1.5 cm, hexane: ethyl acetate, 2: 1) gave the title compound as a yellow paste (39 mg, 100 %).

R_F: 0.55 (hexane: ethyl acetate, 1: 1), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

mass spectrum ($APCI^+$): m/z 722 (M^+ - 3) 96 %, 723 (M^+ - 2) 36 %, 724 (M^+ - 1) 12 %, 725 (M^+) 3 %

Depending on the batches of lanthionine **(24)** used, two types of compounds were synthesised in the course of these reactions, having the same mass spectroscopy but slightly different n.m.r. spectra.

Compound A:

^{1}H n.m.r.: δ ($CDCl_3$, 300 MHz) 1.47 (9H, s, -C(C**H**$_3$)$_3$) 2.95 (1H, m, tBuFmoc-CH-C**H**$_2$-S-) 3.23 (1H, m, tBuFmoc-CH-C**H**$_2$-S-) 3.67 (2H, m, NVOC-CH-C**H**$_2$-S-) 3.82 (1H, m, NVOC-C**H**-CH_2-S-) 3.96 (6H, m, -OC**H**$_3$) 4.19 (1H, t, -C**H**-CH_2-OCONH-, *J* 7.0 Hz) 4.39 (2H, m, -CH-C**H**$_2$-OCONH-) 4.47 (1H, m, tBuFmoc-C**H**-CH_2-S-) 5.54 (2H, m, -CO_2-C**H**$_2$-Ar) 5.69 (1H, m, -N**H**-) 7.02 (1H, s, Ar**H 6**) 7.29 (2H, m, Ar**H 2**) 7.39 (2H, t, Ar**H 3**, *J* 7.4 Hz) 7.58 (2H, d, Ar**H 1**, *J* 7.1 Hz) 7.68 (1H, s, Ar**H 5**) 7.74 (2H, d, Ar**H 4**, *J* 7.5 Hz)

Compound B:

^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 1.48 (9H, s, -C(C**H**$_3$)$_3$) 2.89 (1H, m, tBuFmoc-CH-C**H**$_2$-S-) 3.08 (1H, m, tBuFmoc-CH-C**H**$_2$-S-) 3.16 (2H, m, NVOC-CH-C**H**$_2$-S-) 3.95 (6H, m, -OC**H**$_3$) 4.20 (1H, t, -C**H**-CH_2-OCONH-, *J* 7.3 Hz) 4.37 (2H, m, -CH-C**H**$_2$-OCONH-)

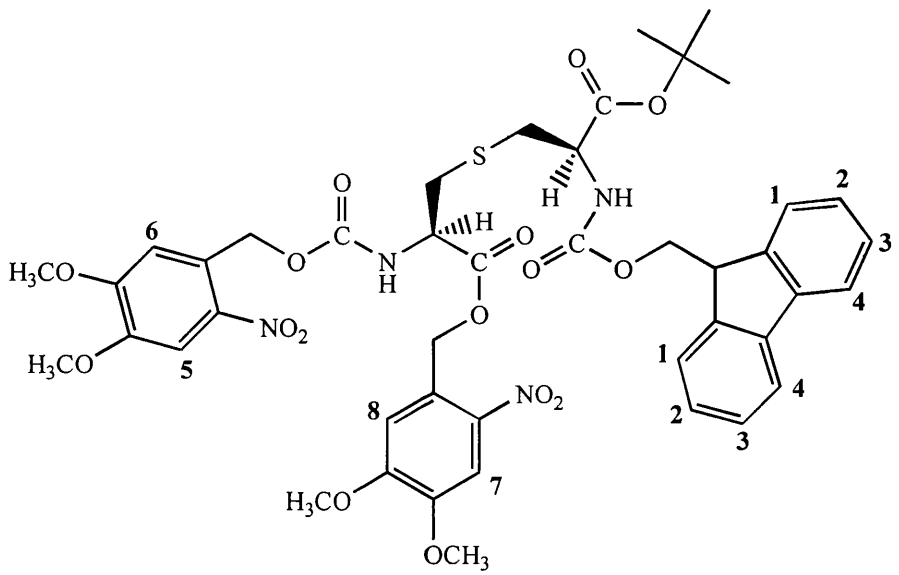
4.44 (1H, m, tBuFmoc-C**H**-CH_2-S-) 4.85 (1H, m, NVOC-C**H**-CH_2-S-) 5.59 (4H, m, -CO_2-C**H**$_2$-Ar + -N**H**-NVOC + -N**H**-Fmoc) 6.98 (1H, s, Ar**H 6**) 7.30 (2H, m, Ar**H 2**) 7.41 (2H, m, Ar**H 3**) 7.57 (2H, d, Ar**H 1**, *J* 7.4 Hz) 7.68 (1H, s, Ar**H 5**) 7.75 (2H, m, Ar**H 4**)

Some test reactions were carried out in the same conditions using compound **(24)**.

(*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-triphenylmethylalanyl (*R*)-*tert*-butyl-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(24)** (4 mg) was dissolved in DCM (1 ml). *N*-Methylmorpholine (0.001 ml, 2 eq.) was added to the solution. The reaction mixture was stirred for 22 hr. T.l.c. analyses revealed that the starting material was not changed after that time.

(*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-triphenylmethylalanyl (*R*)-*tert*-butyl-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(24)** (4 mg) was dissolved in DCM (1 ml). *N*-Methylmorpholine (0.001 ml, 2 eq.) and 4,5-dimethoxy-2-nitrobenzyl chloroformate (2 mg, 2.5 eq.) dissolved in DCM (0.5 ml) were added to the solution. The reaction mixture was stirred for 22 hr. T.l.c. analyses revealed that the starting material was not changed after that time.

6. 4. (R)-*(4,5-Dimethoxy-2-nitrobenzyl)*-N-*(4,5-dimethoxy-2-nitrobenzyloxy-carbonyl)alanyl* (R)-tert-*butyl*-N-*(9-fluorenylmethyloxycarbonyl)alanyl sulfide* (27)



(*R*)-(4,5-dimethoxy-2-nitrobenzyl)-*N*-triphenylmethylalanyl (*R*)-*tert*-butyl-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(24)** (262 mg, 0.284 mmol) was dissolved in dry dichloromethane (8 ml). Trifluoroacetic acid (0.088 ml, 1.14 mmol, 4 eq.) was added to the solution and the reaction mixture was stirred for 4 hr 30 at room temperature. Toluene was added to the mixture and the solvents were removed *in vacuo*. The crude mixture was used in the next step without further purification, after repeated co-evaporation with toluene to remove the last traces of trifluoroacetic acid.

(*R*)-(4,5-Dimethoxy-2-nitrobenzyl)alanyl (*R*)-*tert*-butyl-*N*-(9-fluorenylmethyloxy-carbonyl)alanyl sulfide **(25)** (crude from the previous step, th. 0.284 mmol) was dissolved in dioxane (2 ml). A solution of sodium bicarbonate (120 mg, 1.42 mmol, 5 eq.) in water (4 ml) was added, followed by a solution of 4,5-dimethoxy-2-nitrobenzyl chloroformate (157 mg, 0.57 mmol, 2 eq.) in dioxane (4 ml). The reaction mixture was stirred for 2 hr at room temperature.

The crude mixture was partitioned between dichloromethane (100 ml) and an aqueous solution of sodium hydrogen sulphate (1 N, 8 ml) and the organic layer was washed three times with an aqueous solution of sodium hydrogen sulphate (1 N, 3 x 8 ml). After drying of the organic layer over sodium sulphate followed by removal of the solvents *in vacuo* and purification by flash column chromatography (2.5 cm, hexane: ethyl acetate, 2: 1), the title compound was obtained as a very pale yellow light solid (261 mg, 0.284 mmol, 100 %).

The yield dropped significantly when the crude mixture was concentrated *in high vacuo* before work-up.

Recrystallisation of the title compound can be carried out in chloroform: methanol (1: 4) at -20°C.

$[\alpha]_D$ +1.1° (18°C, c 9.15 mg/ml, $CHCl_3$)

Found: C, 56.9; H, 5.3; N, 5.9; S, 3.2. $C_{44}H_{49}N_4O_{16}S$ requires C, 57.4; H, 5.3; N, 6.1; S, 3.5

ν_{max} ($CHCl_3$) 3431 (NH), 3029 (CH stretching), 1729 (C=O, broad), 1584 (aromatic), 1524 (C-NO_2)

^{13}C n.m.r.: δ ($CDCl_3$, 75.47 MHz) 28.3, 34.7, 42.1, 47.1, 47.3, 54.8, 56.7, 56.8, 57.0, 64.2, 64.6, 67.8, 83.8, 108.4, 108.6, 110.2, 110.9, 111.1, 120.3, 125.4, 127.4, 128.1,

128.4, 140.2, 140.3, 141.6, 144.0, 144.1, 148.3, 148.8, 153.9, 154.1, 156.3, 169.5, 170.7, 171.6

mass spectrum ($APCI^+$): m/z 196 (NV^+) 100 %, 865 (M^+ - tBu + 2H) 11 %, 921 (M^+ + H) 5 %, 938 ($M^+ + H_2O$) 8 %

LC-MS reverse-phase: 12.31 min + 12.53 min (60 % to 90 % B in 20 min) ($APCI^+$): m/z 921 (M^+ + H) 28 %, 943 (M^+ + Na) 72 % and 921 (M^+ + H) 25 %, 943 (M^+ + Na) 100 %

LC-MS normal-phase: 7.80 min + 9.62 min (50 % B') ($APCI^+$): m/z 943 (M^+ + Na) 5 % and 921 (M^+ + H) 1 %, 943 (M^+ + Na) 5 %

Found: (M^+ + Na), 943.2684. $C_{44}H_{48}N_4O_{16}SNa$ requires 943.2700 (FAB)

Melting point: 76-78°C

Two types of lanthionine **(27)** (A: B, 9: 2) were synthesised in the course of these reactions, having the same mass spectroscopy but slightly different n.m.r. spectra.

Compound A: R_F: 0.85 (neat ethyl acetate), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

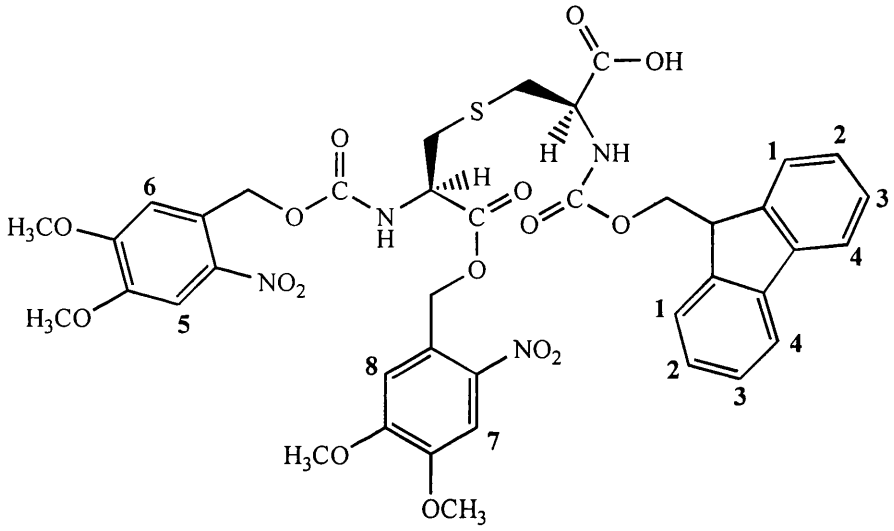
^{1}H n.m.r.: δ ($CDCl_3$, 300 MHz) 1.47 (9H, m, -C(C**H**$_3$)$_3$) 3.02 (1H, m, tBuFmoc-CH-C**H**$_2$-S-) 3.24 (1H, m, tBuFmoc-CH-C**H**$_2$-S-) 3.64 (2H, m, NVOCNV-CH-C**H**$_2$-S-) 3.75 (1H, m, NVOCNV-C**H**-CH_2-S-) 3.93 (12H, m, -OC**H**$_3$) 4.18 (1H, m, -C**H**-CH_2-OCONH-) 4.32 (2H, m, -CH-C**H**$_2$-OCONH-) 4.52 (1H, m, tBuFmoc-C**H**-CH_2-S-) 5.46 (4H, m, -CO_2-C**H**$_2$-Ar) 5.72 (1H, m, -N**H**-, *J* 7.7 Hz) 5.81 (1H, d, -N**H**-, *J* 7.2 Hz) 6.92 + 6.97 (1H, 2s, Ar**H 8**) 7.04 (1H, s, Ar**H 6**) 7.27 (2H, m, Ar**H 2**) 7.37 (2H, t, Ar**H 3**, *J* 6.9 Hz) 7.55 (2H, d, Ar**H 1**, *J* 7.1 Hz) 7.68 (4H, m, Ar**H 4** + Ar**H 5** + Ar**H 7**)

Compound B: R_F: 0.9 (neat ethyl acetate), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

^{1}H n.m.r.: δ ($CDCl_3$, 300 MHz) 1.51 (9H, m, -C(C**H**$_3$)$_3$) 2.91 (1H, dd, tBuFmoc-CH-C**H**$_2$-S-, *J* 14.0, 6.7 Hz) 3.14 (3H, m, tBuFmoc-CH-C**H**$_2$-S- + NVOCNV-CH-C**H**$_2$-S-) 3.94 (12H, m, -OC**H**$_3$) 4.20 (1H, t, -C**H**-CH_2-OCONH-, *J* 6.8 Hz) 4.37 (2H, m, -CH-C**H**$_2$-OCONH-) 4.51 (1H, m, tBuFmoc-C**H**-CH_2-S-) 4.62 (1H, m, NVOCNV-C**H**-CH_2-S-) 5.50 (4H, m, -CO_2-C**H**$_2$-Ar) 5.72 (1H, d, -N**H**-, *J* 7.6 Hz) 6.12 (1H, d, -N**H**-, *J* 7.4 Hz)

6.93 + 6.98 (1H, 2s, Ar**H 8**) 7.04 (1H, s, Ar**H 6**) 7.27 (2H, m, Ar**H 2**) 7.38 (2H, t, Ar**H 3**, *J* 7.4 Hz) 7.56 (2H, d, Ar**H 1**, *J* 7.1 Hz) 7.68 (4H, m, Ar**H 4** + Ar**H 5** + Ar**H 7**)

6. 5. (R)-*(4,5-Dimethoxy-2-nitrobenzyl)*-N-*(4,5-dimethoxy-2-nitrobenzyloxy-carbonyl)alanyl* (R)-N-*(9-fluorenylmethyloxycarbonyl)alanyl sulfide* (28)



(*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-(4,5-dimethoxy-2-nitrobenzyloxycarbonyl)-alanyl (*R*)-*tert*-butyl-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(27)** (243 mg, 0.264 mmol) was dissolved in dry dichloromethane (4 ml), trifluoroacetic acid was added to the solution (4 ml) and the reaction mixture was stirred for 2 hr.

After removal of the solvents *in vacuo*, a yellow powder was obtained as the title compound (228 mg, 0.264 mmol, 100 %).

Further purification of the title compound can be carried out by reverse-phase column chromatography (3 cm, acetonitrile: sodium hydrogen carbonate saturated solution: water, 0 %: 20 %: 80 % to 35 %: 13 %: 52 %)

R_F: 0.63 (neat ethyl acetate + traces of TFA), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

$[\alpha]_D$ +22.2° (18°C, c 12.9 mg/ml, $CHCl_3$)

Found: C, 55.9; H, 4.8; N, 6.6; S, 3.2. $C_{40}H_{40}N_4O_{16}S$ requires C, 55.6; H, 4.7; N, 6.5; S, 3.7

ν_{max} ($CHCl_3$) 3022 (CH stretching), 1724 (C=O broad), 1583 (aromatic), 1522 (C-NO_2)

^{1}H n.m.r.: δ (DMSO, 400 MHz) 2.91 (1H, m, Fmoc-CH-C**H**$_2$-S-) 3.13 (1H, m, Fmoc-CH-C**H**$_2$-S-) 3.39 (1H, m, NVOCNV-CH-C**H**$_2$-S-) 3.48 (1H, m, NVOCNV-CH-C**H**$_2$-S-) 3.79 (1H, m, NVOCNV-C**H**-CH_2-S-) 3.86 (12H, m, -OC**H**$_3$) 4.20 (3H, m, -C**H**-CH_2-OCONH Fmoc + -CH-C**H**$_2$-OCONH- Fmoc) 4.25 (1H, m, Fmoc-C**H**-CH_2-S-) 5.32 (2H, s, -CO_2-C**H**$_2$-Ar NV) 5.42 (3H, m, -CO_2-C**H**$_2$-Ar NV + -N**H**-) 7.17 (2H, m, Ar**H 6** + Ar**H 8**) 7.31 (2H, t, Ar**H 2**, *J* 7.4 Hz) 7.41 (2H, t, Ar**H 3**, *J* 7.4 Hz) 7.67 (4H, m, Ar**H 1** + Ar**H 5** + Ar**H 7**) 7.87 (2H, d, Ar**H 4**, *J* 7.5 Hz)

^{13}C n.m.r.: δ (DMSO, 100 MHz) 46.8, 55.1, 56.2, 56.3, 62.9, 63.6, 66.0, 108.2, 110.4, 120.3, 125.5, 126.4, 127.2, 127.8, 128.0, 139.2, 139.3, 140.9, 143.9, 144.0, 147.8, 148.0, 153.6, 156.0, 156.3, 170.4, 170.8, 172.1, 172.4

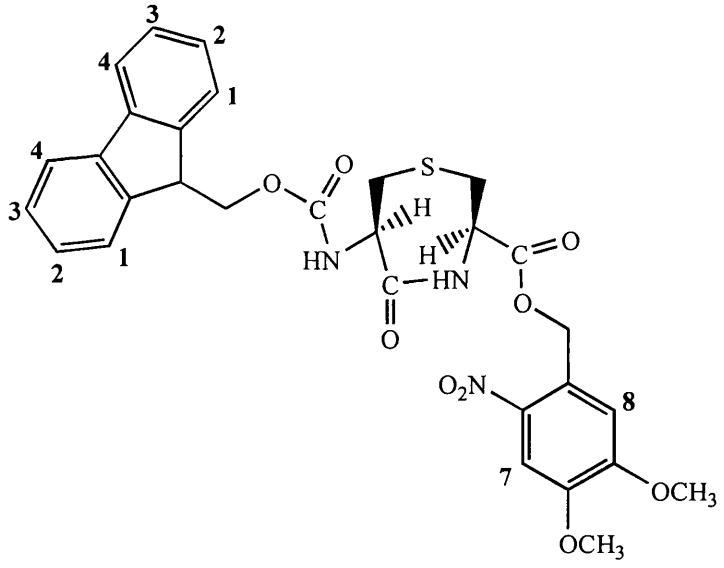
mass spectrum (APCI$^-$): m/z 863 (M^- - H) 100 %, 864 (M^-) 37 %

LC-MS: 15.85 min (50 % to 90 % B in 30 min) (APCI$^+$): m/z 865 (M^+ + H) 1 %, 887 (M^+ + Na) traces

Found: (M^+ + Cs), 997.1214. $C_{40}H_{40}N_4O_{16}SCs$ requires 997.1230 (FAB)

Melting point: 92-95°C

6. 6. (R, R)-*(4,5-Dimethoxy-2-nitrobenzyl)*-N-*(9-fluorenylmethyloxycarbonyl)-cyclolanthionine* (29)



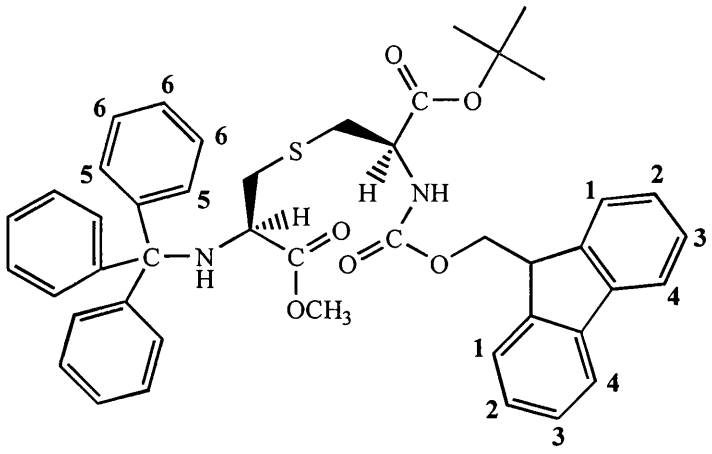
(*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-triphenylmethylalanyl (*R*)-*tert*-butyl-*N*-(9-fluorenylmethyloxycarbonylalanyl) sulfide **(24)** (68 mg, 0.0736 mmol) was dissolved in DCM (1.5 ml) and trifluoroacetic acid (1.5 ml) was added to the solution. After stirring for 30 min, the solvents were removed *in vacuo* and the crude residue was suspended in DMF (3 ml). Diisopropylethylamine (0.026 ml, 0.15 mmol, 2 eq.) was added dropwise to the solution. The pH of the solution changed from 4-5 to 8-9. Carbonyldiimidazole (24 mg, 2 eq.) was dissolved in DMF (1 ml) and this solution was added to the basic solution. The reaction mixture was stirred for 24 hr at room temperature.

The crude mixture was partioned between ethyl acetate (20 ml) and an aqueous solution of citric acid (10 %, w/v, 20 ml) then the aqueous layer was extracted again with ethyl acetate (20 ml) and ether (2 x 20 ml). The combined organic layers were washed with an aqueous solution of citric acid (10 %, w/v, 2 x 10 ml), water (2 x 10 ml) and brine (10 ml). They were then dried over sodium sulphate. After removal of the solvents *in vacuo*, a yellow paste was recovered. Purification was carried out by flash column chromatography (1.5 cm, hexane: ethyl acetate, 3: 2, gradient). Several fractions were recovered which contained the title compound and several other by-products, as identified by mass spectroscopy.

mass spectrum ($APCI^+$): m/z 608 ($M^+ + H$) 2 %, 729 (starting material after cleavage of the NV ester) 2 %

7. Photolabile cleavage studies

7. 1. Photolabile cleavage studies on lanthionine (24)



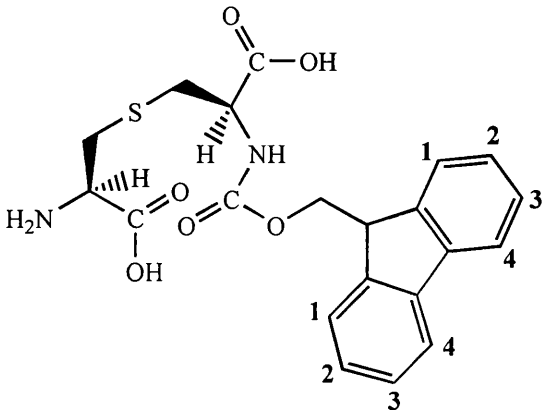
(*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-triphenylmethylalanyl (*R*)-*tert*-butyl-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(24)** (93 mg, 0.1 mmol) was dissolved in dry methanol (5 ml) inside **lamp 1** and the solution was irradiated for 1 hr, then 2 hr, then 6 hr 30 until t.l.c. indicated a change in the solution. After removal of the solvents *in vacuo*, purification of the crude product was carried out by flash column chromatography (1.5 cm, hexane: ether, 2:1, gradient). (*R*)-Methyl-*N*-triphenylmethylalanyl (*R*)-*tert*-butyl-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(30)** was recovered as a yellow paste (9 mg, 12 %).

R_F: 0.33 (hexane: ether, 1: 1), visualisation by absorption of U.V. light and by spraying with basic permanganate solution

^{1}H n.m.r.: δ ($CDCl_3$, 400 MHz) 1.46 (9H, m, -C(C**H**$_3$)$_3$) 2.43 (1H, m, TrtMe-CH-C**H**$_2$-S-) 2.61 (1H, m, TrtMe-CH-C**H**$_2$-S-) 2.89 (1H, m, -S-C**H**$_2$-CH-tBuFmoc) 3.02 (1H, m, -S-C**H**$_2$-CH-tBuFmoc) 3.51 (1H, m, TrtMe-C**H**-CH$_2$-S-) 3.72 (3H, m, -COOC**H**$_3$) 4.22 (1H, m, -C**H**-CH$_2$-OCONH-) 4.37 (2H, m, -CH-C**H**$_2$-OCONH-) 4.49 (1H, m, tBuFmoc-C**H**-CH$_2$-S-) 5.58 (1H, d, -N**H**-, *J* 8.6 Hz) 5.69 (1H, d, -N**H**-, *J* 8.4 Hz) 7.17 (2H, m, Ar**H 2**) 7.23-7.29 (9H, m, Ar**H 6**) 7.38 (2H, t, Ar**H 3**, *J* 7.4 Hz) 7.45 (6H, m, Ar**H 5**) 7.56 (2H, m, Ar**H 1**) 7.75 (2H, d, Ar**H 4**, *J* 7.3 Hz)

mass spectrum (FAB): m/z 243 (Trt^+) 100 %, 765 (M^+ + Na) 41 %

7. 2. Photolabile cleavage studies on lanthionine (28)



(a) (*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-(4,5-dimethoxy-2-nitrobenzyloxy-carbonyl)alanyl (*R*)-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(28)** (10 mg, 0.016 mmol) was dissolved in methanol (2 ml) inside **lamp 1** and the solution was irradiated for 3 hr. As there was no significant change by t.l.c., solvents were evaporated *in vacuo* and the crude mixture was dissolved in a mixture of acetonitrile and methanol (3 ml and 170 ml) the following day and set up again in a similar manner for 5 hr 30. After removal of the solvents *in vacuo*, the crude mixture obtained was purified by HPLC. After 8 hr 30, among impurities, (*R*)-(4,5-dimethoxy-2-nitrobenzyl)alanyl (*R*)-*N*-(9-fluorenylmethyl-oxycarbonyl)alanyl sulfide was identified, as well as (*R*)-alanyl (*R*)-*N*-(9-fluorenyl-methyloxycarbonyl)alanyl sulfide **(31)**.

mass spectrum (APCI$^+$): m/z 626 ((*R*)-(4,5-dimethoxy-2-nitrobenzyl)alanyl (*R*)-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide, M^+) 1 %, 478 (**(31)**, $M^+ + H + 2 Na$) 7 %

(b) (*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-(4,5-dimethoxy-2-nitrobenzyloxy-carbonyl)alanyl (*R*)-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(28)** (10 mg, 0.016 mmol) was dissolved in acetonitrile and methanol (5 ml and 150ml) inside **lamp 1** and the solution was irradiated with **lamp 1** and **lamp 2** for 7 hr 30. Solvents were evaporated *in vacuo* and the crude mixture obtained was purified by HPLC. Among numerous impurities, lanthionine **(31)** was revealed by mass spectrometry.

mass spectrum (APCI$^+$): m/z 430 (**(31)**, M^+) 1 %, 608 (by-product, M^+) 2 %

(c) (*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-(4,5-dimethoxy-2-nitrobenzyloxy-carbonyl)alanyl (*R*)-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(28)** (8.6 mg, 0.01 mmol) was dissolved in a mixture of acetonitrile (3 ml) and an aqueous solution of potassium acetate (5 ml, 0.033M) inside **lamp 1** and the solution was irradiated for 1 hr 15, then 2 hr after purging the solution for 45 min with nitrogen. Solvents were evaporated *in vacuo* and the remaining crude lyophilised overnight. This crude mixture was dissolved in a mixture of acetonitrile and water (5 ml and 5 ml) for 3 hr 30 the day after and the lamp set up again. After a total of 6 hr 45, analyses revealed some photolabile cleavage had taken place.
mass spectrum (FAB): m/z 429 (**(31)**, M^+ - H) 14 %

(d) (*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-(4,5-dimethoxy-2-nitrobenzyloxy-carbonyl)alanyl (*R*)-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(28)** (10 mg, 0.016 mmol) was dissolved in a mixture of acetonitrile (5 ml) and an aqueous solution of potassium acetate (5 ml, 0.0385 M) inside **lamp 1** and the solution was irradiated for 1 hr, then 1 hr. Solvents were evaporated *in vacuo*. This crude mixture was dissolved in a mixture of acetonitrile and water (8 ml, 1: 1) and set up again in a similar manner for 4 hr 30. After 2 hr, analyses revealed no change had taken place. After 6 hr, analyses revealed that a partial photolabile cleavage had taken place.
mass spectrum ($APCI^+$): m/z 478 (**(31)**, $M^+ + H + 2 Na$) 18 %

(e) (*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-(4,5-dimethoxy-2-nitrobenzyloxy-carbonyl)alanyl (*R*)-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(28)** (10 mg, 0.016 mmol) was dissolved in acetonitrile (10 ml) and an aqueous solution of potassium acetate (10 ml, 0.38 %) inside **lamp 1** and the solution was irradiated with **lamp 2** for a total of 12 hr, with samples taken every 2 hr and the solvents evaporated *in vacuo* before the night and freshly replaced the following day. The samples were analysed by HPLC. Among numerous impurities, the disappearance of the starting material was clearly visible (analytical HPLC, retention time 24 min, 40 to 80 % B in 35 min, only 3 % left

after 12 hr). Very weak or non-existent signals were seen with $APCI^+$ maybe due to the presence of TFA used for HPLC, so more analytical HPLC analyses were carried out with the same solvents but with 0.01 % TEA instead of TFA. Lanthionine **(31)** was identified alongside the starting material **(28)** after 4 hr and 8 hr of photolabile cleavage. After 12 hr, only lanthionine **(31)** was visible.
LC-MS: 19.82 min and 22.02 min (5 % to 70 % B in 35 min, 0.01% TEA in solvents) ($APCI^-$): m/z 428 (**(31),** M^- - 2H) 7 % and 863 (**(28),** M^- - H) 40 %

(f) (*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-(4,5-dimethoxy-2-nitrobenzyloxy-carbonyl)alanyl (*R*)-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(28)** (5 mg, 0.008 mmol) was dissolved in dioxane (10 ml) and an aqueous solution of potassium acetate (2 ml, 0.5 %) inside **lamp 1** and the solution was irradiated with **lamp 2** for 5 hr 30. Analytical HPLC showed that no starting material remained after this time.

8. Preparation for SPPS: tests on the basic cleavage of NV ester

(a) (*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-(4,5-dimethoxy-2-nitrobenzyloxy-carbonyl)alanyl (*R*)-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(28)** (36 mg, 0.0416 mmol) was dissolved in 20 % piperidine in DMF (0.2 ml/0.8ml). The reaction mixture was stirred for 20 min.

The reaction mixture was partioned between ethyl acetate (80 ml) and a phosphate buffer (pH = 5.8, 7.9 ml of 1 M Na_2HPO_4 + 92.1 ml of 1 M NaH_2PO_4, 5 ml). The organic layer was washed with the same buffer (5 ml) and the combined aqueous layers were extracted once more with ethyl acetate (20 ml). The combined organic layers were dried over sodium sulphate and after removal of the solvents *in vacuo*, the crude obtained was analysed without further purification.

(b) (*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-(4,5-dimethoxy-2-nitrobenzyloxy-carbonyl)alanyl (*R*)-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(28)** (38 mg, 0.0439 mmol) was dissolved in 2 % piperidine and 2 % DBU in DMF (0.02 ml/0.02 ml/0.96 ml). The reaction mixture was stirred for 20 min.

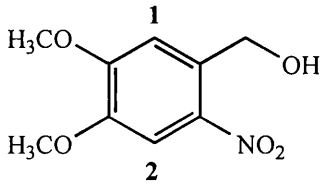
The reaction mixture was partioned between ethyl acetate (80 ml) and a phosphate buffer (pH = 5.8, 7.9 ml of 1 M Na_2HPO_4 + 92.1 ml of 1 M NaH_2PO_4, 5 ml). The organic layer was washed with the same buffer (5 ml) and the combined aqueous layers were extracted once more with ethyl acetate (20 ml). The combined organic layers were dried over sodium sulphate and after removal of the solvents *in vacuo*, the crude obtained was analysed without further purification.

(c) (*R*)-(4,5-Dimethoxy-2-nitrobenzyl)-*N*-(4,5-dimethoxy-2-nitrobenzyloxy-carbonyl)alanyl (*R*)-*N*-(9-fluorenylmethyloxycarbonyl)alanyl sulfide **(28)** (10 mg, 0.0116 mmol) was dissolved in DMF (0.95 ml). DBU was then added to the solution (5 %, 0.05 ml). The reaction mixture was stirred for 10 min.

The reaction mixture was partioned between ethyl acetate (80 ml) and water (10 ml). The organic layer was washed with water (3x 10 ml) and the combined aqueous layers were extracted once more with ethyl acetate (20 ml). The combined organic layers were dried

over sodium sulphate and after removal of the solvents *in vacuo*, the crude obtained was analysed without further purification.

In all cases, 6-nitroveratryl alcohol was identified by n.m.r. analyses.



^{1}H n.m.r.: δ ($CDCl_3$, 500 MHz) 3.94 (3H, s, OC**H$_3$**) 3.99 (3H, s, OC**H$_3$**) 4.96 (2H, s, -C**H$_2$**-OH) 7.00 (1H, s, Ar**H 1**) 7.74 (1H, s, Ar**H 2**)

9. SPPS of a sandostatin analog

9. 1. Batchwise SPPS

9. 1. 1. First attempt

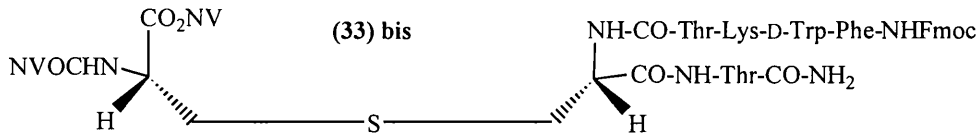
The synthesis was carried out on a 0.1 mmol scale in a Merrifield bubbler, on a Rink Amide MBHA resin (0.1 mmol, 0.54 mmol/g loading). The following amino acid side-chain protecting groups were used: Thr(OtBu), Lys(Boc), Trp(Boc).

The first threonine (Thr 8) and lanthionine **(28)** were both double-coupled as their HOBt-active esters (0.25 mmol, HOBt 2.5 eq., 2.5 M in DMF). DIC (2.5 eq., 2.5 M in dioxane) was used as the activating agent (activation 15 to 20 min before addition of the solution to the resin). Coupling times were of 30 to 45 min for Thr 8 and of 1 hr for lanthionine **(28)**. Pentafluorophenyl activated esters of the other residues were used for the rest of the synthesis alongside HOBt (2.5 mmol, HOBt 2.5 eq., 2.5 M in DMF). Coupling times were of 30 to 45 min. Most couplings were double coupling, except for Lys and Phe when a poor Kaiser test after the second coupling made a third attempt necessary.

Each coupling step except the last one (for D-Phe) was followed by a capping step using a solution of acetic anhydride (0.5 M Ac_2O, 0.125 M DIEA, 0.2 % HOBt in DMF, 2 ml). The Fmoc group was then removed with 20 min treatment with 20 % piperidine in DMF (2 ml), with extensive washing with DMF between each deprotection.

A qualitative Kaiser test was performed after each incorporation of amino acid and after each Fmoc deprotection. It was made with a small amount of resin in 2 drops of solutions A, B, C respectively (solution A: phenol (20 g) in absolute ethanol (5 ml), solution B : an aqueous solution of potassium cyanide (1 mM, 1 ml) in pyridine (49 ml), solution C : ninhydrin (500 mg) in absolute ethanol (10 ml)) mixed together and heated at 100°C for 2 min. A blue colour indicates presence of free amine, a yellow colour absence of free amine.

The linear peptide **(33)** was successfully synthesised by this method. Peptide **(33) bis** (peptide **(33)** after TFA treatment, cleaved from the resin and with no more side-chain protecting groups) was clearly identified by mass spectrometry.



mass spectrum (ESP^+): m/z 1528 ($M^+ + H$) 4 %, 1551 ($M^+ + H + Na$) 3 %, 1574 ($M^+ + H + 2Na$) 3 %

The photolabile cleavage was carried out in the vessel of **lamp 1**, with **lamp 2** projected on it. The resin was suspended in a mixture of dioxane (30 ml) and an aqueous solution of potassium acetate (1 ml, 1 %, w/v) and nitrogen was bubbled through the solution for at least 30 min prior to the experiment to degas the solution and throughout the experiment to agitate the resin. At the end of each day, the solution was removed from the vessel with a cannula and the resin stored under Ar overnight.

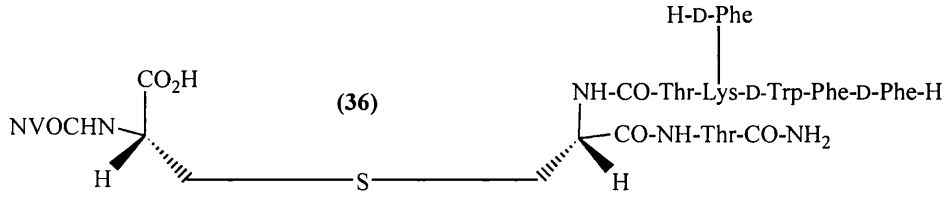
Photolabile cleavage was carried out for a total of 10 hr, at the end of which a red burgundy colour was clearly visible with a Kaiser test. The resin was partioned between two halves and cyclisation and incorporation of D-Phe were carried out on one half of the resin only, the other half being kept under Ar in the fridge. Cyclisation was carried out using HATU/HOAt/DIEA (2 eq., 0.2 M/2 eq., 0.2 M/4 eq., 0.4 M) for 3 hr.

After 10 hr of photolabile cleavage, only small changes were observed by HPLC and the subsequent mass spectroscopy revealed that the photolabile cleavage was only partial, indeed peptide **(33) bis** was still identified.
mass spectrum (ESP^+): m/z 1551 ($M^+ + H + Na$) 3 %

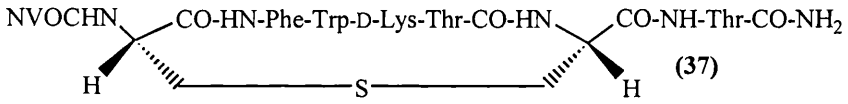
Cleavage of the peptide from the resin and final deprotection was carried out using a 96 % TFA / 2 % water / 2 % thioanisole solution in the following manner: TFA and water (5 x 9.8 ml) were added to the resin and bubbled with the resin for 10 min at a time, then this solution was collected on thioanisole (1 ml). The collected solution was stirred for 4 hr after cleavage of the peptide from the resin, for cleaving and scavenging

the side-chain protections on the peptide. The solvents were removed *in vacuo* and the residue obtained was recrystallised in ether. The crude peptide was purified by HPLC.

Analyses revealed that among other impurities, peptide **(36)** was probably present in this crude product as a peak at 1404 was observed.



A peak at 1093 revealed that a small amount of peptide **(37)** was probably also present in the crude mixture.



mass spectrum (FAB): m/z 1404 (**(36)**, M^+) 1 %

mass spectrum (ESP^+): m/z 1093 (**(37)**, (M^+ + 2H) 4 %, 1405 (**(36)**, (M^+ + H) 6 %

As it was evident from these results that the photolabile cleavage was only partial, further photolabile cleavage was carried out on the second half of the resin, in a similar manner, for a total of 30 hr, at the end of which a darker shade of burgundy was observed with the Kaiser test. Cyclisation and incorporation of D-Phe were carried out as before.

Only a small amount of crude product (15 mg) was recovered from TFA treatment and lyophilisation from an aqueous solution of acetic acid (40 %). Mass spectra and HPLC analyses were too noisy to enable any proper identification of the compounds made.

9. 1. 2. Second attempt

The linear peptide **(33)** was synthesised in a similar manner as before. Each residue was triple-coupled (3 x 45 min), except lanthionine **(28)** which was double-coupled (2 x 1 hr). Peptide **(33) bis** was clearly identified by mass spectroscopy.

mass spectrum (ESP^+): m/z 394 (**(33) bis**, (M^+ + 4H + 2Na)/4) 40 %

Photolabile cleavage was carried out in a similar manner as before, for a total of 70 hr, monitoring the presence of NVOC group left on the resin and the presence of NVOC released in the dioxane solution, by U.V. spectrometry, monitoring the presence of free amine by Kaiser test and monitoring both growing peptide and photolabile filtrate by HPLC.

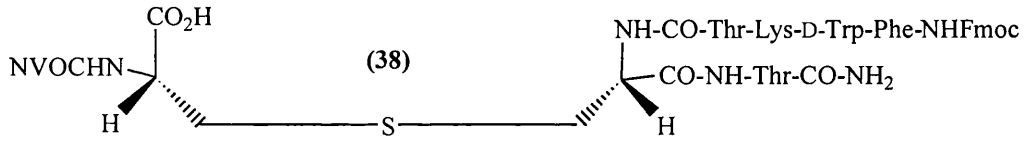
The Kaiser test was still yellow after 50 hr, pale brown after 60 hr and burgundy brown after 70 hr.

U.V. monitoring indicated that there was still some NV/NVOC released in the filtrate after 60 hr (characteristic absorption at 250 nm), but none after 70 hr. No NV/NVOC was identified on the resin either after 70 hr.

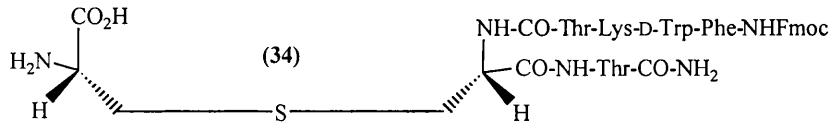
It was quite difficult to draw conclusions on the HPLC results, as a large amount of by-products were continuously present in the filtrate or small amount of test crude product obtained from acidic cleavage from the resin.

However, mass spectroscopy revealed that the photolabile cleavage was this time carried out to completion.

Peptide **(38)** was present on the resin after 45 hr.



Peptide **(34)** was also identified after 45 hr and remained present after 70 hr.



mass spectrum (ESP^+): after 15 hr photolabile cleavage: m/z 394 (**(33) bis**, (M^+ + 4H + 2Na)/4) 58 %

mass spectrum (ESP^+): after 45 hr photolabile cleavage: m/z 452 (**(38)**, (M^+ + 3H + Na)/3) 5 %, 570 (**(34)**, (M^+ + 2H + 2Na)/2) 3 %, 1527 (**(33) bis**, (M^+ + H) 2 %

mass spectrum (ESP^+): after 60 hr photolabile cleavage: m/z 570 (**(34)**, (M^+ + 2H + 2Na)/2) 4 %

mass spectrum (ESP^+): after 70 hr photolabile cleavage: m/z 570 (**(34)**, (M^+ + 2H + 2Na)/2) 0.5 %

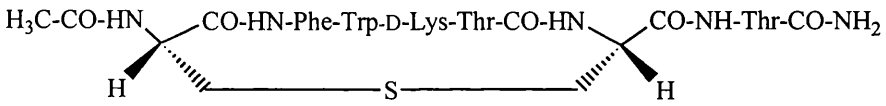
Cyclisation was carried out using PyAOP/HOAt/DIEA (4 eq., 0.4 M/4 eq., 0.4 M/8 eq., 0.8 M) for 2 hr. After incorporation of D-Phe in a similar manner as before, the final peptide was cleaved from the resin by TFA treatment and after lyophilisation, a crude product was purified in four portions by preparative HPLC (10% to 90 % B in 40 min).

Two peptides **(39)** and **(40)** (11 to 13 min on preparative HPLC) were identified by mass spectroscopy.

Peptide **(39)** derived from an unsuccessful cyclisation reaction.

(39)

Whereas peptide **(40)** derived from a successful cyclisation reaction.



(40)

mass spectrum (ESP^+): m/z 895 (**(40)**, (M^+ + H) 1 %, 1083 (**(39)**, (M^+ + H + Na) 1 %

9. 2. Tests on Tentagel resin

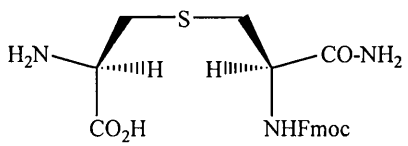
Lanthionine **(28)** (35.4 mg, 0.041 mmol) dissolved in DMF (1.3 ml) was coupled to NovaSyn TGR® resin (78 mg, 0.016 mmol, 1 eq., 0.21 mmol/g loading, previously swollen in DMF (0.5 ml)) as its HOBt-active ester (0.041 mmol, 2.5 eq., 2.5 M in DMF). DIC (0.041 mmol, 2.5 eq., 2.5 M in dioxane) was used as the activating agent (activation

20 min before addition of the solution to the resin). The mixture was kept under nitrogen for 2 hr with occasional agitation.
The resin was washed with DMF (3 x 5 ml) then ether (2 x 5 ml) then dried and kept in the fridge under Ar overnight.

Quantitation of the resin loading was carried out as follows: 20 % piperidine in DMF (0.5 ml) was added to the dried resin (9 mg). The mixture was left for 30 min. The mixture was then diluted with ethanol (19.5 ml) and allowed to stand for 5 min. An aliquot of the mixture was then placed in a U.V. cuvette (width 0.5 cm). The absorbance of this mixture was then measured at 300 nm (blank neat ethanol). The substitution level was found to be 0.174 mmol/g (substitution level (mmol/g) = 3.05 x A_{300} / m, with m: weight of dried resin and A_{300} the absorbance recorded at 300 nm).

The remaining amount of resin was placed in a U.V. cuvette and the cuvette was filled with the following solvent system (3 ml of 30 ml dioxane: 1 ml water: 10 mg KOAc solution). **Lamp 2** was projected on the cuvette for a total of 10 hr. A green coloration was observed with the Kaiser test.

The resin was placed in a sinter funnel and acidic treatment (96 % TFA / 2 % water / 2 % thioanisole) and lyophilisation of the crude peptide were carried out as before. The crude brown mixture obtained was then partially dissolved in chloroform. The residue which did not dissolve in chloroform was separated from the chloroform solution and precipitated in ether. Lanthionine residue **(41)** was identified by mass spectroscopy from this precipitate.



mass spectrum (ESP^+): m/z 860 ($2M^+ + H$) 3 %, 906 ($2M^+ + H + 2Na$) 5 %

9. 3. Continuous-flow SPPS

The synthesis was carried out on a 0.1 mmol scale on a Millipore 9050 PepSynthesizer^{TM}, on a NovaSyn TGR® resin (0.1 mmol, 0.21 mmol/g loading). The following amino acid side-chain protecting groups were used: Thr(OtBu), Lys(Boc), Trp(Boc).

Lanthionine **(28)** was double-coupled (2 x 30 min) as its HOBt active ester (200 mg, 2.3 eq., 0.23 mmol; HOBt 2.3 eq., 2.3 M in DMF). DIC (2.3 eq., 2.3 M in DMF) was used as the activating agent (activation 15 min before manual syringing of activated lanthionine **(28)** into the machine).

Pentafluorophenyl activated esters of the other residues were used for the synthesis alongside HOBt (4 mmol, HOBt 4 eq.). Coupling times were of 45 min. Each coupling step was followed by a capping step using *N*-acetyl imidazole. The Fmoc group was removed with 10 min treatment with 20 % piperidine in DMF. U.V. monitoring of the incorporation of each residue was carried out by detection of DBF in the reaction medium during incorporation and after Fmoc cleavage.

The linear peptide **(33)** was successfully synthesised and peptide **(33) bis** was identified by mass spectrometry.
mass spectrum (ESP^+): m/z 1528 ($M^+ + H$) 1 %

Photolabile cleavage was carried out with **lamp 3** for 30 hr. The resin was suspended in a mixture of dioxane and potassium acetate/acetic acid buffer (30 ml dioxane and 1 ml buffer); the solution was changed and the resin thoroughly rinsed every 5 hr. After 30 hr, both peptide **(33) bis** and **(34)** were identified by mass spectrometry.
mass spectrum (ESP^+): m/z 579 (**(34)**, ($M^+ + 2H + H_2O + 2Na$)/2) 2 %, 776 (**(33) bis**, ($M^+ + 2H + Na$)/2) 8 %

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