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Library of Norcoclaurine Synthases and Their Immobilization for Biocatalytic Transformations

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Norcoclaurine synthases (NCS), catalyzing a Pictet–Spengler reaction in plants as one of the first enzymes in the biosynthetic benzylisoquinoline pathway, are investigated for biocatalytic transformations. The library of NCS available is extended by two novel NCSs from *Argemone mexicana* (AmNCS1, AmNCS2) and one new NCS from *Corydalis saxicola* (CsNCS); furthermore, it is shown that the NCS from *Papaver bracteatum* (PbNCS) is a highly productive catalyst leading to the isoquinoline product with up to >99% e.e. Under certain conditions lyophilized whole *Escherichia coli* cells containing the various overexpressed NCS turned out to be suitable catalysts. The reaction using dopamine as substrate bears several challenges such as the spontaneous non-stereoselective background reaction and side reactions. The PbNCS enzyme is successfully immobilized on various carriers whereby EziG3 proved to be the best suited for biotransformations. Dopamine showed limited stability in solution resulting in the coating of the catalyst over time, which could be solved by the addition of ascorbic acid (e.g., 1 mg ml^{-1}) as antioxidant.

1. Introduction

Benzylisoquinoline alkaloids display various bioactivities and are therefore used for pharmaceutical applications. Probably the most well-known compounds in this class are the narcotic analgesic morphine, the cough suppressant codeine, the muscle relaxant papaverine, and sanguinarine and berberine, the latter two showing antimicrobial activities. These compounds can be found as natural products in secondary metabolic pathways of plants thought to serve as self-defense agents against animals, viruses, and bacteria. Their biosynthesis is achieved via sophisticated enzymatic pathways, starting from the amino acid tyrosine.[1,2]

Norcoclaurine synthase (NCS, EC 4.2.1.78) is one of the first enzymes located in these pathways, catalyzing a stereospecific Pictet–Spengler reaction between dopamine and 4-hydroxyphenylacetaldehyde yielding the benzylisoquinoline (*S*)-norcoclaurine (**Figure 1**).[3,4] The Pictet–Spengler reaction is enabled in nature by a handful of enzymes with different types of substrates.[5] Additionally to the NCS, there is the strictosidine synthase,[6–10] the salsolinol synthase,[11] and McbB from *Marinactinospora thermotolerans*.[12]

Recently, the substrate scope of the NCS from *Coptis japonica* (CjNCS2)[13,14] and from *Thalictrum flavum* (TfNCS)[15–18] was elucidated and showed that a broad scope of aldehydes is accepted, while the number of possible amines is more restricted.[14–16]

The NCS reactions were typically carried out on small scale and with low substrate concentrations (<10 mM). Two attempts to upscale this reaction have been reported: The synthesis of (*S*)-norcoclaurine has been described using purified enzyme and 10 mM substrate concentrations[19] at a 1 L scale with a yield of 81% and an e.e. of 93% for the (*S*)-enantiomer. Another report described the synthesis of two derivatives using, instead of 4-hydroxyphenylacetaldehyde, hydrocinnamaldehyde or 1-butyraldehyde employing a cell-free extract, and a dopamine concentration of 65.3 mM, giving conversions of between 86% and >99% with e.e.s >95% on a 1 ml scale.[14]

During most of these studies several challenges associated with this reaction were reported. One of the major problems is the spontaneous background reaction catalyzed, for example, by phosphate (often present as buffer ion) leading to racemic product.[20] Furthermore, when performing the reaction in the presence of air, the oxidative degeneration of dopamine as well as the polymerization of 4-hydroxyphenylacetaldehyde at a broad pH-range diminishes the yield. Here we report on attempts to address these challenges and on the extension of the library of NCSs.

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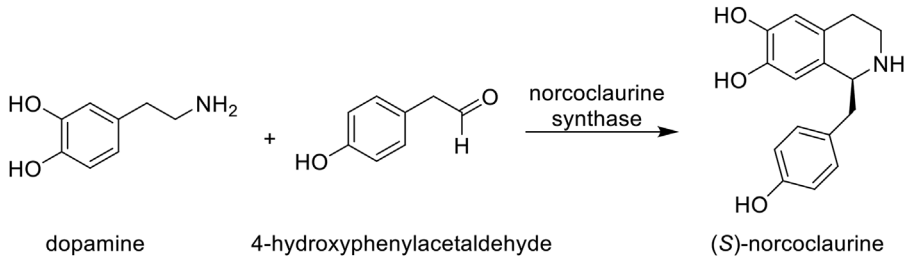


Figure 1. The Pictet–Spengler reaction carried out by norcoclaurine synthase.

2. Experimental Section

2.1. Materials

All chemicals, sodium alginate and Diaion HP-2MG were obtained from Sigma–Aldrich. Phenylacetaldehyde was distilled prior to use. ABT High Density Nickel (cross-linked 6% alginate beads, loading capacity 20–40 μmol Ni^{2+} ml^{-1} gel) from was obtained from Agarose Bead Technologies; YTONG from OBI Graz; Amberlite FPC3500 from AcrosOrganics and EziG1-3 from EnginZyme.

2.2. Synthesis of Substrates and Reference Compounds

4-hydroxyphenylacetaldehyde: 4-Hydroxyphenylacetic acid ethyl ester was prepared by the esterification of 4-hydroxyphenylacetic acid (1.12 g, 7.4 mmol) with ethanol (40 ml) and sulfuric acid (2 ml) under reflux for 3 h yielding 4-hydroxyphenylacetic acid ethyl ester (1.10 g, 6.6 mmol, 90% yield, according to GC-MS >99% pure). 4-Hydroxyphenylacetaldehyde: The reduction with DIBAL-H was carried out under argon atmosphere at a temperature between −90 and −70 °C. Ester (0.91 ml, 5.5 mol) was added to 40 ml toluene (anhydrous >99.8%). DIBAL (1 M in toluene, 15 ml, precooled to −80 °C) was added dropwise at −80 °C. After 5 h of stirring at −80 °C the reaction was quenched by adding methanol (1 ml) dropwise. To the resulting white emulsion aqueous HCl (5 ml, 1 M) was added, the cooling bath was removed and the reaction was allowed to warm to 0 °C and additional aqueous HCl (7 ml, 1 M) was added. The solution was stirred at room temperature until a phase separation with two clear phases was achieved. The aqueous solution was extracted with EtOAc (2 × 20 ml). Combined organic phases were extracted with a NaCl-solution (sat., 20 ml), dried over Na_2SO_4 and concentrated. The product (0.74 g, 5.4 mmol, according to GC-MS 90% pure) was purified with a silica column chromatography (40 g silica gel, 0.04–0.063 mm, dichloromethane/MeOH = 99:1) yielding 4-hydroxyphenylacetaldehyde (0.580 g, 4.3 mmol, 78% yield). The NMR data was in accordance with the literature.[16] ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.73 (*t*, *J* = 2.3 Hz, 1H), 7.08 (*d*, *J* = 7.5 Hz, 2H), 6.83 (*d*, *J* = 6.5 Hz, 2H), 5.43 (s, 1H), 3.65 (*d*, *J* = 2.1 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.4, 155.1, 130.9, 123.6, 116.0, 49.7.

rac-Norcoclaurine: *N*-(3,4-dimethoxyphenethyl)-2-(4-methoxyphenyl)acetamide: 4-methoxyphenylacetyl chloride (5.0 g, 27 mmol) in $CHCl_3$ (20 ml) was added dropwise to a solution of 2-(3,4-dimethoxyphenyl)ethylamine (4.9 g, 27 mmol) in $CHCl_3$ (40 ml) overlaid with NaOH solution (aqueous, 150 ml, 3%) with a syringe. After 4 h, the organic phase was separated. The aqueous phase was extracted two times with CH_2Cl_2 (2 × 20 ml), dried over Na_2SO_4, and evaporated. The yellowish solid was dissolved in hot ethyl acetate and precipitated when cooled down. After filtration, a white solid was identified as *N*-(3,4-dimethoxyphenethyl)-2-(4-methoxyphenyl)acetamide (6.164 g, 18.7 mmol, yield 70%), mp = 120–122 °C (lit[21]: 125–127 °C). The NMR data was in accordance with the literature.[22] ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.08 (*d*, *J* = 8.6 Hz, 2H), 6.84 (*d*, *J* = 8.6 Hz, 2H), 6.73 (*d*, *J* = 8.1 Hz, 1H), 6.66–6.51 (m, 2H), 5.45 (s, 1H), 3.90–3.77 (m, 9H), 3.52–3.36 (m, 4H), 2.68 (*t*, *J* = 6.9 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.3, 158.8, 149.0, 147.6, 131.1, 130.5, 126.7, 120.6, 114.3, 111.7, 111.2, 55.9, 55.8, 55.3, 42.9, 40.7, 35.0.

6,7-Dimethoxy-1-(4-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline: $POCl_3$ (5.0 ml, 55 mmol) was added to a solution of *N*-(3,4-dimethoxyphenethyl)-2-(4-methoxyphenyl)acetamide (6.02 g, 18.3 mmol) in dry acetonitrile (MS 3 Å, 100 ml) and the mixture was heated at reflux for 9 h under an argon atmosphere. The solvent and excess $POCl_3$ was evaporated under reduced pressure and the residue was dissolved under an argon atmosphere in dry MeOH (MS 3 Å, 100 ml). The mixture was kept under an argon atmosphere and cooled with an ice-water bath. Triethylamine (100 μl) and $NaCNBH_3$ (5.78 g, 92 mmol) were added. The mixture was slowly warmed to room temperature overnight. After quenching with aqueous HCl (120 ml, 1 M), MeOH was evaporated and the aqueous residue was neutralized with NaOH (aqueous, 5 M). The aqueous phase was extracted with CH_2Cl_2 (3 × 50 ml), dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. 6,7-Dimethoxy-1-(4-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline was obtained as a yellow oil (5.78 g, 18.3 mmol, yield >99%, purity according to HPLC >95%), rf = 0.27 (silica, 95/5 CH_2Cl_2/MeOH, 0.1% TEA). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.18 (d, *J* = 8.6 Hz, 2H), 6.88 (d, *J* = 8.6 Hz, 2H), 6.63 (s, 1H), 6.60 (s, 1H), 4.20–4.05 (m, 1H), 3.90–3.74 (m, 9H), 3.31–3.09 (m, 2H), 3.02–2.58 (m, 4H), 2.36–2.07 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.3, 147.4, 146.9, 131.0, 130.4, 130.4, 127.3, 114.0, 111.8, 109.4, 56.9, 56.0, 55.9, 55.8, 55.8, 55.3, 55.3, 41.7, 40.7, 29.4. Norcoclaurine: 6,7-dimethoxy-1-(4-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline (640 mg, 2 mmol) was dissolved in dry CH_2Cl_2 (MS 3 Å, 20 ml) under an argon atmosphere. After cooling to −75 °C with an EtOH/N_2 liq. bath BBr_3 (1 M solution in CH_2Cl_2, 18.3 ml, 18.3 mmol) was added. The reaction was warmed slowly to 21 °C and stirred for further 2 h. Then the reaction was quenched with H_2O (100 ml). The aqueous phase

was separated and evaporated under reduced pressure. Norcoclaurine*HBr was obtained as a mixture with salt (1.836 g). Purification of the salt via preparative HPLC (100% H_2O, +0.1% TFA, Nucleodur C18-ec 100 5 µm 250 × 21, flow 8 ml min^{-1}) did not improve purity. The product content in the salt was calculated via internal standard to be 26% (weight norcoclaurine) (calculated yield 0.4774 g, 1.8 mmol, 88%) mp = 200–202 °C (foaming, lit. 220–222 °C).[20] NMR is in accordance to literature.[20] ^{1}H NMR (300 MHz, MeOD) δ 7.14 (*d*, *J* = 8.5 Hz, 2H), 6.81 (*d*, *J* = 8.5 Hz, 2H), 6.66–6.59 (m, 2H), 4.58 (dd, *J* = 8.7, 5.7 Hz, 1H), 3.52–3.17 (m, 2H), 3.08–2.83 (m, 4H). ^{13}C NMR (75 MHz, CD_3OD) δ 158.1, 146.8, 145.7, 131.7, 127.0, 123.7, 123.7, 117.0, 116.2, 114.2, 57.9, 40.9, 40.5, 25.7.

rac-1-Benzyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol: *N*-(3,4-dimethoxyphenethyl)-2-phenylacetamide: phenylacetyl chloride (0.315 g, 2.03 mmol) in $CHCl_3$ (20 ml) was added dropwise to a solution of 2-(3,4-dimethoxyphenyl)ethylamine (0.370 g, 2.03 mmol) in $CHCl_3$ (30 ml) overlaid with NaOH solution (aqueous, 150 ml, 3%) with a syringe into the organic phase. After 16 h, the organic phase was separated. The aqueous phase was extracted two times with CH_2Cl_2 (3 × 15 ml), dried over Na_2SO_4 and evaporated. The yellowish solid was dissolved in hot ethyl acetate which precipitated when cooled down. After filtration, the white solid was identified as *N*-(3,4-dimethoxyphenethyl)-2-phenylacetamide (320 mg, 1.07 mmol, 53%), rf = 0.51 (silica, hexanes: ethyl acetate 3:7). Mp = 106–108 °C (lit[23]: 108–109 °C), the NMR data was in accordance with the literature.[23] ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33–7.10 (m, 3H), 7.10–6.99 (m, 2H), 6.59 (*d*, *J* = 8.1 Hz, 1H), 6.51–6.36 (m, 2H), 5.24 (s, 1H), 3.73 (s, 3H), 3.69 (s, 3H), 3.41 (s, 2H), 3.32 (dd, *J* = 12.8, 6.8 Hz, 2H), 2.55 (*t*, *J* = 6.9 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.9, 149.0, 147.6, 134.8, 131.1, 129.4, 129.0, 127.3, 120.6, 111.7, 111.3, 55.8, 43.9, 40.8, 35.0. 1-Benzyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline: $POCl_3$ (0.5 ml, 5.5 mmol) was added to a solution of *N*-(3,4-dimethoxyphenethyl)-2-phenylacetamide (320 mg, 1.07 mmol) in dry acetonitrile (over MS 3 Å, 10 ml) and the mixture was heated at reflux for 9 h under an argon atmosphere. The solvent and excess $POCl_3$ were evaporated under reduced pressure and the residue was dissolved under an argon atmosphere in dry MeOH (MS 3 Å, 10 ml). The mixture was kept under argon atmosphere and cooled with an ice-water bath. Triethylamine (100 µl) and $NaCNBH_3$ (500 mg, 9.6 mmol) were added. The mixture was slowly warmed to room temperature overnight. After quenching with HCl (10 ml, aqueous 1 M), MeOH was evaporated and the aqueous residue was neutralized with NaOH (aqueous, 5 M). The aqueous phase was extracted with CH_2Cl_2 (3 × 15 ml), dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. 1-Benzyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline was obtained as a yellow oil (267 mg, 0.94 mmol, yield 88%, rf = 0.34 (silica, ethyl acetate + 2% TEA). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40–7.19 (m, 6H), 6.61 (s, 1H), 6.60 (s, 1H), 4.16 (dd, *J* = 9.3, 4.5 Hz, 1H), 3.86 (s, 3H), 3.81 (s, 3H), 3.29–3.14 (m, 2H), 3.00–2.86 (m, 2H), 2.74 (dd, *J* = 13.4, 6.3 Hz, 2H), 1.82 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 147.4, 146.9, 139.1, 130.4, 129.4, 128.6, 127.3, 126.5, 111.8, 109.4, 77.4, 77.0, 76.6, 56.9, 55.9, 42.8, 40.7, 29.5. 1-Benzyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol: 1-benzyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (257 mg, 0.91 mmol) was dissolved in dry CH_2Cl_2 (over MS 3 Å, 10 ml) under an argon atmosphere. After cooling to −78 °C with an EtOH/N_2 liq. Bath, BBr_3 (1 M solution in CH_2Cl_2, 9.0 ml, 9 mmol) was added. The reaction was warmed slowly to 21 °C and stirred for a further 2 h. Then the reaction was quenched with H_2O (100 ml). The aqueous phase was separated and evaporated under reduced pressure. The solid obtained was dissolved in MeOH and diethyl ether was added until precipitation of a solid. 1-Benzyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol was obtained as a yellowish solid (223 mg, 0.66 mmol, 73%) mp = 216–220 °C (foaming, lit. 208–214 °C).[20] The NMR data was in accordance with the literature.[20] ^{1}H NMR (300 MHz, D_2O) δ 7.50–7.27 (m, 5H), 6.76 (s, 1H), 6.70 (s, 1H), 4.70 (dd, *J* = 9.1, 5.6 Hz, 1H), 3.64–3.40 (m, 2H), 3.36–3.18 (m, 1H), 3.14–2.85 (m, 3H). ^{13}C NMR (75 MHz, D_2O) δ 144.0, 142.8, 135.0, 129.5, 129.2, 27.8, 123.8, 123.2, 115.7, 113.7, 56.1, 39.3, 39.2, 24.0.

2.3. Expression of NCS

Genes of NCS were cloned in a pET21(a) or in a pET29(a) (for PbNCS1, CjNCS2) expression vector and expressed in *Escherichia coli* BL21 (DE3). Expression of NCS was performed in LB medium (330 ml). The medium containing the required antibiotic(s) was inoculated with 3 ml L^{-1} of an overnight culture. The culture was grown at 37 °C, 120 rpm until an OD_{600} of 0.6–0.8 was reached. Expression of the target protein was started by adding 1 mM IPTG (final concentration). Cells were grown overnight at 20 °C, 120 rpm. The cells were harvested by centrifugation at 8000 rpm, 20 min at 4 °C, washed with HEPES buffer (pH 7, 50 mM), and stored after centrifugation (8000 rpm, 20 min at 4 °C) at −20 °C. For co-expression employing chaperones, the Takara Chaperone Kit manual was used and expression was carried out with the standard method. MagicMedia™ for expression with autoinduction was used as follows: 6 h at 37 °C followed by 20 °C for 18 h. Autoinduction media (4ZY-LAC) was prepared as described in the literature[24,25] and the cultures were grown for 24 h at 37 °C followed by 20 °C for 18 h.

2.4. Immobilization of NCS

Alginate: The alginate beads were produced by dropping an alginate solution (3% weight) containing the whole cells in a $CaCl_2$ solution (100 ml, 0.2 M) using a syringe with a needle (80 × 0.8 mm). Beads with a diameter of 2–3 mm were produced by this method. Amberlite FPC-3500, Diaion HP-2MG, YTONG: Whole cells were immobilized via adding the carrier material to the growing cells during induction (1 g carrier/330 ml LB medium) to obtain biofilm formation as reported before[26] and lyophilized after decantation of the medium and washing with HEPES buffer. ABT HIGH Density NICKEL: The carrier material (1 ml containing 500 mg beads [wet weight]) was centrifuged and washed with HEPES-buffer (pH 7, 1 ml, 50 mM, three times). Cell lysate of his-tagged PbNCS (1 ml) was added to the beads. The mixture was incubated on ice for 30 min, centrifuged (Epifuge, 15000 rpm, 21 °C), and the liquid phase was discarded. EziG1-3: The beads were incubated in the lysate of 0.5 g wet cells/100 mg carrier for 30 min, according to the

manual using HEPES buffer (50 mM, pH 7, + NaCl 500 mM + Imidazole 20 mM). After centrifugation and washing of the beads with HEPES buffer (50 mM, pH 7), the material was freeze dried and stored at 4 °C for further usage.

2.5. Purification of PbNCS

His-tag purification was carried out using a GE Fast Trap FF column (5 ml when using 3 L cell culture).The following buffers were used: Binding buffer: sodium phosphate (20 mM containing 0.5 M NaCl and 20 mM imidazole, pH 7.4). Elution buffer: sodium phosphate (20 mM containing 0.5 M NaCl and 500 mM imidazole, pH 7.4). The protein was eluted using a gradient of binding buffer/elution buffer 100/0 to 0/100 in 20 min with a flow rate of 5 ml min^{-1}. The purified enzyme was concentrated using a Sartorius Stedim (Vivaspin 6, 10 000 MWCO) tube and dialyzed against HEPES buffer (2 L, 50 mM, pH 7).

2.6. Determination of Initial Activity of PbNCS

Dopamine and phenylacetaldehyde as substrates (each 10 mM) were added to PbNCS (1 μg ml^{-1}) in HEPES buffer (50 mM, pH 7). The reaction was quenched after 15 or 30 s by adding MeOH (50% v/v containing 1% anisole as external standard) and analyzed by HPLC. Measurements were performed in triplicate (Figure S6, S7, S11 Supporting Information).

2.7. Analytics

HPLC methods: Determination of conversion for products on HPLC: Phenomenex Luna 5u C18(2) 250 × 4.6: Gradient 10–100% MeCN 0.1% TFA/water 0.1% TFA in 8 min, hold 2 min, 10:90 MeCN 0.1% TFA/water 0.1% TFA for 5 min at 20 °C with a flow of 1 ml min^{-1}. Retention times: norcoclaurine: 6.1 min, 1-benzyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol: 6.7 min. The conversions were determined using a calibration curve (Supporting Information). Determination of the optical purity of products was carried out on HPLC with an Astec Chirobiotic T 150 × 4.6 column. Method: Isocratic MeOH [containing 0.2% acetic acid (AA) and 0.1% triethylamine (TEA)]/MeCN (containing 0.2% AA + 0.1% TEA) in a ratio of 65/35 at 1 ml min^{-1} at 25 °C. Retention times: 4.5 min ([*S*]-norcoclaurine), 5.7 min ([*R*]-norcoclaurine), 4.6 min ([*S*]-1-benzyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol), 5.1 min ([*R*]-1-benzyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol). The absolute configurations were deduced from the elution order in the literature and/or reported stereopreference of the enzymes for already performed transformations with the specific substrate.[13,15,17]

3. Results

3.1. Evaluation of NCSs Expressed in *E. coli* as Whole Cell Catalyst

In a first step a BLAST search[27] was performed to extend the library of norcoclaurine synthases (NCS) and to identify alternative NCSs. As a template the gene of the previously investigated NCS from TfNCS was employed.[15,16] From the eight NCSs identified by BLAST three have not been described before, namely two NCSs from *Argemone mexicana* (AmNCS1, AmNCS2) and one from *Corydalis saxicola* (CsNCS) (Table S1, Supporting Information). Additionally related NCSs already described in literature were used for comparison or to test their substrate scope, such as the aforementioned NCS from CjNCS2[8,14,16,17,28,29] and from TfNCS[4,15,16,28,30,31] as well as one from *Papaver bracteatum* (PbNCS)[30] and two NCSs from *Papaver somniferum* (PsNCS1, PsNCS2).[32] Furthermore, a ninth NCS was tested, which has no homology to the others and originates from CjNCS1 showing ambiguous activity.[29,30] The genes of the novel NCSs (AmNCS1, AmNCS2, CsNCS, PbNCS) as well as selected other genes (CjNCS2, PsNCS2, TfNCS) were ordered as codon-optimized genes and expressed in *E. coli*.

The nine NCSs (Table S1, Supporting Information) were expressed in *E. coli* following first a standard protocol and activity was tested using lyophilized whole cells. Using HEPES buffer allowed minimization of the spontaneous background reaction in the assay between dopamine and 4-hydroxyphenylacetaldehyde (**Table 1**, Entry 11), as it has been described that various buffer ions,[20] especially phosphate, catalyzes spontaneous Pictet–Spengler reactions of these substrates leading to racemic product. Nevertheless, just using *E. coli* cells void of NCS led to significant conversions after 4 h, albeit with negligible optical purity (Table 1, Entry 10). Most of the NCS preparations led to excellent optical purities with e.e.s >98% together with high conversions, indicating that the enzymatic reaction outperforms the *E. coli* induced non stereoselective background reaction (Entries 1-3,5-6,8-9). The lowest e.e. was observed for *E. coli*/CjNCS1 (Entry 4). CjNCS1 belongs to another structural family showing low Pictet–Spengerlase activity as already previously described.[29] The *E. coli*/PsNCS1 preparation led to reasonable 93% e.e. (Entry 7). The results clearly indicated, that *E. coli*/NCS preparations can be used for the Pictet–Spengler reaction.

Table 1. Enantiomeric excesses and conversions of the recombinant NCSs applied as *E. coli* lyophilized cell preparations.

Entry	*E. coli* lyophilized cell catalyst with	Conversion[a] (%)	e.e.[b] (%)
1	AmNCS1	90	>98
2	AmNCS2	>99	>98
3	CsNCS	90	>98
4	CjNCS1	35	41
5	CjNCS2	>99	>98
6	PbNCS	>99	>98
7	PsNCS1	85	93
8	PsNCS2	>99	>98
9	TfNCS	>99	>98
10	Blank/whole cells	57	rac
11	Blank/buffer only	3	n.d.

[a)]Reaction conditions: Dopamine (5 mM), 4-hydroxyphenylacetaldehyde (5 mM), lyophilized whole cells (10 mg ml^{-1}), HEPES buffer (pH 7, 50 mM), 4 h, 500 rpm, 40 °C; [b)]Measured via HPLC on a chiral phase.

Since during expression it was recognized that a significant part of the NCSs was present (as inclusion bodies) in the non-soluble fraction after lysis, optimization studies to improve expression of the NCSs were started using chaperones, fusion proteins, and media engineering to increase activity per milligram of the whole cell catalyst even further (Figure S1–S3, Supporting Information). To test under preparative reaction conditions, the substrate concentration was increased in the assays to 50 mM for dopamine and 62 mM for the aldehyde. In the initial tests (Table 1) as well as in the literature low concentrations (<10 mM) had been used. The different preparations for each NCS were tested as lyophilized cells as well as cell-free lysate. Additionally, the non-soluble fraction was investigated as well. Interestingly all three types of preparations showed enzymatic activity, including the non-soluble one, as indicated by the measured optical purity. This observed activity can be due to inefficient lysis, but might occur as activity of possible inclusion bodies as well and was not investigated further.

Nevertheless, the preparation as lyophilized cells displayed in all cases the best activity when testing comparable amounts. Notably, the use of chaperones led to an increase of the activity per mg of lyophilized whole cells as observed for instance with the PbNCS preparation when comparing expression with and without the chaperone 1 of the Takara Kit (dnaK-dnaJ-grpE-groES-groEL chaperone) (**Figure 2**, 5th and 6th column). This catalyst preparation turned out to be the most active one of all those tested. Interestingly, although the NCSs are rather similar, it turned out that for each NCS another method/chaperone worked best. The conditions leading to the most suitable whole cell preparations were for AmNCS1 and AmNCS2 using chaperone 2 (groES-groEL), CjNCS2 with chaperone 5 (tig), CsNCS and PbNCS employing chaperone 1 (dnaK-dnaJ-grpE-groES-groEL), PsNCS2 with chaperone 4 (groES-groEL-tig), and TfNCS using autoinduction media [25] (for slow expression).

Although the aim was to optimize reaction conditions for a substrate concentration of 50 mM or higher to allow a high space time yield, it was noticed that increased substrate concentrations also led to an increase in the spontaneous background reaction (Figure 2, pET21(a)-lyo, Figure S4, Supporting Information). At 50 mM substrate concentration, a background reaction was even detectable in water (Figure S5).

PbNCS and AmNCS1 displayed the highest activities as lyophilized whole cell catalysts while PbNCS (1 mg ml^{-1} whole cells containing chaperone 1, dnaK-dnaJ-grpE-groES-groEL chaperone) led to the highest conversion (60%) at 50 mM dopamine giving norcoclaurine in optical pure form (e.e. >98%) within 30 min.

Since the apparent activity of the whole cell catalyst depends on the expression level as well as on the amount of (soluble) enzyme in the cells, the highly active PbNCS was purified and the specific activity was determined to be 137 μmol mg^{-1} min^{-1} (2280 nkatal mg^{-1}) with dopamine and phenylacetaldehyde as substrates which corresponds to twofold the activity of the TfNCS with dopamine and 4-hydroxyphenylacetaldehyde (Figure S10, Supporting Information).[17] Comparison with literature data is in general difficult, since earlier reports[4,28] described experiments using phosphate buffer leading to a significant background activity.[20]

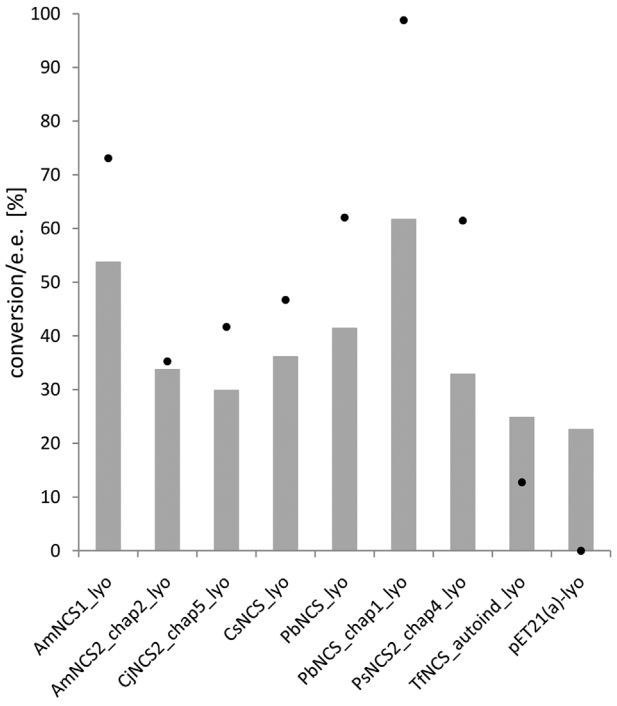


Figure 2. Enantiomeric excess and conversion of optimized lyophilized whole cell preparations at 50 mM dopamine concentration (black dots indicate e.e., gray bars indicate conversion). Reaction conditions: Dopamine (50 mM), 4-hydroxyphenylacetaldehyde (62 mM), lyophilized cells (1 mg ml^{-1}) were added to HEPES buffer (pH 7, 50 mM) and the reaction was stopped after 30 min, 500 rpm at 40 °C by adding acetonitrile (50% v/v, containing 0.1% anisole as external standard).

3.2. Immobilization for Batch Reactions as Well as in Flow

To minimize the non-stereoselective background reaction at elevated substrate concentrations, we anticipated that this may be achieved using immobilized enzyme in flow. Advantages and methods of enzyme immobilization are reviewed in recent articles.[33–41] Immobilized enzymes have been reported to display improved stability, reusability, enable simplified product purification, and therefore more efficient processes compared to the reaction with free enzymes.

The immobilization was tested for the most active identified PbNCS using *E. coli* cells containing the recombinant enzyme as well as purified enzyme on various carrier materials (Table S2, Supporting Information). For carrier materials in whole cells were immobilized (Table S2, Supporting information; Entry 1–4), while some carriers bind only his-tagged enzyme (Table S2, Supporting information; Entry 5–8).

The preparations using Amberlite FPC-3500 or YTONG (**Table 2**, entry 2, 4) did not lead to stereoselective product formation. *E. coli*/PbNCS immobilized on Diaion HP-2MG led to a low e.e. (30%, entry 3), while using MeOH as cosolvent gave high e.e (>98%) although at low conversion (28%, entry 5).

Immobilization of the purified enzyme via a His-tag led to the most promising results. Although an alginate-based carrier linked to Ni^{2+} gave moderate e.e. (56% e.e., entry 7), the best results were obtained with EziG3, a carrier with a semi-

Table 2. Conversions and e.e. achieved with different preparations of immobilized catalyst.

Entry	Carrier	PbNCS preparation immobilized	Conditions[a]	Catalyst amount (mg ml^{-1})	Conv. (%)[b]	e.e. (%)[b]
1	Alginate	Cells	2 h	50	31	65
2	None	Cells	2 h	10	27	>98
3	Diaion HP-2MG	Cells	0.5 h	50	20	30
4	Amberlite FPC-3500	Cells	0.5 h	50	17	*rac*
5	Diaion HP-2MG	Cells	10% MeOH, 0.5 h	50	28	>98
6	YTONG®	Cells	0.5 h	100	12	rac
7	ABT high density nickel	Purified	0.5 h	15	16	56
8	EziG1	Purified	0.5 h	50	33	66
9	EziG2	Purified	0.5 h	50	43	86
10	EziG3	Purified	0.5 h	50	46	88

[a]Reaction conditions: Corresponding amount of catalyst was dissolved in HEPES buffer (50 mM, pH 7). Dopamine (50 mM) and 4-hydroxyphenylacetaldehyde (50 mM) was added. The reaction was shaken at 500 rpm at 30 °C. The transformation was stopped after the time indicated by adding acetonitrile (containing 0.1% anisole as external standard); [b]determined by HPLC on a chiral phase.

hydrophilic surface (Table 2, entry 10 Figure S8, Supporting Information). The carrier EziG,[42] based on glass beads, was used to overcome the compressibility of the alginate beads which may cause problems when used in flow. The carrier links the enzyme via Fe^{2+} ions at the surface which possess according to the supplier enhanced binding strength toward the His-tag. The EziG carrier was loaded with 1 mg of enzyme on 10 mg carrier as quantified via the determination of purified protein concentration before and after loading.

In a next step, a time course study was performed using purified PbNCS immobilized on EziG3. For comparison, also non-immobilized *E. coli*/PbNCS cells and purified PbNCS were tested. Additionally, we added ascorbic acid as antioxidant or MeOH as additive to the EziG3 immobilized enzyme to see their influence the course of the reaction. Ascorbic acid has previously been used in NCS reactions to reduce problems due to the oxidation of dopamine or products.[16,18,19]

All preparations showed very high activity within the first 30 min. After 2 h, the reaction slowed down to a reaction rate comparable to that of the background reaction (**Figure 3**). This could also be seen to effect the e.e. values, which started to decrease from a high level to moderate values (**Table 3**), indicating that the catalyst lost activity. Thus, the reaction taking place after the 2 h corresponds mainly to the background reaction.

The use of ascorbic acid and methanol led to slower initial rates, but after 25 h they showed similar conversions compared to the reactions without additives. Interestingly, comparing the

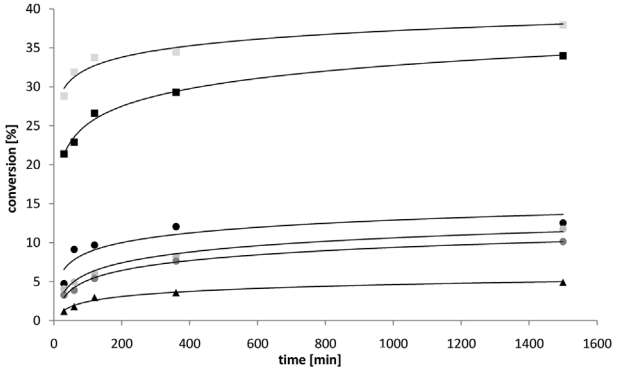


Figure 3. Time course for the Pictet–Spengler reaction catalyzed by various PbNCS preparations. (black square: purified PbNCS 1 mg ml^{-1}, gray square: lyophilized whole cell catalyst (10 mg ml^{-1}), black dots: EziG3 immobilized PbNCS (10 mg ml^{-1}), light gray dots: EziG3 immobilized PbNCS (10 mg ml^{-1}) with ascorbic acid (1 mg ml^{-1}), dark gray dots: EziG3 immobilized PbNCS (10 mg ml^{-1}) with MeOH (10% v/v), black triangle: buffer blank). Reaction conditions: Corresponding amount of catalyst was dissolved in HEPES buffer (50 mM, pH 7). Dopamine (50 mM) and phenylacetaldehyde (50 mM) was added. The reaction was shaken at 500 rpm at 30 °C. The transformation was stopped after the time indicated by adding acetonitrile (containing 0.1% anisole as external standard). E.e. determined by HPLC on a chiral phase.

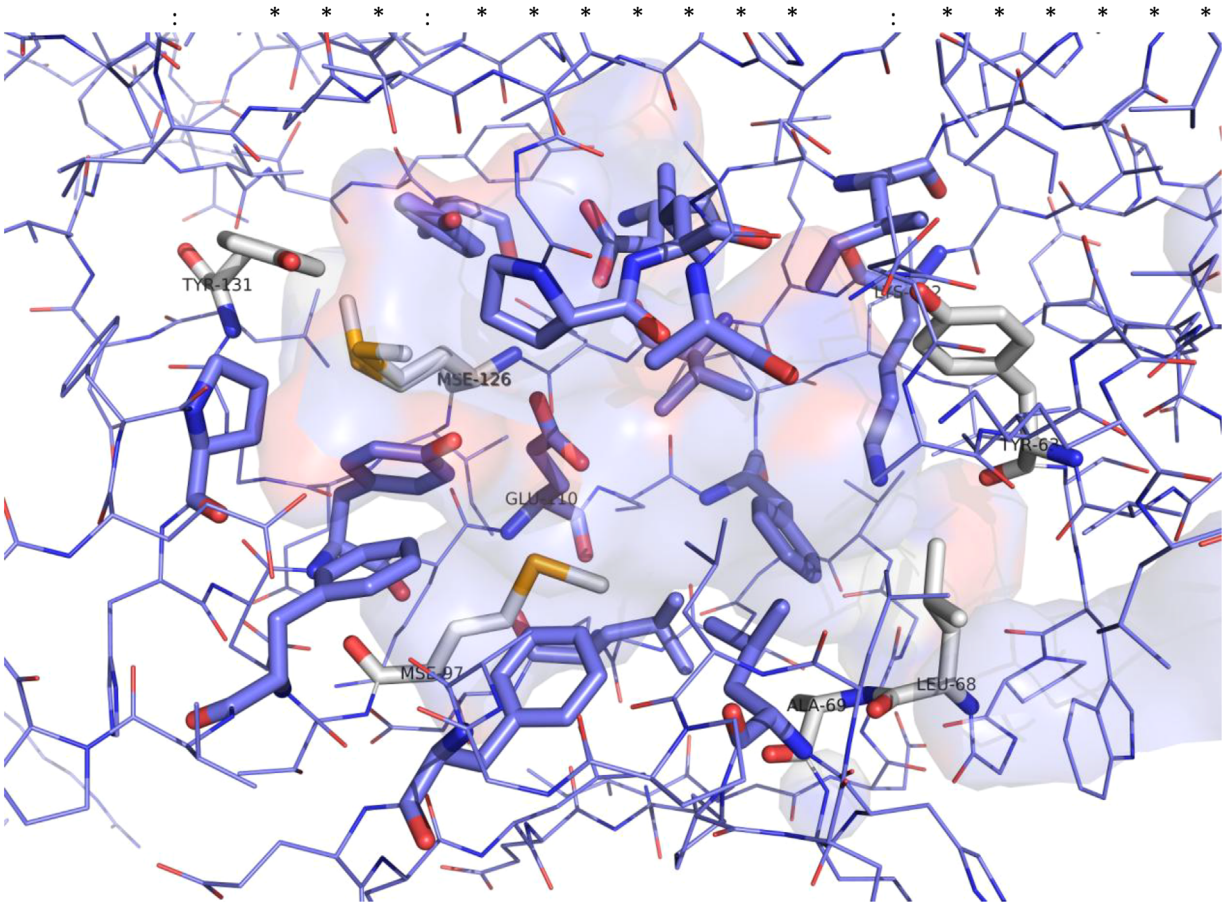
optical puritiy of the product obtained with EziG3 under different conditions (Table 3, entries 5–7) showed that additives (ascorbic acid as well as MeOH), but especially MeOH led to decreased e.e. values. It has to be mentioned that the catalyst preparations turned black over time due to the formation of a precipitate. This is most probably caused by the formation of insoluble polydopamine and eulmelanin derivatives as a result of a side reaction of the substrate [43,44]. By the addition of ascorbic acid as antioxidant no precipitate could be detected visually (Figure S9, Supporting Information).[19]

Other organic solvents were tested as well (Table S3, Supporting Information) to improve the solubility of the aldehyde and therefor the conversion to the product. All of

Table 3. e.e. measured during the time course using different preparations of immobilized catalyst.[a]

		Reaction time (min)		
Entry	Catalyst	30 e.e.(%)	120 e.e.(%)	1500 e.e.(%)
1	Purified PbNCS (1 mg ml^{-1})	>98	94	93
2	*E. coli*/PbNCS (10 mg ml^{-1})	>98	>98	>98
3	No catalyst (blank)	*rac*	*rac*	*rac*
4	EziG3 PbCNS (10 mg ml^{-1})	87	85	82
5	EziG3 PbCNS (10 mg ml^{-1}) + ascorbic acid (1 mg ml^{-1})	92	77	70
6	EziG3 PbCNS (10 mg ml^{-1}) + MeOH (10% v/v)	72	65	55

[a]Reaction conditions: see **Figure 3**. e.e. was determined by HPLC on a chiral phase.



Enzyme	Residue[b] 63	68	69	72	80	95	97	99	105	108	110	112	122	124	126	131	139	141	143	179	180	182
AmNCS1	Y	I	P	L	F	L	I	F	P	Y	E	F	K	V	M	**H**	Y	D	I	P	L	M
AmNCS2	Y	I	P	L	F	L	I	F	P	Y	E	F	K	V	M	Y	Y	D	I	P	L	M
CsNCS	Y	I	P	L	F	L	I	F	P	Y	E	F	K	V	M	Y	Y	D	I	P	L	M
CjNCS2	**E**	**L**	**G**	L	F	L	**M**	F	P	Y	E	F	K	V	**Q**	Y	Y	D	I	P	L	M
PbNCS	Y	I	P	L	F	L	I	F	P	Y	E	F	K	V	M	Y	Y	D	I	P	L	M
PsNCS1	Y	I	P	L	F	L	I	F	P	Y	E	F	K	V	M	Y	Y	D	I	P	L	M
PsNCS2	Y	I	P	L	F	L	I	F	P	Y	E	F	K	V	M	Y	Y	D	I	P	L	M
TfNCS	Y	**L**	**A**	L	F	L	**M**	F	P	Y	E	F	K	V	M	Y	Y	D	I	P	L	M

Figure 4. Active site and sequence comparison of the amino acids lining the active site pocket of TfNCS[a] (pdb: 2vne). The residues in the active site are shown as violet sticks, labeled residues are the catalytically active ones (GLU-110, LYS-122). The residues colored gray are the ones, which differ in NCSs according to the sequence alignment. [a]Variations of the consensus sequence are indicated in bold. [b]The numbering refers to TfNCS (pdb: 2vne), which was used as a basis for alignment.

them are water-miscible. Protic solvents like ethanol and methanol lead to higher conversions, but in the case of ethanol to a low e.e., which indicates increased non-stereoselective background reaction. 2-Propanol and the aprotic solvent 1,2-dimethoxyethane showed decreased activities with e.e. comparable to the reaction without solvent. Acetonitrile led to loss of activity.

DMSO and MeOH led to comparable enantiomeric excess of the product, which was higher than without solvent. While the conversion in DMSO was a little bit decreased, MeOH increases conversion. Hence, DMSO might suppress the background reaction a bit, but harms the enzyme or inhibits the reaction while MeOH seems have no effect on stability if used up to 10%.

Since all preparations lost activity after a rather short time, inhibition of the enzyme by the product over time was suspected. Inhibition by a an unnatural aldehyde (citronellal) has been suggested in the literature.[17] To test our hypothesis of product inhibition we used *rac*-norcoclaurine at different concentrations as an additive and phenylacetaldehyde and dopamine as substrates. Interestingly, the activity decreased at all used concentrations of

norcoclaurine employed. Already 1 mM *rac*-norcoclaurine inhibited the enzyme leading to a significantly reduced product formation (Figure S12, Supporting Information).

To minimize the spontaneous background reaction by the catalyst preparation as well as to address possibly product inhibition, a flow reaction system was tested using a packed-bed column with the immobilized enzyme. In this case the product was synthesized continuously yielding a process easy to scale. [45,46] Since EziG3 showed best results in the previous experiments (Table 2, entry 10), this material was used for the flow experiments. Performing the flow experiment at 42 mM dopamine and 30 mM of phenylacetaldehyde (supplied from separate reservoirs and mixed prior column) in the presence of 10% v/v MeOH and ascorbic acid (1 mg ml^{-1}) using 500 mg of catalyst preparation (in a 100 × 4.5 mm column, 19 µl min^{-1} flow) led to 28 mM of product formation with 98% e.e. at 30 °C.

4. Discussion

The library of NCS was successfully extended by identifying two novel NCSs from AmNCS1, AmNCS2, one new NCS from CsNCS and it was shown that the NCS from PbNCS is a highly suitable catalyst for the Pictet–Spengler reaction. Comparing the sequences of the different NCSs (except CjNCS1, since it is not a structural homologue), they are highly similar when considering the 22 residues in and close to the active site (Figure 2). For instance, CjNCS2 differs in four out of the 22 residues while TfNCS displays three changes in relation to the consensus sequence. Comparing the sequence of TfNCS with the other NCSs, Leu68 of TfNCS is in most cases an isoleucine (except for CjNCS2, where it is Leu), Ala69 is in most cases a proline (except for CjNCS2, where it is glycine), Met97 is often isoleucine, and Tyr131 is only in the case of AmNCS1 a histidine (**Figure 4**).

Lichman et al.[18] identified a loop in TfNCS responsible for the carbonyl substrate tolerance of the NCS. It consists of the residues 76–80 (LPGAF). Interestingly, this loop is conserved as LPGVF in all tested NCSs except CjNCS2 and TfNCS. TfNCS shows a valine to alanine substitution and a valine (beween 74 and 75) is missing in a helix before the loop, while CjNCS2 is missing this residue as well, but has an additional alanine in the loop (LPAGIF) plus an valine to isoleucine modification.

For synthetic applications it is advantageous to use a preparation of the catalyst, which is as simple as possible to be prepared. Thus, using for instance a freeze dried whole cell catalyst[47] containing the overexpressed enzyme will avoid time-consuming, cost- and labor-intensive protein purification and might improve the apparent stability of the enzyme of interest.[48] The apparent activity of the whole cell catalyst is then a result of the enzyme activity and the expression level of the enzyme as well as the permeability of the cell. The permeabilization by freeze drying should ensure, that the cell membrane does not represent a (major) barrier for the substrate/product. Lyophilized *E. coli* cells containing the overexpressed NCS turned out to be suitable catalysts. The highest activities of lyophilized cells were observed when the enzymes were co-expressed with chaperones supporting correct folding. Using, for example, PbNCS norcoclaurine co-expressed with dnaK-dnaJ-grpE-groES-groEL chaperone, the isoquinoline product, was obtained within 30 min with up to >99% e.e and 60% conversion in a 50 mM scale.

The reaction using dopamine as substrate bears several challenges including the spontaneous non-stereoselective background reaction. Additionally, product inhibition was encountered. This can be (partly) overcome using low substrate concentrations and suitable buffer salts. For elevated substrate concentrations a flow set-up might be advantageous. The PbNCS enzyme was successfully immobilized on various carriers whereby a His-tag based immobilization on glass beads (EziG3) proved to be best suited. As a prove-of concept this immobilized catalyst was further used in a flow-based system leading to promising results. Although the procedure in flow still needs further optimization, the results indicated that in flow a high optical purity and reasonable product concentrations can be achieved. Furthermore, dopamine showed limited stability in solution resulting in coating the catalyst over time. This can be resolved as previously described[16,18,19] by the addition of ascorbic acid (e.g., 1 mg ml^{-1}) as antioxidant.

As conclusion it can be stated, that the norcoclaurine synthase is a highly stereoselective catalyst. The catalyzed Pictet–Spengler reaction with the natural (highly activated) substrates bears challenges concerning oxidation and low reaction barrier leading easily to racemic products. These problems were addressed using different methods and solutions were presented.

Abbreviations

AmNCS1, AmNCS2, norcoclaurine synthases from Argemone Mexicana; CjNCS1, norcoclaurine synthase from Coptis japonica; CjNCS2, norcoclaurine synthase from Coptis japonica; CsNCS, norcoclaurine synthase from Corydalis saxicola; DIBAL, diisobutylaluminium hydride; e.e., enantiomeric excess; MS, molecular sieve; NCS, norcoclaurine synthase; PbNCS, norcoclaurine synthase from Papaver bracteatum; PsNCS1, PsNCS2, norcoclaurine synthases from Papaver somniferum; TfNCS, norcoclaurine synthase from Thalictrum flavum.

Chemical Compounds

Norcoclaurine (PubChem CID: 114840), (S)-norcoclaurine (PubChem CID: 440927), dopamine (PubChem CID: 681), 4-hydroxyphenylacetaldehyde (PubChem CID: 440113), phenylacetaldehyde (PubChem CID: 998).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

Keywords

biocatalysis, immobilization, Pictet–Spengler-reaction, norcoclaurine synthase, flow chemistry

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