An enzymatic, stereoselective synthesis of (*S*)-norcoclaurine†

Alessandra Bonamore,[a,b] Irene Rovardi,[c] Francesco Gasparrini,[a,c] Paola Baiocco,[b] Marco Barba,[c] Carmela Molinaro,[c] Bruno Botta,[a,c] Alberto Boffi[a,b] and Alberto Macone*[b]

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An efficient, stereoselective, green synthesis of (*S*)-norcoclaurine (higenamine) has been developed using the recombinant (*S*)-norcoclaurine synthase (NCS) enzyme, starting from the cheap tyrosine and dopamine substrates in a one-pot, two step process. Key steps in the biotransformation consist of the oxidative decarboxylation of tyrosine by stoichiometric amounts of sodium hypochlorite in order to generate 4-hydroxyphenylacetadehyde, followed by the addition of enzyme and dopamine substrate in the presence of ascorbate, a necessary ingredient in order to avoid oxidation of the catechol moiety. Quantitative extraction of the product from an aqueous solution was achieved by adsorption onto active charcoal dispersed in the reaction mixture. The optimized process afforded enantiomerically pure (*S*)-norcoclaurine (93%) in a yield higher than 80% and allowed good recovery of the enzyme for recycling. The process thus developed represents the first example of a green Pictet–Spengler synthesis, which may pave the way to novel strategies in benzylisoquinoline alkaloid synthesis.

Introduction

Benzylisoquinoline alkaloids are among the most important plant secondary metabolites since they include a number of biologically-active substances that are widely employed as pharmaceuticals. The rich bioactive potential of these compounds is being rigorously explored, as demonstrated by the recent start of clinical trials on higenamine ((*S*)-norcoclaurine) derivatives for the treatment of septic shock syndrome.[1] (*S*)-Norcoclaurine is also an efficient β1-adrenergic drug, with a pharmacokinetic profile that competes favourably with dobutamine, the currently accepted best in its class. Nevertheless, the low availability of natural (*S*)-norcoclaurine and the expensive stereoselective synthesis of this compound with respect to dobutamine (currently marketed as a racemic mixture) have hampered its clinical development. (*S*)-Norcoclaurine is thus the first example of a series of particularly interesting secondary metabolites of the benzylisoquinoline pathway that may provide a handful of therapeutically-useful natural products, once their synthetic strategy has been redesigned on the basis of cheaper and cleaner synthetic routes.

The synthesis of benzylisoquinoline alkaloids is based on the Pictet–Spengler reaction, which entails the acid-catalyzed electrophilic addition of an iminium ion to a substituted benzyl species.[2–4] The reaction mechanism consists of a two step process, in which the iminium ion is generated first from a condensation between the aldehyde carbonyl and the phenylethyl amine moiety, followed by a Mannich-type cyclization to yield tetrahydrobenzylisoquinoline racemic mixtures.[5] In order to obtain the biologically-active (*S*)-isomer, diverse synthetic strategies have been employed based on asymmetric catalytic approaches involving the enantioselective hydrogenation of the corresponding dihydroisoquinoline intermediates by appropriate chiral metal catalysts.[1,2] These synthetic routes are highly efficient and guarantee a very good enantioselectivity. Nevertheless, large scale preparations entail the extensive use of organic solvents and metal catalysts, which involve the need for solvent and metal recycling. Alternatively, an enzymatic synthesis may offer the advantage of a clean and green synthetic process in the absence of organic solvents and metal catalysts, provided that the efficiency of the process is at least comparable to that of the traditional route. The enzymatic pathway leading to benzylisoquinoline derivatives has been shown to originate from a common route, in which the first committed step consists of the stereospecific Pictet–Spengler condensation of dopamine with 4-hydroxyphenylacethaldehyde (4-HPAA) to yield the benzylisoquinoline central precursor (*S*)-norcoclaurine.[6] The enzyme (*S*)-norcoclaurine synthase (NCS) has been recently identified[7,8] and shown to be an efficient bifunctional reaction catalyst that is able to bind and activate the *p*-hydroxybenzaldehyde substrate and subsequently drive the Mannich-type condensation with the incoming dopamine substrate in an exquisitely enantioselective manner.[9–11] However, the reaction conditions for NCS catalysis reported thus far have limited synthetic advantage in that they are limited to analytical scale (*S*)-norcoclaurine preparation. In particular, both the generation of the aldehyde and the reaction with the dopamine substrate suffer from insufficient yields and are affected by parasitic reactions, such as aldehyde polymerization and dopamine oxidation, that take place in aqueous solutions, thus rendering the whole synthesis impractical. In this paper, the overall NCS-catalyzed reaction has been revisited in

[a]*MOLIROM s.r.l, via Carlo Bartolomeo Piazza no 8, 00161, Rome, Italy*
[b]*Dipartimento di Scienze Biochimiche and Istituto di Biologia e Patologia Molecolari (IBPM), CNR, Università "Sapienza", P. Aldo Moro 5, 00185, Rome, Italy. E-mail: alberto.macone@uniroma1.it*
[c]*Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, Università "Sapienza", P. Aldo Moro 5, 00185, Rome, Italy*

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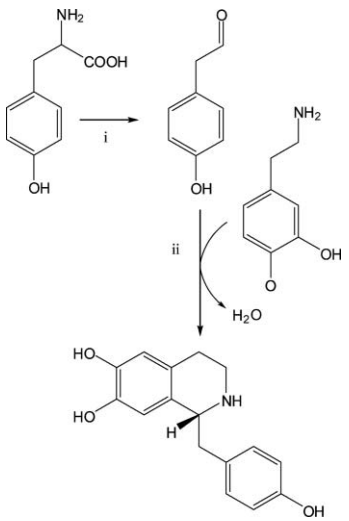
order to provide an environmentally-friendly, easily scalable and robust process that expands the scope of the biotransformation to a multigram level. The present approach is a first attempt to exploit the potential of a Pictet–Spengler enzyme in the direct synthesis of chiral benzylisoquinolines, thus suggesting that many other enzymes belonging to this family might be used for the synthesis of precious plant secondary metabolites.

Results and discussion

The present data highlight a method for the efficient enzymatic synthesis of (*S*)-norcoclaurine, the key precursor of benzylisoquinoline alkaloids. In order to develop a green and easily scalable process, a one-pot, two-step synthesis was set up starting from tyrosine and dopamine in the presence of NCS (Scheme 1).



Scheme 1 The stereospecific chemoenzymatic synthesis of (*S*)-norcoclaurine from tyrosine and dopamine. Reagents and conditions: (i) NaClO, phosphate buffer pH 7.0, 1 h, 37 °C; (ii) NCS 0.5 μM, ascorbate 5 mM, phosphate buffer 0.1 M pH 7.0, 0.5 h, 25 °C.

NCS was recombinantly expressed in *Eschericha coli* in high yield using a codon-optimized synthetic gene (Geneart AG), as described previously.[9,10] *E. coli* cells were fermented in a 2 L Sartorius fermentor by a feed batch procedure in minimal medium at 25 °C. The yield of wet bacterial paste was about 50 g L^{-1} fermented medium with a raw yield of 30 mg protein per gram of bacterial paste. The his-tagged protein was easily purified in batch by a standard procedure on a nickel nitriloacetate resin. The present expression and purification protocol allows one to obtain ten-fold larger amounts of purified protein with respect to previously reported methods,[10] which consisted of low density growth in shake flasks suitable only for analytical scale preparation. The large amount of enzyme obtained represents the pre-requisite for scaling-up (*S*)-norcoclaurine production.

The major limiting step in the large scale production of (*S*)-norcoclaurine is represented by the low stability of both dopamine and the aldehydic substrate in aqueous solution at neutral pH values. In fact, 4-HPAA is not commercially available, and all efforts to produce it enzymatically using plant monoamino oxidases starting from tyramine or alcohol dehydrogenase starting from tyrosol were unsuccessful (data not shown). In turn, the chemical synthesis of the pure aldehyde is impractical in that it entails anhydrous preparations to be stored at low temperature.[12] Thus, 4-HPAA was conveniently generated by the oxidative decarboxylation of tyrosine in aqueous solutions in the presence of an equimolar amount of hypochlorite, followed by its immediate use in the enzymatic reaction. The product yield was estimated to be greater than 99%, with less than 0.1% chlorinated by-products (by GC-MS). The reaction was carried out in the same phosphate buffer as needed for NCS catalysis, thus suggesting that the whole (*S*)-norcoclaurine synthesis could be optimized as a one pot reaction by quickly adding dopamine and NCS to the solution containing the newly synthesized aldehyde.

It is important to focus on the critical step concerning the low stability of dopamine in air-equilibrated aqueous solutions, which is responsible of the low overall yields of the intermediates in benzylisoquinoline alkaloid synthesis.[13] Dopamine is in fact easily oxidized to melanine-like pigments in a second order reaction with oxygen. The dopamine oxidation reaction severely impairs the possibility of reaching synthetically convenient concentrations higher than 1–2 mM. Thus, several attempts were made in order to improve the effective concentration of the dopamine substrate in solution, including de-aeration (N_2 purging into the reaction batch) or by the use of solvent mixtures (water/isopropanol) in the presence of reductants (sodium dithionite, menadiol, vitamin E). Among the different reductants used, ascorbate was observed to be the most efficient, easiest to use and cheapest. Thus, in order to prevent dopamine oxidation during the reaction course, the reaction mixture had 5 mM ascorbate added in the absence of cosolvents. This strategy allowed ten-fold higher dopamine concentrations (10 mM) to be used.

On the basis of the experimental findings outlined above, (*S*)-norcoclaurine synthesis was achieved from 10 mM dopamine and 4-HPAA substrates in a yield of 2.2 g isolated product (81% overall yield) from 1 L of solution and 30 min incubation at 37 °C. Product formation was followed by GC-MS, as reported in Fig. 1 (see also the ESI, Fig. S1†). The enzyme concentration was adjusted to achieve relatively fast reaction kinetics in order to limit the competing non-enantioselective chemical coupling between dopamine and 4-HPAA, which would dominate the NCS reaction for longer periods of incubation. The optimal enzyme concentration was found to be 0.5 μM, corresponding to 10 mg L^{-1} enzyme in the solution.

In Fig. 2, the chiral HPLC chromatograms of standard, racemic norcoclaurine and the reaction mixture are reported, and the enantiomeric excess of (*S*)-norcoclaurine was found to be 93%. This result was confirmed by CD analysis, indicating the presence of two peaks with opposite Cotton effects (due to the presence of both enantiomers) in standard racemic norcoclaurine (Fig. 3A), whereas the reaction mixture shows a very large excess of the expected (*S*)-isomer (Fig. 3B). The newly synthesized (*S*)-norcoclaurine did not racemize after three months storage as a dry powder at room temperature.

An extraction strategy that avoids the use of organic solvents was developed based on the use of activated carbon (NORIT, multipurpose activated charcoal). The adsorption/desorption properties of activated carbon towards phenolic compounds

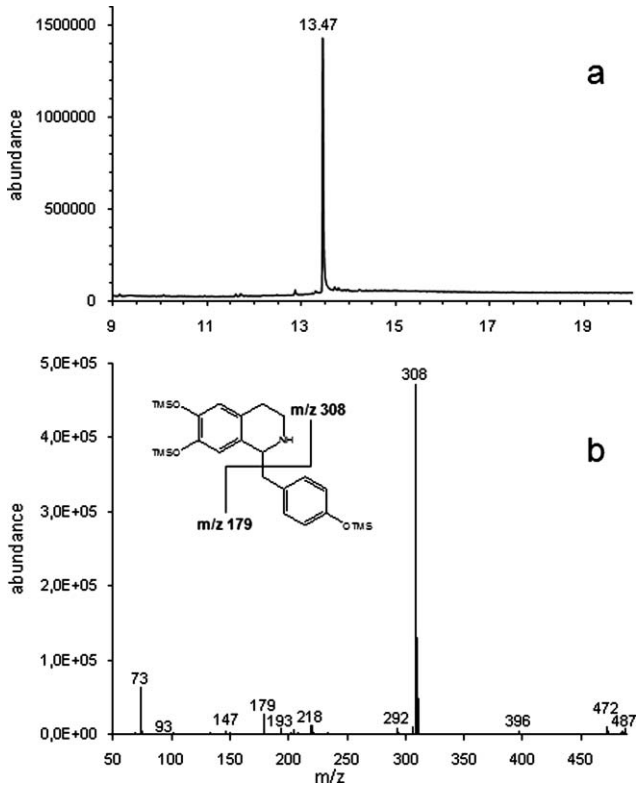


Fig. 1 (A) A GC-MS chromatogram of the reaction mixture after extraction and derivatization with TMS-chloride. (B) The EI mass spectrum of TMS-derivatized (*S*)-norcoclaurine. The inset shows the two major ions derived from TMS-(*S*)-norcoclaurine fragmentation (m/z = 308 and 179).

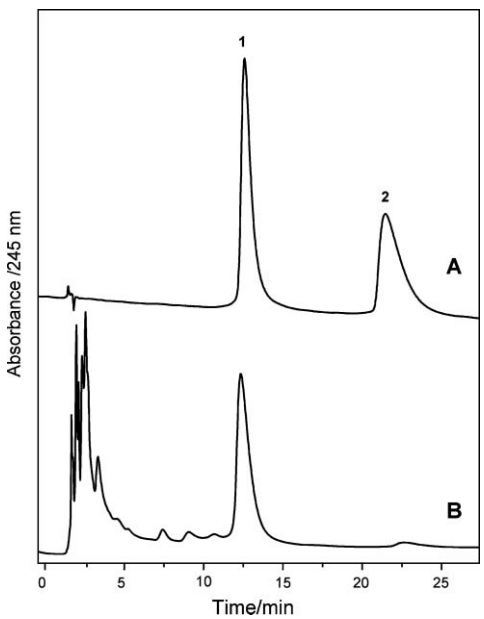


Fig. 2 (A) Chiral HPLC chromatogram of a standard racemic mixture of norcoclaurine. Acquisition was performed with a UV detector at 254 nm. (B) HPLC chromatogram of the reaction mixture, showing the presence of the expected (*S*)-norcolaurine enantiomer (peak 1).

have been widely characterized,[14] and conditions for optimal binding/desorption of (*S*)-norcoclaurine were investigated as a function of temperature and solvent composition. The absorption was achieved by shaking carbon granules directly added to the aqueous phase at room temperature (30 min shaking). Control experiments indicated that the dopamine substrate is not absorbed onto the activated carbon beads, whereas the aldehyde substrate is strongly absorbed. The best desorption conditions for (*S*)-norcoclaurine were obtained in ethanol at 40 °C in the presence of a slight molar excess of NaOH. This procedure allowed a very good yield of (*S*)-norcoclaurine to be obtained in the absence of unreacted aldehyde contaminant, which remained adsorbed on the carbon matrix. This yield is comparable to that achieved by extraction with a four volume excess of diethyl ether (81% *vs.* 79%). The procedure based on activated carbon has the notable advantage of recovering about 70% of the active NCS enzyme from the reaction mixture once the carbon has been removed by filtration. In recycling experiments (Fig. 4), the same enzyme solution could be used up to five times, with an overall product recovery of about

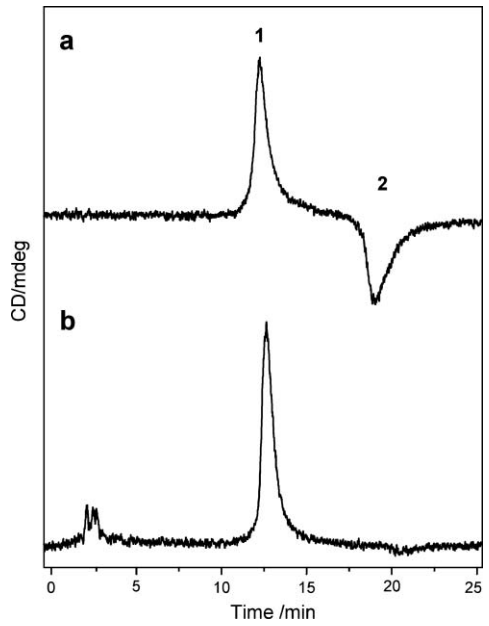


Fig. 3 (A) Chiral HPLC chromatogram of a standard racemic mixture of norcoclaurine. Acquisition was performed with a CD detector at 280 nm. (B) HPLC chromatogram of the reaction mixture, showing the presence of the expected (*S*)-norcolaurine enantiomer (peak 1).

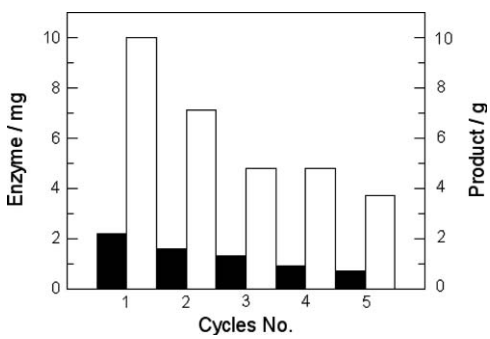


Fig. 4 Reaction recycling: Five recycling cycles were performed by monitoring the enzyme activity after each step (white bars) and the product yield (black bars). The enzyme concentration in centricon 20 mL tubes was obtained before each cycle.

670 mg of product (isolated) per mg of enzyme. However, further optimization may be necessary to make this recycling cost-effective. The current limitation of the process resides mainly in the relatively low stability of the aldehyde substrate. In turn, the aldehyde might be conveniently generated *in situ* by means of an appropriate amino acid or amine oxidase. With this aim in mind, several enzymes bearing appropriate specificities for tyrosine or tyramine are presently being tested.

Conclusions

In conclusion, a new, easily scalable process for the synthesis of (*S*)-norcoclaurine has been reported according to a one-pot, two-step reaction by using norcoclaurine synthase enzyme, produced in *E. coli* and purified in high yield. The stereoselectivity of the reaction was 93% and the reaction yields were found to be about 80%.

The results reported in this study provide an example of a chemoenzymatic synthetic strategy as a novel, efficient and green tool for manufacturing plant-derived metabolites, particularly benzylisoquinoline alkaloids.

Experimental section

Chemicals

Reagents obtained from commercial suppliers were used without further purification. (*R*,*S*)-norcoclaurine was obtained from Sequoia Research Products (UK).

NCS expression and purification

A synthetic gene coding for norcoclaurine synthase (NCS) from *Thalictrum flavum* has been constructed by GENEART (GmbH, Germany) with optimised *E. coli* codons. The NCS protein truncated at the first 19 amino acids with a His-tag at the C-terminus was expressed in *E. coli* BL21 (DE3) cells under fermentative conditions. The cells were harvested by centrifugation (4000*g* for 10 min at 4 °C). Cell pellets were frozen overnight, then resuspended and sonicated in 10 mL of 50 mM phosphate buffer pH 8 containing 300 mM NaCl (buffer A) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The extracts were centrifuged at 16000*g* for 20 min at 4 °C. NCS was purified in batch using a nickel-chelating resin (Protino, Ni-TED, Macherey-Nagel). After washing with buffer A, the protein was eluted with buffer A containing 500 mM imidazole (buffer B).

One-pot preparation of norcoclaurine from 4-hydroxyphenylacetaldehyde and dopamine

4-Hydroxyphenylacetaldehyde was prepared according to the method of Hazen *et al*.[15] A solution of tyrosine (10 mmol) was mixed with a solution of NaClO (10 mmol) in phosphate buffer 50 mM pH 7.0 (1 h at 37 °C) to a final volume of 1 L. Aliquots of 50 µL of the reaction mixture were extracted with 200 µL of diethyl ether and directly injected into the GC-MS instrument (1 µL) to follow the aldehyde formation. Once tyrosine had been completely converted in 4-hydroxyphenylacetaldehyde (10 mM final concentration), dopamine (10 mmol) and norcoclaurine synthase (10 mg, corresponding to a final concentration of 0.5 µM) were added to the reaction mixture in the presence of ascorbate (5 mM) and incubated for 30 min at 37 °C. Activated carbon NORIT (Sigma-Aldrich 93067) was used as the adsorbent for the purification of (*S*)-norcoclaurine. Briefly, 10 g of adsorbent were added to the aqueous reaction mixture (1 L). After 30 min shaking at room temperature, the mixture was filtered. The adsorbent was recovered and washed twice with 50 mL of distilled water. It was then transferred into a conical flask and treated with 100 mL of ethanolic sodium hydroxide (0.005 N NaOH in 99% ethanol). Desorption was carried out by shaking for 2 h at 40 °C. The (*S*)-norcoclaurine-enriched organic fraction was neutralized with 0.005 N HCl, evaporated to dryness under reduced pressure and the resulting solid characterized by means of GC-MS, ESI/MS, ^{1}H NMR and ^{13}C NMR. Enantiomeric excesses were determined by chiral HPLC. The specific rotations of MeOH solutions of norcoclaurine were measured using a JASCO P-1030 polarimeter at 25 °C (cell path 10 cm).

ESI-MS

1 mg of (*S*)-norcoclaurine, obtained as described above, was dissolved in 1 mL of acetonitrile and analyzed by ESI-MS (Thermo Finnigan LXQ). The spectrometer had an electrospray ion source and a linear ion trap analyzer. The ESI capillary temperature was 275 °C and the analysis flow was 5 µL min^{-1}. The positive ESI-MS of norcoclaurine was observed with an $m/z = 272$ $[M + H]^+$.

GC-MS analysis

An Agilent 6850A gas chromatograph coupled to a 5973 N quadrupole mass selective detector (Agilent Technologies, Palo Alto, CA, USA) was used. Chromatographic separations were carried out on an Agilent HP5 ms fused-silica capillary column (30 m × 0.25 mm i.d.) coated with 5% phenyl 95% dimethylpolysiloxane (film thickness 0.25 µm) as a stationary phase. Injection mode: splitless at a temperature of 260 °C. Column temperature program: 100 °C (1 min), then to 300 °C at a rate of 15 °C min^{-1} and then held for 5 min. The carrier gas was helium at a constant flow rate of 1.0 mL min^{-1}. Spectra were obtained in electron impact mode at 70 eV ionization energy and a mass scan range from $m/z = 50$ to 500; ion source 280 °C; ion source vacuum 10^{-5} Torr.

(*S*)-Norcoclaurine formation was followed by withdrawing aliquots of the reaction mixture (20 µL) until the increase in yield was negligible. Each aliquot was dried under an N_2 stream and directly derivatized with trimethylsilyl chloride in order to obtain the corresponding trimethylsilyl ether.

HPLC analysis for enantiomeric excess determination

Separation of the norcoclaurine enantiomers was performed by a HPLC analysis carried out on a chiral stationary phase with Teicoplanin as the selector, obtained from Gasparrini F. (CSP-Teicoplanin, 250 × 4.0 mm); the mobile phase was methanol–acetonitrile (70/30 v/v) containing 0.25% Et_3N and 0.25% CH_3COOH; flow rate: 1.00 mL min^{-1} at 25 °C. UV and CD detections were performed at λ = 254 and 280 nm,

respectively. The enantiomeric excess of the product was determined by chiral HPLC. The retention times of the (*S*)- and (*R*)-norcoclaurine isomers were 12.5 and 21.5 min, respectively. The $[\alpha]_D^{25}$ value obtained was −24.7 in methanol. The compound is thus assigned as (*S*)-(−) norcoclaurine, in agreement with ref. 16.

NMR analysis

NMR measurements were carried out in CD_3OD at room temperature on a Varian Unity Inova instrument at 400 MHz and 296 K. Chemical shifts are expressed in δ values relative to tetramethylsilane (TMS) as an internal reference.

^{1}H NMR (CD_3OD, 400 MHz): δ 7.09 (d, J = 7.5 Hz; H-2′, H-6′), 6.77 (d, J = 7.5 Hz; H-3′, H-5′), 6.64 (s, H-5), 6.55 (s, H-8), 4.21 (br s, H-1), 3.21 (m, H_2-3), 2.89 (m, H_2-9), 2.76 (m, H_2-4); ^{13}C NMR (CD_3OD, 400 MHz): δ 156.4 (C-4′), 144.6, 143.8 (C-6, C-7), 130.3 (C-2′, C-6′), 127.8, 126.1, 124.3 (C-4a, C-8a, C-1′), 115.4 (C-3′, C-5′), 115.1, 113.0 (C-5, C-8), 56.7 (C-1), 40.3, 39.9 (C-3, C-9), 34.0 (C-4).

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