Mechanistic Studies on Norcoclaurine Synthase of Benzylisoquinoline Alkaloid Biosynthesis: An Enzymatic Pictet−Spengler Reaction†

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ABSTRACT: Norcoclaurine synthase catalyzes an asymmetric Pictet−Spengler condensation of dopamine and 4-hydroxyphenylacetaldehyde to give (*S*)-norcoclaurine. This is the first committed step in the biosynthesis of the benzylisoquinoline alkaloids that include morphine and codeine. In this work, the gene encoding for the *Thalictrum flavum* norcoclaurine synthase is highly overexpressed in *Escherichia coli* and the resulting His-tagged recombinant enzyme is purified for the first time. A continuous assay based on circular dichroism spectroscopy is developed and used to monitor the kinetics of the enzymatic reaction. Dopamine analogues bearing a methoxy or hydrogen substituent in place of the C-1 phenolic group were readily accepted by the enzyme whereas those bearing the same substituents at C-2 were not. This supports a mechanism involving a two-step cyclization of the putative iminium ion intermediate that does not proceed via a spirocyclic intermediate. The reaction of [3,5,6-^{2}H]dopamine was found to be slowed by a kinetic isotope effect of 1.7 ± 0.1 on the value of k_{cat}/K_M. This is interpreted as showing that the deprotonation step causing rearomatization is partially rate determining in the overall reaction.

The benzylisoquinoline alkaloids are a large group of plant secondary metabolites that contain more than 2500 compounds with widely divergent structural scaffolds and pharmacological properties (Figure 1) (*1*). They are formed by a group of five related plant families that include the *Papaveraceae* (poppy family) and the *Ranunculaceae* (buttercup family). The opiates morphine and codeine are well-known benzylisoquinoline alkaloids that have long been used for their analgesic properties. Other examples include the muscle relaxants papaverine and tubocurarine (the main active component in the South American arrow poison curare) as well as the antimicrobials berberine and sanguinarine.

The biosynthetic pathway that produces all benzylisoquinoline alkaloids begins with the conversion of the amino acid tyrosine into both dopamine and 4-hydroxyphenylacetaldehyde by four enzymes that catalyze hydroxylation, decarboxylation, and transamination reactions (*2*). The first committed step of the pathway is catalyzed by the enzyme norcoclaurine synthase that condenses dopamine and 4-hydroxyphenylacetaldehyde to give (*S*)-norcoclaurine (Figure 1) (*3*−*5*). This is formally an asymmetric Pictet−Spengler reaction and sets up the characteristic benzylisoquinoline core that defines this family of alkaloids. The (*S*)-configuration is required for further enzymatic steps and is retained in several of the alkaloids of this pathway (such as the key branch-point intermediate (*S*)-reticuline), but is often inverted in later biosynthetic steps (morphine bears the (*R*)-configuration at this position).

The asymmetric synthesis of isoquinoline alkaloids has been a long-standing goal for organic chemists, and the extensive work in this field has recently been reviewed (*6*). One strategy involves the Pictet−Spengler reaction that is an electrophilic cyclization between an iminium ion and an aromatic ring (*7*−*9*). This addition generates a new stereogenic center, and recent efforts have focused on the development of catalysts that promote the asymmetric Pictet−Spengler reaction with indole substrates (*10*, *11*). The use of "Pictet−Spenglerases" provides an attractive option in this quest and may lead to novel products with high stereochemical purity. Three such enzymes have been identified in alkaloid biosynthetic pathways and include strictosidine synthase, norcoclaurine synthase, and deacetylipecoside synthase. Strictosidine synthase catalyzes the condensation of secologanin and tryptamine to form strictosidine, which is the first commited step in the bisosynthesis of the terpene indole alkaloids (Figure 2). This enzyme was first purified from *Catharanthus roseus*, and a recombinant form of the *Rauvolfia serpentina* enzyme was subsequently produced in *Escherichia coli* (*12*, *13*). More recently, the structure of strictosidine synthase has been reported, and studies on investigating and manipulating the substrate specificity of the enzyme have appeared (*14*, *15*). Significantly less detailed information is available on norcoclaurine synthase. The activity of this enzyme was first detected in a variety of plant extracts in 1981, and it was originally characterized as a (*S*)-

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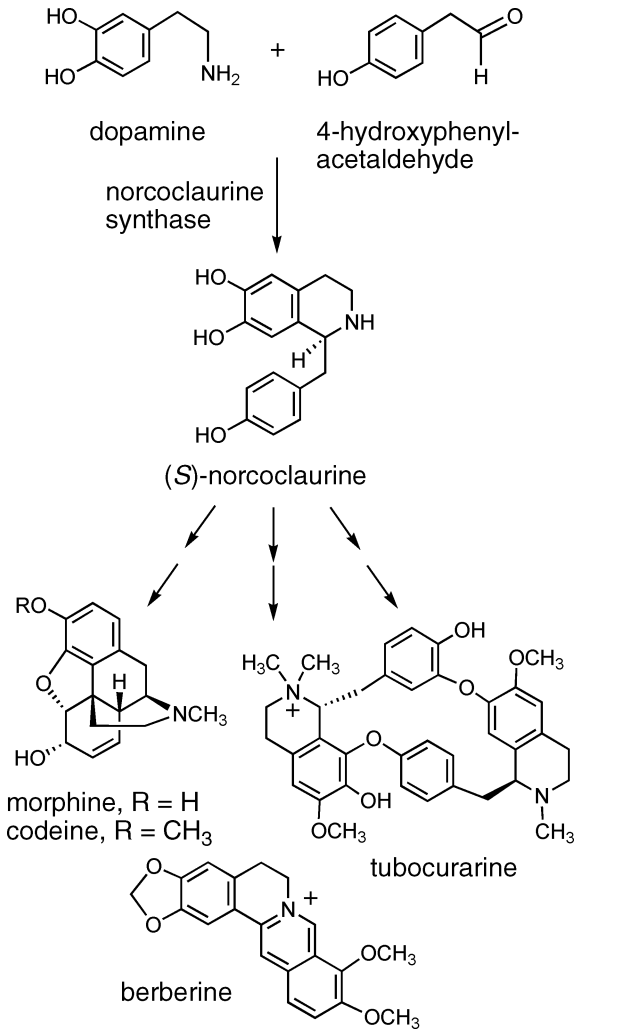
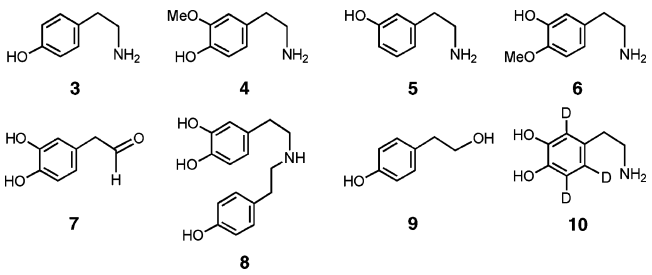


FIGURE 1: The reaction catalyzed by norcoclaurine synthase and representative structures of benzylisoquinoline alkaloids.

norlaudanosoline synthase based on its ability to use 3,4-dihydroxyphenylacetaldehyde **7** as an alternate substrate (*3*−*5*, *16*). More recently, norcoclaurine synthase has been



purified from *Thalictrum flavum* ssp. glaucum and kinetically characterized (*17*). The gene encoding for a truncated form of the enzyme (lacking a putative signal peptide) has been cloned and overexpressed in *E. coli*. While the recombinant enzyme was found to active, low expression levels precluded its purification (*18*). Notably, no sequence homology is detected between strictosidine synthase and norcoclaurine synthase, suggesting that they may have arisen from different ancestors as products of convergent evolution (*14*). Finally, deacetylipecoside synthase catalyzes the Pictet−Spengler condensation of secologanin and dopamine to give deacetylipecoside (*19*). This enzyme has been purified from *Alangium*

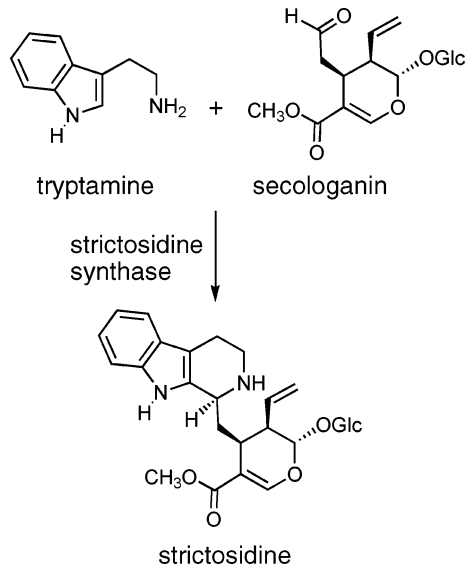


FIGURE 2: The reaction catalyzed by strictosidine synthase.

lamarckii Thw.; however, the gene that encodes for it has yet to be identified.

Two general mechanistic possibilities may be considered for the enzymatic Pictet−Spengler reaction (Figure 3). In each case, the first step involves formation of an iminium ion intermediate that either may be enzyme-catalyzed or may spontaneously occur in solution. The most direct path to product involves an ionization of the phenolic group at C-2 of the catechol moiety to generate the zwitterionic intermediate **1** that cyclizes to give the σ-intermediate (Figure 3, path A). A subsequent deprotonation causes rearomatization and product formation. A second possibility involves ionization of the phenolic group at C-1 of the catechol moiety to generate the zwitterionic intermediate **2** that cyclizes to form a spirocyclic intermediate (Figure 3, path B). A subsequent ionization of the C-2 hydroxyl and protonation of the ketone induces a semi-pinacol rearrangement to give the σ-intermediate that proceeds to product as described. While the second mechanism involves an additional step and a potentially unfavorable 5-*endo-trig* cyclization, it should not be discounted as several lines of evidence point toward an analogous mechanism for nonenzymatic Pictet−Spengler reactions involving tryptamine (*8*, *20*−*22*). While this mechanism has only been postulated for indole-based reactions in the literature, it is possible that catechol-based cyclizations could also proceed in this manner.

In this work, we describe the high level overexpression of the *T. flavum* norcoclaurine synthase gene in *E. coli* and the purification of the resulting His-tagged recombinant enzyme. A kinetic assay based on circular dichroism spectroscopy is described and used to kinetically characterize the reaction with both natural and alternate substrates. Data that supports the direct cyclization mechanism (path A, Figure 3) is presented and a kinetic isotope effect is measured that indicates rearomatization is a partially rate determining step of catalysis.

EXPERIMENTAL PROCEDURES

Materials and General Methods. All chemicals were purchased from Sigma-Aldrich and used without further refinement unless otherwise noted. 4-Hydroxyphenylacetal-

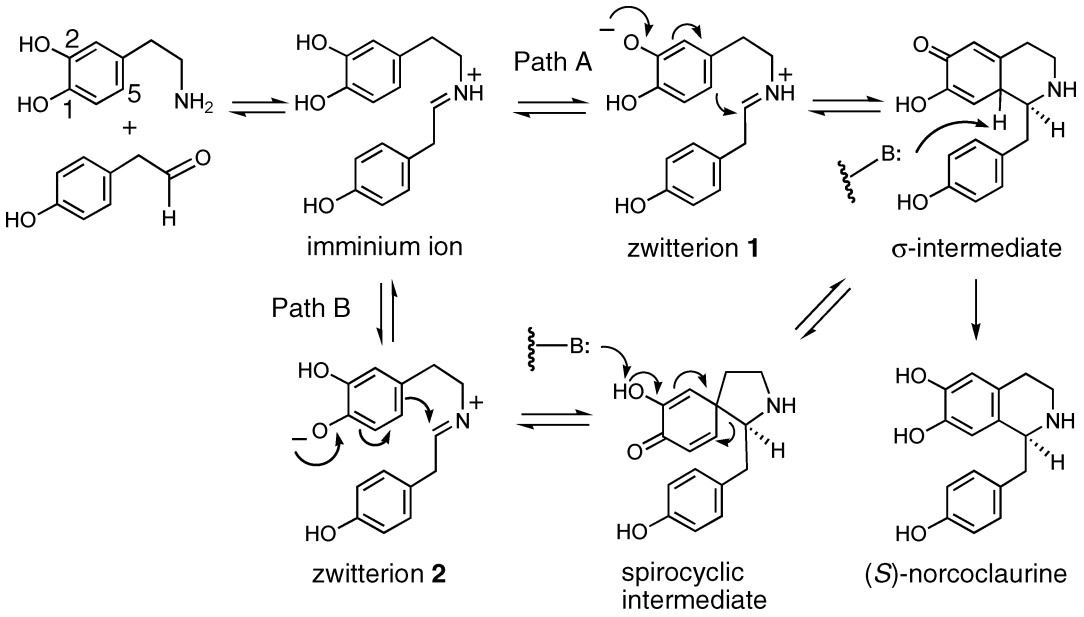


FIGURE 3: Potential mechanisms for the reaction catalyzed by norcoclaurine synthase.

dehyde was synthesized by the Doering−Parikh oxidation of 2-(4-hydroxyphenyl)ethanol as described previously (*23*). It was stable indefinitely if stored at −78 °C as a neat oil. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard (*24*). ^{1}H NMR spectra were obtained on a Bruker AV300 and AV400 spectrometer at a field strength of 300 or 400 MHz, respectively. Mass spectrometry was performed by the Mass Spectrometry Center at UBC by electrospray ionization (ESI-MS) using a Waters Micromass LCT mass spectrometer.

Overexpression and Purification of Norcoclaurine Synthase (TfNCSΔ19). The recombinant TfNCSΔ19/pET29b plasmid (*18*) was transformed into BL21 (DE3) RIL chemically competent *E. coli* (Stratagene). The cells were incubated overnight at 37 °C in 10 mL Terrific Broth (TB) medium containing 37 μg mL^{-1} chloramphenicol and 30 μg mL^{-1} kanamycin with shaking at 225 rpm. The overnight cultures were poured into 1 L of Luria−Bertani (LB) medium containing 37 μg mL^{-1} chloramphenicol and 30 μg mL^{-1} kanamycin and grown at 37 °C with shaking at 225 rpm until an OD_{600} of 0.6 was reached. Cells were induced for overexpression by addition of 238 mg (1 mM) of isopropyl β-D-galactopyranoside (IPTG), and the cultures were allowed to continue growth at 24 °C until an OD_{600} of 3.0−4.0 was reached (∼24 h). Cells were harvested by centrifugation at 6000 rpm for 30 min and resuspended in 20 mL of 200 mM potassium phosphate buffer (pH 7.5) containing 1 μg mL^{-1} pepstatin A and 1 μg mL^{-1} aprotinin. The cells were lysed at 20 000 psi in an ice-cooled French pressure cell. The cell lysate was clarified by centrifugation at 6000 rpm for 60 min and filtered through a 0.22 μm membrane prior to affinity chromatography.

A 10 mL column containing Chelating Sepharose Fast Flow resin (Pharmacia Biotech) was charged with 2 column volumes (CV) of 100 mM $NiSO_4$ followed by washing with 2 CV of distilled H_2O and 3 CV of phosphate buffer (200 mM potassium phosphate buffer, pH 7.5) containing 5 mM imidazole. The filtered cell lysate was loaded at 2 mL min^{-1}, and phosphate buffer containing 5 mM imidazole (∼10 CV) was passed through the column at 3 mL min^{-1} until no more flow-through protein eluted, as determined by monitoring A_{280}. A wash with phosphate buffer containing 125 mM imidazole (∼5 CV) was used to remove nonspecifically bound proteins. Histidine-tagged protein was finally eluted with 3−4 CV of phosphate buffer containing 500 mM imidazole. The enzyme was concentrated by ultrafiltration (Amicon Ultra-4, 10 000 MWCO) to 5−30 mg mL^{-1}, and 25−50 μL aliquots were flash-frozen with liquid N_2 in the presence of 10% glycerol. The enzyme could be stored at −70 °C for at least 6 months without significant loss of activity.

^{1}H NMR Assay of Norcoclaurine Synthase Activity. A solution of deuterated potassium phosphate buffer (200 mM, pD 7.5, 1.00 mL) containing dopamine (10 mM) and 4-hydroxyphenylacetaldehyde (10 mM, prepared from the addition of 10 μL of a 1 M stock in d^4-methanol) was placed in an NMR tube, and an initial ^{1}H NMR spectrum was taken. To the solution was then added 100 μg of norcoclaurine synthase (TfNCSΔ19), and after incubating the reaction mixture for 30 min at 25 °C a ^{1}H NMR spectrum was taken.

Isolation and Characterization of (S)-Norcoclaurine. To a solution of potassium phosphate buffer (200 mM, pH 7.5, 10 mL) containing dopamine (50 mM) and 4-hydroxyphenylacetaldehyde (50 mM, prepared from the addition of 500 μL of a 1 M stock in methanol) was added 220 μg of norcoclaurine synthase (TfNCSΔ19), and the mixture was incubated for 30 min at 37 °C. Enzyme was removed by centrifugal ultrafiltration, and the resulting filtrate was evaporated to dryness under reduced pressure. In order to isolate (*S*)-norcoclaurine, the resulting solid was suspended in 100 mL of ice-cold EtOH and filtered to remove the phosphate salts. Removal of the solvent under reduced pressure gave (*S*)-norcoclaurine that was free of phosphate salts as analyzed by ^{31}P NMR spectroscopy. When analyzed by circular dichroism spectroscopy, material isolated in this fashion retained the same molar ellipticity as was originally observed during the enzymatic reaction. In order to purify the compound away from minor impurities it was possible to run a silica gel column, however, the product obtained in this fashion was found to be racemic. The solid obtained

following ultracentrifugation and evaporation was dissolved in 10 mL of MeOH, and 600 mg of silica gel was added. The MeOH was then removed under reduced pressure. The silica gel-adsorbed sample was dry loaded onto a silica gel column (50 mL). The column was washed with 1000 mL of 100:1 acetone/acetic acid, and the product was eluted with 200 mL of 50:50:1 acetone/methanol/acetic acid. The resulting (*R*,*S*)-norcoclaurine acetate salt was characterized using ^{1}H NMR in D_2O and positive ESI-MS mass spectrometry: ^{1}H NMR (D_2O) δ 1.96 (s, 3H), 2.96 (m, 3H), 3.28 (m, 1H), 3.47 (m, 2H), 4.60 (dd, 1H, J= 9.2 Hz, J = 5.5 Hz), 6.68 (s, 1H), 6.74 (s, 1H), 6.90 (d, 2H, J = 8.5 Hz), 7.18 (d, 2H, J = 8.5 Hz); +ve ESI-MS (MeOH) *m*/*z* 272 ($M - H^+$).

Reactions with Substrate Analogues. To test the four dopamine analogues **3**−**6** as alternate substrates, solutions containing 30 mM of the analogue and 30 mM 4-hydroxyphenylacetaldehyde in 200 mM potassium phosphate buffer (pH 7.5, 5 mL total volume) were incubated with 150 μg of norcoclaurine synthase (TfNCSΔ19) at 37 °C for 24 h. The reaction progress was monitored by positive ESI-MS. To test 3,4-hydroxyphenylacetaldehyde **7** as an alternate substrate, a similar incubation containing 30 mM **7** and 30 mM dopamine was monitored. Products from the successful reactions (obtained with analogues **5**−**7**) were isolated in an identical manner as described for (*S*)-norcoclaurine. 7-Deoxynorcoclaurine acetate (product from analogue **5**): ^{1}H NMR (D_2O) δ 1.96 (s, 3H), 3.04 (m, 3H), 3.50 (m, 3H), 4.70 (dd, 1 H, J = 8.5 Hz J = 6.2 Hz), 6.77 (s, 1H), 6.79 (m, 1 H, J = 8.5 Hz), 6.91 (d, 2 H, J = 8.3 Hz), 7.12 (d, 1 H, J = 8.5 Hz), 7.19 (d, 2 H, J = 8.3 Hz); +ve ESI-MS (MeOH) *m*/*z* 256 ($M - H^+$). 7-Methoxynorcoclaurine acetate (product from analogue **6)**: ^{1}H NMR (D_2O) δ 1.96 (s, 3H), 3.05 (m, 1H), 3.30 (m, 3H), 3.50 (m, 2H), 3.65 (s, 3H), 4.60 (t, 1H, J = 8.5 Hz), 6.68 (s, 1H), 6.74 (s, 1H), 6.90 (d, 2H, J = 8.5 Hz), 7.18 (d, 2H, J = 8.5 Hz); +ve ESI-MS (MeOH) *m*/*z* 286 ($M - H^+$).

Deuterium Incorporation Experiment. The intermediate analogue, *N*-(4-hydroxyphenethyl)-*N*-(3,4-dihydroxyphenethyl)amine hydrochloride **8**, was synthesized as previously described (*25*). To three NMR tubes containing 900 μL of deuterated potassium phosphate buffer (200 mM, pD 7.5) was added either 50 μL of a solution containing 0.1 M dopamine, or 50 μL of a solution containing 0.1 M dopamine and 0.1 M 2-(4-hydroxyphenyl)-ethanol **9**, or 50 μL of a solution containing 0.1 M of compound **8** (final concentrations of 5 mM). The incubations were initiated by the addition of 50 μL of norcoclaurine synthase (TfNCSΔ19, 150 μg) and incubated at 37 °C. ^{1}H NMR spectra and ESI mass spectra (following dilution of an aliquot into MeOH) were acquired at 0 and 24 h.

Circular Dichroism Spectroscopic Assay. Enzyme kinetics were measured by monitoring the formation of (*S*)-norcoclaurine using circular dichroism spectroscopy. An initial determination of the molar ellipticity of (S)-norcoclaurine was made by incubating 600 μM dopamine and 700 μM 4-hydroxyphenylacetaldehyde in 200 mM phosphate buffer, pH 7.5, with 30 μg of norcoclaurine synthase (TfNCSΔ19) for 30 min at 37 °C. The enzyme was removed by ultracentrifugation and a circular dichroism spectrum was taken that showed a maximal signal at 285 nm with $[\theta]$ = 12541 mdeg cm^{-1} M^{-1}. Control reactions run in D_2O and monitored by ^{1}H NMR spectroscopy confirmed that the enzymatic reaction had proceeded to completion during this incubation time.

To measure the enzyme kinetics, a 0.5 cm cuvette containing 200 mM potassium phosphate buffer (pH 7.5), 4-hydroxyphenylacetalehyde (4-HPAA) (variable) and dopamine (variable) was thermally equilibrated for 3 min at 37 °C. The enzymatic reaction was initiated by the addition of norcoclaurine synthase (TfNCSΔ19, 2.1 μg) for a total assay volume of 1000 μL, and the enzymatic rate was calculated from the observed increase of signal at 285 nm (using $[\theta]$ = 12541 mdeg cm^{-1} M^{-1}). The K_m value for 4-hydroxyphenylacetaldehyde was measured in the presence of 2 mM dopamine (saturating) with the concentration of the aldehyde varying between 100 μM and 2000 μM. The K_m value for dopamine was measured in the presence of 2 mM 4-hydroxyphenylacetaldehyde (saturating) with the concentration of dopamine varying between 100 μM and 2000 μM. Kinetic parameters were determined from initial velocities fit to the Michaelis−Menten (4-hydroxyphenylacetaldehyde) and sigmoidal (dopamine) kinetics using the programs GraFit and Sigma Plot, respectively.

Kinetic constants for the dopamine analogues **5** and **6** were measured under identical conditions except that they were monitored at 280 and 285 nm and the molar ellipticities of the products were found to be $[\theta]$ = 6590 and 6175 mdeg cm^{-1} M^{-1}, respectively. Kinetic constants for the 4-hydroxyphenylacetaldehyde analogue **7** were measured under identical conditions except that the reaction was monitored at 280 nm and the molar ellipticity of the product was found to be $[\theta]$ = 5963 mdeg cm^{-1} M^{-1}.

Kinetic Isotope Effect Studies. [3,5,6-^{2}H]Dopamine **10** was prepared according to the procedure of Vining et al. (*26*). The extent of ^{2}H incorporation was determined to be ≥97% by ^{1}H NMR spectroscopy and ESI-MS. To a solution of an approximately 1:1 molar ratio of unlabeled dopamine and [3,5,6-^{2}H]-dopamine (12.5 mM each) and 30 mM 4-hydroxyphenylacetaldehyde in 200 mM potassium phosphate buffer (pH 7.5, 5 mL total) was added norcoclaurine synthase (TfNCSΔ19, 340 μg in 100 μL of 200 mM potassium phosphate buffer, pH 7.5). 100 μL aliquots were collected at 0, 2.5, 5, 10, and 15 min time intervals. To each sample was added 200 μL of 1 M HCl to inactivate the enzyme, followed by neutralization with 200 μL of 1 M NaOH. The samples were immediately diluted with 20 mL of H_2O, flash frozen with liquid N_2, and lyophilized. Final ^{1}H NMR (D_2O containing 1 mM dioxane) and ESI-MS spectra were acquired. The KIE[1] on k_{cat}/K_m was determined from the initial and final spectra using the equation KIE = $\ln(1 - F_H)/\ln[(1 - F_H)R/R_0]$ (*27*), where F_H is the fractional conversion of the protiated species to products and R_0 and R are the initial and final ratios of protiated to deuterated substrate, respectively. F_H was calculated from the NMR spectra before and after addition of the enzyme by integration of the benzylic dopamine signals at 3.11 ppm relative to the internal standard of 1 mM dioxane. R_0 and R were calculated from the mass spectra before and after addition of the enzyme using the relative peak intensities of the protiated substrate [*m*/*z* 154 ($M - H^+$)] to the deuterated substrate [*m*/*z* 157 ($M - H^+$)]. The reported value represents the average of three indepen-

[1] Abbreviation: KIE, kinetic isotope effect.

dent determinations. A control reaction lacking enzyme showed that <5% product was formed from nonenzymatic background reaction during these incubation times.

RESULTS

Gene Expression and Enzyme Purification. In past work on norcoclaurine synthase from *T. flavum*, the full length cDNA encoding for the enzyme was cloned and sequenced (*18*). Expression of this gene in *E. coli* did not result in the production of any detectable level of recombinant protein, however, expression of constructs encoding for an N-terminally truncated protein (missing either the first 10, pNCSΔ10, or first 19, pNCSΔ19, amino acids) did generate small amounts of active enzyme. Kinetic studies on crude bacterial lysate containing the truncated enzyme revealed Michaelis constants and Hill coefficients that were similar to those obtained with enzyme isolated form wild type sources, indicating that the truncations had not greatly affected its catalytic properties.

In this work, extensive attempts were made to optimize the expression levels of pNCSΔ19 and permit isolation of the recombinant protein. A notable increase in protein production was observed upon switching to *E. coli* cells that carry extra copies of the *argU*, *ileY*, and *leuW* tRNA genes (BL21 (DE3) RIL) in order to compensate for codon bias during heterologous expression. Further increases were observed when Terrific Broth was used in the growth of the starter cultures and when the bacteria were grown at 25 °C following induction. Ultimately it was possible to obtain the recombinant synthase at levels of 15 mg/L under optimal conditions. Since the expression of pNCSΔ19 generates a protein bearing a C-terminal hexahistidine tag, it was possible to purify the synthase using metal ion affinity chromatography. In this fashion protein could be isolated that was >90% pure as estimated by SDS−PAGE analysis (Figure 4). This protein was quite stable and could be stored at −70 °C for 6 months without significant loss of activity.

Kinetic Studies. In past studies the kinetics of the reaction catalyzed by norcoclaurine synthase were analyzed using a stopped radioactive assay employing either [2,6-^{3}H]dopamine or [8-^{14}C]dopamine (*16*, *18*). In the former case the amount of tritium release into solution was monitored, and in the latter case a TLC separation of the reactants/products was employed. A continuous assay that does not require radiolabeled substrate or a separation step could greatly facilitate studies on this enzyme. In particular, it would allow one to measure the kinetics of the reaction with dopamine analogues that are not available as isotopically labeled compounds. Since the substrates dopamine and 4-hydroxyphenylacetaldehyde are achiral and the product (*S*)-norcoclaurine is chiral, circular dichroism spectroscopy presents an attractive method for following product formation. Indeed, a CD spectrum of the (*S*)-norcoclaurine formed in the enzymatic reaction was found to exhibit a maximum at 285 nm with a molar ellipticity of 12 541 mdeg cm^{-1} M^{-1}. Thus, it was possible to determine the enzyme kinetics in a continuous fashion using a CD assay. In past studies with enzyme obtained from natural sources, all kinetic data had to be corrected for a low background reaction that can occur nonenzymatically (*17*, *28*). In this work, the background reaction did not pose a problem as the relative abundance of enzyme permitted a much more rapid kinetic analysis (5 min) during which time no significant nonenzymatic reaction could occur. Furthermore, any norcoclaurine produced in a nonenzymatic fashion would be racemic and would not affect the observed ellipticity values. The fact that a CD signal is observed indicates that one enantiomer of norcoclaurine, presumably (*S*), is formed in excess of the other. We expect that the enzyme is highly stereoselective, but have not yet established the enantiomeric excess of the product formed. Nevertheless, the kinetic assay will still be valid as long as the ratio of enantiomers is constant throughout the reaction.

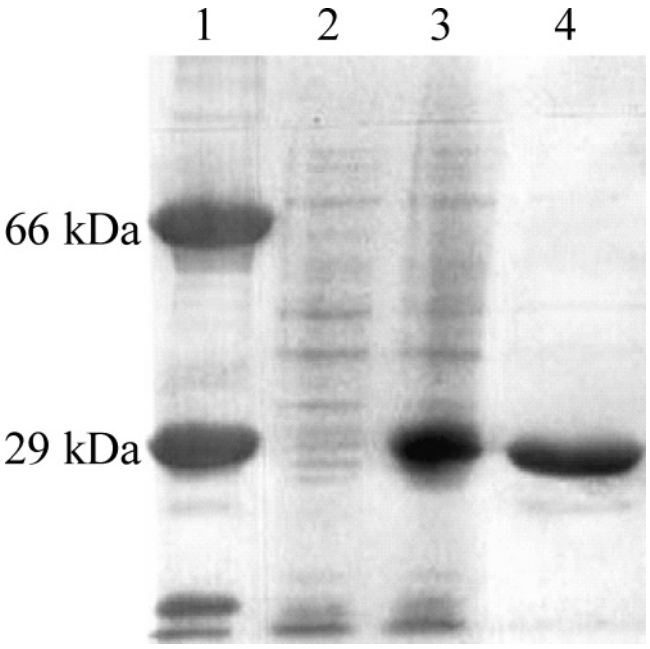


FIGURE 4: SDS−PAGE gel showing the purification of norcoclaurine synthase (TfNCSΔ19). Lane 1 contains molecular mass standards of 66 and 29 kDa, lane 2 shows crude cell extract before induction, lane 3 shows crude cell extract after isopropyl β-D-1-thiogalactopyranoside induction, and lane 4 shows the purified norcoclaurine synthase.

Determination of the kinetic constants at saturating levels of both substrates gave a value of $k_{cat} = 6.4 \pm 0.3$ s^{-1}. When the concentration of dopamine was held constant and the concentration of 4-hydroxyphenylacetaldehyde was varied, a hyperbolic kinetic profile was observed that led to a value of $K_M = 288 \pm 38$ μM (Figure 5A, Table 1). When the concentration 4-hydroxyphenylaldehyde was held constant and the concentration of dopamine was varied, however, a sigmoidal kinetic profile was observed indicative of positive cooperativity (Figure 5B, Table 1). Fitting this data to the Hill equation gave values of $K_{Mapp} = 350 \pm 48$ μM and $n = 1.8 \pm 0.2$. One limitation to the circular dichroism assay was that accurate initial velocity measurements were difficult to obtain at substrate concentrations lower than 100 μM due to low signal-to-noise ratios. This led to errors in the values of K_M that were typically ±15%. The value of K_M determined for 4-hydroxyphenylaldehyde agreed reasonably well with those previously reported for the wild type enzyme (335 μM) and the crude recombinant enzyme (700 μM) (*17*, *18*). The sigmoidal dependence of rate versus dopamine concentration was also observed in previous studies and Hill coefficients of 1.8 and 1.98 were reported for the wild type and recombinant enzymes, respectively. A notable difference was observed in the apparent K_M values for dopamine that were

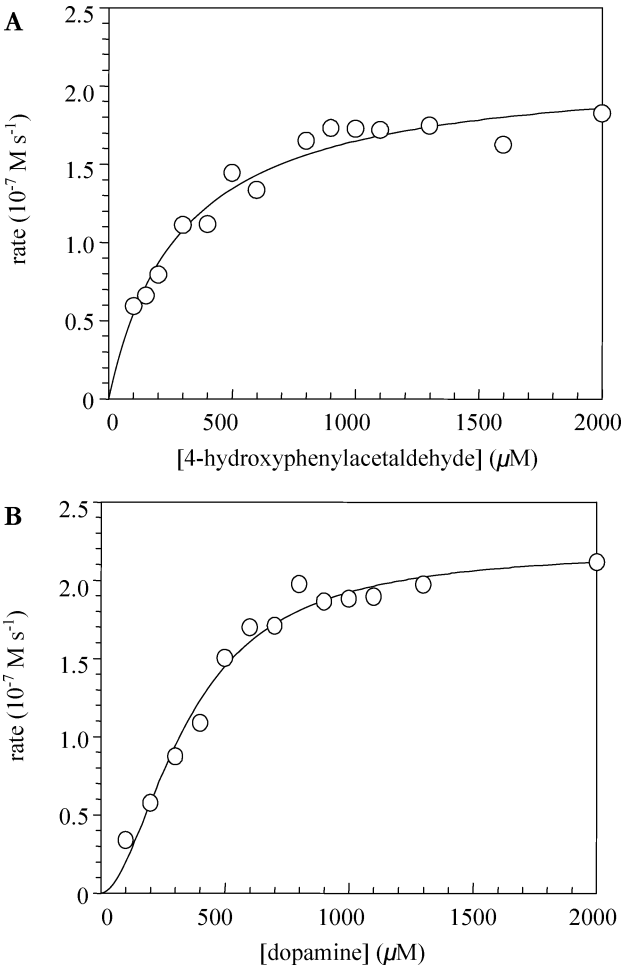
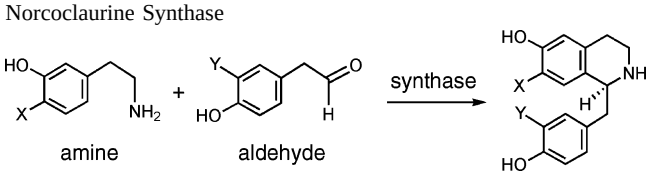


FIGURE 5: Enzyme kinetic plots of initial velocity vs substrate concentration: (A) Dopamine as the variable substrate with saturating 4-hydroxyphenylacetaldehyde (2 mM). Data was fit to the Hill equation. (B) 4-Hydroxyphenylacetaldehyde as the variable substrate with saturating dopamine (2 mM). Data was fit to the Michaelis−Menten equation.

Table 1: Kinetic Constants for the Reactions Catalyzed by Norcoclaurine Synthase



X	Y	k_{cat} (s^{-1})	K_{Mapp} (μM) amine	Hill coeff amine	K_M (μM) aldehyde
OH	H	6.4 ± 0.3	350 ± 48	1.8 ± 0.2	288 ± 38
H (**5**)	H	6.0 ± 0.5	407 ± 54	1.9 ± 0.5	
OMe (**6**)	H	6.0 ± 0.5	303 ± 54	1.9 ± 0.5	
OH	OH (**7**)	7 ± 2			335 ± 221

previously reported to be 40 μM for the wild type enzyme and 2.5 μM for the recombinant enzyme. These differences may be partially due to the narrow range of dopamine concentrations that were used in the previous kinetic assays (55−280 μM and 20−70 μM, respectively) and could lead to errors. In addition, differences in pH and buffer between the two assays could account for some of the discrepancy.

Alternate Substrate Testing and Mechanistic Analysis. In order to investigate the mechanism and substrate specificity of norcoclaurine synthase, several substrate analogues were tested in the enzymatic reaction. In both proposed mechanisms (Figure 3), deprotonation of the C-2 phenol plays a key step in catalysis. To test for the importance of this hydroxyl group, dopamine analogues lacking the hydroxyl at C-2 or bearing a methoxy group at this position (**3** (tyramine) and **4**, respectively) were tested as alternate substrates. In the spirocyclic intermediate mechanism (path B, Figure 3), the C-1 hydroxyl group plays a key role in the first step of the reaction. To test for this possibility, analogues lacking the C-1 hydroxyl or bearing a methoxy group at this position (**5** and **6**, respectively) were also analyzed. In each case the reactions were first monitored by ^{1}H NMR spectroscopy and mass spectrometry to determine whether the compounds served as alternate substrates. Successful reactions were repeated on a larger scale, and the products were isolated and characterized. Kinetic constants were obtained for compounds that behaved as alternate substrates and are listed in Table 1. Compounds **3** and **4** did not serve as substitutes for dopamine, and no condensation products could be observed upon prolonged incubations. This result is expected for compound **3** since it lacks the electron-donating oxygen substituent at C-2 of dopamine that would be required for either reaction mechanism. With compound **4**, however, the C-2 oxygen is present in the form of an ether, and compounds of this type are known to be good substrates for acid-catalyzed Pictet−Spengler reactions (*29*). A likely scenario is that the enzymatic reaction requires a phenolate to be formed at C-2 during catalysis. Alternatively, steric interactions with the methyl group of the ether may impede binding to the enzyme. Compounds **5** and **6** both served as substrates for the enzymatic reaction and displayed catalytic constants that were essentially identical to those obtained with dopamine. This observation rules out the mechanism involving a spirocylic intermediate (path B, Figure 3), as its formation would be greatly accelerated by an oxygen substituent at C-1. In addition to the dopamine analogues described above, one analogue of 4-hydroxyphenylacetaldehyde, 3,4-dihydroxyphenylacetaldehyde **7**, was investigated as an alternate substrate. This compound is of historic interest as it was first thought to be the natural substrate of the enzyme (*3*−*5*, *16*). It is also of synthetic interest as many benzylisoquinoline alkaloids are hydroxylated at this position in steps subsequent to the Pictet−Spengler condensation. As expected from previous studies on crude plant extracts, compound **7** is an excellent substrate for the synthase and displays kinetic constants quite similar to those of 4-hydroxyphenylacetaldehyde.

Test for Solvent Isotope Incorporation and Kinetic Isotope Effect Study. To test the notion that the enzyme may facilitate electrophilic addition to the aromatic ring of dopamine, nonreactive substrate analogues were incubated with the synthase in D_2O buffer and then analyzed for solvent-derived isotope incorporation. Compound **8** was tested as an analogue of the putative imine intermediate, and dopamine itself was incubated in both the presence and absence of the 4-hydroxyphenylacetaldehyde analogue, **9**. In all three cases no incorporation of deuterium was observed upon extensive incubation. The lack of incorporation may simply reflect the absence of a suitably positioned acidic residue in the active site of the enzyme, or it could be explained by a stereospecific protonation/deprotonation event that does not result in an overall exchange of isotope.

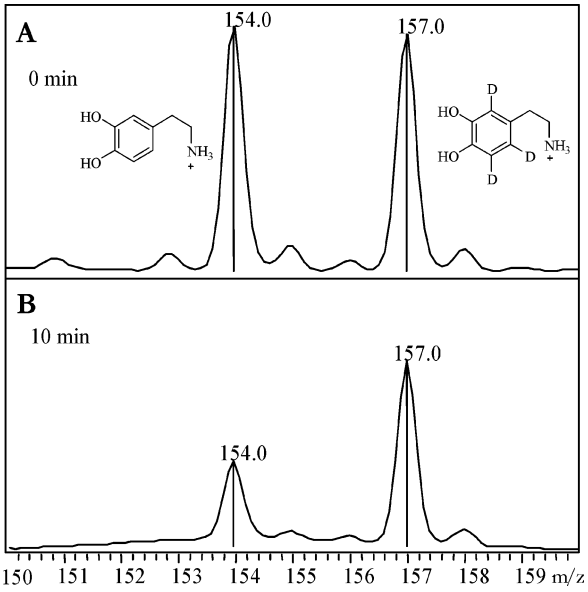


FIGURE 6: Mass spectra following the consumption of a mixture of unlabeled dopamine and [3,5,6-^{2}H]dopamine in the reaction catalyzed by norcoclaurine synthase: (A) Spectrum taken prior to the addition of enzyme. (B) Spectrum taken after 86% of the unlabeled dopamine has been converted to product.

In order to probe the rate-determining step of the enzymatic reaction, a kinetic isotope effect measurement was made that compares the rate of reaction between [3,5,6-^{2}H]-dopamine **10** and unlabeled dopamine. The trilabeled compound was chosen because it is readily synthesized and the assumption is made that only the deuterium at the site of alkylation (C-5) will affect the reaction rate. The direct circular dichroism spectroscopic assay was not employed in this analysis as the inherent errors in this method may mask small secondary isotope effects. Instead, a competitive method was employed that gives the isotope effect on the value of k_{cat}/K_M. A mixture of the deuterated dopamine **10** and unlabeled dopamine was enzymatically converted into norcoclaurine, and the ratio of the two isotopically labeled starting materials was monitored as a function of the reaction progress. Equation 1 was used to calculate the isotope effect

$$(V/K)_H/(V/K)_D = \ln(1 - F_H)/\ln[(1 - F_H)R/R_0] \quad (1)$$

on $(V/K)_H/(V/K)_D$ where F_H is the fractional conversion of the protiated species to products and R_0 and R are the initial and final ratios of the protiated to deuterated substrate, respectively (*27*). The value of F_H was determined using ^{1}H NMR spectroscopy and comparing the integrals obtained for the dopamine benzylic signals with those for an internal standard of dioxane as a function of time. The values of R_0 and R were determined using +ESI mass spectrometry and comparing the relative peak intensities for the labeled and unlabeled substrates as a function of time. The competitive method of determining KIEs is much more accurate than direct methods as separate rates do not have to be individually measured and many sources of error cancel. Figure 6 shows representative mass spectral traces used in determining the kinetic isotope effect. Prior to the addition of enzyme the ratio of labeled to unlabeled dopamine was 51:49 (Figure 6A). After 86% of the unlabeled material had been converted to product, the ratio changed to 32:68 (Figure 6B). It is clear that the unlabeled material is reacting faster than the deuterated material, and by applying eq 1 to the data obtained, a value of $(V/K)_H/(V/K)_D = 1.7 \pm 0.1$ was determined.

The observation of a kinetic isotope effect indicates that the electrophilic alkylation steps are rate-determining in the overall reaction. If substrate binding or imine formation were cleanly rate determining, one would not expect to see any isotope effect. If the initial electrophilic attack were cleanly rate determining, one would expect to see an inverse or much smaller positive isotope effect (see Discussion). The value of $(V/K)_H/(V/K)_D = 1.7$ suggests that the rearomatization step (deprotonation of the σ-intermediate) is partially rate determining and that a masked primary kinetic isotope is observed. Since *V/K* isotope effects only report on steps up to and including the first irreversible step in a reaction sequence, it appears that rearomatization is the first irreversible step in norcoclaurine formation.

DISCUSSION

Enzymes that catalyze Friedel−Crafts alkylation reactions are somewhat rare in nature, and few have been studied in detail (*30*). Dimethylallyltryptophan synthase is one such enzyme that alkylates tryptophan using dimethylallyl diphosphate in an apparent electrophilic aromatic substitution reaction (*31*, *32*). Similarly, porphobilinogen deaminase is thought to catalyze three sequential electrophilic aromatic substitution reactions in the biosynthesis of tetrapyrroles (*33*, *34*). The enzymes histidine ammonia-lyase and phenylalanine ammonia-lyase do not catalyze overall substitution reactions, yet are thought to use electrophilic addition of an alkyl group to an aromatic ring as a key step of catalysis (*35*). The Pictet−Spengler reaction is an electrophilic aromatic substitution reaction that involves a cyclization of an iminium ion and an electron rich phenolic or indole ring. Norcoclaurine synthase, strictosidine synthase, and deacetylipecoside synthase are three enzymes that are known to catalyze this reaction (vide supra). In a somewhat related sense, a few enzymes are known that generate aryl-*C*-glycosides via the formal addition of an electron rich aromatic onto an oxocarbenium ion intermediate (*36*−*38*).

The core of norcoclaurine synthase from *T. flavum* shows highest sequence homology to the pathogenesis-related (PR)-10 and Bet v 1 protein families (*18*). The biochemical context for this homology is unclear, since definitive catalytic functions for most PR10 and Bet v 1 family members have not yet been identified. The closest appears to be the HYP1 enzyme from St. John's wort (*Hypericum perforatum*) that is reported to be responsible for the biosynthesis of hypericin (*39*). Unlike other (PR)10/Bet v 1 family members, norcoclaurine synthase bears an N-terminal extension containing a putative 19 amino acid signal peptide. This suggests that the enzyme is associated with a subcellular compartment other than the cytosol, such as the endoplasmic reticulum (*18*). Nococlaurine synthase shows no sequence homology with the only other "Pictet−Spenglerase" of known sequence, strictosidine synthase (*12*, *14*). Recently, another enzyme from *Coptis japonica* has been reported to catalyze the synthesis of (*S*)-norcoclaurine from dopamine and 4-hydroxyphenylacetaldehyde, however, this enzyme appears to be related to 2-oxoglutarate-dependent dioxygenases (*40*). This homology is curious given that the Pictet−Spengler reaction

is overall redox neutral. Furthermore, the identification is questionable given the report that the product of the *C. japonica* enzyme travels with a different HPLC retention time than that of norcoclaurine produced from a close homologue of the *T. flavum* norcoclaurine synthase when the two compounds are coinjected.

In this work, a method for the high level overexpression of the norcoclaurine synthase gene was developed and the recombinant enzyme was purified for the first time. A continuous kinetic assay was developed that relied on CD spectroscopy and did not have a requirement for radiolabeled substrates. The observation of a hyperbolic kinetic profile with varying levels of 4-hydroxyphenylacetaldehyde and a sigmoidal kinetic profile with varying levels of dopamine is in agreement with previous studies using crude plant extracts or purified exogenous enzyme from several sources. The value of the Hill coefficient ($n = 1.8$) obtained when varying dopamine concentrations indicates positive cooperativity in which the binding of the first dopamine molecule increases the affinity for a second dopamine molecule. The report that this enzyme exists as a homodimer in solution helps to explain how this cooperativity can occur as binding to one subunit could affect binding to the other subunit (*17*).

The mechanism employed by norcoclaurine synthase likely involves a direct electrophilic aromatic substitution without a spirocyclic intermediate (Figure 3, path A). This is evident by the fact that dopamine analogues lacking a C-1 hydroxyl (**5** and **6**) were found to serve as alternate substrates with catalytic constants comparable to those of dopamine itself. In the direct electrophilic substitution mechanism, the substitution of the C-1 hydroxyl group for a hydrogen (analogue **5**) would be expected to increase the rate of addition at C-5 slightly since the hydroxyl group is somewhat electron-withdrawing ($\sigma_m = 0.12$) (*41*). This effect was likely not observed because the addition step is not rate limiting in the enzymatic reaction (vide infra). The inability of analogues **3** and **4** to serve as alternate substrates points out the requirement for a hydroxyl group at the C-2 position of dopamine and suggests that the enzymatic reaction occurs via a C-2 phenolate ion that attacks the imminium ion in the first step of the substitution process. The observation of a deuterium kinetic isotope effect of 1.7 is interpreted as being due to a masked primary kinetic isotope effect on the step involving deprotonation of the σ-intermediate that causes rearomatization. In nonenzymatic electrophilic aromatic substitution reactions (alkylations, chlorinations, and nitrations) the observed kinetic isotope effects are usually inverse or quite small ($k_H/k_D = 0.7-1.2$) (*42*, *43*). In these cases, the difference in rates is generally ascribed to a secondary isotope effect where the electrophilic addition step is rate determining. For those reactions showing inverse kinetic isotope effects, the change in hybridization of the carbon bearing the isotopic substituent from sp^2 to sp^3 is expected to increase the zero point energy and result in a faster reaction for the heavier isotope (*43*). In cases where the measured kinetic isotope effect is close to zero, arguments involving hyperconjugation have been presented (*43*, *44*). Hyperconjugation between the out-of-plane carbon−hydrogen bond and the p-orbitals of the other five ring carbons should decrease the zero point energy and oppose the effects due to rehybridization at the transition state. In many cases, such as the alkylation of anisole with the diphenylmethyl cation ($k_H/k_D = 1.0$), these effects simply cancel one another (*45*). In the case of norcoclaurine synthase, the $(V/K)_H/(V/K)_D$ isotope effect of 1.7 is notably larger than normally observed in nonenzymatic reactions, and is not likely due to a secondary isotope effect on the electrophilic addition step. Instead, it likely reflects a primary isotope effect on the step involving deprotonation of the σ-intermediate. The value is somewhat lower than what is expected for a cleanly rate-determining primary kinetic isotope effect (≈2−6), however, previous steps such as imine formation may also be partially rate determining and result in a partially masked kinetic isotope effect. Alternatively, the observed value may reflect an intrinsic primary kinetic isotope that is smaller than expected because of an asymmetric transition state in an exothermic step of the reaction (*46*−*48*). The difference in the rate-determining step between the enzymatic reaction and the nonenzymatic reactions is likely due to the increased nucleophilicity of a phenolate as compared to neutral aromatic ring systems. Most electrophilic aromatic substitution reactions are carried out under acidic conditions and the resulting σ-intermediate bears a positive charge. In the reaction catalyzed by norcoclaurine synthase, however, one anticipates that the catechol moiety of dopamine will bear a negative charge and the σ-intermediate will be neutral. This would lower the barrier to the addition step relative to that of the deprotonation step. With dopamine in aqueous solution the first pK_a of the catechol moiety is expected to be near 9.5 and this may account for the ability of a background nonenzymatic reaction to occur at measurable rates (*49*). In the case of the enzyme-catalyzed reaction, this pK_a value is likely perturbed to a lower value since maximal activity is observed at a pH of 7.0 (*18*).

Further work on norcoclaurine synthase will include attempts to determine the stereochemistry of the intermediates formed and to probe the scope of alternate substrates that are accepted in the reaction. The use of this enzyme in the preparation of chiral benzylisoquinoline alkaloids will provide an attractive alternative to conventional asymmetric syntheses.

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