Mechanistic Studies on the Tyrosinase-Catalyzed Formation of the Anachelin Chromophore

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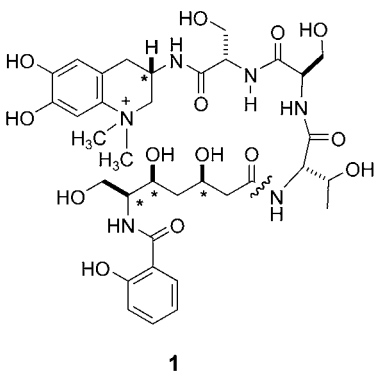
The complex secondary metabolite anachelin, isolated from the freshwater cyanobacterium Anabaena cylindrica*, is believed to act as siderophore, facilitating iron uptake. Its structure is characterized by a fascinating blend of polyketide, peptide, and alkaloid fragments. In particular, the tetrahydroquinolinium-derived chromophore is unique among natural products, and its biosynthesis is unknown. We propose a hypothesis for the biogenesis of the anachelin chromophore starting from a C-terminally bound* L*-Tyr residue. It is proposed that this amino acid is reductively aminated, methylated, and hydroxylated. Oxidation of this catechol diamine substrate by a tyrosinase would lead to an* o*-quinone, which would react by intramolecular aza-annulation and tautomerization to give the anachelin chromophore. In order to evaluate this hypothesis, a model substrate related to the proposed biogenetic precursor was prepared. It was shown that the enzyme tyrosinase is able to transform this substrate into an anachelin chromophore derivative, which corroborates the biogenetic hypothesis. In order to gain further insight into the mechanism of this transformation, we performed spectrophotometric reaction monitoring, allowing the formation of the expected product to be observed. In addition, a rise in absorption at around 250 nm might be due to the presence of a spiro five-membered ring intermediate resulting from an alternative 1,4-addition to the* o*-quinone. Lastly, we were able to show that the action of tyrosinase on this substrate follows Michaelis–Menten kinetics ($k_{cat} = 123\ s^{-1}$ and $K_m = 8.66$ mM). Interestingly, the catalytic efficiency is decreased only by a factor of 30 relative to the natural substrate* L*-DOPA.*

Introduction

Cyanobacteria constitute one of the most successful life forms on earth, populating habitats as diverse as soil, rocks, and plants to the open ocean. Especially in such marine environments, the bioavailability of iron is very low, so Fe can become a growth-limiting nutrient.[1–3] The acquisition of iron is therefore crucial for cyanobacterial growth, and understanding of the molecular mechanism of iron transport in cyanobacteria constitutes a significant challenge.[4–7] Under iron-deficient conditions, many bacteria secrete small organic compounds into their extracellular surroundings, these being used to bind and to sequester iron (so called *siderophores*).[8–10] For cyanobacteria, however, only simple compounds such as hydroxamic acids and shizokinen had been identified as siderophores until recently.[3,11,12] This changed four years ago, however, when a complex secondary metabolite was isolated from the freshwater cyanobacterium *Anabaena cylindrica* and was postulated to act as a siderophore.[13–15] Budzikiewicz, Walsby, and co-workers obtained mixtures of anachelin H (**1**) and anachelin 1 (**2**), and determined the constitution of the former by spectroscopic methods.[13] Later, Murakami et al. isolated anachelin 1 (**2**) and anachelin 2 (**3**) and determined their constitutions.[16] All compounds are characterized by a fascinating blend of polyketide, peptide, and alkaloid fragments. In particular, the quaternary aminotetrahydroquinolinium ring, which acts as a fluorescent chromophore, is unprecedented in other natural products. The unique structure of this chromophore raises questions about its biosynthesis, which remains unknown for anachelin and its constituents. Therefore, in the context of our research program directed towards the elucidation of the molecular mechanism of cyanobacterial iron acquisition,[15] we wanted to investigate the biosynthesis of both the peptide/polyketide fragment and the alkaloid chromophore.



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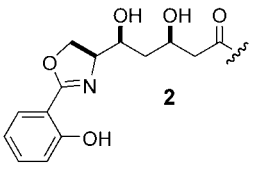

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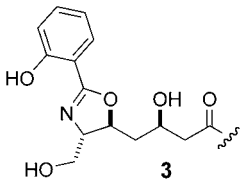

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The cationic tetrahydroquinolinium ring of the anachelin chromophore reminded us of the corresponding *dihydroindolinium* systems found in, for example, the pigments betanidin[17,18] and the melanins.[19] The biosynthesis of melanins (melanogenesis) has been under investigation for the last 80 years[20,21] and is still under intense mechanistic scrutiny.[22,23] In these transformations, L-Tyr and/or L-DOPA is oxidized to dop-

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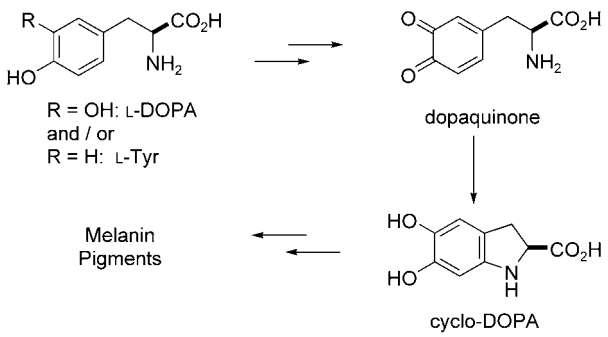
aquinone, which can react in an intramolecular 1,4-addition to form *cyclo*-DOPA (Scheme 1). This oxidation to the *o*-quinone



Scheme 1. *The early steps of melanin biogenesis.*[20–23]

is catalyzed by tyrosinase, which serves a dual function of either polyphenol oxidase or catecholoxidase.[24] In the course of their investigations into melanogenesis, Ramsden and colleagues also published studies on the formation of several homologues of *cyclo*-DOPA.[25–27]

This pathway shown in Scheme 1 led us to postulate a biosynthesis of the anachelin chromophore. We propose that the final steps of anachelin biosynthesis start from the precursor **A**, containing a *C*-terminal Tyr residue, which is attached to peptide and polyketide fragments (labeled *peptide*; see Scheme 2). Reductive transamination and methylation would result in the amine **B**. Hydroxylation of the aromatic ring would then give the dihydroxyphenylalanine derivative **C**, which would serve as substrate for the postulated key step. We propose that a transformation similar to that observed in the biosynthesis of melanins (cyclization of DOPA to DOPA-chrome) is operative in the biogenesis of the anachelin chromophore, shown in Scheme 2. Therefore, oxidation of the amine **C** to the *o*-quinone **D** with subsequent intramolecular 1,4-addition could provide the six-membered ring annulation product **E**, which, after tautomerization and aromatization, would give the final chromophore **F**.

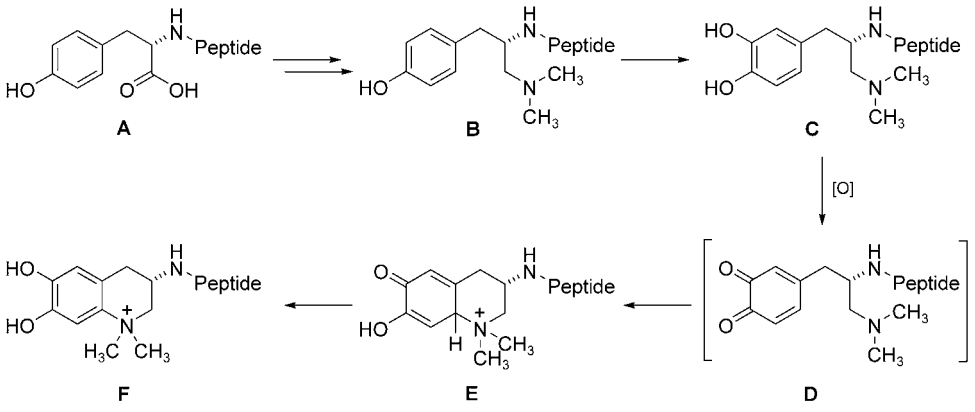
In order to corroborate this biogenetic hypothesis in the laboratory, we prepared model substrates and investigated the mechanism by a combination of spectroscopic and kinetic techniques.[28]

Results

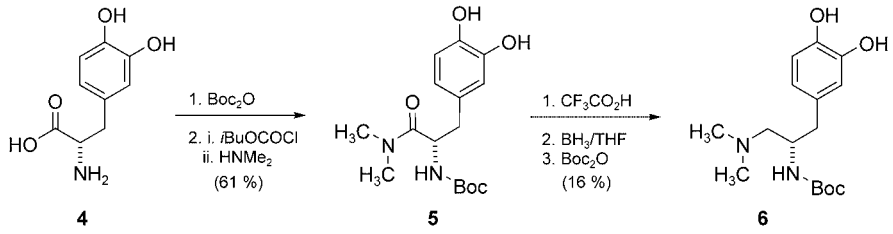
We first had to identify a model compound that would allow us to probe the biosynthetic hypothesis presented above. We hypothesized that the peptide group in compound **C** could be replaced by a smaller group without any effect on the cyclization, and so we chose the carbamate-protected diamine **6** (Scheme 3) as a model substrate in which the carbamate group allows for differentiation between the two amines, thus rendering the tertiary amine more reactive. This compound was prepared from L-DOPA (**4**), which was protected at the N atom with a Boc group. Subsequent activation to provide the mixed anhydride was followed by conversion into the dimethyl amide **5**. Removal of the Boc group with trifluoroacetic acid, borane reduction, and subsequent reprotection (again Boc) gave the desired diamine **6**. The low yield can be attributed to problems in isolation and purification, as **6** decomposes on silica.

As discussed in the Introduction, the enzyme tyrosinase is the key enzyme in the early stages of melanin biosynthesis and is known to catalyze the conversion of DOPA into *cyclo*-DOPA, serving as a catechol oxidase. We therefore wondered whether this enzyme would also catalyze the analogous formation of the anachelin chromophore (i.e. **C**→**F** in Scheme 2). The diamine **3** was therefore used as substrate in phosphate buffer at pH 6.8 in the presence of tyrosinase (Scheme 4).

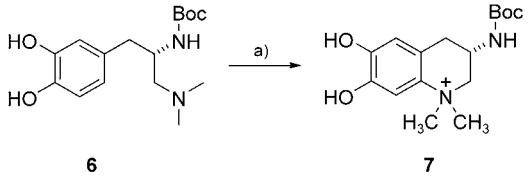
Interestingly, the anachelin chromophore **7** could be isolated after a 3 h reaction time in nearly quantitative yield on a preparative scale. The structure of **7** was verified by NMR spectros-



Scheme 2. *Proposed biogenesis of the anachelin chromophore* ***F*** *from the Tyr-peptide* ***A*** *via the* o*-quinone* ***C*** *and subsequent oxidative aza-annulation.*



Scheme 3. *Preparation of the model substrate* ***6****.*

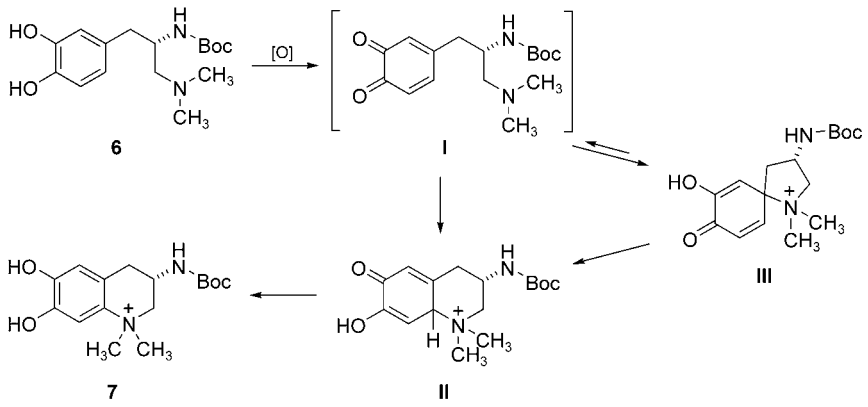


6 7

Scheme 4. *Preparative-scale transformation of the model substrate **6** into the anachelin chromophore derivative **7** by the enzyme tyrosinase. a) Mushroom tyrosinase, O_2; 350 units enzyme per mL, 0.3 mM substrate, 18 mM phosphate buffer, pH 6.8.*

copy and mass spectroscopy, and the isolated compound was shown to be identical to a reference compound obtained by chemical oxidation. Therefore, we had shown that tyrosinase is indeed able to catalyze the transformation **6**→**7**, thus corroborating the biosynthetic hypothesis outlined in Scheme 2. This result led us to investigate the mechanism of this transformation, in particular the reaction of **6** to give chromophore **7**, in more detail.

The mechanism of this remarkable oxidative aza-annulation differs from that for the natural substrate DOPA, due to the additional CH_2 unit, which results in a six-membered annulated ring (instead of a five-membered ring for DOPA). We think that the first step is the oxidation of the catechol in the substrate **6** to the *ortho*-quinone **I**, catalyzed by tyrosinase with dioxygen consumption (Scheme 5). Related studies on the tyrosinase-mediated oxidation of catechols suggest this step to be rate-limiting.[29,30] Interestingly, this quinone has two electrophilic carbon atoms available for nucleophilic 1,4-addition. Addition to the C atom at the C(1) position would furnish the five-membered ring spiro compound **III**, a reaction that has been postulated for the biosynthesis of some natural products such as FR901483[31,32] or TAN1251 A.[33b] On the other hand, the six-membered ring annulation product **II** would result from addition to C(6), a reaction analogous to the cyclo-DOPA formation in Scheme 1. Whereas the six-membered ring compound **II** can quickly tautomerize into the isolable aromatic compound **7**, the spiro compound **III** cannot aromatize. It can either undergo a dienone–phenol rearrangement to give **II**,[33] or eliminate the amino group again to provide the quinone **I**. Eventually, both **II** and **III** will result in the final compound **7**, as this compound is the thermodynamically most stable one.



6 I III 7 II

Scheme 5. *A possible mechanism for the formation of the anachelin chromophore derivative **7** from the corresponding diamine **6**.*

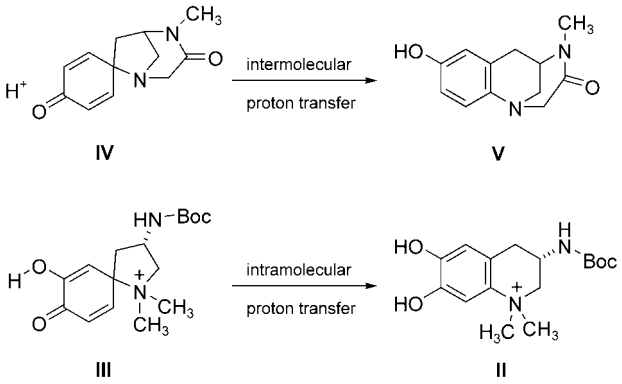
On a preparative scale, several attempts to isolate either the spiro compound **III** or its six-membered ring counterpart **II** have so far proven unsuccessful. It must be pointed out that, according to a recent literature search, compounds of type **III** have not been isolated so far. This is in contrast to derivatives of type **III** *lacking the hydroxy group in the 3-position*, which constitute fragments of natural products and have thus been prepared and isolated (for examples, see refs. [31, 32, 33b]). Apparently, the presence of an enol hydroxy group in **III** facilitates the dienone–phenol rearrangement through intramolecular proton transfer to give **II** (Scheme 6).

In addition, the presence of the quaternary ammonium group in **III** should facilitate decay of this adduct to the starting quinone **I**. On the other hand, the six-membered ring annulated compound **II** is believed to tautomerize quickly and thus aromatize to **7**. For all these reasons, compounds **I**–**III** are not likely to be isolable.

Additional information concerning the mechanism can be gathered by UV-spectrophotometric reaction monitoring. A typical time-dependent UV spectrum is shown in Figure 1. Several facts become evident on analysis of the data. Firstly, a gradual shift of the maximum from 280 nm to 284 nm is observed during the course of the reaction. This is due to the formation of the product **7**, which displays $\lambda_{max} = 284$ nm. In addition, the UV spectrum of the product also displays a shoulder at 308 nm; again, an increase in the UV spectrum shown in Figure 1 at this wavelength is a consequence of product formation.

Interestingly, an unexpected and rather strong increase in absorbance at 250 nm can be observed. As the extinction coefficients of both **6** and **7** at this wavelength are rather small (e.g. ε(**7**, 250 nm) = 1200 $M^{-1}cm^{-1}$), a different species must be considered for this UV signal at 250 nm. In fact, Ramsden and colleagues attributed this increase to a related spiro intermediate, which should display strong absorption at this wavelength ($\varepsilon \approx 10\,000$ $M^{-1}cm^{-1}$).[27] Calculations of the UV absorption of compound **III**, which point to a maximum at around 250 nm, support this hypothesis. Therefore, the increase observed at around 250 nm in Figure 1 is suggestive of the formation of the spiro intermediate **III**.

As the conversion of **II**→**7** involves a intramolecular proton transfer leading to aromatization, which can be considered a very fast process, we postulate that the three species **6**, **7**, and **III** can be observed by UV spectroscopy. Kinetic analysis with



Scheme 6. *The dienone–phenol rearrangement[33a] from **IV** to **V** according to Honda and co-workers,[33b] featuring an intermolecular proton transfer. The additional enol group in **III** might induce an intramolecular transfer, resulting in instability of **III**.*

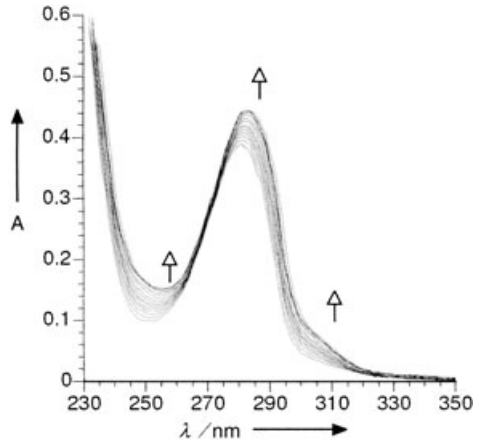


Figure 1. *Time-dependent UV spectrophotometry of the tyrosinase-catalyzed formation of the anachelin chromophore. "A" denotes the absorbance measured.*

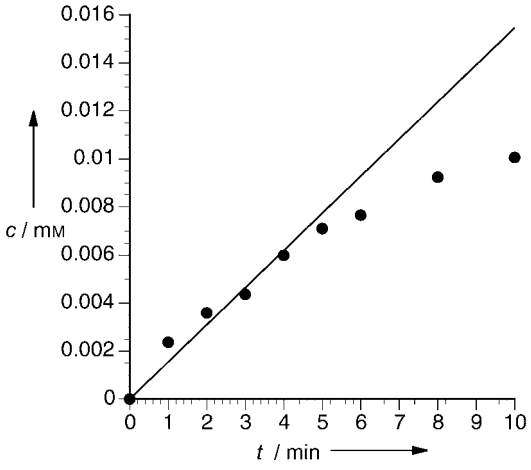


Figure 2. *Concentration of product **7** (circles) as a function of time; the initial rate is shown as a solid line.*

the given molar extinction coefficients allows for calculation of the absolute concentrations of **6** and **7** (and in principle **III**) over the course of the reaction by UV-spectroscopic analysis.

The concentration of product **7** at a given time in the reaction can be calculated from data extracted at 284 nm and use of Equation (1), where c denotes the concentration, A the absorbance, and ε the respective molecular extinction coefficients:

$$c^{\mathrm{prod}} = \frac{A_{\mathrm{obs}} - c^{\mathrm{tot}} \cdot \varepsilon^{\mathrm{subst}}}{\varepsilon^{\mathrm{prod}} + \varepsilon^{\mathrm{subst}}} \qquad (1)$$

From the data extracted at 284 nm from Figure 1, one can calculate the concentration of product **7** and plot it as a function of time, as shown in Figure 2.

From this kinetic analysis, two facts concerning the mechanism become evident:

1) The product **7** is directly formed from the *o*-quinone **I** without any lag phase. This is interesting to point out, as tyrosinase displays complex kinetics for different substrates.[22,23]
2) The initial reaction rate decreases after about 5 minutes, probably reflecting consumption of substrate.

After we had investigated the intramolecular decay reactions of the *o*-quinone **I**, we turned our attention to the tyrosinase-mediated oxidation of catechol **6** to the *o*-quinone **I**. It is well known that the conversion of the natural substrate DOPA by tyrosinase follows the kinetic model described by the Michaelis–Menten equation.[21–24] In order to investigate the kinetics of the conversion **6**→**I**, we measured the initial velocities of the oxidation of substrate **6** at different substrate concentrations, and the dependence of initial rate of oxidation of **6** on the concentration of **6** is shown in Figure 3. Interestingly, this graph demonstrates that the oxidation of substrate **6** by tyrosinase exhibits Michaelis–Menten kinetics. We fitted this data

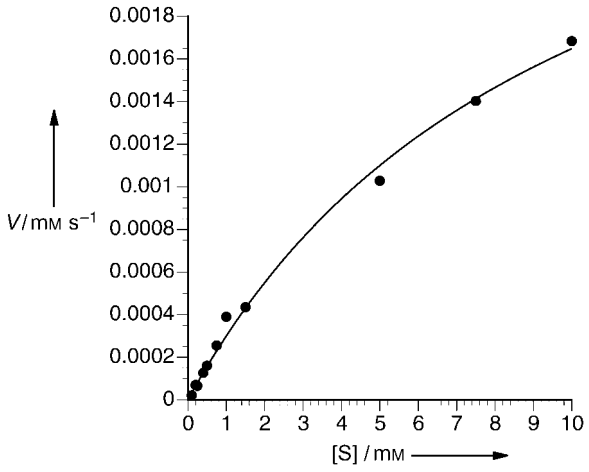


Figure 3. *Michaelis–Menten plot of the tyrosinase-catalyzed formation of the anachelin chromophore by the oxidation of substrate **6**. The solid line represents the fit of the data with the Michaelis–Menten equation and the kinetic constants shown in Table 1.*

with a nonlinear Michelis–Menten equation and obtained the kinetic parameters given in Table 1.

Table 1. *Kinetic constants characterizing the action of tyrosinase on different substrates.*

	6	L-DOPA	4-Me-catechol[34]	Dopamine[34]
k_{cat} [s^{-1}]	123	107	841	439
K_M [mM]	8.66	0.20	2.36	2.22
k_{cat}/K_M [$M^{-1}s^{-1}$]	1.4×10^4	5.3×10^5	3.5×10^5	2.0×10^5

When we compare the data obtained for the anachelin chromophore substrate **6** to those relating to the reference substrate DOPA, it becomes evident that although the K_m is decreased by a factor of about 40, the catalytic efficiency is only about 30 times lower. It is surprising to see that the catalytic efficiency of tyrosinase for this substrate is so high, given the steric demands of the Boc group and thus of substrate **6**. In comparison with other substrates such as 4-methylcatechol and dopamine, the anachelin chromophore substrate **6** follows the same trends, such as higher k_{cat} and higher K_m values, which results in decreased catalytic efficiency.

Conclusion

In conclusion, we have postulated a mechanism featuring tyrosinase catalysis for the formation of the anachelin chromophore. In order to evaluate this hypothesis we prepared a model substrate and were able to demonstrate that the enzyme tyrosinase is a catalyst for the putative key step in the biosynthesis of the anachelin chromophore. Moreover, a mechanistic analysis combining spectrophotometric reaction monitoring and kinetic analysis was performed. Several conclusions can be drawn from the data obtained:

1) The oxidation of the catechol substrate **6** to the DOPA quinone is the rate-determining step in this transformation.
2) The reaction kinetics can be followed by a Michaelis–Menten model.
3) The K_m obtained is 8.66 mM and the k_{cat} 123 s^{-1}, resulting in a k_{cat}/K_m ratio of 1.4×10^4 $M^{-1}s$. The catalytic efficiency of this process is therefore only about 30 times lower than for the natural reference substrate L-DOPA.
4) By spectrophotometry, we were able to observe an increase at 250 nm, which might be due to the presence of an intermediate featuring a spiro five-membered ring originating from a different 1,4-addition of the amine to the quinone.

From these experimental observations, we were able to corroborate our mechanistic hypothesis for the tyrosinase-catalyzed formation of the unique anachelin chromophore. Moreover, these experiments pave the way for in vivo feeding experiments with *A. cylindric*a and synthetic substrates.

Experimental Section

Abbreviations: Boc = *tert*-butoxycarbonyl; DOPA = 3,4-dihydroxyphenylalanine; ether refers to diethyl ether; NMM = *N*-methylmorpholine.

General: Unless otherwise stated, chemicals were purchased from Fluka, ABCR, or Acros and were used without further purification. L-DOPA was purchased from Acros. Dianisyltellurium oxide was prepared according to the literature.[35] Isobutyl chloroformate was distilled prior to use. Solvents for workup and chromatography were distilled from technical quality. Solvents used for chemical transformations were either puriss. quality or were dried over columns of dry aluminium oxide.

Reactions were run under Ar in dry glassware (at least 24 h in an oven at 140 °C, followed by heating with a heat gun under high vacuum). Analytical thin layer chromatography (TLC) was performed on Merck silica gel 60 F254 plates (0.25 mm thickness) precoated with a fluorescent indicator. The developed plates were examined under UV light and stained with ceric ammonium molybdate (CAM) or with $KMnO_4$ followed by heating. Flash chromatography was performed on silica gel 60 (230–400 mesh) from Fluka. All ^1H and ^{13}C NMR spectra were recorded with Varian Gemini (300 MHz (^1H) or 75 MHz (^{13}C)), Varian Mercury (300 MHz (^1H) or 75 MHz (^{13}C)), Bruker AMX (500 MHz (^1H) or 125 MHz (^{13}C)), or Bruker DMX (500 MHz (^1H) or 125 MHz (^{13}C)) FT spectrometers at ambient temperature, chemical shifts are given in ppm, coupling constants (J) are in Hz. IR spectra were recorded as $CHCl_3$ solutions with a Perkin–Elmer RX I FTIR spectrometer, absorptions are given in cm^{-1}. Optical rotations were measured in a 1 mL cell with a 1 dm path length in a Jasco DIP 1000 digital polarimeter, the concentration (c) is given in g per 100 mL. Elemental analyses were performed by the Mikroanalyse Labor of the Laboratorium für Organische Chemie der ETH Zürich. All mass spectra were recorded by the Mass Spectroscopy Service of the Laboratorium für Organische Chemie der ETH Zürich on an Ion spec Ultima 4.7 spectrometer in a 2,5-DHB matrix by MALDI, fragment ions are given in m/z with relative intensities (%) in parentheses. Nomenclature is according to Autonom 2.0, Beilstein Informationssysteme GmbH.

(*S*)-[2-(3,4-Dihydroxy-phenyl)-1-dimethylcarbamoyl-ethyl]carbamic acid *tert*-butyl ester (5): L-DOPA (5 g, 25.4 mmol) was dissolved in dioxane (40 mL), NaOH solution (1 M, 28 mL), and H_2O (25 mL). Boc_2O (6.1 g, 1.1 equiv) dissolved in dioxane (10 mL) was added to the above orange solution, and the mixture was stirred at room temperature. The pH was monitored after 30 minutes, 1 h, 2 h, and 10 h, and, if necessary, adjusted to 9–10 by addition of NaOH solution (1 M). After 16 h, the resulting brown solution was concentrated under reduced pressure and acidified to pH 2 with HCl solution (1 M). The resulting brown suspension was extracted four times with EtOAc. The combined organic phases were dried ($MgSO_4$) and the solvent was removed under reduced pressure. The resulting brown slurry was dissolved in THF (50 mL) and cooled to −40 °C. *N*-Methylmorpholine (2.91 mL) and *i*BuOCOCl (3.44 mL, 26.5 mmol, 1.1 equiv) were added, and the resulting suspension was stirred at −35 °C for 0.5 h. A solution of $HNMe_2$ in THF (2 M, 38.1 mL, 3 equiv) was added, and the reaction mixture was allowed to warm to room temperature and was stirred overnight. The solvent was removed under reduced pressure and the residue was dissolved in EtOAc. The organic phase was washed three times with citric acid (10%), three times with saturated $NaHCO_3$ solution, and three times with saturated NaCl solution. The organic layer was dried ($MgSO_4$) and the solvent was removed under reduced pressure. Recrystallisation from EtOAc gave the title com-

pound **5** (5.03 g, 15.5 mmol, 61%). Colorless crystalline solid. M.p. = 156–157 °C; R_f = 0.5 (EtOAc); $[\alpha]_D$ = +56.8 (c = 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.40 (s, 9H), 2.62 (s, 3H), 2.74–2.81 (m, 2H), 2.85 (s, 3H), 4.77 (q, J = 6.2 Hz, 1H), 5.55 (d, J = 8.7 Hz, 1H), 6.56 (dd, J_1 = 1.9, J_2 = 8.1 Hz, 1H), 6.74 (d, J = 1.9 Hz, 1H), 6.77 (d, J = 8.1 Hz, 1H), 7.02 (br s, 1H), 7.95 ppm (br s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 28.4, 36.0, 37.2, 39.4, 51.7, 80.2, 115.2, 116.1, 121.1, 127.8, 143.5, 144.0, 155.3, 172.3 ppm; IR: $\tilde{\nu}$ = 3431 w, 3278 w, 3022 m, 1703 s, 1637 s, 1497 s, 1369 cm^{-1} s; MS: 347.2 (79, $[M+Na]^+$), 225.1 (100, $[M-Boc]^+$); HRMS calcd for $C_{16}H_{24}N_2O_5Na$ $[M+Na]^+$: 347.1577; found 347.1578; elemental analysis calcd (%) for $C_{16}H_{24}N_2O_5$ (387.43): C 59.24, H 7.46, N 8.64; found: C 59.29, H 7.51, N 8.53.

(*S*)-[1-(3,4-Dihydroxy-benzyl)-2-dimethylamino-ethyl]carbamic acid *tert*-butyl ester (6) Boc-L-DOPA-dimethylamide (1.2785 g, 3.94 mmol) was suspended in CH_2Cl_2 (4 mL) and cooled under Ar to 0 °C. Trifluoroacetic acid (4 mL) was added dropwise, and the resulting solution was stirred for 1 h at 0 °C and for 1 h at room temperature. The solvent was removed under reduced pressure, and the residue was twice dissolved in toluene and the solvent removed under reduced pressure. The residue was dried under high vacuum and used without further purification.

The residue was dissolved in THF (8 mL) and cooled under Ar to 0 °C and BH_3·THF complex (1 M in THF, 20 mL) was added dropwise (gas evolution). The solution was stirred at 0 °C for 2 h, warmed to room temperature, and stirred overnight. The reaction mixture was cooled to 0 °C, HCl solution (6 M, 3 mL) was added dropwise (gas evolution), and the system was stirred at 0 °C for 3 h. The solvent was removed under reduced pressure. The resulting diamine hydrochloride was used without further purification.

The oily residue was dissolved in dioxane (4 mL) and H_2O (1 mL), and the pH was adjusted to ≈9–10 by addition of NaOH solution (1 M). Boc_2O (860 mg, 3.94 mmol) was dissolved in dioxane (1 mL) and added to the reaction mixture. Gas evolution started, and the pH was monitored and adjusted to ≈9–10 by addition of NaOH solution (1 M) every 30 min for 4 h. The reaction mixture was then stirred overnight at room temperature. The solvent was removed under reduced pressure, and the residue was partitioned between EtOAc and saturated $NaHCO_3$ solution. The layers were separated, and the aqueous layer was extracted three times with EtOAc. The combined organic layers were dried ($MgSO_4$) and filtered, and the solvent was removed under reduced pressure. Flash chromatography (10 g SiO_2, CH_2Cl_2/MeOH 7:1→1:1) gave the title compound **6** (195 mg, 0.63 mmol, 16%) as a colorless oil. R_f = 0.05 (CH_2Cl_2/MeOH 7:1); $[\alpha]_D$: −4.2 (c = 1, MeOH); ^{1}H NMR (CD_3OD, 300 MHz): δ = 1.37, 1.40 (s, 9H, Boc-rotamers), 2.20 (s, 6H), 2.25 (dd, J_1 = 12.4, J_2 = 5.0 Hz, 1H), 2.39 (dd, J_1 = 12.4, J_2 = 8.7 Hz, 1H), 2.46 (br m, 2H), 3.80 (br m, 1H), 6.48–6.56 (m, 2H), 6.63–6.65 ppm (m, 1H); ^{13}C NMR (CD_3OD, 75 MHz): δ = 28.0, 28.5 (Boc rotamers), 40.2, 45.4, 51.0, 63.3, 79.5, 115.7, 117.1, 121.3, 130.7, 144.4, 145.7, 157.4 ppm; IR: $\tilde{\nu}$ = 3432 w, 3019 m, 1766 w, 1700 s, 1492 s, 1368 cm^{-1} s; MS: 643.4 (72) $[2\times M+Na]^+$, 333.2 (40) $[M+Na]^+$, 311.2 (100) $[M+H]^+$; HRMS calcd for $C_{16}H_{27}N_2O_4$ $[M+H]^+$: 311.1965; found 311.1967.

(S)-(6,7-Dihydroxy-1,1-dimethyl-1,2,3,4-tetrahydro-quinolin-3-yl)-carbamic acid tert-butyl ester (7)

Procedure A: (*S*)-[1-(3,4-Dihydroxy-benzyl)-2-dimethylamino-ethyl]-carbamic acid *tert*-butyl ester (**6**, 8.37 mg, 27 μmol) was dissolved in phosphate buffer (1.8 mL, 15 mM, pH 6.8), and tyrosinase (100 units) was added. The colorless solution was shaken in an open flask exposed to air for three hours. The color changed from colorless to orange. The reaction mixture was lyophilized, and the crude compound was purified on Sephadex (methanol) to give—after evaporation, dissolving in water, and lyophilization—the title compound **7** (7.68 mg, 24.9 μmol, 92%) as an orange-red, fluffy powder.

Procedure B: (*S*)-[1-(3,4-Dihydroxy-benzyl)-2-dimethylamino-ethyl]-carbamic acid *tert*-butyl ester (**6**, 16 mg, 51 μmol) was dissolved in CH_2Cl_2 (0.5 mL), and dianisyltellurium oxide (20.3 mg, 56 μmol) was added. The reaction mixture was stirred for 45 minutes and a color change from colorless to orange-red was observed. The reaction was quenched with H_2O, and the organic layer was extracted three times with H_2O. The combined organic layer were washed with CH_2Cl_2 three times. Lyophilization gave the title compound **7** (12.0 mg, 38.9 μmol, 76%) as an orange-red, fluffy powder.

Analytical data for compound 7: ^{1}H NMR (D_2O, 500 MHz): δ = 1.44 (s, 9H), 2.79 (dd, J_1 = 11.1, J_2 = 16.2 Hz, 1H), 3.07 (dd, J_1 = 4.6, J_2 = 16.2 Hz, 1H), 3.55 (s, 3H), 3.59 (s, 3H), 3.51–5.60 (m, 1H), 3.85 (d, J = 12.0 Hz, 1H), 4.30 (br s, 1H), 6.73 (s, 1H), 7.16 ppm (s, 1H); ^{13}C NMR (D_2O, 125 MHz): δ = 30.2, 33.9, 43.7, 60.6, 61.0, 68.4, 84.5, 110.2, 118.8, 122.3, 135.5, 147.9, 149.5, 159.8 ppm; UV (H_2O): λ_{max} = 284 nm, ε = 3960 $M^{-1}dm^{-1}$, shoulder at λ = 305 nm, ε = 850 $M^{-1}dm^{-1}$; MS 309.2 (100) $[M]^+$, 253.1 (12) $[M-t\text{Bu}+H]^+$; HRMS calcd for $C_{16}H_{25}N_2O_4$ $[M]^+$: 309.1809; found 309.1815.

Spectrophotometric reaction monitoring: The enzyme concentration was determined according to the literature.[36] The phosphate buffer was prepared from KH_2PO_4 and adjusted with KOH solution to pH 6.8. The following solutions were prepared immediately before the experiment and stored on ice: Buffer **A** (50 mM phosphate buffer), substrate solution **B** (1 mM substrate **6** in H_2O), enzyme solution **C** (350 units tyrosinase per mL). A reaction cocktail containing H_2O (4.5 mL), buffer **A** (5 mL), and solution **B** (5 mL) was prepared. As a control, a blank run was performed by charging a 3 mL UV cuvette with reaction cocktail (2.9 mL), oxygenation was carried out for 5 min by bubbling pure O_2 through the solution, and buffer solution **A** (0.1 mL) was added. For the experiment shown in Figure 1, a 3 mL UV quartz cuvette was charged with reaction cocktail (2.9 mL), oxygenation was carried out for 5 minutes by bubbling pure O_2 through the solution, and enzyme solution **C** (0.1 mL) was added. The reaction was monitored on a UV spectrophotometer from 800 nm to 200 nm every minute for 40 minutes. The resulting spectra are shown in Figure 1.

Michaelis–Menten kinetics: The enzyme concentration was determined according to the literature.[36] In addition, the enzyme activity was determined according to the literature.[24] The phosphate buffer was prepared from KH_2PO_4 and adjusted with KOH solution to pH 6.8. The following solutions were prepared immediately before the experiment and stored on ice: Buffer **A** (50 mM phosphate buffer), substrate solution **B** (15 mM substrate **6** in H_2O), enzyme solution **C** (66 units tyrosinase per mL). For the experiments shown in Figure 3, a 3 mL UV quartz cuvette was charged with buffer **A** ([2−x] mL) and substrate (x mL) solution **B**. The reaction was oxygenated for 5 minutes by bubbling pure O_2 through the solution, and enzyme solution **C** (1 mL) was then added. The reaction was monitored on a UV spectrophotometer at 305 nm for 5 min and the initial rates for varying substrate concentration x were determined. These initial rates plotted against the substrate concentration are shown in Figure 3.

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Keywords: biosynthesis · cyanobacteria · enzyme kinetics · enzyme mechanism · natural products

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