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Directed Biosynthesis of Phytotoxic Alkaloids in the Cyanobacterium *Nostoc* 78–12A

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Nostocarboline, a chlorinated and N-methylated carbolinium alkaloid, displays potent and selective inhibition of photoautotrophic organisms as well as the malaria parasite *Plasmodium falciparum*, while showing very low toxicity to bacterial and fungal pathogens, rat myoblasts and crustaceans. New derivatives of nostocarboline incorporating Br, F or methyl substituents have been obtained through precursor-directed biosynthesis in *Nostoc* 78–12A (identical to *Nostoc sp.* ATCC 43238) by feeding this cyanobacterium with differently substituted tryptophan derivatives or 6-Br-norharmane (eudistomin N). These experiments substantiate the biosynthetic hypothesis and validate the inherent flexibility of the corresponding enzymes for metabolic engineering. The new derivatives inhibit the growth of the toxic-bloom-forming cyanobacterium *Microcystis aeruginosa* PCC 7806 above 1 μM. The mode of action of nostocarboline was investigated by using chlorophyll-*a* fluorescence imaging, and it was demonstrated that a decrease in photosynthesis precedes cell death, thus establishing the phytotoxic properties of this alkaloid.

Introduction

Cyanobacteria constitute a promising source for novel bioactive metabolites that employ a structurally diverse chemical framework.[1] These prokaryotic photoautotrophs face ecological pressure from both competing organisms and grazers and have thus evolved sophisticated chemical defense strategies through secondary metabolites. Whereas many depsipeptides and cyclopeptides have been identified as major bioactive compounds,[2] relatively few alkaloids have been described from cyanobacteria.[3] We have recently isolated the carbolinium alkaloid nostocarboline from the freshwater cyanobacterium *Nostoc* 78–12A[4] and found strong algicidal effects against competing eukaryotic and prokaryotic photosynthetic organisms.[5] The allelochemical activity of this chlorinated carbolinium[6] is thought to offer competitive advantage to the producing organism through selective inhibition of competitors populating the same habitats.[7]

As recognized in recent years, such ecological implications of natural products can open up new avenues for potential therapeutic applications, if corresponding pathways in competitors or grazers and pathogens are targeted.[8] In the context of the research of our laboratories, the ecological implications of nostocarboline provided a chemical ecology rationale for its evaluation against the malaria parasite *Plasmodium falciparum*, as this parasite contains an organelle of photoautotrophic (cyanobacterial) origin, the apicoplast.[9] Nostocarboline was found to be active against *P. falciparum* at submicromolar concentrations, yet it was also shown to be selective, as little cytotoxicity ($IC_{50} > 100$ μM against rat myoblasts) was determined.[10] The potent and selective biological profile of this compound thus indicates that similar pathways in algae and *Plasmodium* might be targeted. Complementing this selective profile, nostocarboline was not determined active against a panel of pathogenic bacteria and fungi.[5] These results substantiated the hypothesis that pathways unique to photoautotrophs and to *Plasmodium*, potentially through the apicoplast, are targeted by nostocarboline. This potent and selective profile of nostocarboline thus clearly warrants the evaluation of additional derivatives.

Precursor-directed biosynthesis is a technology for the production of modified natural products in the producing organism itself.[11] This strategy offers certain advantages when compared to combined approaches such as mutasynthesis or combinatorial biosynthesis.[11] Existing biosynthetic pathways in the native organisms can be exploited and no modification of the relevant enzymes is required. Moreover, questions about the biosynthesis of metabolites can be investigated and the promiscuity of enzymes for unnatural substrates can be probed. For cyanobacteria, relatively few studies documented the successful implementation of precursor-directed strategies in vivo for the generation of novel natural product analogues.[12] This might be due in part to the challenges associated with feeding potentially toxic precursors to prokaryotic photoautotrophic organisms. In addition, some cyanobacteria such as *Nostoc* or *Anabaena* are able to carry out nitrogen fixation, thus further decreasing their need for xenobiotic uptake. Lastly, the produc-

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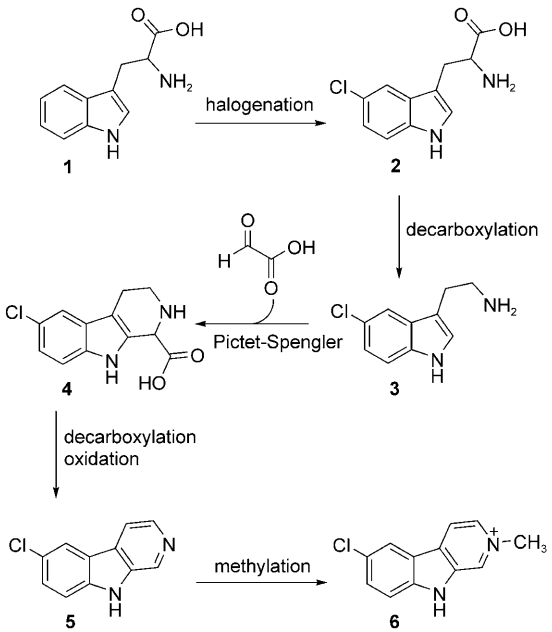
tion of novel algicidal or phytotoxic compounds in a prokaryotic cyanobacterium constitutes a serious problem for the producer. In this study, we demonstrate that all these challenges can be successfully addressed, and that the bioproduction of novel algicidal nostocarboline derivatives can be carried out in *Nostoc* 78–12A. Moreover, chlorophyll-*a* fluorescence imaging was used to investigate the mode of action of nostocarboline.

Results and Discussion

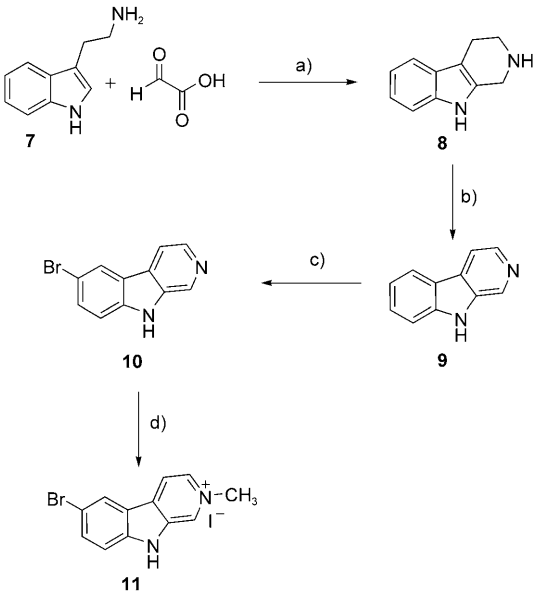
We postulate that the biosynthesis of nostocarboline follows the well-investigated route for β-carboline alkaloids (Scheme 1).[13] The halogenation of tryptophan likely proceeds



Scheme 1. Postulated biosynthesis of nostocarboline.

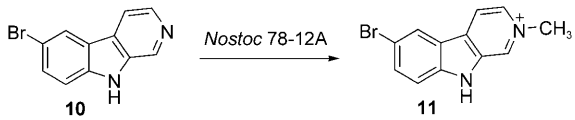
through a tryptophan halogenase; this is well-precedented in the biosynthesis of halogenated indole alkaloids such as rebeccamycin[14] and pyrroindomycin.[15] Decarboxylation of 5-Cl-tryptophan (**2**) would generate 5-Cl-tryptamine (**3**), which reacts with glyoxylic acid in a Pictet-Spengler-type reaction to give the tetrahydro-β-carboline derivative **4**.[13,16] Subsequent decarboxylation and oxidation would generate the 6-Cl-β-carboline (**5**), which is proposed to be finally N-methylated by SAM-dependant methyltransferase to nostocarboline (**6**). We wanted to investigate two distinct processes of this proposed biosynthetic scheme, that is, the final methylation and the possibility of employing different starting materials (derivatives of **1** or **2**) to carry analogues through the whole biosynthetic pathway.

At first, reference compounds (standards) as well as required precursors for feeding experiments needed to be synthesized. The brominated derivative **11** of nostocarboline as well as the precursor utilized for feeding were prepared starting from tryptamine **7** following literature procedures.[17–20] 1,2,3,4-Tetrahydro-β-carboline **8** was obtained via a Pictet-Spengler reaction between **7** and glyoxylic acid followed by an acidic decarboxylation.[17] Compound **8** was then oxidized with Pd/C to give norharmane **9** in high yield (98%).[18,19] Bromination of **9** with NBS afforded eudistomin N (**10**) in good yield (75%).[20] Finally, the methylation of compound **10** to Br-nostocarboline (**11**) with CH_3I was realized according to the procedure developed for the total synthesis of nostocarboline[4] (Scheme 2).



Scheme 2. a) 1. H_2O, HCl, RT; 2. KOH, 3. HCl, pH ≈ 4, RT, 49%; b) Pd/C, *p*-xylene, 98%; c) NBS, AcOH, 75%; d) MeI, *i*PrOH, 40%.

Having the required standards at hand, we wanted to investigate whether the enzyme mediating the putative terminal N-methylation step would recognize a different carboline substrate and if it would tolerate the larger Br substituent. Therefore, 6-Br-norharmane **10** (0.25 mM, final overall concentration, previously dissolved in DMSO) was added to BG11 medium, and inoculated with *Nostoc* 78–12A (identical to *Nostoc sp.* ATCC 43238).[21] The culture was allowed to grow for four weeks in a 250 mL Erlenmeyer flask exposed to a 12 h:12 h light/dark cycle. The culture was harvested by lyophilization and the metabolites extracted with 60% EtOH. The extract was analyzed by HPLC-MS and the presence of 6-Br-nostocarboline (**11**) in the mixture was clearly identified upon comparison to the totally synthetic standard (Scheme 3). This result showed that feeding experiments with compounds similar to putative biosynthetic intermediates are possible in *Nostoc* 78–12A, corroborated the hypothesis that methylation of a halogenated carboline such as **10** is a biosynthetically viable operation, and



Scheme 3. Biotransformation of Br-carboline **10** to **11** mediated by *Nostoc* 78–12A.

demonstrated that the corresponding enzyme tolerates a modified substrate.

Encouraged by this result, we next tried to use different precursors to evaluate the promiscuity of all enzymes along the putative biosynthetic pathway towards substrate modifications. Different tryptophan derivatives were first dissolved in DMSO and then diluted in BG11 medium to a final concentration of 0.25 mM before inoculation with *Nostoc* 78–12A in 500 mL Erlenmeyer flasks. The cultures were allowed to grow during four weeks exposed to a 12 h:12 light/dark cycle, and then harvested and extracted with 60% EtOH. We could unequivocally demonstrate by HPLC-MS analysis and comparison to a synthetic standard that 5-Br-Trp was converted by *Nostoc* 78–12A to 6-Br-nostocarboline (**11**). In addition, a series of fluorinated Trp derivatives was transformed by *Nostoc* to the corresponding nostocarboline derivatives **17** and **18** (Scheme 4). Interestingly, substitution on both positions 5 and

12 R^1 = Br, R^2 = H
13 R^1 = F, R^2 = H
14 R^1 = H, R^2 = F
15 R^1 = CH_3, R^2 = H
16 R^1 = H, R^2 = CH_3

11 R^1 = Br, R^2 = H
17 R^1 = F, R^2 = H
18 R^1 = H, R^2 = F
19 R^1 = CH_3, R^2 = H
20 R^1 = H, R^2 = CH_3

Scheme 4. Conversion of different Trp derivatives by *Nostoc* 78–12A.

6 on the Trp indole ring was tolerated by the native enzymes along the biosynthetic pathway. This is noteworthy, as different steric and electronic consequences result from this substitution. Likewise, the 5-CH_3-Trp and the 6-CH_3-Trp were successfully employed, and the corresponding dehalogenated methylnostocarboline compounds **19** and **20** were isolated. The compounds were purified by HPLC (characterized by ^{1}H NMR and HiRes-MS) with isolation yields between 0.5 and 1.2 mg L^{-1}. Feeding experiments with electron-rich substrates such as 5-methoxy and 5-hydroxy-Trp were less successful: In the case of the MeO-Trp, the corresponding nostocarboline derivative could be identified by HPLC but not isolated. Feeding of 5-hydroxy Trp was not possible, and the medium turned brown after a short amount of time. We also tried to utilize tryptamine derivatives, but it appeared that tryptamine was inhibitory to *Nostoc* 78–12A at the fed concentration (0.25 mM), as chlorosis was induced within a short amount of time.

Several interesting observations warrant further discussion. 1) Intermediates from the postulated pathway, for example the halogenated norharmane derivatives related to **5**, could not be observed in any of the feeding experiments. This would likely be the case if one of the involved enzymes would display lower tolerance for modification, resulting in the buildup of a biosynthetic intermediate. 2) No chlorinated derivatives of compounds **18** and **20** could be observed; this suggests that the putative Trp halogenase does not accept halogenated or methylated tryptophan derivatives (at different positions on the ring) as substrates. This observation thus supports the hypothesis that the employed precursors enter the biosynthetic pathway en lieu of **2** (and not of **1**). In contrast, nostocarboline itself was always observed, albeit in rather different concentrations with respect to the newly produced derivative. In the case of precursor-directed biosynthesis using Trp derivatives halogenated at the 5-position, the observed ratio was in favor of nostocarboline (Table 1), reaching ratios up to 5:1 in the

Table 1. Precursor directed biosynthesis starting from tryptophan derivatives.

Nr.	R^1	R^2	λ_{max}	Yield[a]	Ratio[b]	MIC[c]
11	Br	H	251, 306, 383	–	0.85	1
17	F	H	250, 302, 385	0.5	0.2	10
18	H	F	243, 307, 360	1.2	1.3	10
19	CH_3	H	258, 309, 389	0.9	3.8	10
20	H	CH_3	252, 314, 376	0.9	3.2	10

[a] Isolated yield in mg L^{-1} of culture suspension. [b] Ratio of produced derivative to nostocarboline as determined by HPLC. [c] Minimal inhibitory concentration in μM against *M. aeruginosa* PCC 7806.

case of 5-F-Trp. This could be explained if the endogenously produced 5-Cl-Trp is favored over the 5-F-Trp in either decarboxylation or Pictet-Spengler cyclization reactions. However, predicting relative rates and substrate preference in vivo without knowledge of substrate concentrations and purely based on product ratios is difficult. Nonetheless, it is interesting to point out that the 6-F-Trp precursor reverts the observed selectivity, and the new derivative **18** is observed in excess. Comparing these two fluorinated substrates thus shows that the steric and electronic properties of 6-F-Trp result in higher incorporation rates thus suggesting higher tolerance of the processing enzymes for 6-F-Trp when compared to 5-F-Trp and the endogenously produced substrate. The methyl substituted Trp precursors are incorporated in even higher ratios of 3:1 to 4:1 versus nostocarboline (**6**).

The nostocarboline derivatives **11** and **17**–**20** were then evaluated for their inhibitory power to the growth of the toxic cyanobacterium *Microcystis aeruginosa* PCC7806. This species is a prototype of a bloom-forming cyanobacterium of the genus *Microcystis*, which is involved in many harmful algal blooms

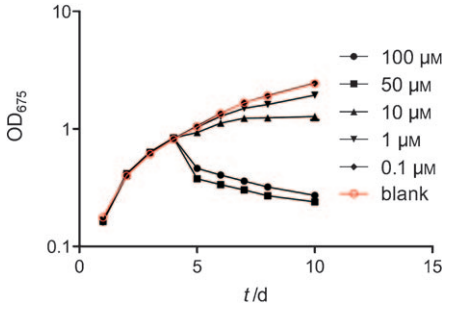


Figure 1. Growth curves of *Microcystis aeruginosa* PCC 7806 before and after exposure to Br-nostocarboline (**11**). The compound was administered on day four. OD refers to the optical density measured at 675 nm.

throughout the world. The derivatives **11** and **17–20** were added to growing cultures on day four following established assays,[5] and the minimal inhibitory concentration was determined as the minimal concentration at which the growth rate of *M. aeruginosa* is decreased compared to controls. Whereas Br-nostocarboline matched the inhibitory power of nostocarboline (MIC = 1 μM), both the fluorinated and methylated derivatives **17–20** displayed lower activities, with the MIC values increased by one order of magnitude (Table 1). Inspection of the growth curves of *Microcystis aeruginosa* PCC 7806 before and after treatment with Br-nostocarboline **11** (Figure 1) demonstrated that addition of 1 μM is sufficient to impact the growth of the toxic cyanobacterium, and concentrations above 50 μM induced cell death. This algicidal activity of Br-nostocarboline **11** was found comparable to the parent nostocarboline (**6**).[5]

After establishing that the new derivatives **11** and **17–20** display algicidal effects at concentrations similar or higher than nostocarboline (**6**), we wanted to investigate whether the observed effects results from inhibition of photosynthesis. In order to test the effects of nostocarboline (**6**) on photosynthesis, we used chlorophyll-*a* fluorescence imaging, a noninvasive technique for probing oxygenic photosynthesis.[22] Necrotic lesions, which became visible about 12-24 h after infiltration of nostocarboline (**6**), occurred in tobacco leaves for concentrations > 10 μM (Figure 2A). Immediately after infiltration, photosynthetic electron transport (PET) was inhibited, dependent upon the nostocarboline concentration used (Figure 2B). Cell death assays demonstrated that the down regulation of PET precedes cell death. In the first hours after nostocarboline treatment in the light, about 10% of all counted cells were found dead (Figure 2C), but photosynthesis was downregulated by about 50% (10 μM nostocarboline) (Figure 2B).

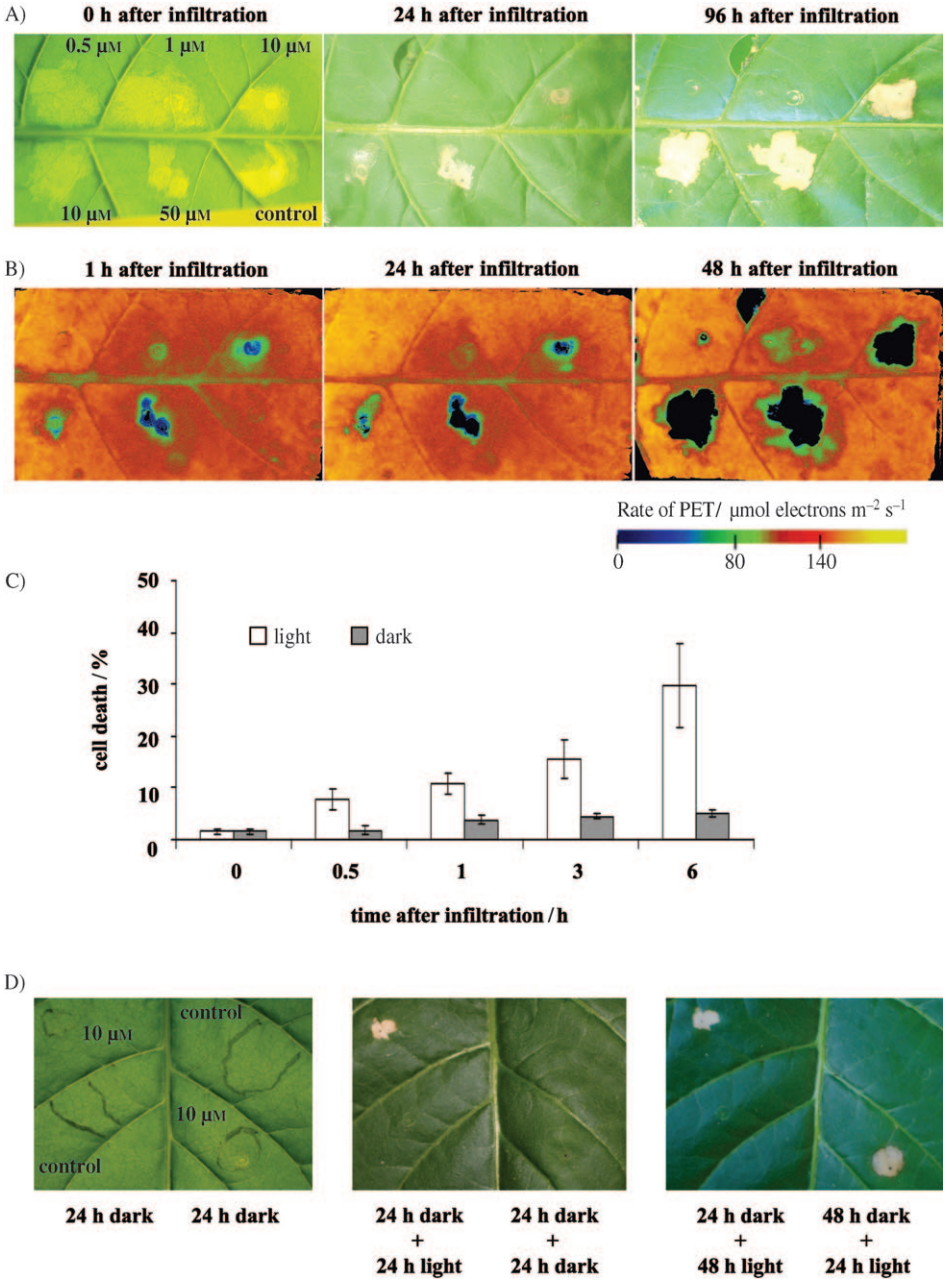


Figure 2. Effects of nostocarboline on photosynthesis and cell death in higher plants A) Representative images of an infiltrated tobacco leaf at the beginning of the light phase. At 0 hour post infiltration (hpi): Leaf areas infiltrated with 2% DMSO (control) or different concentrations of nostocarboline. Occurrence of necrotic lesions 24 and 48 h after infiltration. B) Chlorophyll-*a* fluorescence imaging picture of the same leaf. C) Cell death at the infiltration site. Data are means ± SE of at least three individual plants at each time point. D) Representative images of an infiltrated tobacco leaf. At 24 h after dark incubation: Marked leaf areas infiltrated with 2% DMSO (control) or 10 μM nostocarboline and occurrence of necrotic lesions after dark-plus-light combinations.

The occurrence of cell death after nostocarboline treatment is light dependent. On the one hand, significantly more dead cells were detected if the leaves had been exposed to light (Figure 2C). On the other hand, leaves which were put in the dark after nostocarboline infiltration showed no formation of necrotic lesions. But if exposed to light subsequently, necrotic lesions occurred (Figure 2D). This also indicates that the effect of nostocarboline is long-lasting (over days) in plant tissue.

Preliminary experiments on the mechanism of photosynthesis inhibition as measured by P700 redox kinetics[23] indicate that the electron transport chain might be restricted upstream of photosystem I (PS I, data not shown). The effect of nostocarboline might be comparable to the non-selective herbicide paraquat (methylviologen, 1,1′-dimethyl-4,4′-bipyridinium), which is a strong autooxidable electron acceptor in PS I; and its presence in light-exposed plants has several drastic consequences, like the production of superoxide.[24] Similarly, downregulation of photosynthesis in the light after nostocarboline treatment could lead to an enhanced production of light-driven reactive oxygen species which could be responsible for the formation of the necrotic lesions.

Conclusions

In conclusion, we have shown that precursor-directed biosynthesis of algicidal and phytotoxic nostocarboline derivatives **11** and **17**–**20** in the cyanobacterium *Nostoc* 78–12A is possible. Several implications concerning the biosynthetic hypothesis outlined in Scheme 1 became evident: 1) The N-methylation of a halogenated precursor is a biosynthetically viable transformation. 2) Whereas tryptophan derivatives can be employed, the corresponding tryptamine derivatives were not successful; this suggests a role for Trp in the biosynthesis of nostocarboline. 3) The enzymes along the biosynthetic pathway are promiscuous to the extent that other halogenated starter units (such as Br and F) as well as dehalogenated Me derivatives can be used. 4) Modified substrates such as 7-substituted Trp derivatives were not found to be halogenated; this lack of halogenation suggests reduced flexibility of the putative halogenase, and the precursors thus enter biosynthesis in lieu of 6-Cl-Trp **2**. Studies on the mode of action of nostocarboline (**6**) using fluorescence imaging demonstrated that downregulation of photosynthesis preceded cell death and that its mechanism is light dependent. The present work thus delivers new potential antiplasmodial agents and encourages the precursor-directed biosynthesis of other cyanobacterial metabolites of indole origin.

Experimental Section

Instruments and methods: BG11 was purchased from Sigma. NMR spectra were acquired on a Bruker AVII-800 equipped with a cryoprobe or a DPX-400 and the chemical shifts are referenced to residual solvent proton and carbon signals (δ_H 3.31, δ_c 49.0 for CD_3OD; δ_H 2.50, δ_c 39.5 for $[D_6]$DMSO). Accurate mass ESI spectra were recorded on a MICROMASS (ESI) Q-TOF Ultima API (Waters Corporation, Milford, MA, USA). HPLC purification and analyses were performed on a Dionex HPLC system (Sunnyvale, CA, USA) equipped with a P680 pump, an ASI-100 automated sample injector, a TCC-100 thermostated column compartment, a PDA-100 photodiode array detector, a Foxy Jr. fraction collector and a MSQ-ESI mass spectrometric detector.

Synthesis

1,2,3,4-Tetrahydro-β-carboline **8**.[17] Glyoxylic acid monohydrate (6.33 g, 68.8 mmol, 1.1 equiv) in water (15 mL) and a few drops of HCl (aq.) were added to a suspension of tryptamine (**7**; 10.0 g, 62.5 mmol, 1.0 equiv) in water (190 mL). The milky, light brown reaction mixture was stirred at RT for 15 min. A solution of KOH (3.40 g, 60.6 mmol, 0.96 equiv) in water (17 mL) was then slowly added and the pH was subsequently adjusted to pH ~ 4 with HCl (aq.). The mixture was stirred at RT for 1 h and then stored at 4 °C for 12 h. The resulting light brown solid was filtered and washed thoroughly with water. The solid was then suspended in water (160 mL) and 25% HCl (24 mL, 0.60 mmol) was slowly added. The mixture was heated to reflux for 30 min, 25% HCl (24 mL, 0.60 mmol) was then added, the mixture was stirred at reflux for another 15 min before it was allowed to cool down to RT. The reaction mixture was stored at 4 °C for 3 days to allow for precipitation. The resulting solid was isolated by filtration and washed with water. The solid was then dissolved in water by heating to 55 °C and the pH was adjusted to pH ~ 12 with 20% KOH (aq.). The resulting white precipitate was filtered, washed with water and dried under reduced pressure to afford compound **8** as a colorless solid (5.22 g, 30.3 mmol, 49%). ^{1}H NMR spectrum (400 MHz, $[D_6]$DMSO): $\delta = 10.62$ (s, 1H, NH), 7.34 (d, ^3J(H,H) = 7.6 Hz , 1H), 7.25 (d, ^3J(H,H) = 7.9 Hz, 1H), 7.00 (m, 1H), 6.92 (m, 1H), 3.84 (s, 2H), 3.32 (s, 1H), 2.96 (t, ^3J(H,H) = 5.7 Hz, 2H), 2.58 ppm (t, ^3J(H,H) = 5.6 Hz, 2H). HPLC-ESI-MS *m/z* $[M+H]^+$ 173.3 (calcd for $C_{11}H_{13}N_2$, 173.1).

β-Carboline **9**.[18,19] Pd/C (10%, three spatulas) was added to a suspension of **8** (5.11 g, 29.7 mmol) in *p*-xylene (150 mL). The mixture was stirred at 145 °C. The course of the reaction was monitored by HPLC-MS. After 24 h the reaction was allowed to cool down to RT and MeOH (300 mL) was added. The mixture was filtered through a pad of celite and the solvent was removed under reduced pressure to afford compound **9** as a white solid (4.86 g, 28.9 mmol, 97%). ^{1}H NMR spectrum (400 MHz, $[D_6]$DMSO): $\delta = 11.55$ (s, 1H), 8.90 (s, 1H), 8.34 (d, ^3J(H,H) = 5.4 Hz, 1H), 8.23 (d, ^3J(H,H) = 8.0 Hz, 1H), 8.09 (d, ^3J(H,H) = 5.4 Hz, 1H), 7.60 (d, ^3J(H,H) = 8.0 Hz, 1H), 7.54 (t, ^3J(H,H) = 8.1 Hz, 1H), 7.24 ppm (t, ^3J(H,H) = 8.0 Hz, 1H).

6-Br-β-carboline **10**.[20] NBS (4.98 g, 27.9 mmol, 1 equiv) was added to a solution of **9** (4.7 g, 27.9 mmol, 1 equiv) in AcOH (180 mL). The reaction mixture was stirred for 2 h at RT before removing the solvent under reduced pressure. The residue was dissolved in CH_2Cl_2 and sequentially washed with saturated solutions of $NaHCO_3$, NaCl and $Na_2S_2O_3$. The solution was dried ($MgSO_4$) and the solvent was removed under reduced pressure. The compound was purified by flash chromatography on SiO_2 (AcOEt, NEt_3 0.1%) to afford a yellow solid (5.13 g, 20.8 mmol, 75%). R_f (AcOEt, NEt_3 0.1%) 0.19; ^{1}H NMR spectrum (400 MHz, $CDCl_3$): $\delta = 8.93$ (s, 1H), 8.49 (d, ^3J(H,H) = 5.4 Hz, 1H), 8.27 (d, ^4J(H,H) = 1.9 Hz, 1H), 7.92 (dd, ^3J(H,H) = 5.3 Hz, ^4J(H,H) = 1.1 Hz, 1H), 7.65 (dd, ^3J(H,H) = 8.6 Hz, ^4J(H,H) = 1.9 Hz, 1H), 7.42 ppm (d, ^3J(H,H) = 8.6 Hz, 1H).

6-Bromonostocarboline **11**. MeI (50 L, 0.79 mmol, 2 equiv) was added at RT to a solution of **10** (0.097 g, 0.39 mmol, 1 equiv) in *i*PrOH (2.5 mL). The solution was heated to reflux for 4 h. The resulting yellow suspension was then cooled to RT and filtered. The filtrate was washed with *i*PrOH and dried under reduced pressure to afford compound **11** as a yellow solid (0.039 g, 0.15 mmol, 40%). ^{1}H NMR (400 MHz, CD_3OD): $\delta = 9.24$ (s, 1H, H-1), 8.69 (d, ^3J(H,H) = 6.4 Hz, 1H, H-4), 8.66 (d, ^4J(H,H) = 1.7 Hz, 1H, H-5), 8.54 (d, ^3J(H,H) = 6.4 Hz, 1H, H-3), 7.93 (dd, ^3J(H,H) = 8.9 Hz, ^4J(H,H) = 1.7 Hz, 1H, H-7) 7.73 (d, ^3J(H,H) = 8.9 Hz, 1H, H-8), 4.54 ppm (s, 3H, *N*-Me); UV (MeOH): λ_{max} ($\log \varepsilon$) = 209 (1.69), 251 (1.13), 306 (0.72), 383 (0.17); HRESI-QqTof-MS calcd for $C_{12}H_{10}BrN_2$: 261.0027 $[M]^+$, found: *m/z*: 261.0028

General procedure for the feeding experiments: *Nostoc* 78–12A (identical to *Anabaena* 78–12A and ATCC 43238) was cultured in

200 mL of BG11 in a 500 mL Erlenmeyer flask at 22 °C with a light/dark cycle of 12 h:12 h for eight weeks. The precursors were dissolved in DMSO (1 mL) and sterilized by filtration prior to addition to the culture to obtain a final concentration of 0.25 mM.

General procedure for the isolation of the metabolites: The lyophilized culture was extracted three times with aqueous EtOH (60%); the cell material was removed by centrifugation and the solvents were removed under reduced pressure. The resulting extract was dissolved in aqueous methanol (80%) and filtered (0.25 μm). The metabolites were first identified by C_{18} RP-HPLC-UV-MS (Phenomenex Gemini C_{18} 150×5 mm; Torrance, CA, USA); Mobile phase A was TFA (0.05%) in acetonitrile and B was TFA (0.05%) in water. The column was stabilized for 10 min with 10% A, then a linear gradient was used to reach 100% A over 40 min, followed by washing for 10 min with 100% A. The flow rate was 1 mL min^{-1}. The compounds were purified by multiple runs of C_{18} RP-HPLC (Phenomenex Gemini C_{18} 150×10 mm). The column was stabilized for 10 min with 10% A, the a linear gradient was used to reach 45% A in 15 min, followed by washing for 5 min with 100% A. The flow rate was 5 mL min^{-1}. The retention times are almost identical on both columns. The solvent was removed from the combined fractions with a stream of N_2. The amount of sample was determined spectrophotometrically with the extinction coefficient of nostocarboline.[4] The fermentation yield was calculated based on the volume of the culture and is given in Table 1, and the chemical yield is based on the amount of precursor employed and is given below.

6-Fluoro-nostocarboline **17**. Retention time 8.4 min, (0.10 mg, 1.0%); UV (MeOH): λ_{max}=250, 302, 385; ^{1}H NMR spectrum (800 MHz, CD_3OD): δ=9.23 (s, 1H, H-1), 8.68 (d, ^3J(H,H)=6.5 Hz, 1H, H-4), 8.50 (d, ^3J(H,H)=6.5 Hz, 1H, H-3), 8.17 (dd, ^3J(H,F)=8.4 Hz, ^4J(H,H)=2.5 Hz, 1H, H-5), 7.80 (dd, ^3J(H,H)=8.9 Hz, ^4J(H,F)=4.0 Hz, 1H, H-8) 7.64 (ddd, ^3J(H,H)=8.9 Hz, ^3J(H,F)=8.9 Hz, ^4J-(H,H)=2.5 Hz, 1H, H-7), 4.54 ppm (s, 3H, *N*-Me); HRESI-QqTof-MS calcd for $C_{12}H_{10}FN_2$: 201.0828 $[M]^+$, found: *m/z* 201.0840.

7-Fluoro-nostocarboline **18**. Retention time 10.6 min, (0.24 mg, 2.4%); UV (MeOH): λ_{max}=243, 307, 360; ^{1}H NMR spectrum (800 MHz, CD_3OD): δ=9.17 (s, 1H, H-1), 8.63 (d, ^3J(H,H)=6.4 Hz, 1H, H-4), 8.51 (d, ^3J(H,H)=6.4 Hz, 1H, H-3), 8.46 (dd, ^3J(H,H)=9.0 Hz, ^4J(H,F)=5.2 Hz, 1H, H-5), 7.48 (dd, ^3J(H,F)=9.1 Hz, ^4J(H,H)=2.1 Hz, 1H, H-8), 7.28 (ddd, ^3J(H,H)=9.0 Hz, ^3J(H,F)=9.0 Hz, ^4J-(H,H)=2.1 Hz, 1H, H-6) 4.52 ppm (s, 3H, *N*-Me); HRESI-QqTof-MS calcd for $C_{12}H_{10}FN_2$: 201.0828 $[M]^+$, found: *m/z* 201.0838.

6-Methyl-nostocarboline **19**. t_R=9.8 min, (0.18 mg, 1.9%); UV (MeOH) λ_{max} 258, 309, 389; ^{1}H NMR spectrum (800 MHz, CD_3OD): δ=9.16 (s, 1H, H-1), 8.64 (d, ^3J(H,H)=6.4 Hz, 1H, H-4), 8.48 (dd, ^3J-(H,H)=6.4 Hz, ^4J(H,H)=1.0 Hz, 1H, H-3), 8.24 (brs, 1H, H-5), 7.70 (m, 2H, H-7 and H-8), 4.53 (s, 3H, *N*-Me), 2.04 ppm (s, 3H, Me); HRESI-QqTof-MS calcd for $C_{13}H_{13}N_2$: 197.1079 $[M]^+$, found: *m/z* 197.1071.

7-Methyl-nostocarboline **20**. t_R=10.1 min, (0.17 mg, 1.7%); UV (MeOH) λ_{max} 252, 314, 376; ^{1}H NMR spectrum (800 MHz, CD_3OD): δ=9.10 (brs, 1H, H-1), 8.58 (d, ^3J(H,H)=6.5 Hz, 1H, H-4), 8.45 (dd, ^3J(H,H)=6.5 Hz, ^4J(H,H)=1.0 Hz, 1H, H-3), 8.28 (d, ^3J(H,H)=8.2 Hz, 1H, H-5), 7.34 (brd, ^3J(H,H)=8.2 Hz, 1H, H-6), 7.34 (brs, 1H, H-8), 4.50 (s, 1H, *N*-Me), 2.01 ppm (s, 1H, Me); HRESI-QqTof-MS calcd for $C_{13}H_{13}N_2$: 197.1079 $[M]^+$, found: *m/z* 197.1079.

Biological evaluation: Growth inhibition experiments against *Microcystis aeruginosa* PCC 7806 were carried out as described in the literature.[5] Effects of nostocarboline on higher plants were tested in tobacco (*Nicotiana tabacum* SNN) by infiltration of different concentrations in 6–8 weeks old leaves, exposed to a 14 h:10 h light/dark cycle, except as noted otherwise. Imaging of photosynthetic parameters from chlorophyll-*a* fluorescence were determined as described previously.[23] Cell death studies were performed with 0.5 mg mL^{-1} propidium iodide. Cells with disrupted membranes allow propidium iodide to enter the cell and fluoresce, indicating cell death. The fluorescence is detected at 590 to 650 nm after excitation at 488 nm using a cLSM (TCS SP2 with inverse DMIRB-microscope, Leica).

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