Nostocarboline: Isolation and Synthesis of a New Cholinesterase Inhibitor from *Nostoc* 78-12A

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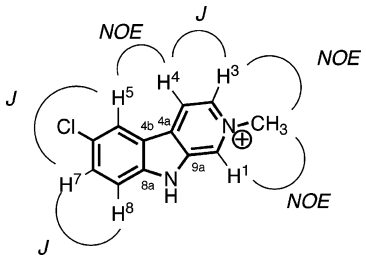
A new quaternary β-carboline alkaloid, nostocarboline (**1**), was isolated from the freshwater cyanobacterium *Nostoc* 78-12A, and its constitution was assigned by 2D-NMR methods. The structure was proven by its total synthesis starting from norharmane via chlorination at C-6 and methylation at N-2. Nostocarboline (**1**) was found to be a potent butyrylcholinesterase (BChE) inhibitor, with an IC_{50} of 13.2 μM. The related 2-methylnorharmane, which is present in the human brain and might be relevant to Parkinson's disease (PD), was also determined to be a BChE inhibitor (11.2 μM). These inhibitory concentrations are comparable to galanthamine, an approved drug for the treatment of Alzheimer's disease (AD). Nostocarboline (**1**) can thus be considered as a lead for the development of novel neurochemicals.

Cyanobacteria are a rich source of novel secondary metabolites with potent biological activities and a wide variety of different structural classes including several indole alkaloids.[1] Interestingly, however, there are only two reports on carboline derivatives isolated from cyanobacteria, the bauerines from *Dichotrix baueriana* and norharmane from *Nodularia harveyana*.[2] This is surprising, as alkaloids containing the carboline skeleton are often encountered in higher plants, animals, and bacteria.[3] In this publication, we report the isolation, structure elucidation, and synthesis of a new quaternary carbolinium compound from *Nostoc* 78-12A, which we have named nostocarboline (**1**). This alkaloid displays potent cholinesterase-inhibitory activity.

Nostoc 78-12A, originally isolated from a wastewater lagoon, has been reported as a producer of an anticyanobiotic.[4] The aqueous methanol extract of the frozen wet biomass was dried and washed with acetonitrile. The bioactive compound was isolated by repeated extraction with aqueous CH_3CN and subsequently purified by HPLC on a Polyamine II column. Nostocarboline (**1**) is soluble in 95% aqueous CH_3CN but not in pure CH_3CN itself. The compound displays strong blue fluorescence ($\lambda_{ex} = 306$ nm, $\lambda_{em} = 456$ nm), and the UV spectrum is reminiscent of that of a carboline system such as in the bauerines.[2a]

The high-resolution matrix-assisted laser desorption/ionization (HRMALDI) spectrum of nostocarboline (**1**) supported the molecular formula, $C_{12}H_{10}ClN_2$, which was corroborated by ^{15}N-labeling, establishing the presence of two N atoms. The ^1H NMR spectrum (CD_3OD, 600 MHz, see Table 1) displayed two different and separate ABX spin systems in the aromatic region as well as a singlet at 4.53 ppm accounting for three additional protons. NMR spectrum analysis by HSQC and HMBC techniques combined with the *J* values extracted from the ^1H NMR spectrum established the presence of a C-6 or C-7 Cl-substituted carbolinium system. The unusual chemical shift of the 3H singlet pointed to the presence of a Me group connected to a quaternary N atom as part of an aromatic system. 1D-NOE difference measurements established the pyrimido-2-N as methylated, as NOEs between the Me group at 4.53 ppm and the protons at 8.52 ppm (H-3) and 9.23 ppm (H-1) could be detected. Further NOEs between resonances at 8.68 and 8.49 ppm demonstrated the protons H-4 and H-5 to be proximal. Tentative structural assignments based upon these spectroscopic data led to either a C-6 Cl substituted β-carbolinium or a C-7 Cl substituted γ-carbolinium system, with the former being supported by the chemical shifts of C-4b, C-8a, and C-9a.[5]

Table 1. NMR Spectroscopic Data of Nostocarboline (**1**, CD_3OD, 600 MHz)[a] (lines in figure denote key *J* couplings or NOEs)



position	δ_H	δ_C	*J* (Hz)	HMBC[a]	NOE
1	9.23	132.4	br s	C-3, C-4a	*N*-Me
3	8.52	134.6	dd, 6.4 (H-4), 0.9 (H-1)	C-4a, C-1, C-4	H-4
4	8.68	119.3	d, 6.4 (H-3)	C-9a	H-5, H-3
4a		134.0			
4b		122.1			
5	8.49	123.8	dd, 2.0 (H-7), 0.8 (H-8)	C-6, C-7, C-8a	H-4
6		128.4			
7	7.80	133.8	dd, 8.9 (H-8), 2.0 (H-5)	C-8a	
8	7.77	115.5	dd, 8.9 (H-7), 0.8 (H-5)	C-4b, C-6	
8a		145.5			
9a		138.4			
N-Me	4.53	48.8	s	C-1, C-3	H-1, H-3

[a] Denotes HMBC correlations from proton at position X to the corresponding carbon.

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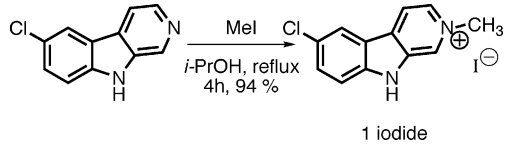
Scheme 1. Synthesis of Nostocarboline Iodide (**1a**)



1 iodide

Table 2. Inhibitory Concentrations (IC_{50}) for 50% Inhibition of Butyrylcholinesterase in an in Vitro Enzyme Assay

inhibitor	IC_{50} (μM) ± SD
nostocarboline (**1**, natural)[a]	19.4 ± 2.8
nostocarboline iodide (**1a**, synthetic)	13.2 ± 2.2
galanthamine hydrobromide	16.9 ± 0.9
2-methylnorharmane iodide	11.2 ± 0.3

[a] The counterion was not determined.

We therefore sought to establish unambiguously the structure of **1** by total synthesis (Scheme 1). Norharmane[6] was converted to 6-Cl-norharmane[7] using a modified literature procedure.[8] This compound was then treated with MeI in *i*-PrOH at reflux for 4 h, and the corresponding, bright yellow, quaternized compound **1** was isolated in excellent yield. The resonances in the ^{1}H NMR spectrum of nostocarboline (**1**) were slightly dependent on concentration and, for the synthetic sample of **1**, the counterion (I^-, Cl^-, $CF_3CO_2^-$).[9] Overall, spectroscopic data (UV, ^{1}H NMR, ^{13}C NMR) of the synthetic sample of **1** were in very good agreement with those of nostocarboline (**1**). The final proof of structure was obtained by mixing natural and synthetic samples, which were thus shown to be identical by ^{1}H NMR spectroscopy.[8] The structure of nostocarboline was thus definitely established as **1** by chemical synthesis.

Nostocarboline (**1**) was then evaluated as an inhibitor of butyrylcholinesterase (Table 2), and an IC_{50} value of 13.2 μM was determined. The small difference in IC_{50} values of synthetic nostocarboline iodide vs the natural sample can be attributed to the different counterion, which however remains unknown for the natural isolate. Overall, the inhibitory activity of nostocarboline (**1**) is comparable to galanthamine, which is an approved drug for the therapy of Alzheimer's disease.[10]

β-Carbolinium derivatives, which are anhydronium bases,[11] are frequently encountered in higher plants[12] and, to a much lesser extent, in marine species.[13] Nostocarboline (**1**) constitutes, to our knowledge, the first quaternary carbolinium derivative isolated from a cyanobacterium. The reason for this as well as the biological role of **1** remains unclear. In this respect it is interesting to point out that the parent compound, norharmane, was shown to possess anticyanobacterial acitivity against *Anabaena cylindrica*, *A. variabilis*, *Anacystis marina*, *Microcystis aeruginosa*, *M. viridis*, and *Oscillatoria agardhii*.[14] We did not find putative biogenetic precursors of **1** such as norharmane or Cl-norharmane itself in the *Nostoc* 78-12A.

Although the physiological role of the related deschloronostocarboline, 2-methylnorharmane, is unknown, it is of interest to note that this alkaloid has been isolated from the post mortem human brain.[15] In addition, methylation of harmane derivatives has been proposed to occur enzymatically in the human brain.[16a] The resulting *N*-methylated β-carbolinium cations are structurally and functionally similar to the neurotoxic 1-methyl-4-phenylpyridinium cation.[16a] Furthermore, β-carbolines have been shown to be capable of inducing apoptosis in neurons.[16b] All these findings support the notion that β-carbolines could be involved in Parkinson's disease.[16c]

We have measured a potent BChE inhibitory activity for 2-methylnorharmane (IC_{50} = 11.2 μM),[17] which is comparable to nostocarboline. These inhibitory activities are of the same order of magnitude as those of galanthamine. The potent biological activity of the BChE inhibitor nostocarboline (**1**), the current therapeutic approach to AD involving cholinesterase inhibitors,[18] and the presence of related endogenous substances in humans might therefore provide a strong rationale for the development of nostocarboline derivatives for the research and treatment of neurological disorders.[19]

Experimental Section

General Experimental Procedures. Chemicals were generally purchased from ABCR, Acros, Aldrich, or Fluka. Norharmane was prepared according to the literature.[6] 6-Chloroharmane was prepared using a modified literature procedure (see Supporting Information).[7] 2-Methylnorharmane was prepared according to van Tamelen et al.[5] IR spectra were recorded as a KBr pellet using a Perkin-Elmer RX I FT-IR spectrometer, and absorptions are given in cm^{-1}. ^{1}H and ^{13}C NMR spectra were recorded using either Varian Gemini [300 MHz (^{1}H) or 75 MHz (^{13}C)], Varian Mercury [300 MHz (^{1}H) or 75 MHz (^{13}C)], or Bruker AV600 [600 MHz (^{1}H) or 150 MHz (^{13}C)] FT spectrometers at ambient temperature with chemical shifts δ given in ppm and coupling constants (*J*) in Hz. Mass spectra were recorded by the MS Service of the Laboratorium für Organische Chemie der ETH Zürich on an IonSpec Ultima 4.7 FT-ICR spectrometer using MALDI with fragment ions given in *m/z* and relative intensities (%) in parentheses. HPLC-ESIMS data were obtained on a LC-MS (LCQ Duo mass spectrometer, Finnigan Thermoquest).

Culture of *Nostoc* 78-12A. The axenic strain *Nostoc* 78-12A (identical to *Anabaena* 78-12A and ATCC 43238) was obtained from C. P. Wolk (MSU, East Lansing, MI) as a liquid culture. It was grown in glass tower-type reactors as previously described.[20] The yield was 2.0 g wet biomass per liter. During centrifugation in a flow-through centrifuge, the cells lyse to a great extent and lose nostocarboline (**1**) to the medium. Therefore, short centrifugation steps are required to retain sufficient amounts of **1** in the cells. The ^{15}N-labeled compound was obtained from cultures in 300 mL Erlenmeyer flasks by replacing the naturally labeled nitrate of the mineral medium by ^{15}N-enriched nitrate (98 atom %, Cambridge Isotope Laboratories, Inc., Andover, MA).

Isolation of Nostocarboline (1). The frozen wet biomass (8.8 g) of *Nostoc* was extracted with methanol. Methanol was added in quantities to obtain a final concentration of 60% aqueous methanol (MeOH−H_2O, 60:40 v/v) assuming 85% water in the wet biomass. The extract was brought to dryness on a rotary evaporator at 40 °C under reduced pressure. To remove the lipid byproducts, the residue was washed with CH_3-CN. The bioactive compound was then extracted four times with 1 mL portions of 95% aqueous CH_3CN (CH_3CN-H_2O, 95:5 v/v). The yellow extract was separated by HPLC on a Polyamine II column (250 × 4.6 mm i.d., S-5 μm, 12 nm, YMC Europe GmbH) using the solvent 30% aqueous CH_3CN at pH 4 (trifluoroacetic acid) and reading the absorption at 306 nm. Nostocarboline (**1**) eluted at 2.5 min. The natural anion (counterion) is not known. The concentration of nostocarboline (**1**) was determined in a standing culture of *Nostoc* (12 μg chlorophyll *a*/mL) to be 0.4 μg nostocarboline (**1**)/μg chlorophyll *a*.

Nostocarboline Iodide (1a). 6-Cl-Norharmane (70.0 mg, 0.35 mmol) was dissolved in *i*-PrOH (2 mL). MeI (45 μL, 0.7 mmol, 2 equiv) was added at room temperature and the resulting suspension heated to reflux for 4 h. The resulting yellow suspension was then cooled to room temperature and filtered. The filtrate was washed with *i*-PrOH and dried under high vacuum. Recrystallization from MeOH−*i*-PrOH gave the title compound **1a** (113 mg, 0.33 mmol, 94%).

1a: bright yellow, thin needles (MeOH); mp > 215 °C; UV (MeOH) λ_{max} (log ϵ) 254 (3.68), 307 (3.46), 385 (2.81); IR (KBr) 2979, 1644, 1572, 1518, 1492, 1451, 1323, 1284, 1152, 1067,

879, 834, 806; ^{1}H and ^{13}C NMR, see Table 1; MS (MALDI, matrix: 3-hydroxypicolinic acid) *m/z* 235.1 (7), 233.1 (15), 219.0 (39), 218.0 (18), 217.0 (100); HRMALDI *m/z* 217.0523 (calcd for $C_{12}H_{10}{}^{35}ClN_2$ 217.0527, $\Delta = 1.84$ ppm); *anal.* C 41.56%, H 2.99%, N 8.00%, calcd for $C_{12}H_{10}N_2ClI$, C 41.83%, H 2.93%, N 8.13%.

Cholinesterase Inhibition Assay. Inhibitors were dissolved in Sørensen phosphate buffer (66.7 mM; pH 7.2). The inhibition of the activity of butyrylcholinesterase (pseudocholinesterase from horse serum, Sigma) was determined with a colorimetric procedure based on the Ellman reaction.[21] A mixture of butyrylthiocholine iodide (5 mM) and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, 0.25 mM) was obtained as Sigma Diagnostics Cholinesterase (BTC) reagent (no longer available). For each assay, 60 μL of this reagent, 30 μL of BChE (approximately 0.001−0.015 U), and 160 μL of the dissolved inhibitor were filled in a microwell of a 96-well polystyrene plate (Corning, Wiesbaden, Germany). Colorimetric measurements (405 nm) were performed on a SpectraMax 190 multichannel spectrophotometer (Molecular Devices, Ismaning/München, Germany) at a temperature of 30 °C. For each inhibitor, three replicates of seven concentrations (between 0.1 and 813 μM) were measured. The inhibition of the enzyme was calculated from the slope of the linear part of the enzyme reaction (absorption vs time) in relation to controls (no inhibition, 100% activity). The concentration of the inhibitor that reduced the activity of the enzyme by 50% (IC_{50}) was determined by interpolation of the data.

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Supporting Information Available: Spectra of synthetic and natural nostocarboline (**1**) and synthetic procedures for the preparation of 6-Cl-norharmane are reported. This material is available free of charge via the Internet at http://pubs.acs.org.

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