# CYTOTOXIC, ANTIVIRAL INDOLOCARBAZOLES FROM A BLUE-GREEN ALGA BELONGING TO THE NOSTOCACEAE

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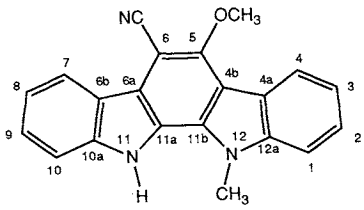
6-Cyano-5-methoxy-12-methylindolo[2,3-*a*]carbazole and 6-cyano-5-methoxyindolo[2,3-*a*]carbazole are responsible for most of the cytotoxicity and antiviral activity associated with the blue-green alga *Nostoc sphaericum* EX-5-1. The compounds are active against HSV II and show weak cytotoxicity against KB and LoVo human carcinoma cell lines.

The heterocyclic indolo[2,3-*a*]carbazole system is found in several biologically active natural products from a wide variety of organisms, *e.g.*, in staurosporin from *Streptomyces staurosporeus*,[1] in the acriflavins from the slime mold *Arcyria denudata*[2] and in rebeccamycin from the bacterium *Nocardia aerocoligenes*.[3] We now find that the extract of a blue-green alga *Nostoc sphaericum* (strain EX-5-1), which shows moderate antiviral activity against herpes simplex virus type 2 and weak, non-selective cytotoxicity against several murine and human tumor cell lines, contains indolo[2,3-*a*]carbazoles, the major one being 6-cyano-5-methoxy-12-methylindolo[2,3-*a*]carbazole (**1**).

The alga was isolated from a mud sample collected on the University of Hawaii campus grounds and grown in mass culture. Compound **1** was obtained from the ethanolic extract of the alga by successive normal and reversed-phase chromatography. The yield was 0.22% based on the dried weight of the alga.



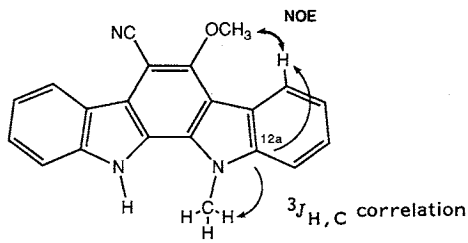

**1**

The UV-spectrum of **1** was typical of an indolo[2,3-*a*]carbazole. The EI mass spectrum revealed a MW of 325 and a HR mass measurement was consistent with the formula $C_{21}H_{15}N_3O$. The $^{13}C$ NMR spectrum confirmed the presence of 21 carbon atoms and showed that the molecule possessed 19 unsaturated carbons. The $^{13}C$ DEPT spectrum established that the two remaining carbons were methyls and the $^1H$ and $^{13}C$ chemical shifts indicated that one methyl was attached to oxygen and the other to nitrogen. The $^1H$ NMR spectrum and $^1H,^1H$ COSY experiment suggested that an indolic NH and two independent *ortho*-disubstituted benzenoid rings were present. The IR spectrum showed a sharp line at 2200 $cm^{-1}$ for a nitrile group.

To solve the structure of **1** the $^1H$ and $^{13}C$ NMR signals were first correlated by performing heteronuclear multiple-quantum correlation (HMQC) and heteronuclear multiple-bond correlation (HMBC) experiments. Next the [2,3-*a*] fusion of the indolocarbazole system was confirmed by detection of an NOE between the signals for the *N*-methyl protons and the indolic NH proton. The relative positions of the methoxyl and *N*-methyl groups were established by (1) detection of a NOE between the signals for the OMe protons and 4-H and (2) correlation of 4-H and the protons of the *N*-methyl group with C-12a

in the HMBC experiment. The placement of the cyano group on C-6 was rigorously established by one-dimensional $^{13}C$-$^{13}C$ decoupling experiments with uniformly $^{13}C$-enriched **1** which showed that C-6 was connected to the cyano carbon, C-5 and C-6a. The relatively high-field chemical shift for C-6 was consistent with the attachment of the cyano group at this position and the location of the methoxyl group on the adjacent carbon (C-5).

Smaller signals in the $^1H$ and $^{13}C$ NMR spectra (each one about one-fourth the intensity of the comparable signal for **1**) suggested that **1** occurs in a 4:1 mixture with its regioisomer. For example, in the $^1H$ NMR spectrum of **1** an unresolved shoulder appeared on the high-field side of the *N*-methyl signal and a broad NH signal could be observed at 12.10 ppm. No other $^1H$ signals could be seen for the minor regioisomer. Attempts to separate the two isomers by HPLC failed.




A second minor component of the extract was isolated and identified by NMR and HR mass spectral data (observed *m/z* 311.1036, 2.2 mmu error) to be 6-cyano-5-methoxylindolo[2,3-*a*]carbazole, the nor-*N*-methyl derivative of **1**. The NMR spectra of the nor compound were essentially identical with those of **1** except that the $^1H$ and $^{13}C$ signals for the *N*-methyl group were missing and two NH proton signals were present.

Unlike all other indolo[2,3-*a*]carbazoles, **1** does not possess the annelated [3,4-*c*]pyrrolo unit on ring C. Since biosynthetic studies[4] indicate that this pyrrolo unit originates from tryptophan, **1** is probably formed by biodegradation of a pyrrolo[3,4-*c*]indolo[2,3-*a*]carbazole.

Compound **1** is responsible for most of the antiviral activity and cytotoxicity associated with the extract of the alga. It is moderately active against herpes simplex type 2; *i.e.*, in infected mink lung cells the virus titer is reduced 95% at 1 $\mu$g/ml. The virus population, however, is not totally eliminated at any concentration below the MIC for cytotoxicity (100 $\mu$g/ml). It is weakly cytotoxic (MIC 5 $\mu$g/ml) against KB and LoVo human carcinoma cell lines, but is probably not selectively cytotoxic against murine and/or human solid tumor cell lines compared with leukemic and normal cell lines, since the crude extract of *Nostoc sphaericum* EX-5-1 shows no selective cytotoxicity in the CORBETT assay.[5] 6-Cyano-5-methoxyindolo[2,3-*a*]carbazole shows similar antiviral activity and cytotoxicity.

## Experimental

$^1H$ NMR spectra were obtained at 500 MHz and $^{13}C$ NMR spectra at 125 MHz in DMSO-$d_6$ on a 11.75 tesla instrument. Chemical shifts are reported in $\delta$ units (ppm) relative to the solvent as internal standard for both $^1H$ (2.49 ppm) and $^{13}C$ (39.5 ppm).

Culture Conditions

*N. sphaericum* Vaucher ex Bornet & Flahault, designated strain EX-5-1, was isolated from a mud sample collected on the campus of the University of Hawaii at Manoa. Clonal cultures were prepared by repeated subculture on solidified media. The alga was cultured in 20 liters glass bottles containing a modified inorganic medium, designated $A_3M_7$.[6] Prior to autoclaving, the pH of the medium was adjusted to 7.0 with sodium hydroxide. Cultures were illuminated continuously at an incident intensity of 300 $\mu$ einstein $m^{-2}$ $s^{-1}$ from banks of cool-white fluorescent tubes, aerated at a rate of 5 liters/minute with a mixture of 0.5% $CO_2$ in air, and incubated at a temperature of 24$\pm$1°C. After 15~22 days the alga was harvested by filtration and freeze-dried. Yields of lyophilized cells ranged from 0.5 to 0.8 g/liter.

Table 1. NMR data for 6-cyano-5-methoxy-12-methylindolo[2,3-*a*]carbazole (**1**) in DMSO-$d_6$.

| $\delta_C$ | Position | $\delta_H$ |
|---|---|---|
| 154.3 s | 5 | |
| 140.4 s | 10a | |
| 140.2 s | 12a | |
| 130.9 s | 11b | |
| 126.0 d | 9 | 7.50 t, $J$=7.4 Hz |
| 125.6 d | 2 | 7.54 m |
| 121.7 s | 11a | |
| 121.6 d | 4 | 8.23 d, $J$=7.4 Hz |
| 121.0 s | 6b | |
| 120.8 s | 4a | |
| 120.7 d | 3 | 7.35 t, $J$=7.4 Hz |
| 119.6 d | 8 | 7.29 t, $J$=7.4 Hz |
| 119.5 d | 7 | 8.44 d, $J$=7.4 Hz |
| 119.0 s | 6a | |
| 117.6 s | CN | |
| 112.3 s | 4b | |
| 112.3 d | 10 | 7.73 m |
| 109.9 d | 1 | 7.74 m |
| 85.9 s | 6 | |
| 62.0 q | OMe | 4.21 s |
| 31.8 q | NMe | 4.34 s |
| | NH | 11.86 |

Table 2. NMR data for 6-cyano-5-methoxyindolo[2,3-*a*]carbazole in DMSO-$d_6$.

| $\delta_C$ | Position | $\delta_H$ |
|---|---|---|
| 154.34 s | 5 | |
| 139.71 s | 10a | |
| 139.02 s | 12a | |
| 129.56 s | 11b | |
| 125.84 d | 9 | 7.50 t, $J$=7.8 Hz |
| 125.62 d | 2 | 7.50 t, $J$=7.8 Hz |
| 122.10 s | 11a | |
| 121.60 d | 4 | 8.21 d, $J$=7.8 Hz |
| 121.60 s | 6b | |
| 121.40 s | 4a | |
| 120.70 d | 3 | 7.34 m |
| 119.59 d | 8 | 7.30 m |
| 119.54 d | 7 | 8.41 d, $J$=8.4 Hz |
| 118.05 s | 6a | |
| 117.62 s | CN | |
| 112.70 s | 4b | |
| 112.41 d | 10 | 7.78 d, $J$=8.4 Hz |
| 112.09 d | 1 | 7.78 d, $J$=8.4 Hz |
| 85.80 s | 6 | |
| 62.09 q | OMe | 4.22 s |
| | NH (11) | 11.41 |
| | NH (12) | 11.73 |

Isolation

Freeze-dried alga (25 g) was extracted with 2 × 2 liter portions of 7 : 3, EtOH - water. The total extract (4.7 g) was subjected to step-gradient flash chromatography on a RP-18 column (200 ml YMC Gel ODS, 12 nm). The chromatogram was developed with 500 ml of each of the following solvents: $H_2O$, 3 : 7, MeOH - $H_2O$, 1 : 1, MeOH - $H_2O$, 3 : 1, MeOH - $H_2O$, 9 : 1, MeOH - $H_2O$, MeOH, $CH_3CN$, 1 : 1, EtOAc - hexane; eight 500-ml fractions were collected. Fraction 4 (80 mg) was separated further by preparative HPLC on a 22.5 × 250 mm reversed-phase column (Alltech Econosil ODS, 10 $\mu$m), using 17 : 3, MeOH - $H_2O$ as the eluant at a flow rate of 6 ml/minute, to give a crude mixture of **1** and 6-cyano-5-methoxyindolo[2,3-*a*]carbazole. Final purification was achieved by additional reversed-phase HPLC (first on the column above with 3 : 2, THF - $H_2O$ and then on a 10 × 300 mm column of Phenomenex Ultracarb ODS (5 $\mu$m) using MeOH at a flow rate of 2.5 ml/minute) to give 14 mg of **1** and 1 mg of 6-cyano-5-methoxyindolo[2,3-*a*]carbazole.

The material from combination of fractions 1, 2, 3 and 6 was passed through a column of silica (200 ml Davisil 60A, 100 ~ 200 mesh) with acetone and subjected to normal-phase chromatography (Davisil 60A, 100 ~ 200 mesh, 4 × 30 cm, THF - hexane (2 : 1), detection of the indolo[2,3-*a*]carbazole fraction by blue fluorescence when irradiated at 365 nm). Normal-phase HPLC (Alltech Econosil Si, 10 $\mu$m, 10 × 250 mm, 1 : 2, THF - hexane + 0.2% $H_2O$, 4 ml/minute, 254 nm) and reversed-phase HPLC (Alltech Econosil ODS, 10 $\mu$m, 22.5 × 250 mm, 60% THF, 6 ml/minute, 254 nm) gave 41 mg of **1** and 6 mg of 6-cyano-5-methoxyindolo[2,3-*a*]carbazole.

6-Cyano-5-methoxy-12-methylindolo[2,3-*a*]carbazole (**1**)

Amorphous white solid, mp > 280°C (dec). HREI-MS *m/z* 325.1211 (calcd for $C_{21}H_{15}N_3O$, 325.1214); UV (THF) $\lambda_{max}$ nm (log $\varepsilon$) 234 (4.407), 252 (4.452), 290 (4.824), 338 (4.176), 354 (4.000), 372 (4.046); IR (THF) $cm^{-1}$ 2200 (CN). $^1H$ and $^{13}C$ NMR data: See Table 1.

HPLC Rt's: 11 minutes (Phenomenex Ultracarb, ODS 5 $\mu$m, 10 × 300 mm, 2.5 ml/minute, MeOH); 14 minutes (Alltech Econosil Si, 10 $\mu$m, 10 × 250 mm, 1 : 2, THF - hexane + 0.2% $H_2O$, 4 ml/minute).

6-Cyano-5-methoxyindolo[2,3-*a*]carbazole

Amorphous white solid. $^1H$ NMR (DMSO-$d_6$): $\delta$ 11.73 (br s, H on N-12), 11.41 (br s, H on N-11),

8.41 (d, $J$=8.4 Hz, 7-H), 8.21 (d, $J$=7.8 Hz, 4-H), 7.78 (d, $J$=8.4 Hz, 1-H and 10-H), 7.50 (t, $J$=7.8 Hz, 2-H and 9-H), 7.34 (m, 3-H), 7.30 (m, 8-H), 4.22 (s, OMe). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 154.3 (s, C-5), 139.7 (s, C-10a), 139.0 (s, C-12a), 129.6 (s, C-11b), 125.8 (d, C-9), 125.6 (d, C-2), 122.1 (s, C-11a), 121.6 (d, C-4), 121.6 (s, C-6b), 121.4 (s, C-4a), 120.7 (d, C-3), 119.6 (d, C-8), 119.5 (d, C-7), 118.1 (s, C-6a), 117.6 (s, CN), 112.7 (s, C-4b), 112.4 (d, C-10), 112.1 (d, C-1), 85.8 (s, C-6), 62.1 (q, OMe).

HPLC Rt: 18 minutes (Alltech Econosil Si, 10 $\mu$m, 10×250 mm, THF-hexane, 1:2+0.2% $H_2O$, 4 ml/minute).

Uniform $^{13}$C Enrichment of **1**

*N. sphaericum* EX-5-1 was grown in a 10-liter glass bottle with 5.3 g of $NaH^{13}CO_3$ (99 atom %) as previously described,[7] except that 4.0 g of unlabeled $NaNO_3$ was used and the aeration rate was 0.1 liter/minute. After 25 days the 8 liters culture was harvested by filtration and the alga lyophilized to give 1.17 g of dried cells. The freeze dried alga was extracted with two 200 ml portions of 7:3, EtOH-$H_2O$ (each for 24 hours) to give 150 mg crude extract. Normal-phase column chromatography on silica with 2:1, THF-hexane followed by normal-phase HPLC on silica with 1:2, THF-hexane+0.2% $H_2O$ as described above and finally reversed-phase HPLC on ODS (Phenomenex Ultracarb, 5 $\mu$m, 10×300 mm, 2 ml/minute, 254 nm, 95% MeOH) resulted in the isolation of 1 mg of $^{13}$C labeled **1**. Inspection of its $^{13}$C NMR spectrum indicated uniform enrichment in $^{13}$C to >80%.

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## References

1) FURASAKI, A.; N. HASHIBA, T. MATSUMOTO, A. HIRANO, Y. IWAI & S. OMURA: X-Ray crystal structure of staurosporine: A new alkaloid from a *Streptomyces* strain. J.C.S. Chem. Comm. 1978: 800~801, 1978
2) STEGLICH, W.; B. STEFFAN, L. KOPANSKI & G. ECKHARDT: Indole pigments from the fruiting bodies of the slime mold *Arcyria denudata*. Angew. Chem. Int. Ed. Engl. 19: 459~460, 1980
3) NETTLETON, D. E.; T. W. DOYLE, B. KRISHNAN, G. K. MATSUMOTO & J. CLARDY: Isolation and structure of rebeccamycin—a new antitumor antibiotic from *Nocardia aerocoligenes*. Tetrahedron Lett. 26: 4011~4014, 1985
4) MEKSURIYEN, D. & G. CORDELL: Biosynthesis of staurosporine. 2. Incorporation of tryptophan. J. Nat. Prod. 51: 893~899, 1988
5) CORBETT, T. H.; L. POLIN, A. J. WOZNIAK, M. BISSERY, P. M. LORUSSO, F. A. VALERIOTE & L. H. BAKER: The use of a multiple-tumor-soft-agar-disk-diffusion-assay to detect agents with selective solid tumor activity. Proc. Am. Assoc. Cancer Res. 29: 533~534, 1988
6) MOORE, R. E.; C. CHEUK, X.-Q. G. YANG, G. M. L. PATTERSON, B. BONJOUKLIAN, T. A. SMITKA, J. S. MYNDERSE, R. S. FOSTER, N. D. JONES, J. K. SWARTZENDRUBER & J. B. DEETER: Hapalindoles, antibacterial and antimycotic alkaloids from the cyanophyte *Hapalosiphon fontinalis*. J. Org. Chem. 52: 1036~1043, 1987
7) MOORE, R. E.; V. BORNEMANN, W. P. NIEMCZURA, J. M. GREGSON, J.-L. CHEN, T. R. NORTON, G. M. L. PATTERSON & G. L. HELMS: Puwainaphycin C, a cardioactive cyclic peptide from the blue-green alga *Anabaena* BQ-16-1. Use of two-dimensional $^{13}$C-$^{13}$C and $^{13}$C-$^{15}$N correlation spectroscopy in sequencing the amino acid units. J. Am. Chem. Soc. 111: 6128~6132, 1989