Dihydrocarbazole Alkaloids from *Aspergillus tubingensis*

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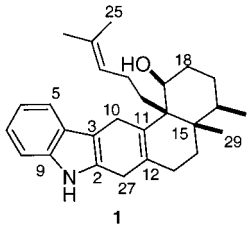
Investigation of the fungus *Aspergillus tubingensis* has led to the isolation and identification of two dihydrocarbazole-containing compounds (**1** and **2**). Details of the purification and structure elucidation of **1** and **2** are described. This is the first known report of dihydrocarbazole-containing compounds to be isolated from a living system.

Investigation of the fungus *Aspergillus tubingensis* (ATCC 76608, Trichocomaceae) has led to the isolation and identification of two new indole diterpenes, dihydrotubingensin A (**1**) and dihydrotubingensin B (**2**). Compounds **1** and **2** were isolated from the methyl ethyl ketone (MEK) extract of *A. tubingensis* along with two previously reported metabolites, tubingensins A (**3**)[1] and B (**4**).[2] As a class, these fungal metabolites hold considerable interest due to the rare dihydrocarbazole unit. In fact, this is the first known report that describes the isolation of a dihydrocarbazole from a living system.[3] The occurrence of **1**−**4** in the extract of *A. tubingensis* suggests that the dihydro compounds **1** and **2** may represent the incipient state of these metabolites, while tubingensins A and B are the nonnatural oxidation products. Details of the isolation and structure elucidation of **1** and **2** are reported herein.



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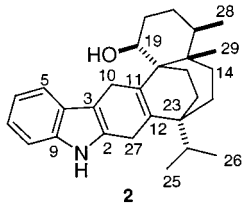

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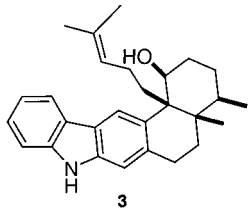

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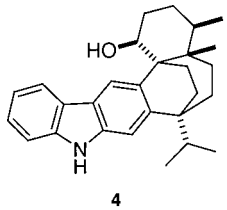

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Aspergillus tubingensis was obtained from the American Type Culture Collection in Manassas, VA. Fermentation was performed on a solid substrate medium as described in the Experimental Section. After 26 days of growth, the solid fungal material (~2.0 L) was extracted with MEK (2.0 L) and filtered, and the filtrate was evaporated under reduced pressure to afford 5.5 g of crude extract. Fractionation of the crude extract was carried out using flash Si gel column chromatography with hexanes−EtOAc. A portion of the fractionated material containing **1**−**4** (40 mg) was subjected to semipreparative reversed-phase HPLC to afford compounds **1**−**4** in the following order of elution: tubingensin A (**3**, 3.0 mg, t_R = 27.5 min); dihydrotubingensin A (**1**, 2.5 mg, t_R = 30.2 min); tubingensin B (**4**, 2.0 mg, t_R = 31.35 min); and dihydrotubingensin B (**2**, 1.5 mg, t_R = 32.8 min).

The molecular formulas of both **1** and **2** were established as $C_{28}H_{37}NO$ from HRESIMS, suggesting they differed from tubingensins A and B (both $C_{28}H_{35}NO$) by one degree of unsaturation. Initially, NMR data for **1** and **2** were acquired in $CDCl_3$; however, chloroform facilitated oxidation of the dihydrocarbazole unit. Therefore subsequent NMR data for all compounds were obtained using CD_3CN as the solvent.[4] The structure of the first new metabolite to be isolated, dihydrotubingensin B (**2**), was determined by 1D and 2D ^1H and ^{13}C NMR experiments (COSY, HMQC, HMBC) and by comparison of the NMR data for **2** to that of isolated tubingensin B (**4**). The ^1H NMR spectra of **2** and **4** indicated the compounds were related structurally. The similarities included the presence of a 2,3-disubstituted indole ring, an isolated isopropyl group, two isolated ethylene moieties, one methyl singlet, and one additional methyl doublet. Noticeably absent from the ^1H NMR spectrum of **2** were the two aromatic singlets corresponding to H-10 and H-27 for tubingensin B (numbering system adapted from ref 2). Other major ^1H NMR spectral differences between tubingensin B and **2** included the presence of two additional downfield-shifted methylene signals in **2** (δ 3.34−3.53). Furthermore, ^{13}C NMR data indicated signals for only eight aromatic carbons, as compared to the 12 seen in tubingensin B, implying that the compounds differed in the carbazole portion of the molecule. Modification of the carbazole unit was also indicated by the UV spectrum of **2**, which more closely resembled that of a substituted indole (UV maxima at 227 and 281 nm), rather than a carbazole (UV maxima for tubingensin B at 218, 237, 260, 299, 326, and 338).[2] The UV data, in conjunction with MS and NMR data, suggested that **2** was the dihydrocarbazole derivative of tubingensin B.

The presence of a dihydrocarbazole unit was established through long-range $^1H-^{13}C$ NMR correlations obtained by HMBC (Table 1). Specifically, the methylene protons for both C-10 and C-27 showed long-range correlations to C-12, C-11, C-3, and C-2, providing evidence for the C-2,C-3-disubstituted dihydrocarbazole substructure. Further structural confirmation was achieved via semisynthesis. Compound **2** was dissolved in $CHCl_3$ and placed under a gentle stream of air for 24 h. This mild air oxidation resulted in 55% conversion of dihydrotubingensin B to tubingensin B. A comparison of the ^1H NMR and optical rotation data of the semisynthetic material to that reported in the literature for tubingensin B[2] confirmed that the product was tubin-

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Table 1. NMR Data for **2** (CD_3CN)

position[a]	^{1}H (m, *J* in Hz)[b]	^{13}C[c]	HMBC[b]
1	8.82 (1H, brs)		
2		137.42	
3		107.24	
4		127.93	
5	7.43 (1H, d, 7.6)	118.43	7, 9
6	7.00 (1H, ddd,1.0, 7.1, 7.6)	119.53	4, 8
7	7.05 (1H, ddd, 1.1, 7.1, 7.9)	121.55	5, 9
8	7.31 (1H, d, 7.9)	111.47	4, 6
9		136.77	
10	3.53 (1H, dt, 20.9, 5.7)	25.46	2, 3, 11, 12
	3.34 (1H, dt, 20.9, 7.8)		
11		133.15	
12		130.70	
13	1.58 (1H, m)	35.92	12, 15, 23
	1.36 (1H, m)		
14	1.33 (1H, m)	38.79	
	0.82 (1H, dt, 6.2, 13.5)		
15		40.68	
16	1.54 (1H, m)	39.41	
17	1.56 (1H, m)	27.72	
	1.46 (1H, m)		
18	2.08 (1H, m)	31.53	
	1.82 (1H, m)		
19	4.17 (1H, m)	72.94	
OH-19	2.44 (1H, d, 4.6)		
20		49.27	
21	2.18 (1H, brt, 12.0)	27.43	
	1.31 (1H, m)		
22	1.74 (1H, brt, 12.9)	26.28	
	1.51 (1H, m)		
23		43.23	
24	1.97 (1H, sept, 6.5)	34.88	22, 23, 25, 26
25	0.92 (3H, d, 6.5)	21.25	23, 24, 26
26	0.92 (3H, d, 6.5)	17.0	23, 24, 25
27	3.66 (2H, m)	28.23	2, 3, 11, 12
28	0.75 (3H, d, 6.5)	16.92	16, 17
29	1.02 (3H, s)	15.38	14, 15, 20

[a] Numbering system adapted from ref 2. [b] Data recorded at 500 MHz. [c] Data recorded at 100 MHz.

gensin B: observed $[\alpha]^{20}_D$ −7.4° (*c* 0.80, $CHCl_3$); lit. $[\alpha]^{20}_D$ −6.7° (*c* 0.80, $CHCl_3$).

Once the structure of dihydrotubingensin B was confirmed, structure elucidation of dihydrotubingensin A (**1**) was straightforward. A comparison of the ^{1}H NMR of **1** to that of isolated tubingensin A (**3**) quickly revealed that compound **1** was the dihydro derivative of tubingensin A, analogous to the relationship between **2** and **4**. To summarize the key spectral differences, compound **1** lacked the H-10 and H-27 aromatic singlets that are characteristic of the carbazole unit of **3** and **4**; compound **1** had two additional methylene signals (δ 3.26−3.41) which are not observed in tubingensin A; and **1** had a UV spectrum that resembled an indole, rather than a carbazole. Though great care was taken to handle this dihydrocarbazole under nitrogen, the conversion of **1** into **3** could not be suppressed; therefore, the structure of dihydrotubingensin A was confirmed by subjecting this compound to mild air oxidation for 1 h, resulting in 40% conversion to tubingensin A. A comparison of the ^{1}H NMR and optical rotation data of the semisynthetic material to that reported in the literature for tubingensin A[1] confirmed that the product was tubingensin A: observed $[\alpha]^{20}_D$ +9.3° (*c* 1.0, $CHCl_3$); lit. $[\alpha]^{20}_D$ +13.6° (*c* 1.0, $CHCl_3$).[1]

The occurrence of dihydrotubingensins A and B in the extract of *A. tubingensis* suggests that the dihydrocarbazole compounds may be the incipient natural products, while tubingensins A and B result from spontaneous oxidation of the unstable dihydrocarbazole unit. HPLC analysis of the Si gel fractions containing **1**−**4** showed tubingensin A and dihydrotubingensin A to be in an approximate ratio of 1:1.4 (254 nm) and tubingensin B and dihydrotubingensin B to be in an approximate ratio of 3:1 (all four compounds are present in freshly extracted crude extract; however HPLC analysis of the crude extract is not adequate for quantitation, as **1**−**4** partially coelute with nonrelated material). Once it was clear that compounds **1** and **2** could spontaneously oxidize, great care was taken to handle and store the material under nitrogen. However, HPLC analysis of the same Si gel fractions, which had been stored at 4 °C for one year, revealed that the relative ratio of tubingensin B and dihydrotubingensin B changed from 3:1 to 25:1, respectively, while dihydrotubingensin A could no longer be detected. Due to the instability of these compounds, bioactivity data are not reported.

Experimental Section

General Experimental Procedures. NMR spectra were determined on Varian Unity 500 and 400 spectrometers operating at 500 and 400 MHz for ^{1}H and 125 and 100 MHz for ^{13}C, respectively. ^{1}H and ^{13}C chemical shifts are referenced to the solvent (CD_3CN) signal at δ 1.93 (^{1}H) and 1.39 ppm (^{13}C). Homonuclear ^{1}H−^{1}H connectivities were determined by 2D COSY. One-bond heteronuclear ^{1}H−^{13}C connectivities were determined by 2D proton-detected HMQC experiments and long-range ^{1}H−^{13}C connectivities by 2D proton-detected HMBC experiments. Standard Varian pulse sequences and parameters were used. Chemical shifts (δ) are in ppm; multiplicities are indicated by s (singlet), d (doublet), brd (broad doublet); t (triplet), brt (broad triplet), sept (septet), or m (multiplet). Coupling constants (*J*) are reported in Hz.

LCMS was performed on a Thermo Quest LCQ instrument using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). High-resolution mass spectral analysis was performed on a Thermo Quest FTMS using electrospray ionization. UV spectra were measured in MeOH at 20 °C on a Beckman DU-20. Optical rotations were measured in MeOH or $CHCl_3$ at 22 °C at the sodium D line on a Perkin-Elmer 241 instrument. Reversed-phase HPLC was performed using a HP1100 with diode array detection. Analytical HPLC conditions: Phenomenex, C_8, 5 μm, 4.5 × 250 mm, 7:3 ACN−H_2O at 1.0 mL/min. Semipreparative HPLC conditions: Phenomenex, C_8, 5μm, 10 × 250 mm, 7:3 ACN−H_2O at 3.5 mL/min.

Isolation and Fermentation. *A. tubingensis* (ATCC 76608) was obtained from the American Type Culture Collection in Manassas, VA. It was maintained as frozen vegetative mycelium (−75 °C) in stock vials (10−20% glycerol). The culture was inoculated into seed flasks by aseptically transferring an aliquot (0.4−1.0 mL) of the frozen vegetative mycelium into a 250 mL Erlenmeyer flask containing seed medium (50 mL) of the following composition (g/L): corn steep powder (2.5); tomato paste (40); oat flour (10); glucose (10); and trace elements solution (10 mL/L; pH to 6.8). The trace elements solution for the seed medium was prepared in 0.6 N HCl and had the following composition (g/L): $FeSO_4 \cdot 7H_2O$ (1.0); $MnSO_4 \cdot H_2O$ (1.0); $CuCl_2 \cdot 2H_2O$ (0.025); $CaCl_2$ (0.1); H_3BO_3 (0.056); $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.019); $ZnSO_4 \cdot 7H_2O$ (0.2). The culture was incubated at 25 °C, 220 rpm, for 2 days.

Fermentations were performed on a solid substrate medium. The culture was grown in roller bottles (4 × 4.0 L) containing large-particle vermiculite (1250 cm^3) (sterilized separately from the liquid), with a liquid nutrient solution poured over it (440 mL). The nutrient solution was formulated as follows (g/L): glucose (150); urea (4); NZ amine Type A (4); K_2HPO_4 (0.5); $MgSO_4 \cdot 7H_2O$ (0.25); KCl (0.25); $ZnSO_4 \cdot 7H_2O$ (0.9); $CaCO_3$ (16.5) (no pH adjustment). The solid and liquid portions of the production medium were combined at the time of inoculation. Each bottle was inoculated with vegetative seed (20 mL) and shaken to coat the vermiculite with the seed growth and nutrient solution. The bottles were then incubated on a Wheaton rolling machine (4 rpm), at 22 °C for 26 days.

After 26 days of growth, each roller bottle was extracted with MEK (0.5 L). The MEK extracts were combined, filtered, and evaporated to dryness under reduced pressure to afford 5.5 g of material. A portion of this material (5.0 g) was dissolved in CH_2Cl_2 (20 mL) and applied to an Si gel column (E. Merck Si gel 60, 5.0 × 20 cm) equilibrated with 9:1 hexane−EtOAc. The column was eluted with 9:1 hexane−EtOAc followed by 1.5:1 hexane−EtOAc. The solvents were evaporated under reduced pressure to afford two fractions, which contained **1**−**4**. The earlier eluting fraction (66 mg) contained tubingensin A and dihydrotubingensin A, and the later eluting fraction (98 mg) contained tubingensin B and dihydrotubingensin B. A portion of these samples (20 mg each) was purified separately using semipreparative reversed-phase HPLC (see General Experimental Section for HPLC conditions).

Dihydrotubingensin A (1): white solid (2.5 mg); ^{1}H NMR (CD_3CN, 500 MHz) δ 8.87 (1H, brs), 7.44 (1H, brd, J = 7.6 Hz), 7.33 (1H, d, J = 8.1 Hz), 7.08 (1H, ddd, J = 1.2, 7.1, 8.1 Hz), 7.02 (1H, ddd, J = 1.0, 7.1, 7.6 Hz), 5.08 (1H, m), 4.31 (1H, dd, J = 3.4, 6.4 Hz), 3.26−3.41 (4H, m), 2.56 (1H, d, J = 4.1 Hz), 2.20 (1H, m), 1.92−1.98 (4H, m), 1.87 (1H, m), 1.73 (1H, dt, J = 6.9, 13.3 Hz), 1.66 (1H, dd, J = 3.7, 12.6 Hz), 1.63−1.67 (2H, m), 1.59 (3H, s), 1.50 (1H, dd, J = 7.2, 14.3 Hz), 1.44 (3H, s), 1.15−1.18 (2H, m), 1.10 (3H, s), 0.87 (3H, d, J = 6.9 Hz); HRESIMS m/z 404.2967 (calcd for $C_{28}H_{38}NO$, 404.2953).

Dihydrotubingensin B (2): white solid; $[\alpha]^{20}_D$ −50° (c 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 206.6 (sh) (4.02), 227 (4.26), 281 (3.57), 289.6 (3.52) nm; ^{1}H and ^{13}C NMR, see Table 1; HRESIMS m/z 404.2955 (calcd for $C_{28}H_{38}NO$, 404.2953).

Oxidation of Dihydrotubingensin B. Dihydrotubingensin B (2.5 mg) was dissolved in $CHCl_3$ (1.0 mL). The material was placed under a stream of air for 24 h. Analytical HPLC indicated 55% conversion of dihydrotubingensin B to tubingensin B. The semisynthetic tubingensin B was purified using semipreparative reversed-phase HPLC (t_R = 31.35 min), and the ^{1}H NMR ($CDCl_3$) and optical rotation were compared to the literature.[2] Observed $[\alpha]_D$ −7.4° (c 0.80, $CHCl_3$); lit. $[\alpha]_D$ −6.7° (c 0.80, $CHCl_3$).

Oxidation of Dihydrotubingensin A. Dihydrotubingensin A (2.5 mg) was dissolved in $CHCl_3$ (1.0 mL). The material was placed under a stream of air for 1 h. Analytical HPLC indicated 40% conversion of dihydrotubingensin A to tubingensin A. The semisynthetic tubingensin A was purified using semipreparative reversed-phase HPLC (t_R = 27.5 min), and the ^{1}H NMR ($CDCl_3$) and optical rotation were compared to the literature. Observed $[\alpha]^{20}_D$ +9.3° (c 1.0, $CHCl_3$); lit. $[\alpha]^{20}_D$ +13.6° (c 1.0, $CHCl_3$).

References and Notes

(1) TePaske, M. R.; Gloer, J. B. *J. Org. Chem.* **1989**, *54*, 4743−4746.
(2) TePaske, M. R.; Gloer, J. B. *Tetrahedron Lett.* **1989**, *30*, 5965−5968.
(3) For a review of carbazole alkaloids see: Chakraborty, D. P.; Shyamali, R. *Fortschr. Chem. Org. Naturst.* **1991**, *57*, 71−152.
(4) The oxidation of dihydrotubingensin B to tubingensin B could not be completely prevented. Though great care was taken to handle and store the material under nitrogen, analytical HPLC and ^{1}H NMR analysis of freshly purified **2** still showed ∼10% conversion of **2** to **4**. This material was used for characterization.

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