Coproverdine, a Novel, Cytotoxic Marine Alkaloid from a New Zealand Ascidian

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Received November 21, 2001

The crude extract of a New Zealand ascidian displayed antitumor activity. Bioassay-directed fractionation yielded a novel alkaloid, coproverdine (**1**). The structure of **1** was assigned on the basis of detailed spectroscopic analysis. Coproverdine (**1**) was responsible for the antitumor activity of the crude extract.

In our continuing search for new anticancer lead compounds from marine organisms, an unidentified New Zealand ascidian was studied. This resulted in the isolation of the novel alkaloid coproverdine (**1**), possessing a carbazole ring. Coproverdine (**1**) was obtained from a rare, poorly identified ascidian collected at a depth of 20−25 m from a rock wall in an exposed but shaded area at Irishman's Garden, Three Kings, 55 km offshore from the northern tip of the North Island of New Zealand. (The taxonomy of this specimen remains unknown. The specimen was initially examined by Mr. M. Page, NIWA, New Zealand, and later by Dr. P. Kott (Mather), Queensland Museum, Australia. The name given to the alkaloid was derived from the descriptor attached to the voucher specimen ("green sheep-shit-like in appearance"), hence coproverdine (*copro* Greek dung, *ovis* Latin sheep, *verde* Latin green).) A voucher specimen is stored in the museum at NIWA, Wellington, New Zealand (NIWA code MNP670). Although the specimen was poorly preserved, it was considered to possibly be a polycitorid. The crude extract of this specimen displayed cytotoxic activity against the P388 (murine leukaemia) cell line and also against the slow growing BSC-1 (African green monkey kidney) cell line. The total, size-limited frozen sample (34 g) was extracted with MeOH/DCM. The extract was initially separated by RP vacuum liquid chromatography, followed by gel permeation chromatography (LH-20). Final purification was achieved using reversed-phase HPLC to yield the major metabolite coproverdine (**1**) (5 mg, 0.01%) as a yellow oil, $[\alpha]_D$ −8°. The molecular formula was established by HRFABMS and HREIMS as $C_{15}H_{11}NO_6$, thus requiring 11 DBE. The ^{13}C NMR spectrum of **1** contained 15 resolved signals (one methyl, six methines, and eight quaternary carbons as supported by a gHSQC NMR experiment). The IR spectrum of **1** had absorptions at 3690 cm^{-1} (sharp) and 3500 cm^{-1} (broad), suggestive of both hydrogen-bonded and free hydroxyl groups. In the ^1H NMR spectrum one of the exchangeable protons [δ 8.50 (bs)] was observed while the other was not, presumably due to rapid exchange.

The UV absorption at 382 nm was suggestive of a conjugated indole moiety,[1] while an IR absorption at 1665 cm^{-1} together with the NMR data (δ 10.13 (s); 192.7) indicated the presence of a formyl group. Also present were a methyl ester grouping (δ 4.06 (s); 53.4 and 168.2) and a ketone (δ 186.4).

Two key structural fragments were identified from the ^1H and COSY NMR data. First, a 1,2,3-trisubstituted aromatic ring [δ 7.84 (dd, J = 1.5, 8.0 Hz), 7.28 (dd, J = 8.0, 8.0 Hz), and 7.39 (dd, J = 1.5, 8.0 Hz)] and, second, a *cis* double bond [δ 6.25 (d, J = 10.2 Hz) and 7.01 (d, J = 10.2 Hz)]. Linkage of the fragments to give the core structure (**2**) arose from interpretation of COSY, gHSQC, and various gHMBC NMR experiments, including a CIGAR HMBC (Table 1).[2] An HMBC correlation from H7 to C11 confirmed that the methyl ester moiety was attached to the 1,2,3-trisubstituted aromatic ring system. Connectivity in the vicinity of the *cis*-olefin present was assembled from the HMBC correlations from H2 to C1, the ketone C4, C9a, and the formyl C10 as well as from H3 to C1 and C4a. The key HMBC correlations that allowed closure of the core structure (**2**) were from H7 to C8a, from H5 to C4a and C8a, and from H10 to C9a. Some of the observed HMBC correlations were weak and required data reacquisition using different coupling constant optimizations.

Table 1. NMR Data for Coproverdine (**1**)[a]

no.	^{13}C[b]	^1H δ [m, J (Hz)][c]	gCOSY	gHMBC[d]
1	90.5			
2	143.6	7.01 (d, J = 10.2)	H 3	C1, C4, C9a, C10[e]
3	128.5	6.25 (d, J = 10.2)	H 2	C1, C2, C4, C4a, C10[e]
4	186.6			
4a	105.7			
4b	127.7			
5	125.1	7.39 (dd, J = 1.5, 8.0)	H 6	C4a,[e] C4b, C6, C8a
6	126.4	7.28 (dd, J = 8.0, 8.0)	H 5, H 7	C4b, C5, C7, C8, C8a
7	125.9	7.84 (dd, J = 1.5, 8.0)	H 6	C4b, C5, C6, C8, C8a,[e] C11
8	118.3			
8a	144.9			
9				
9a	157.2			
10	192.7	10.13 (s)		C1, C4a, C9a
11	168.2			
12	53.4	4.06 (s)		C11
1-OH		8.50 (bs)[f]		
9-OH		8.50 (bs)[f]		

[a] Spectra were recorded in CD_3OD. [b] ^{13}C NMR at 75 MHz, referenced to CD_3OD (δ 49.3), and assignments are supported by a gHSQC NMR experiment. [c] ^1H NMR at 300 and 500 MHz, referenced to residual solvent CHD_2OD (δ 3.3). [d] gHMBC NMR experiments were run using J = 140 and 160 Hz and J_{nxh} = 2, 4, 8, 9, 10 Hz. [e] These HMBC correlations were weak. [f] This signal may be interchanged, and only one exchangeable proton was observed.

The core structure (**2**) accounted for all but one of the degrees of unsaturation and all atoms in the molecular formula except H_2NO_2. On the basis of the UV data and carbon chemical shift calculations the core structure (**2**) was configured as a carbazole system (see **1**) and the remaining atoms (H_2O_2) were assigned as two −OH groups attached at positions C1 and N9 to give coproverdine (**1**).[3] The

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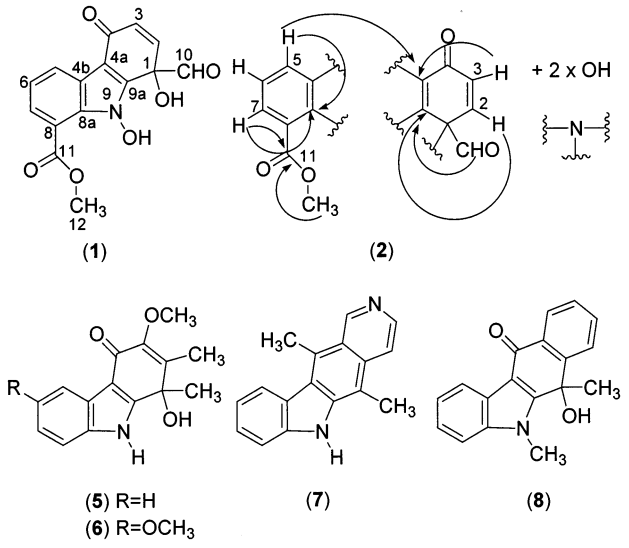
10.1021/np010594z CCC: $22.00
Published on Web 08/27/2002

deshielded quaternary carbon (δ 90.5) was consistent with the substitution at C1 of a hydroxyl group and a deshielding functionality such as a formyl.[4]

Although the carbazole skeleton of coproverdine (**1**) had been explicitly defined by the NMR and UV data, a computer-assisted constitutional assignment of coproverdine (**1**) was made using the structure calculation program COCON.[5] The initial 510 structures that were calculated were reduced to eight that fitted the experimental data. Of these eight structures, that proposed for coproverdine (**1**) best fitted the experimental data in terms of the HMBC correlations and the closest ^{1}H and ^{13}C NMR shifts. The absolute stereochemistry of coproverdine (**1**) has not been assigned.

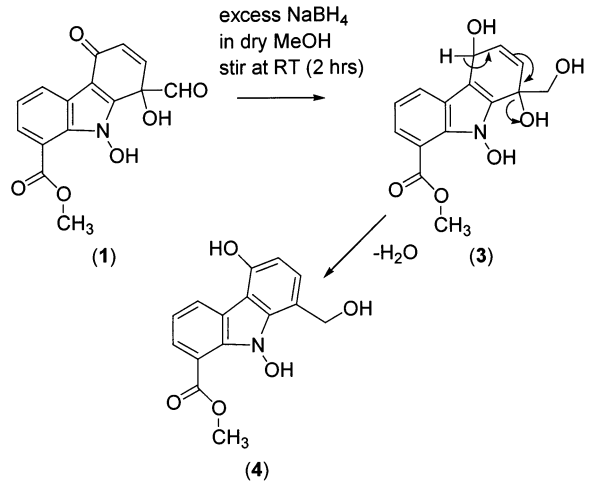
Due to the limited amount of coproverdine (**1**) isolated (5 mg), only small-scale (<0.5 mg) chemical degradations were attempted in an effort to gain support for the proposed structure. Attempted methylation and acetylation reactions resulted only in complex mixtures or decomposition products. Reaction of **1** with acid resulted in interesting color changes (from yellow to green); however, the nature of this interconversion could not be clarified by mass spectrometry. Some limited support for the proposed structure came from the reduction of **1** with excess $NaBH_4$ in dry MeOH. A single compound was formed. On the basis of ESIMS analysis (MH^+ 288) it is postulated that compound **4** was formed from the initial reduction product **3** by the loss of water (Scheme 1).

Biological evaluation of coproverdine (**1**) against a variety of murine and human tumor cell lines, which included P388, A549, HT29, MEL28, and DU145, established a cytotoxicity profile with IC_{50} values of 1.6, 0.3, 0.3, 0.3, and 0.3 μM, respectively. The closest related compounds are carbazomycins G (**5**) and H (**6**), which were isolated from the culture broth of *Streptoverticillium ehimense* in 1988.[6] Carbazomycin G (**5**) was found to have some antimicrobial properties, and the total synthesis of both (**5**) and (**6**) was reported in 1997.[6,7] Of interest is the plant alkaloid ellipticine (**7**), well-known for its cytostatic activity.[8,9] Carba analogues of **7** have been synthesized, resulting in an analogue (**8**), which has structural similarities with coproverdine (**1**).[10]



In the search for anticancer compounds the isolation of coproverdine (**1**) is a satisfactory outcome. A novel, bioactive structural type has been identified that is of low molecular weight, is a basic compound, has calculated log

Scheme 1. The Reduction of Coproverdine (**1**)



P values of −0.17, 0.12, or −2.14,[11,12] and meets Lipinski's rules of five[13] (< five H-bond donors, molecular weight < 500, the log *P* is < 5, sum of N's and O's < 10 and should have good absorption or permeation properties). An independent total synthesis of coproverdine (**1**) and analogues for SAR studies is underway.[14]

Experimental Section

General Experimental Procedures. All solvents were redistilled or of HPLC grade. BakerBond Octadecyl C18 (40 μm) reversed-phase packing was used for vacuum liquid chromatography and Sephadex LH-20 for gel permeation chromatography. NMR spectra were recorded variously on Varian UNITY, XL 300 MHz, or INOVA 500 MHz spectrometers. Electrospray mass spectra (ESIMS) were recorded in both the positive and negative modes on a Micromass LCT mass spectrometer, and electron impact mass spectrometry (EIMS) was carried out on a Kratos MS 80 RFA mass spectrometer. FABMS and HRFABMS were also recorded on the Kratos MS 80 RFA mass spectrometer using glycerol and *m*-nitrobenzyl alcohol (NOBA) as the matrix. LC/ESIMS was carried out on an Agilent technologies 1100 HPLC-MSD system using a Zorbax C8 column (2.1 mm × 15 cm) at 40 °C. The mass spectrometer was run using API-ES (+ve). The UV spectrum was obtained on a Hewlett-Packard 8452A diode array spectrophotometer, while the IR spectra were recorded on a Shimadzu FTIR-8201PC spectrophotometer. The optical rotation was recorded on a Perkin-Elmer 341 polarimeter (*c* g/100 mL) set to the Na wavelength (589 nm). Analytical HPLC was carried out on a Shimadzu VP system on a Phenomenex Prodigy 5 μ ODS column (100 Å; 250 × 4.6 mm) using a flow rate of 1 mL/min. Semipreparative reversed-phase HPLC was carried out on a Shimadzu LC-4A HPLC. The final purification conditions were carried out on a Phenomenex Prodigy 5 μ ODS column (100 Å; 250 × 10 mm) using a flow rate of 4 mL/min.

Collection, Extraction, and Isolation. A specimen of a rare, unidentified ascidian was collected from Irishman's Garden at the Three Kings, New Zealand. Collection was by scuba at a depth of 20−25 m from a rock wall in an exposed but shaded area of Irishman's Garden in March 1997. The specimen was described as epizoic on an *Anchorina* sp. sponge.

Collector's Description of the Organism. A colonial dark green/dark brown ascidian with round 1 cm high zooids with a common stolon was observed.

A voucher specimen is stored in the museum at NIWA, Wellington, New Zealand (NIWA code MNP670). The specimen had been poorly preserved, and even after examination by Dr. Patricia Kott (Mather) (Queensland Museum, Australia) an opinion could be given only that is was possibly a polycitorid. The crude extract of this specimen displayed cytotoxic activity against fast growing P388 (murine leukaemia) cells (IC_{50} =

0.95 μg/mL) and also showed cytotoxicity against slow growing BSC-1 (African green monkey kidney) cells (3+, 8). The frozen specimen (34 g) was exhaustively extracted with MeOH/DCM (3:1; 700 mL) and filtered through a pad of Celite, and the extract (1.4 g) was subjected to C18 vacuum liquid chromatography, followed by gel permeation chromatography on Sephadex LH-20 eluting with MeOH. The final purification was achieved using reversed-phase HPLC (CH_3CN/H_2O gradient (30% to 75%) over 30 min) with UV detection at 254 nm to yield the major metabolite coproverdine (**1**) [5 mg, 0.01% (based on mass of crude extract)].

Coproverdine (1) [8-formyl-8,9-dihydroxy-5-oxo-8,9-dihydro-5*H*-carbazole-1-carboxylic acid methyl ester]: yellow oil; $[\alpha]^{20}_D$ −8° (*c* 0.36, EtOH); IR ($CHCl_3$) ν_{max} 3690 (sharp), 3500 (br), 1665, 1603, 1556, 1290 cm^{-1}; UV (EtOH) λ_{max} (ϵ) 208 (20 000), 270 (6700), 302 (4800), 382 (16000) nm; ^1H NMR data (CD_3OD, 300 MHz), see Table 1; ^{13}C NMR data (CD_3OD, 75 MHz), see Table 1; ESIMS (−ve) (30 V) *m/z* 300 $[MH]^-$, 272 $[(MH) - CO]^-$; ESIMS (+ve) (20 V) *m/z* 942 $[M_3K]^+$, 926 $[M_3Na]^+$, 641 $[M_2K]^+$, 625 $[M_2Na]^+$, 472 $[M_3HK]^{2+}$, 340 $[MK]^+$, 324 $[MNa]^+$, 302 $[MH]^+$; EI (70 eV) *m/z* 301 (M^+, 35), 285 (75), 273 (24), 253 (48), 225 (100), 213 (28), 197 (26), 169 (28), 146 (20); FABMS (glycerol matrix, positive ion) *m/z* 302 $[MH]^+$, 277, 185; LC/MS *m/z* 625 $[M_2Na]^+$, 324 $[MNa]^+$, 302 $[MH]^+$; HREIMS *m/z* 301.05910 ($[M]^+$, calculated for $C_{15}H_{11}NO_6$, 301.05864); HRFABMS *m/z* 302.0673 ($[MH]^+$, calculated for $C_{15}H_{12}NO_6$, 302.0665).

Reduction of Coproverdine (1). Coproverdine (0.2 mg) together with $NaBH_4$ (3.5 mg) and dry MeOH (3 mL) were stirred at room temperature for 2 h. The reaction mixture was concentrated under reduced pressure, redissolved in MeOH (2 mL), and analyzed by RPHPLC and mass spectrometry. The HPLC analysis indicated the presence of two compounds, which were more polar than coproverdine (**1**) but with the same UV profile. These compounds were suspected to be **4** as well as **3**, but in the electrospray mass spectrum of the mixture the only product detected was that with a mass corresponding to **4** (low-resolution ESIMS (+ve) (30 V) *m/z* 288.21 $[MH]^+$, suggesting a possible molecular formula of $C_{15}H_{13}NO_5$, calculated 287.2675).

Acknowledgment. We thank PharmaMar SA for providing financial support in the form of a Postdoctoral Research Fellowship (S.U.), as well as for further biological evaluation of coproverdine; Ms. G. Ellis (University of Canterbury) for biological assays; Mr. B. M. Clark (University of Canterbury) for mass spectral analyses; Dr. L. K. Pannell (NIDDK/NIH) for LC/MS; Mr. A. Duckworth (NIWA, New Zealand) for the collection of the specimen; and Dr. P. Kott (Mather) (Queensland Museum, Australia) for examining the voucher specimen.

Supporting Information Available: ^1H NMR in CD_3OD as well as low-resolution ESI, EI, and FAB mass spectrometry data for coproverdine. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP010594Z