VOLUME 69, NUMBER 23

JOC Articles

NOVEMBER 12, 2004

Lihouidine, a Novel Spiro Polycyclic Aromatic Alkaloid from the Marine Sponge *Suberea* n. sp. (Aplysinellidae, Verongida)

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Received January 20, 2004

An investigation of a new species of sponge from the genus *Suberea* collected at Lihou Reef in the Coral Sea afforded lihouidine, an unprecedented cytotoxic spiro nonacyclic polyaromatic alkaloid. The structure of the alkaloid, which was racemic, was determined by a combination of 1D and 2D NMR techniques and single-crystal X-ray structural analysis.

Alkaloids isolated from marine sponges of the family Aplysinellidae are generally based on bromotyrosine. Sponges of the genus *Suberea* (Aplysinellidae, Verongida) are typical in that they have been reported to produce metabolites that incorporate functionalized bromotyrosine dipeptide moieties, with one phenolic group etherified by 3-hydroxypropylamine (or a related amino acid derivative).[1−4] To our knowledge, no examples of the occurrence of fused polyaromatic alkaloids have previously been reported from the family Aplysinellidae.

We report here the isolation and characterization of lihouidine, a cytotoxic alkaloid pigment from a new species of *Suberea* sponge, obtained from Lihou reef in the Coral Sea. Lihouidine contains two modified aaptamine structural units joined by a six-membered ring that contains a spiro carbon atom. In addition, one of these has a fused quinoline system attached. Quinolones have been reported from a bacterium cultured from *Suberea creba* from New Caledonia.[4] Aaptamine (**1**)[5−8] is a known benzo[*de*](1,6)naphthyridine alkaloid that was proposed as a potential taxonomic marker for sponges of the order Hadromerida.[9]

Results and Discussion

A crimson *Suberea* sponge was collected at Lihou Reef from −13 m in 1996. Extraction of the dried sponge material with dichloromethane and methanol afforded a blood-red extract that yielded the crystalline optically inactive alkaloid lihouidine (**2**) after vacuum liquid chromatography on silica gel.

The formula of lihouidine was determined to be $C_{36}H_{27}O_6N_5$ from high-resolution mass spectrometric measurement of the protonated molecular ion produced by electrospray ionization. The proton NMR spectrum contained two sets of mutually coupled doublets characteristic of two 2,3,4-trisubstituted pyridine groups (6.73 and 8.70 ppm, J = 5.6 Hz; 6.98 and 8.93 ppm, J = 5.3 Hz). In addition, signals for an ortho-disubstituted benzene ring (7.82 ppm, J = 8.4, 1.1 Hz; 7.61 ppm, J = 8.4, 6.8, 1.1 Hz; 7.38 ppm, J = 8.6, 6.8, 1.1 Hz; 8.85 ppm, J = 8.6, 1.1 Hz), and an isolated aromatic proton (6.55 ppm) were observed. Signals for six methyl groups on heteroatoms (3.42, 3.56, 3.71, 3.89, 4.05, 4.05 ppm) were also observed. HMQC correlations were used to assign protons to their attached carbon atoms (see Table 1). This process identified the six methyl groups as two *N*-methyl groups (^{13}C NMR: 29.7, 30.7 ppm) and four *O*-methyl groups (^{13}C NMR: 57.8, 60.2, 61.5, 61.9 ppm). Connectivities, deduced from HMBC correlations, readily established the presence of a functionalized aaptamine residue (Figure 1).

The likely presence of a second modified aaptamine unit was deduced from the NMR spectral data that contained evidence for another 4-methylamino-2,3-

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(1) Gao, H.; Kelly, M.; Hamaan, M. T. *Tetrahedron* **1999**, *55*, 9717.
(2) Hirano, K.; Tsuda, M.; Watanabe, K.; Fromont, J.; Kobayashi, J. *Tetrahedron* **2000**, *56*, 8107.
(3) Tsuda, M.; Sakuma, Y.; Kobayashi, J. *J. Nat. Prod.* **2001**, *64*, 980.
(4) Debitus, C.; Guella, G.; Mancini, I.; Waikedre, J.; Guemas, J.; Nicolas, J. I.; Pietra, F. *J. Mar. Biotechnol*. **1998**, *6*, 136.
(5) Nakamura, H.; Kobayashi, J.; Ohizumi, Y.; Hirata, Y. *Tetrahedron lett*. **1982**, *23*, 5555.
(6) Nakamura, H.; Kobayashi, J.; Ohizumi, Y.; Hirata, Y. *J. Chem. Soc., Perkin Trans. 1* **1987**, 173.
(7) Fedoreev, S. A.; Prokofeva, N. G.; Denisenko, V. A.; Rebachuk, N. M. *Khim. Farm. Zh*. **1988**, *22*, 943.
(8) Shen, Y. C.; Lin, T. T.; Sheu, J. H.; Duh, C. Y. *J. Nat. Prod*. **1999**, *62*, 1264.
(9) Bergquist, P. R.; Cambie, R. C.; Kernan, M. R. *Biochem. Syst. Ecol*. **1991**, *19*, 289.

10.1021/jo0498819 CCC: $27.50 © 2004 American Chemical Society
Published on Web 05/11/2004

TABLE 1. NMR Assignments and Observed HMBC Correlations for Lihouidine (2) in $CDCl_3$

carbon	δ^1H, mult, *J*(Hz)	δ ^{13}C[a]	HMBC ^{1}H to ^{13}C (δ^{13}C) correlations
2	8.70, d, 5.6	152.1	101.3, 144.7, 146.3
3	6.73, d, 5.6	101.3	114.4
4		146.3	
5-CH_3	3.89, 3H, s	30.7	146.3, 148.0
6		148.0	
8		148.4	
9	7.82, dd, 8.4, 1.1	128.0	122.2, 127.6
10	7.61, ddd, 8.4, 6.8,1.1	130.4	126.3, 148.4
11	7.38, ddd, 8.6, 6.8, 1.1	127.6	122.2, 128.0
12	8.85, dd, 8.6, 1.1	126.3	123.5, 130.4, 148.4
13		122.2	
14		123.5	
15		118.0†	
16		131.5†	
17		114.4	
18		144.7	
19		148.3	
19-OCH_3	4.049, 3H, s	61.9*	148.3
20		150.1	
20-OCH_3	3.42, 3H, s	60.2	150.1
21		121.6†	
22		63.2	
23		195.0	
24		167.7	
25-CH_3	3.71, 3H, s	29.7	144.9, 167.7
26		144.9	
27	6.98, d, 5.3	102.6	111.0, 144.9, 152.1
28	8.93, d, 5.3	152.1	102.6, 144.2, 144.9
30		144.2	
31		144.1	
31-OCH_3	4.053, 3H, s	61.5*	144.1
32		152.1	
32-OCH_3	3.56, 3H, s	57.8	152.1
33	6.55, s	112.4	63.2, 111.0, 126.8, 144.1, 152.1
34		126.8	
35		111.0	

[a] Assignments denoted by the same symbol (*,†) may be interchanged within each group.

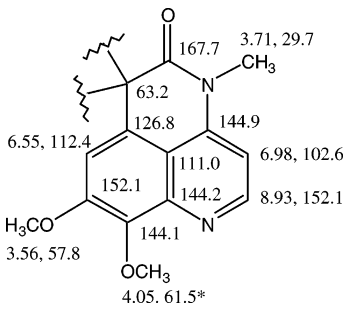


FIGURE 1. ^{1}H and ^{13}C NMR assignments for a functionalized aaptamine structural unit in lihouidine. *Resolution was insufficient to distinguish between 61.5 and 61.9 ppm in HMQC and HMBC spectra.

substituted pyridine residue and two additional methoxyl groups. However, it was only possible to confirm the presence of the 4-methylamino-2,3-substituted pyridine residue and to assign the NMR signals for the methoxyl groups with their point of attachment on the aromatic ring from HMBC correlations, as the methoxyl substituents were located on a fully substituted ring (Figure 2).

In addition to the two modified aaptamine residues, a conjugated ketone carbonyl group (^{13}C NMR: 195.0 ppm) and a 2,3,4-trisubstituted quinoline residue (Figure 3) appeared to constitute the remainder of the structure.

The two modified aaptamine residues accounted for $C_{28}H_{23}N_4O_5$, the ketone added CO, and the quinoline

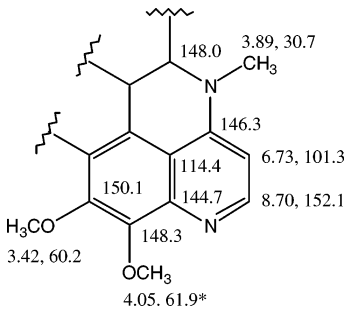


FIGURE 2. Proposed NMR assignments for a second functionalized aaptamine unit in lihouidine. The location and NMR assignments for methoxyl groups are by analogy with those in Figure 1. *Resolution was insufficient to distinguish between 61.5 and 61.9 ppm in HMQC and HMBC spectra.

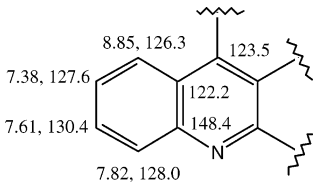
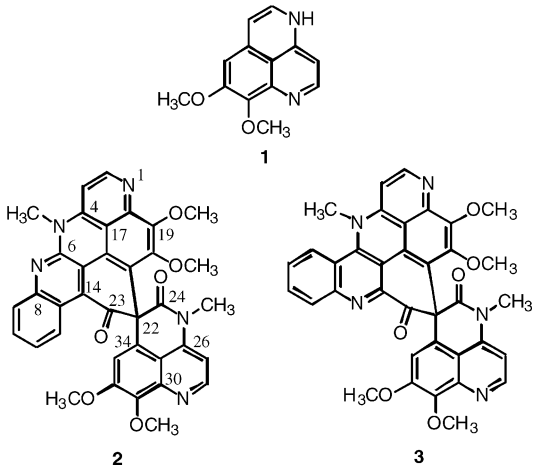


FIGURE 3. NMR assignments for the functionalized quinoline structural unit in lihouidine.

added C_9H_4N. This totals 38 carbon atoms and accounts for all H, O, and N atoms. Lihouidine, however, only contains 36 carbon atoms, so the second aaptamine unit and the quinoline must be directly fused (2C atoms in each substructure must be common to both substructures). The formula of lihouidine, together with the number of identified sp^2 carbon atoms, implies the presence of nine rings, only eight of which to date have been discussed. The ninth ring must involve the quaternary sp^3 carbon atom in the first discussed aaptamine unit (Figure 1), the ketone carbonyl group, and the two substituted aromatic positions not involved in fusing the quinoline and aaptamine moieties. The aaptamine can be fused at either the 3 and 4 or 2 and 3 positions of the quinoline (with two possible orientations of the aaptamine for each fusion position). These four arrangements, however, were reduced to two, since only one of the arrangements at each of the fusion locations had the remaining two aromatic positions close enough to complete the ninth ring. The carbonyl could either be attached adjacent to the methoxyl on the aaptamine aromatic ring or directly to the quinoline. Placement on the aaptamine aromatic ring, however, would afford a vinylogous amide carbonyl group (utilizing the aaptamine N−CH_3 group) for either fused quinoline system, rather than the observed ketone functionality (195.0 ppm), so only those structures with the carbonyl group attached to the quinoline—**2** and **3**—were considered as probable structures. (See the Supporting Information for the two discarded structures that contain vinylogous amide functionality.)

NOESY experiments were not useful to distinguish between the structures. Because of the spiro ring fusion, distances between the two planar aromatic systems precluded significant internuclear Overhauser effects between substituents on the two ring systems. The molecule must contain a spiro ring junction, so the absence of optical activity was puzzling.



1 **2** **3**

As discrimination between structures **2** and **3** was not readily achieved solely by use of spectroscopic techniques, a crystal was grown to allow structural determination from single-crystal X-ray data. Crystals that had been grown from acetone, acetonitrile, methanol, or bromobenzene proved unsuitable for data collection either because crystals were too small or twinned or because of solvent evaporation during data collection. Ultimately, a suitable crystal grown from DMF was used for X-ray data collection. The overall X-ray structure determination of lihouidine relied on information from the NMR study to place the nitrogen atoms. The X-ray crystallographic study showed that lihouidine has structure **2** (Figure 4).[10] It also confirmed the racemic nature of lihouidine, since the two molecules present in the unit cell were related by an inversion center (i.e., two enantiomeric molecules were present in the cell). Analytical chromatography on a chiral HPLC column also indicated that both enantiomers were present in equal amounts (data not presented).

Several other interesting observations were evident from the X-ray structure:

(a) The crystal contained one molecule of DMF for each lihouidine molecule. The solvent was located in an otherwise vacant region of the unit cell; it did not adequately fill the space, and large thermal parameters were observed for the solvent molecules. The cells were stacked such that these solvent regions transversed from one side of the crystal to the other, so the crystals are effectively micro sieves. Solvent loss is probably the explanation for difficulties encountered in obtaining useful data sets with crystals obtained from more volatile solvents.

(b) The ketone carbonyl group was distorted from the plane of the rest of the aromatic system by approximately 20−30° (torsion angles C21−C22−C23−O23, −152.3°; C15−C14−C23−O23, 154.7°; C13−C14−C23−O23, −20.2°).

(c) As a result of the nonplanarity of the carbonyl group, the angle between the two pseudoplanar polyaromatic systems in lihouidine was significantly distorted from 90°.

The biogenesis of lihouidine presumably involves enolate attack by a β-dicarbonyl intermediate in a Michael (1,4) fashion on an *o*-quinonoid ring to produce a spiro ring junction that would be expected to be racemic. Proton shifts would result in an ortho substituted diphenol that could then be methylated to afford the functionalized aaptamine moiety that contains the spiro linkage (see the Supporting Information for this proposed partial biosynthetic route).

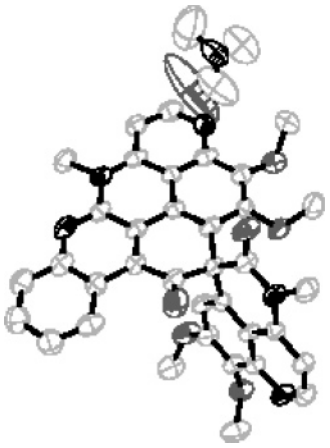


FIGURE 4. ORTEP diagram of lihouidine and DMF in the asymmetric unit.

Cytotoxicity assays were carried out against a cultured suspension of $P388D_1$ mouse lymphoma cells. Despite limited solubility in the cell culture medium, lihouidine (**2**) exhibited moderate cytotoxicity with an IC_{50} of approximately 3 μg/mL (5 μM).

Experimental Section

Isolation Procedures. The crimson *Suberea* n. sp. sponge was collected by scuba (−13 m) at Lihou Reef (17.6°S, 152.4°E) in 1996. A photograph of the sponge has been included in the Supporting Information, and a voucher specimen (G25099) has been deposited in the Museum of Tropical North Queensland, Townsville.

The freeze-dried sponge (4.16 g) was extracted with dichloromethane/methanol (1:1, 5 × 10 mL). Solvent removal from the combined extracts afforded a red gum (176 mg, 4.2%) that gave a crimson spot (R_f 0.45) on a TLC plate developed in acetone. Vacuum liquid chromatography on silica gel[11] afforded crude lihouidine (**2**) after elution with EtOAc/CH_2Cl_2 (1:4). The alkaloid was further purified by vacuum liquid chromatography on silica gel with acetone to afford a powder (45 mg, 1.08%) that crystallized from acetonitrile as purple-red optically inactive prisms: mp >330 °C; UV (EtOH) λ_{max} 210 nm (ϵ 54 400), 246 nm (ϵ 74 200), 287(s) nm (ϵ 24 000), 314 nm (ϵ 19 000), 340 nm (ϵ 14 700), 406 nm (ϵ 5100), 430 nm (ϵ 5600), 444 nm (ϵ 5600), 514 nm (ϵ 10 500), 543(s) nm (ϵ 10 200); IR ν_{max} ($CHCl_3$) 1676 (s), 1669, 1592 cm^{-1}; high-resolution mass measurement (electrospray ionization) 626.2034, $C_{36}H_{28}O_6N_5$ (MH^+) requires 626.2036.

Acknowledgment. We thank Taya Pitt for assistance with extraction, Dame Patricia Bergquist for sponge taxonomy, Mr. Stephen Wright for cytotoxicity assays, and Dr. Lyndon Llewellyn from AIMS for access to a recollection of the organism.

Supporting Information Available: Photograph of the sponge *Suberea* n.sp.; general experimental conditions; the four structures initially considered; the proposed biogenetic route to lihouidine; ^1H, ^{13}C, HMQC, and HMBC spectra; X-ray crystallographic data and tables including ORTEP and packing diagrams. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) Crystal/refinement data: $C_{36}H_{27}O_6N_5 \cdot C_3H_7NO$, M 698.74, triclinic space group $P\bar{1}$, a 9.6376(17) Å, b 13.194(2) Å, c 14.228(3) Å, α 67.068(3)°, β 82.044(3)°, γ 79.640(3)°, V 1634.4(5) Å^3, $D_c(Z = 2)$ 1.420 g cm^{-3}, $F(000)$ 732, μ_{Mo} 0.71073 Å, specimen 0.80 × 0.80 × 0.15 mm, $2\theta_{max}$ 46.5°, N_{tot} 4567, N 4567, R_1 0.0816, wR_2 (all data) = 0.3007.

(11) Coll, J. C.; Bowden, B. F. *J. Nat. Prod.* **1986**, *49*, 934.