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APLYSILLIN A, A THROMBIN RECEPTOR ANTAGONIST FROM THE MARINE SPONGE *APLYSINA FISTULARIS FULVA*[1]

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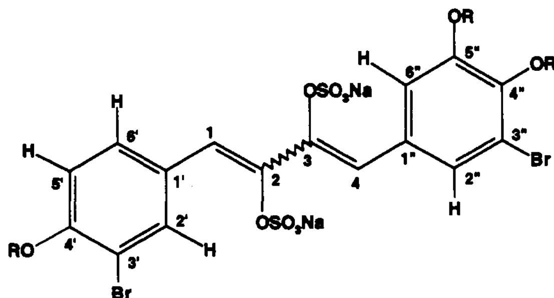
ABSTRACT.—Aplysillin A [**1**], an unusual disulfate ester of a 1,4-diphenyl-1,3-butadiene, was isolated from a deep water specimen of a sponge of the genus *Aplysina*. Its structure was determined through spectroscopic methods. The compound weakly inhibited binding to the thrombin receptor with an IC_{50} value of 20 μM.

As part of our continuing research program to find new enzyme inhibitors and receptor antagonists with therapeutic potential, we have focused on thrombin receptor antagonists. Thrombin is an important component in the clotting cascade, which binds to the thrombin receptor and causes blood clotting. Therefore, development of a low-molecular-weight thrombin receptor antagonist would be of therapeutic interest in preventing blood clotting. During our initial assays, an *Aplysina* sp. (family Aplysinidae) collected in the Bahamas showed moderate thrombin receptor antagonist activity. Sponges of the genus *Aplysina* are typically known for their production of metabolites derived from bromotyrosine such as fistularin-1 (1), cavernicolin-1 (2), and aplysamine-1 (3). Here we report the bioassay-guided isolation and structure elucidation of aplysillin A [**1**], a weak inhibitor of thrombin receptor binding.

The sponge, which has been identified as *Aplysina fistularis fulva* (Pallas), was collected using the Johnson-Sea-Link manned submersible at a depth of 369 feet off Sweetings Cay, Grand Bahama Island. The frozen sponge was successively extracted with EtOH and MeOH and the combined extracts were concentrated to dryness. The dried extract was triturated with absolute MeOH. Repeated chromatography of the MeOH solubles on a Sephadex LH-20 stationary phase using MeOH as the eluent led to the purification of aplysillin A [**1**] (0.07% of wet wt). Compound **1** is very unstable and rapidly decomposes after the final purification step. All attempts at devel-



1 R=H
2 R=CH_3

[1]Dedicated to Prof. Paul J. Scheuer on the occasion of his 80th birthday.

TABLE 1. Nmr Data of Aplysillin A [**1**] in CD_3OD and in DMSO-d_6.[a]

Carbon	CD_3OD			DMSO-d_6		
	δ_C	δ_H	HMBC correlations	δ_C	δ_H	HMBC correlations
1	118.3	6.71 (s)	H-2′,H-6′	115.5	6.66 (s)	H-2′
2	144.3	—	H-4	144.7	—	H-4
3	144.3	—	H-1	144.5	—	H-1
4	118.6	6.65 (s)	H-2″	116.1	6.60 (s)	H-6″
1′.	129.7	—	H-5′	128.3	—	H-5′
2′.	135.7	7.94 (d,2.1)	H-1,H-6′	133.6	7.90 (d,1.9)	H-1,H-6′
3′.	110.4	—	H-2′,H-5′	108.8	—	H-2′,H-5′
4′.	154.6	—	H-2′,H-6′ H-5′[b]	152.4	—	H-2′,H-6′
5′.	116.7	6.83 (d,8.4)	—	115.5	6.83 (d,8.7)	—
6′.	131.5	7.72 (dd,8.4,2.1)	H-1,H-2′	130.0	7.55 (dd,8.7,1.9)	H-1,H-2′
1″.	129.1	—	—	128.9	—	-
2″.	127.0	7.32 (d,1.7)	H-4,H-6″	124.3	7.55 (d,1.7)	H-4,H-6″
3″.	110.0	—	H-2″	109.4	—	H-2″
4″.	143.9	—	H-2″,H-6″	141.7	—	H-2″,H-6″
5″.	146.5	—	H-6″	145.1	—	—
6″.	116.5	7.48 (d,1.7)	H-4,H-2″	116.4	7.04 (d,1.7)	H-4,H-2″
OH-4′	—	—	—	—	10.12 (br s)	—
OH-4″	—	—	—	—	9.00 (br s)	—
OH-5″	—	—	—	—	9.52 (br s)	—

[a]Chemical shifts are given in δ (ppm) and J values are reported in Hz.
[b]Observed when $^{2,3}J_{CH}$ was optimized for 5 Hz.

oping a suitable hplc system for the final purification of **1** failed due to its high polarity and unstable nature. The majority of the spectroscopic characterization was carried out on the parent compound, but final confirmation was achieved by studying the nmr spectra and negative-ion hrfabms of a trimethyl derivative of aplysillin A [**2**] prepared by treatment of **1** with CH_2N_2.

The aromatic substitution patterns were determined by analysis of the ^{1}H-^{1}H scalar couplings and confirmed through interpretation of the HMQC and HMBC data observed for **1** (Table 1). A 1,3,4-trisubstituted aromatic ring was suggested by the ABX system observed at δ 7.72 (dd,J=8.4 and 2.1 Hz, H-6′), 6.83 (d,J=8.4 Hz, H-5′), and 7.94 (d,J=2.1 Hz, H-2′). The relative placement of the substituents was assigned based upon the following HMBC correlations: H-1: C-2′, and C-6′; H-2′: C-1, C-3′, C-4′, and C-6′; H-6′: C-1, C-2′, and C-4′; and H-5′: C-1′, and C-3′. The presence of a 1,2,3,5-tetrasubstituted aromatic ring was suggested by the observation of two meta-coupled protons in the ^{1}H-nmr spectrum observed at δ 7.32 (d,J=1.7 Hz, H-2″) and 7.48 (d, J=1.7 Hz, H-6″). The relative placement of the substituents was assigned based upon the following HMBC correlations: H-4, C-2″ and C-6″; H-2″: C-4, C-4″, and C-6; and H-6″, C-2″, C-4″, and C-5″. Typical ^{13}C-nmr signals of bromine-bearing aromatic carbons ortho to an oxygenated aromatic carbon appear at about δ 110 (6). Therefore, the bromine substituents were placed at C-3′ and C-3″. The carbons and hydrogens which remained to be placed in **1** were four sp^2 hybridized carbons and two olefinic protons [δ 118.3 (d, C-1), 144.3 (s, 2C, C-2, C-3); 118.6 (d, C-4); 6.71 (s, H-1); and 6.65 (s, H-4)]. These could be assigned to a 1,3-butadiene unit with oxygen substitution at the 2 and 3 positions. HMBC correlations between H-1 and C-2′ and C-6′ and between H-4 and C-2″ and C-6″ firmly established the lo-

cations of the aromatic rings on the butadiene unit. HMBC correlations observed between H-1 and C-3 and between H-4 and C-2 were consistent with the proposed dioxygenated butadiene structure.

Methylation of **1** with CH_2N_2 led to a trimethoxy derivative, **2** [^{1}H: δ 3.92 (3H, s), 3.87 (3H, s), and 3.79 (3H, s)] which suggested the presence of three acidic hydroxyl groups in **1**. The negative-ion hrfabms of **2** was observed as a triplet at *m/z* 666.8370 which suggested a molecular formula of $C_{19}H_{16}O_{11}S_2{}^{79}Br^{81}BrNa$ for the $[M-Na]^+$ ion (Δ 0.8 mmu from calcd). Comparison of the ms data with the nmr data suggested the presence of two sulfate units in **1**. The similarity of the chemical shifts of C-1/C-4 and C-2/C-3 indicated some degree of symmetry in the molecule. It was therefore predicted that the two sulfate units should be either on C-2 and C-3 or on the aromatic rings. The chemical shifts of C-2 and C-3 are closer to those observed for the enol sulfate carbon found in suvanine [^{13}C: δ 133.3] (4). In addition, the observed ^{13}C-nmr chemical shifts are similar to those observed for acetylated enols (5). Past experiments have shown that the effect of sulfate substitution on carbon chemical shifts is similar to that observed for acetate substitution (6). The chemical shifts of an enolized diketone, which can undergo keto-enol tautomerization, would be expected to occur at significantly lower fields [^{13}C: δ 190–174] than those observed for **1** (7–9). Based upon the observed chemical shifts, the sulfates were assigned to C-2 and C-3, finalizing the carbon skeleton of **1**. Additional evidence for the structure was obtained by 1D nOe experiments where the irradiation of the phenolic ^{1}H-nmr signals at δ 10.12 (OH-4′) and δ 9.52 (OH-5″) enhanced the signals at δ 6.83 (H-5′) and δ 7.04 (H-6″), respectively, indicating the presence of phenolic OH groups in **1**. The geometry of the C-2 and C-3 double bonds was not determined due to the inavailability of functional groups on which nmr techniques could be applied. Chemical methods were not used due to the instability and the general reactivity of the compounds.

Aplysillin A [**1**] has the same carbon skeleton as xanthocillin (10) and emerin (11), which were isolated from *Penicillium notatum* and *Aspergillus nidulans*, respectively. The compounds of the xanthocillin class have isonitrile or nitrile substituents at the C-2 and C-3 positions. Recent literature has revealed that the alga *Colpomenia sinuosa* also contains a brominated 1,4-diphenyl-but-2-ene, colpol (12), which belongs to this same class of compound. Aplysillin A [**1**] weakly inhibited the binding of [^{125}I]-thrombin to platelet membranes with an IC_{50} value of 20 μM.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The 1D and 2D nmr measurements were recorded on Bruker AM-360 and AMX-500 instruments, respectively. The ^{1}H-nmr chemical shifts were referenced to the residual solvent signals at δ 3.30 for CD_3OD and δ 2.49 for DMSO-d_6. The ^{13}C-nmr chemical shifts were referenced to the residual solvent signals at δ 49.0 for CD_3OD and δ 39.5 for DMSO-d_6. Proton-detected heteronuclear multiple-quantum coherence (HMQC) spectra recorded with a BIRD pulse (13) were optimized to observe single-bond correlations with $^1J_{CH}$ of 143 Hz (delay=3.5 msec). Proton-detected multiple bond correlation (HMBC) spectra were optimized for $^{2,3}J_{CH}$ of 10 Hz (delay=50 msec) (14) unless otherwise specified. The Ft-ir spectra were recorded on a Midac M 1200 instrument. Mass spectra were recorded on a Kratos Concept IH instrument in the fab (negative) mode.

ANIMAL MATERIAL.—The sponge, *Aplysina fistularis fulva*, was collected off Sweetings Cay, Grand Bahama Island, Bahamas, in June 1984, at a depth of 369 feet using the Johnson-Sea-Link manned submersible. The sponge was blackish-green and multilobate, with a finely conulose surface. Microscopically, the sponge has a reticulate-polygonal fiber skeleton, and the fibers are heavily pithed. The sponge is most closely related to *Aplysina fistularis fulva* (Van Soest, 1978) (15). A taxonomic voucher specimen has been deposited in the Harbor Branch Oceanographic Museum (catalog number: 003:00882).

EXTRACTION AND ISOLATION.—The sponge

(52 g) was extracted with EtOH (300 ml), blended and filtered. The residue was reblended with MeOH (300 ml) and filtered. The combined filtrates were concentrated to dryness (5 g) and triturated with absolute MeOH and filtered. One half of the filtrate was chromatographed on a Sephadex LH-20 column (4.5×31 cm) with MeOH. One half of the active fraction (eluted in 750–925 ml) was rechromatographed on another Sephadex LH-20 column (1×75 cm) with MeOH to yield aplysillin A [**1**] (eluted in 120–140 ml, 9.7 mg, 0.07% wet wt): ir (film on KBr) ν max 3454 (br d), 1631, 1257 (br d), 1059, 1018; ^{1}H- and ^{13}C-nmr data, see Table 1.

METHYLATION OF **1**.—A sample of **1** (6.4 mg) was dissolved in MeOH (2 ml) in a vial and reacted with an etheral solution of excess CH_2N_2. The reaction mixture was kept at room temperature for 3 h and quenched with HOAc (5 drops). The solvent was removed under reduced pressure and the residue was chromatographed on a Sephadex LH-20 column (1×75 cm) to yield the pure trimethyl ether of aplysillin A [**2**] (eluted in 105–115 ml, 2.2 mg): fabms (negative-ion) *m/z* 667 $(M-Na^+)^-$, 587 $(M-SO_3Na)^-$, 565 $(M-SO_3-2Na+H)^-$; ^{1}H nmr (CD_3OD) δ 8.03 (1H, d, J=1.7 Hz, H-6″), 7.88 (1H, d, J=2.1 Hz, H-2′), 7.86 (1H, dd, J=8.8 and 2.1 Hz, H-6′), 7.35 (1H, d, J=1.8 Hz, H-2″), 6.96 (1H, d, J=8.8 Hz, H-5″), 6.90 (1H, s, H-1), 6.83 (1H, s, H-4), 3.92 (3H, s, OMe), 3.87 (3H, s, OMe), 3.79 (3H, s, OMe).

THROMBIN BIOASSAY.—Binding of [^{125}I]thrombin to human platelet membranes was measured according to the following procedure. Platelets (4 units platelet concentration) were obtained from New Jersey Blood Services (New Brunswick, NJ). Platelets were combined into a plastic beaker with a magnetic stirrer and adjusted to pH 6.5 with 0.1 M citric acid. A solution (1/10 volume) of 100 μM indomethacin and 50 mM EDTA was added to the platelets and incubated for 15 min at room temperature. The solution was then centrifuged at 2000×g for 20 min using 250-ml conical plastic tubes. The pellet was resuspended in 25-ml ice-cold platelet preparation buffer (5 mM Tris, 10 mM EGTA, 1 mM PMSF, 5 μg/ml leupeptin at pH 7.4). The pellet was homogenized in a Dounce tissue homogenizer using 20–30 strokes. The solution was centrifuged in 5-ml fractions using a Beckman 20.1 rotor for 1 h at 20,000 rpm. The pellets were quickly frozen and stored at −70°.

On the day of the assay, the platelet membrane pellet was resuspended in 10 ml assay buffer (10 mM imidazole, 150 mM NaCl, 6% PEG 8000, 0.1% BSA at pH 7.4). The membranes were diluted 1:5 in assay buffer on the day of the assay. Tubes in duplicate containing 200 μl platelet membranes, 25 μl [^{125}I]thrombin (NEN-Dupont, Boston, MA; final concentration 25 pM), and 25 μl unlabeled human thrombin (1 μM), drug or buffer were incubated for 1 h at 25°. Bound radioactivity was isolated by filtration through BetaPlate A filtermats presoaked for 1 h in assay buffer using a TomTec Harvester 96 (TomTec, Orange, CT). The filters were washed three times with wash buffer (5 mM Tris, 0.9% NaCl, pH 7.4) to remove unbound radioactivity. The amount of bound radioactivity was measured using an LKB 1205 Betaplate scintillation counter.

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