Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 4, No. 20, pp. 2447-2450, 1994

0960-894X/94 $7.00+0.00

0960-894X(94)00356-4

Penazetidine A, an Alkaloid Inhibitor of Protein Kinase C

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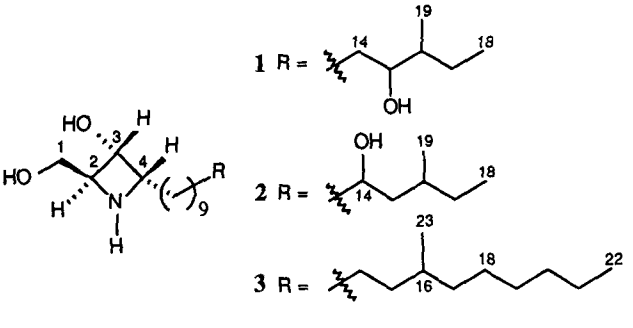
Abstract: The Indo-Pacific marine sponge *Penares sollasi* yielded a new protein kinase C inhibitor, penazetidine A (3). The structure of this compound was determined by spectroscopic data, including 1D and 2D NMR measurements as well as tandem mass spectrometry.

INTRODUCTION

Inhibitors of protein kinase C (PKC) could be important as leads for new cancer chemotherapeutics[1] as the PKC family of enzymes mediate signal transduction across membranes and are implicated in the regulation of cell proliferation and gene expression.[2] Currently, mostly three very potent natural product PKC inhibitors are mentioned, including the terrestrial microbial alkaloids staurosporine[3] (IC_{50} = $6x10^{-10}$ M) and K252a[4] (IC_{50} = $2.5x10^{-8}$ M) along with the marine ascidian alkaloid 11-hydroxystaurosporine[5] (IC_{50} = $2.2x10^{-9}$ M). We have recently demonstrated that new active alkaloids[6] can be discovered by employing a rapid primary assay using recombinant PKCβ1 to evaluate sponge crude extracts of biosynthetically rich Indo-Pacific marine sponges.

This report concerns our isolation and characterization of the PKC active constituent of a potato-shaped Indo-Pacific sponge, *Penares sollasi*.[7] It was targeted for further study because the crude extract exhibited an IC_{50} = 0.3 μg/mL against PKCβ1 while showing inactivity against protein tyrosine kinase (PTK).[8] Semi-purified fractions indicated that potential interfering compounds such as alkyl sulfates were not present. In addition, a search of our sponge natural products database revealed a very short list of diverse bioactive metabolites known from the genus *Penares* including: penasterol,[9] an unusual triterpene possessing antileukemic activity, penaresin,[10] an indole which acts as a sarcoplasmic reticulum inducer, and penaresidins A (**1**) and B (**2**),[11] azetidine derivatives exhibiting ATPase activation. The structure of a new azetidine (**3**), along with its PKC inhibition and cytotoxic properties, are described below.



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RESULTS AND DISCUSSION

An assay directed evaluation of the methanol extract[12] of the sponge *Penares sollasi* obtained from the Milne Bay Province of Papua New Guinea, was carried out as follows. Solvent partitioning of the MeOH extract oil was followed by Sephadex LH-20 chromatography (1:1 MeOH:CH_2Cl_2) of the CH_2Cl_2 fraction. Initial attempts at purifying the Sephadex fractions were by HPLC (ODS 2% aq MeOH) but this led to a mixture of C_{23} isomers. The resolution was increased without changing the retention time by using a ternary MeOH/THF/H_2O mixture. The last Sephadex fraction was purified by HPLC (ODS, first MeOH:THF:H_2O 8:1:1 followed by MeOH:THF:H_2O 7:2:1), which afforded pure penazetidine A (**3**).[13]

Early in the structure elucidation work, a monocyclic alkaloid functionality was indicated by the HRFABMS $[M+1]^+$ *m/z* 370.3685 molecular formula of $C_{23}H_{47}NO_2$ (Δ 0.5 mmu of calcd). This was further supported by the ^{13}C NMR spectrum which contained no resonances lower than δ 70. Three hetero atom protons (APTMF = $C_{23}H_{44}$ determined by ^{13}C-^1H COSY) were apparent (two IR bands at 3650 and 3590 cm^{-1}) as NH and OH moieties. The ^1H-^1H COSY NMR spectrum revealed successive correlations between protons at δ 3.86/3.82 (H1/1'), 4.10 (H2), 4.54 (H3), 4.27 (H4) and 1.92/1.86 (H5/5'), with the five low-field H's each attached to hetero atom bearing carbons identified from ^1H-^{13}C COSY NMR cross peaks to resonances at δ 59.5 (C1), 70.0 (C2), 66.3 (C3) and 65.4 (C4). All four of these ^{13}C NMR shifts were similar to those of **1** and **2** (e.g., 62.3-C1, 66.6-C2, 67.4-C3, 64.8-C4) indicating that **3** was a trisubstituted azetidine and not an oxetane. The two ring substituents were seen to be a $HOCH_2$ and a methyl substituted *n*-C_{18} chain. Kobayashi[11] established the relative stereochemistry for the azetidine ring protons in **1** - **2** as 2/3 *trans* and 3/4 *cis* from the *J* values observed between H2/H3 (4.2 - 5.2 Hz) and H3/H4 (8.7-9.0 Hz),[14] and an analogous arrangement was apparent for **3** also based on *J* data between H2/H3 (5.5 Hz) and H3/H4 (7.0 Hz).

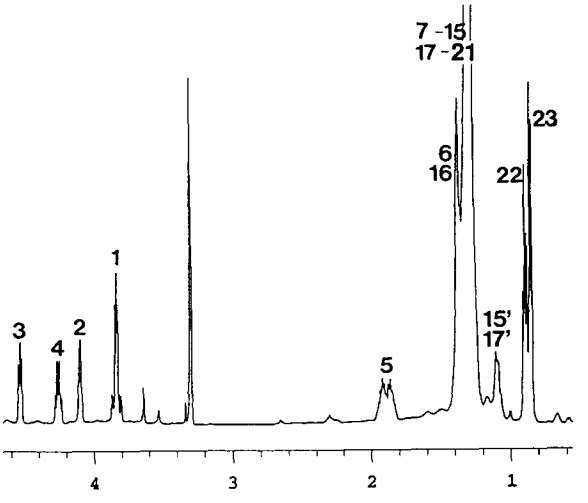

Figure 1. ^1H NMR of **3** in CD_3OD at 500 MHz.

What remained was to position the $CHCH_3$ substructure among the seventeen possible attachment sites on the *n*-C18 chain. Using ^{13}C substituent increment values to evaluate the shifts of C6 (δ 26.2) and C20 (δ 33.1) relative to model compounds indicated that the methyl group could be attached anywhere between C9 and C17 of the long aliphatic chain. Figure 1 shows that the assignment of this regiochemistry was not possible by NMR because a large number of aliphatic resonances overlapped in the ^1H and ^{13}C NMR spectra. This problem was finally solved by tandem mass spectrometry. The intense fragment peak at 296 ($C_{20}H_{42}N$) was selected as the seed ion for further fragmentation and the array of fragmentation ions observed in the second mass analysis region is shown in Figure 2. A gap of 28 Daltons was observed between 210 and 182 indicating the $CHCH_3$ to be at C16 (Figure 2). Similar fragmentation results were obtained by Jensen and Gross in their MS/MS study of *iso* and *anti iso* fatty acids.[15]

Penazetidine A (**3**) is one of the most active PKC inhibitors we have isolated to date as it exhibited an IC_{50} = 1μM.[16] The positive control, staurosporine, showed an average percent inhibition of 100% at 0.1 μM. In addition, **3** showed no inhibitory activity against PTK which suggested that it might bind to the lipid-binding regulatory site of PKC rather than at the ATP binding site. This was confirmed by observing dose response curve shifts to the right as increasing concentrations of phosphatidylserine were included in the assay. In

addition, penazetidine A showed *in vitro* cytotoxicity against human and murine cell lines with IC_{50}'s (μg/mL) of 0.099 (A549), 0.13 (HT-29), 0.079 (B16/F10) and 0.22 (P388).

A simple amino acid analog of **3**, *L*-azetidine-2-carboxylic acid has been previously isolated from two Australian sponges, *Haliclona* sp. and *Chalinopsilla* sp.[17] The biogenesis of **3** could involve a union of serine and a C_{20} polyketide, indicating that it belongs to the ketide-amino acid biosynthetic class.[18]

Acknowledgment: Assistance in the collection of sponges was provided by Ms. Maria Cristina Diaz (UCSC), Ms. Lisa Hunter (UCSC), Dr. Larry Manes (Syntex), and Mr. Rob Vanderloos (Skipper of the M/V Tania, Milne Bay, PNG). Ms. Diaz generously provided expert sponge taxonomic analysis. Thanks go to Drs. Furong Sun and Steve Mullen who obtained MS/MS data in the MS lab at University of Illinois on a 70-SE-4F mass spectrometer purchased in part with funds from the NIGMS (GM27029). Financial support was from NIH grant CA52955. Finally, we thank Mr. Don Niles of the Institute of Papua New Guinea Studies for assistance on behalf of the PNG government.

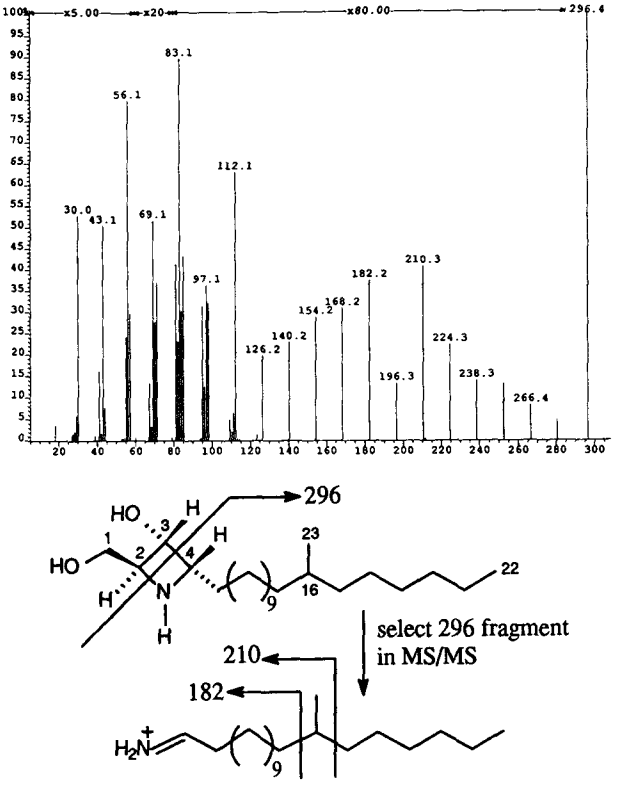

Figure 2. FABMS/MS data at *m/z* 296.

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7. Collections were from outside of Milne Bay, Papua New Guinea and the identification as *Penares*

sollasi (Thiele, 1903), [family Ancorinidae (Schmidt, 1862), order Choristida or Astrophorida] was made by Ms. Maria Cristina Diaz (UCSC) and R. W. M. van Soest (University of Amsterdam). *Penares sollasi* is a semi-globular sponge (4-15 cm in diameter) typically inhabiting shallow (3-10 m) protected caves where they are often found covered in silt. The color alive is brown to dark tan externally and lighter tan internally. The surface is smooth with one to several compound oscules (≈ 1 mm) aggregated on the top of the sponge. The sponge is compressible alive but the internal body collapses and partially disintegrates both in alcohol (50%) or after drying. The ectosome, present as a detachable cortex, is made up of closely packed tangential oxeas (type I: 100-130 x 5-8 μM and type II: 180-330 x 10-20 μM) and less abundant microspined oxyasters (16-20 μM in diameter, 4-6 μM rays). The internal skeletal arrangement could not be clearly observed due to the collapse of the choanosome after drying. Nevertheless, the choanosomal spiculation consisted mainly of large oxea (type III: 900-1000 x 15-20 μM) and orthotriaenes (shaft 400 x 20 μM, rhabd. 200-250 x 20 μM), with oxyasters loosely packed among them.

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13. *Penazetidine A* (**3**): Hydroscopic solid 30.7 mg, [α] = -16.9°(c = 0.04, MeOH). IR ($CHCl_3$): 3650, 3590, 2998, 2852, 1267 cm-1. $^{13}C/^{1}H$ NMR data 125/500 MHz (CD_3OD) δ 59.5 t, 3.86/3.82 ABX, J_{AB} = 12.5, J_{AX} = J_{BX} = 4.0 Hz ($CH_2$1); 70.0 d, 4.10 m (CH2); 66.3 d, 4.54 dd, J = 7.0, 5.5 Hz (CH3); 65.4 d, 4.27 q, J = 7.0 Hz (CH4); 27.6 t, 1.92 m/1.86 m ($CH_2$5); 26.2 t, 1.37 m ($CH_2$6); 30.8 t, 1.28 m ($CH_2$7-12); 31.1 t, 1.28 m ($CH_2$13); 28.2 t, 1.28 m ($CH_2$14); 38.2 t, 1.28 m/1.10 m ($CH_2$15); 33.9 d, 1.37 m (CH16); 38.2 t, 1.28 m/1.10 m ($CH_2$17); 28.2 t, 1.28 m ($CH_2$18); 30.5 t, 1.28 m ($CH_2$19); 33.1 t, 1.28 m ($CH_2$20); 23.8 t, 1.28 m ($CH_2$21); 14.5 q, 0.88 t, J = 7.0 Hz (Me22); 20.2 q, 0.85 d, J = 7.0 Hz (Me23).
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(*Received in USA* 22 *July* 1994; *accepted* 14 *September* 1994)