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Spermatinamine, the first natural product inhibitor of isoprenylcysteine carboxyl methyltransferase, a new cancer target

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Abstract—Isoprenylcysteine methyltransferase (Icmt) catalyzes the carboxyl methylation of oncogenic proteins in the final step of a series of post-translational modifications. The inhibition of Icmt provides an attractive and novel anticancer target. A natural product high-throughput screening campaign was conducted to discover inhibitors of Icmt. The Australian marine sponge, *Pseudoceratina* sp., yielded spermatinamine, a novel alkaloid with a bromotyrosyl-spermine-bromotyrosyl sequence, as the bioactive constituent. Its structure was determined by 1D and 2D NMR spectroscopy. Spermatinamine is the first natural product inhibitor of Icmt.

CAAX proteins have a diverse range of functions inside cells and many of these proteins are involved in intracellular regulatory processes important in oncogenesis.[1,2] CAAX proteins undergo a series of post-translational modifications important for their localization and function (cell membrane association, protein–protein interactions, protein stability, and protein biological activities). The modifications are initiated by the addition of an isoprenoid lipid (farnesyl or geranylgeranyl) to the cysteine of the carboxyl terminal of the CAAX motif by protein farnesyltransferase (FTase) or protein geranylgeranyltransferase-1 (GGTase-1), respectively. Following the attachment of the isoprenoid, the AAX tripeptide is removed in a reaction that is catalyzed by a prenyl-protein specific protease known as RCE1, and now the C-terminal prenylcysteine is methylated by isoprenylcysteine carboxyl methyltransferase (Icmt). Icmt and RCE1 are polytopic membrane proteins localized to the endoplasmic reticulum.[3] The best studied example of CAAX proteins is the RAS family of regulatory proteins. In its control of cellular behavior RAS is a very important molecular switch for a variety of signal pathways that control such processes as cytoskeletal integrity, proliferation, cell adhesion, apoptosis, and cell migration. Mutational activation of RAS is associated with various human cancers. In addition, many cancers contain alterations upstream of RAS in signaling cascades and the resultant hyperactivation of RAS is thought to contribute to tumorigenesis.[4] The protein prenyltransferases, most notably FTase, have been targets of major drug discovery programs for the last decade, however, FTase inhibitors, although showing significant activity in a number of clinical trials, have given overall response rates in patients less than expected.[5] Recent studies using genetic disruption of RCE1 and Icmt, particularly that of Icmt-catalyzed methylation, indicate that inhibition of these post-prenylation processing steps might provide a better approach to cancer-cell proliferation. The studies provided strong evidence that blocking Icmt activity significantly mislocalizes RAS proteins and tumorigenesis is markedly impaired in cells that lack Icmt. With emerging evidence for the importance of Icmt-catalyzed CAAX protein methylation in oncogenesis, there is a clear need for specific pharmacological agents to target this process. Analogues of the substrate prenylcysteine or the product *S*-adenosylhomocysteine have been reported as inhibitors.

Keywords: Isoprenylcysteine methyltransferase (Icmt); Cancer target; Natural products; *Pseudoceratina* sp.; Spermine alkaloid; Structure elucidation.
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The only reported small-molecule selective inhibitor of Icmt, which is other than an analogue of the substrate prenylcysteine or the product *S*-adenosylhomocysteine, is the indole-based, cysmethynil.[2]

Marine sponges from the order Verongida are characterized by bromotyrosine-derived chemotaxonomic markers, and many of these compounds possess potent antimicrobial and cytotoxic activities.[6] Chemical modification occurs both in the side chain and the aromatic ring of the brominated tyrosine precursors, giving rise to a broad range of biosynthetically related compounds. In a natural product high-throughput screening (HTS) campaign to find inhibitors of Icmt a CH_2Cl_2–MeOH (4/1) extract from a Verongida sponge, *Pseudoceratina* sp., was found to be active. Bioassay guided purification of this extract afforded the novel polyamine alkaloid, spermatinamine (**1**) (Fig. 1), containing a bromotyrosyl-spermine-bromotyrosyl sequence, which has not been discovered before. Compound **1** is also a rare example of a spermine alkaloid from a marine source. The compounds with structures closest to **1** are philanthotoxin-433 (butyryl-tyrosyl-spermine) (**2**) (Fig. 1) and its synthetic analogues.[7–9] Philanthotoxin-433 (**2**) is a wasp toxin that antagonizes ionotropic glutamate receptors and nicotinic acetylcholine receptors non-selectively. Interestingly, in a pathway different from Icmt, polyamines have been associated with cell growth and cancer, and now polyamine analogues are being developed as anticancer drugs to target polyamine metabolic enzymes and inhibit polyamine biosynthesis.[10,11] This paper reports the isolation, structure elucidation, and Icmt inhibitory activity of spermatinamine (**1**).

HTS of a natural product extract library (64, 271 extracts) was conducted to identify small-molecule inhibitors of Icmt. The assay measures the transfer of a methyl group from ^{3}H-*S*-adenosyl-L-methionine (^{3}H-SAM) to biotin-*S* -farnesyl-L-cysteine (BFC) by Icmt catalysis.[12] BFC is a synthetic substrate, which provides cysteine sites for methylation. Polyvinyl toluene (PVT) SPA beads capture the product, BFC ^{3}H-methyl ester, which is monitored by a scintillation counter. A CH_2Cl_2–MeOH (4/1) extract of the marine sponge *Pseudoceratina* sp.[13] was shown to inhibit Icmt.

The marine sponge *Pseudoceratina* sp. (300 mg) was ground and extracted sequentially with *n*-hexane, CH_2Cl_2–MeOH (4/1), and finally by MeOH. Once dried, the extract was resuspended in 1200 μL DMSO The biologically active CH_2Cl_2–MeOH (4/1) extract was further purified by injecting 150 μL onto a semi-preparative C_{18} HPLC column (Betasil C_{18} 5 μm 10×250 mm id). A gradient from H_2O/1% TFA to MeOH/1% TFA in 17 min, then isocratic for 5 min (flow 4 mL/min) was used and 22 fractions were collected. Fraction 17 was bioactive and contained spermatinamine (**1**) (2.3 mg). In order to isolate more of **1** a larger quantity of the sponge (11.22 g) was similarly ground and extracted. The CH_2Cl_2–MeOH (4/1) extract (1.09 g) was further purified, by preadsorbing the extract onto C_{18} (04K-4348 Sepra C_{18} End-Capped Silica), and loaded into a refillable preparative guard column (30×10 mm id), in line with a semi-preparative C_{18} HPLC column (Betasil C_{18} 5 μm 21.2×150 mm id). A gradient from H_2O/1% TFA to MeOH/1% TFA in 95 min, then isocratic for 25 min (flow 5 mL/min) was used and 60 fractions were collected. MS analysis indicated fractions 39–47 contained **1** and these fractions were combined (226 mg). A portion of this (149 mg) was further purified by C_{18} HPLC using a sharp gradient from H_2O/1% TFA to H_2O/1% TFA–MeOH/1% TFA (2/3) in 2 min, then a slower gradient to MeOH/1% TFA in 43 min, and finally isocratic for 15 min (flow 10 mL/min). MS analysis of these fractions showed that fractions 22 (16 mg), 23 (19 mg) and 24 (10 mg) contained **1**. These were separately purified by C_{18} HPLC using the following conditions. A sharp gradient from H_2O/1% TFA to H_2O/1% TFA–MeOH/1% TFA (3/7) in 3 min, followed by isocratic for 42 min, and finally a gradient to MeOH/1% TFA in 15 min (flow 10 mL/min). Spermatinamine (**1**)

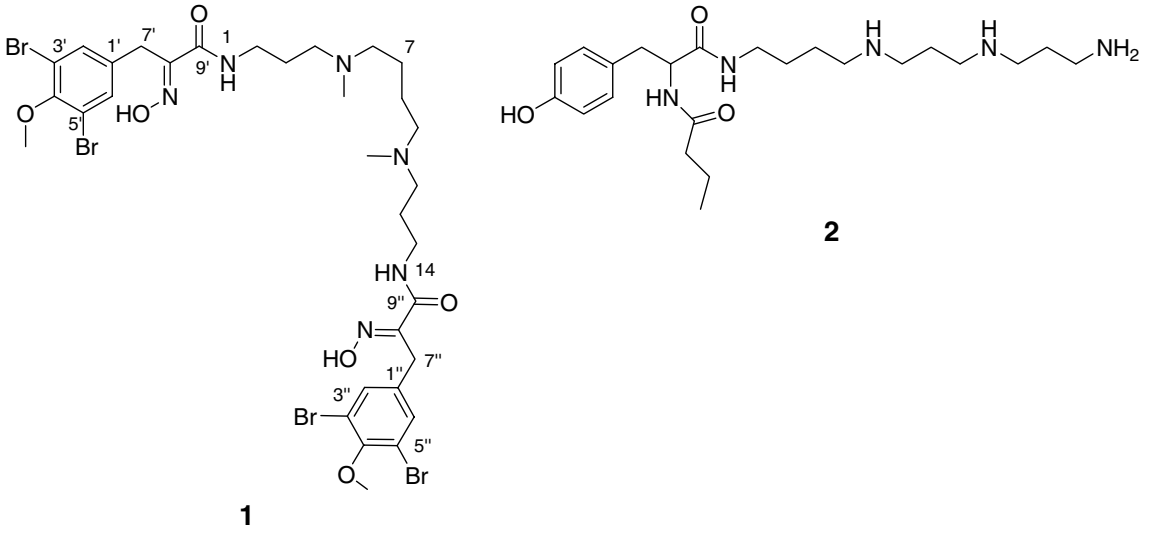

Figure 1. Spermatinamine (**1**) and philanthotoxin-433 (**2**).

Table 1. ^{1}H (600 MHz), ^{13}C (125 MHz), gCOSY, and gHMBC NMR data for spermatinamine (**1**) in DMSO-d_6[a]

Position	δ_C	δ_H (mult, J Hz)	COSY (H No.)	$^{2,3}J_{CH}$ HMBC (C No.)
1/14 (N)		8.22 (t, 6.0, 2H)	2/13	2/13, 9′/9″
2/13	36.0 t	3.21 (q, 6.0, 4H)	1/14, 3/12	3/12, 4/11, 9′/9″
3/12	23.9 t	1.83 (m, 4H)	2/13, 4a/11a, 4b/11b	2/13, 4/11
4/11	53.1 t	3.07 (m, 2H)a	3/12	
		2.98 (m, 2H)b	3/12	
5/10 (N)		9.41 (m, 2H)	5/10-NMe	
5/10-NMe	39.0 t[b]	2.73 (br d, 4.6, 6H)	5/10	4/11, 6/9
6/9	54.2 t	3.10 (m, 2H)a	7/8	
		3.00 (m, 2H)b	7/8	
7/8	20.5 t	1.62 (m, 4H)	6a/9a, 6b/9b	7/8
1′/1″	136.2 s			
2′/2″	132.9 d	7.47 (s, 2H)		1′/1″, 3′/3″, 4′/4″, 6′/6″, 7′/7″
3′/3″	117.0 s			
4′/4″	151.7 s			
4′/4″-OMe	60.3 q	3.76 (s, 6H)		4′/4″
5′/5″	117.0 s			
6′/6″	132.9 d	7.47 (s, 2H)		1′/1″, 3′/3″, 4′/4″, 6′/6″, 7′/7″
7′/7″	27.9 t	3.78 (s, 4H)		1′/1″, 2′/2″, 6′/6″, 8′/8″, 9′/9″
8′/8″	150.9 s			
8′/8″-NOH		12.04 (s, 2H)		8′/8″
9′/9″	163.3 s			

[a] NMR spectra were recorded at 30 °C on Varian Inova 500 and 600 MHz NMR spectrometers. Samples were dissolved in DMSO-d_6 (residual ^{1}H δ 2.50 and ^{13}C δ 39.5 ppm). Multiplicity determined by DEPT (s = C, d = CH, t = CH_2, q = CH_3). Standard parameters were used for the 2D experiments, which included gradient COSY, HSQC ($^{1}J_{CH}$ = 140 Hz), and HMBC ($^{n}J_{CH}$ = 8.3 Hz).

[b] Chemical shift obtained from DEPT as signal obscured by DMSO peak.

(14.3 mg, 0.13% dry wt) eluted with a retention time of 25 min.

Spermatinamine (**1**) was an optically inactive compound with molecular formula $C_{32}H_{44}Br_4N_6O_6{\cdot}2CF_3COOH$ obtained from HRESIMS.[14] It showed 1:4:6:4:1 ion cluster peaks at *m/z* 925/927/929/931/933 $[M+H]^+$ and 463/464/465/466/467 $[M+2H]^{2+}$, indicating the presence of four bromine atoms. The ^{13}C NMR data (Table 1) revealed that **1** contained 14 carbon resonances, indicating a molecule with symmetry. The signals in the ^{1}H NMR spectrum integrated for six methylenes, an *N*-methyl (δ_H 2.73), an aromatic methoxyl (δ_H 3.76), two aromatic methines (δ_H 7.47, 2H), an amide proton (δ_H 8.22), and an oxime proton (δ_H 12.04). There was also an aminium proton (δ_H 9.41) as the compound was isolated as its trifluoroacetate salt. A 4-alkyl-2,6-dibromophenol moiety was implied from the ^{1}H and ^{13}C chemical shifts as well as 2D data (Table 1).[6] The position of the methoxyl (δ_H 3.76) was confirmed by gHMBC correlations from the aromatic protons (δ_H 7.47) and the methoxyl protons into the quaternary aromatic carbon at δ_C 151.7. The ^{13}C NMR signals at δ_C 150.9 and 163.3 were assigned to an amide-oxime conjugated system.[6] This was supported by the proton signals at δ_H 8.22 and 12.04. The amide-oxime moiety was attached to the aromatic ring through a methylene group (δ 3.78/27.9). This linkage was established from gHMBC correlations between δ_H 3.78 (s, 2H) and δ_C 136.2, 132.9, 150.9, and 163.3. The upfield ^{13}C NMR chemical shift at δ_C 27.9 suggested *E* configuration for the oxime as the corresponding value for a (*Z*)-oxime would be >35 ppm.[15] Two spin systems were identified from the gCOSY spectrum: NH–CH_2–CH_2–CH_2–N and N–CH_2–CH_2. The gHMBC correlations from the N–Me (δ_H 2.73) to δ_C 53.1 and 54.2 clearly linked these units together. With a linkage to the methylene at δ 20.5/1.62 remaining to be identified, the gHMBC correlation between δ_H 1.62 and δ_C 20.5, and with only half of the molecular formula accounted for, it was obvious that the structure was dimeric. Thus, the structure for spermatinamine was established as **1**.

Spermatinamine (**1**), with a novel bromotyrosyl-spermine-bromotyrosyl sequence, showed inhibition of Icmt at an IC_{50} of 1.9 μM (assay performed in duplicate on five independent days). It showed no activity in the artifact assay up to 100 μM, ruling out assay technology interference.[16] Spermatinamine (**1**) is the first natural product inhibitor of Icmt. In fact the synthetic compound, cysmethynil,[2] is the only reported selective small-molecule inhibitor other than analogues of the substrate or the product. Although spermatinamine (**1**) is chemically unattractive as a drug lead it has suitable potency to be a useful pharmacological tool for the Icmt target in anticancer-drug research.

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12. *Icmt assay*: Test substances in a DMSO solution (final concentration 1%) were added to microplates. Then to each well was added 5 μL of Icmt enzyme and BFC (final concentrations of 20 μg/mL and 3μM, respectively) in assay buffer (50 mM HEPES, 10 mM $MgCl_2$, and 3 mM DTT, pH 7.5). The reaction was initiated with addition of 10 μL of [^{3}H]SAM (final concentration 6.2 μCi/mL) and unlabeled SAM (final concentration 0.6 μM). The plate was incubated at ambient temperature (~22 °C) for 60 min. The reaction was stopped with the addition of 20 μL of SAM (0.1 mM) and Streptavidin SPA beads (12 mg/mL) added to capture the product BFC ^{3}H-methyl ester. Following overnight incubation at ambient temperature radioactivity was counted on a Microbeta Trilux™ (Perkin-Elmer, Turku, Finland). The percentage inhibition relative to the reference compound SAH was then calculated.
13. The sponge sample *Pseudoceratina* sp. 2196 (phylum Porifera, class Demospongiae, order Verongida, family Pseudoceratinidae) was collected by scuba diving at a depth of 14 m from N.E. Erskine Island, Capricorn-Bunker Group, Great Barrier Reef, Queensland, Australia, in August 1996. A voucher sample, QMG307576, was lodged at the Queensland Museum, South Brisbane, Queensland, Australia.
14. Spermatinamine (**1**), (2*Z*,2′*Z*)-*N*,*N*′-{butane-1,4-diyl-bis[(methylimino)propane-3,1-diyl]}bis[3-(3,5-dibromo-4-methoxyphenyl)-2-(hydroxyimino)propanamide]: isolated as a gum; UV λ_{max} (log ε) 270 sh (3.49), 207 (4.46) nm; IR ν_{max} (film) 3412, 3047, 1677, 1539, 1472, 1422, 1203, 1136, 1002, 722 cm^{-1}; ^{1}H and ^{13}C NMR: see Table 1; positive-LRESIMS *m*/*z* 463.0 $[C_{32}H_{44}Br_4{}^{79}N_6O_6+2H]^{2+}$ (10), 464.0 $[C_{32}H_{44}Br_3{}^{79}Br^{81}N_6O_6+2H]^{2+}$ (55), 465.0 $[C_{32}H_{44}Br_2{}^{79}Br_2{}^{81}N_6O_6+2H]^{2+}$ (100), 466.0 $[C_{32}H_{44}Br^{79}Br_3{}^{81}N_6O_6+2H]^{2+}$ (75), 467.0 $[C_{32}H_{44}Br_4{}^{81}N_6O_6+2H]^{2+}$ (35), 924.9 $[C_{32}H_{44}Br_4{}^{79}N_6O_6+H]^+$ (5), 926.9 $[C_{32}H_{44}Br_3{}^{79}Br{}^{81}N_6O_6+H]^+$ (36), 928.9 $[C_{32}H_{44}Br_2{}^{79}Br_2{}^{81}N_6O_6+H]^+$ (70), 930.9 $[C_{32}H_{44}Br{}^{79}Br_3{}^{81}N_6O_6+H]^+$ (50), 932.9 $[C_{32}H_{44}Br_4{}^{81}N_6O_6+H]^+$ (15). positive-HRESIMS *m*/*z* 465.007569 $[C_{32}H_{44}Br_2{}^{79}Br_2{}^{81}N_6O_6+2H]^{2+}$ (calcd 465.00807, Δ—1.1 ppm).
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16. *Artifact assay*: To determine if activity of test compounds was due to interference with the assay reagents or technology, an artifact assay in which [^{3}H]*S*-adenosylmethionine was replaced with [^{3}H]biotin was used. The assay measures the binding of ^{3}H-biotin to streptavidin on the SPA beads. The test substances were added to microtiter plates. The assay reagents, Icmt enzyme, BFC, and [^{3}H]biotin (final concentration 1.25 μCi/mL) were dispensed into the 384 plate. The plate was incubated for 60 min at room temperature. Streptavidin coated PVT SPA beads were added to each well to capture [^{3}H]biotin and the amount of radioactivity measured. The percentage inhibition relative to the reference compound tartrazine was then calculated. The artifact assay was performed using the Icmt assay method.