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Pentathiepins and Trithianes from two *Lissoclinum* species and a *Eudistoma* sp.: inhibitors of Protein Kinase C

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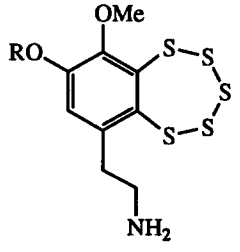
Abstract: The ascidian *Lissoclinum japonicum* from Palau contained the antimicrobial and antifungal metabolites N,N-dimethyl-5-(methylthio)varacin (**6**) and 3,4-dimethoxy-6-(2'-N,N-dimethylaminoethyl)-5-(methylthio)benzotrithiane (**7**), both of which were isolated as the trifluoroacetate salts. An inseparable 2:3 mixture of 5-(methylthio)varacin (**8**) and the corresponding trithiane **9** was isolated from a different *Lissoclinum* species from Pohnpei and 3,4-desmethylvaracin (**10**), isolated as the trifluoroacetate salt, was obtained from a species of *Eudistoma* from Pohnpei. The pentathiepins and trithianes selectively inhibit protein kinase C.

Among the many metabolites from ascidians that are derived from amino acids[2] are a small number of cyclic polysulfides that have interesting biological activities. The first compound in this series was varacin (**1**), an antifungal and cytotoxic metabolite of *Lissoclinum vareau*.[3] A similar compound, lissoclinotoxin A (**2**), an antibacterial and antifungal constituent of *L. perforatum*, was originally reported to have the cyclic trithiane structure **3**[4] but was subsequently shown to be a pentathiepin.[5] A New Zealand ascidian of the genus *Aplydium* contained the unstable trithiane **4**.[6] The structure of varacin (**1**) has been



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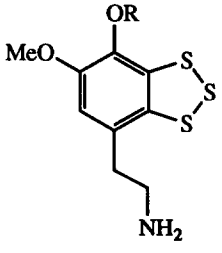

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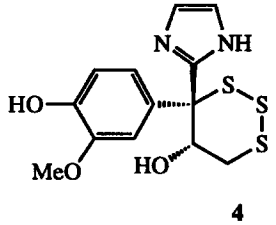

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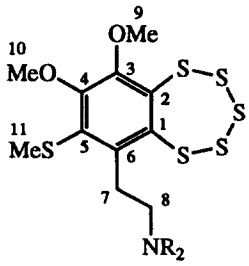
confirmed by two syntheses,[7,8] one of which also produced the corresponding trithiane **5**,[8] which could readily be distinguished from **1** by comparison of their ^{1}H NMR spectra.

Protein kinase C (PKC) is a family of isozymes that are activated, to various extents, by calcium, phospholipids, and diacylglycerol. These properties distinguish PKC from other serine/threonine protein kinases.[9,10] PKC activation can result from agonist interactions with cell surface receptors, leading to inositol phosphate turnover or phosphatidyl choline hydrolysis.[11] PKC-mediated phosphorylation of target proteins is believed to be critical to many aspects of cellular physiology that depend on control of mitosis or selective gene expression.[9,12] Consequently, inhibitors of PKC are actively being sought and developed as novel therapeutic agents for cardiovascular, inflammatory, CNS, and neoplastic diseases.[10,13] In this paper, we report the isolation and protein kinase C inhibitory activity of N,N-dimethyl-5-(methylthio)varacin (**6**) and the corresponding trithiane **7** from the Palauan ascidian *Lissoclinum japonicum*, an inseparable 2:3 mixture of 5-(methylthio)varacin (**8**) and the corresponding trithiane **9** from a different *Lissoclinum* species[14] from Pohnpei, and 3,4-desmethylvaracin (**10**) from a *Eudistoma* sp.[14] from Pohnpei.

The thinly-encrusting light brown ascidian *L. japonicum* was collected by hand using scuba (-25m) at Malakal Passage, Palau and was kept frozen for 6 months. The crude methanolic extract of *L. japonicum* showed good activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Candida albicans*. The hexane-soluble portion of a methanolic extract of the ascidian was subjected to a bioassay-directed fractionation using *S. aureus* as the test organism and was eventually purified by chromatography on a C8 reversed phase column to obtain N,N-dimethyl-5-(methylthio)varacin (**6**, 40 mg, 1.04×10^{-2} % wet wt.) and 3,4-dimethoxy-6-(2′-N,N-dimethylaminoethyl)-5-(methylthio)benzotrithiane (**7**, 30 mg, 7.8×10^{-3} % wet wt.), stabilized as their trifluoroacetate salts.

The trifluoroacetate salt of N,N-dimethyl-5-(methylthio)varacin (**6**) was isolated as a pale yellow oil. The high resolution mass spectrum showed a positive molecular ion for $C_{13}H_{20}NO_2S_6$ at *m/z* = 413.9823 $(M+H)^+$. The UV absorption at 211 nm (ε 16500) and IR band at 1678 cm^{-1} provided little structural information. The ^{1}H NMR spectrum contained four singlets at δ 2.47 (s, 3 H, SMe), 2.88 (s, 6 H, NMe_2), 3.87 (s, 3 H, OMe), and 3.95 (s, 3 H, OMe) together with signals at 3.06 (m, 2 H), 3.67 (m, 1 H), and 3.77 (m, 1 H) that were assigned to two adjacent methylene groups. The ^{13}C NMR spectrum, together with the HMQC experiment, confirmed the presence of one S-methyl group (δ 19.0), two N-methyl groups (42.6), two O-methyl groups (60.8, 62.0), and two methylene groups (29.5 and 56.7) and also contained six signals at δ 135.6, 139.9 (2 C), 141.1, 154.2, and 157.1 that were assigned to a fully substituted benzene ring. The remaining five sulfur atoms can only be accommodated in a benzopentathiepin ring system similar to that of varacin (**1**). The substitution pattern around the benzene ring was determined by analysis of the HMBC experiment. The two O-methyl signals at δ 3.87 and 3.95 showed correlations to the aromatic carbon signals at 154.2 and 157.1, respectively. The S-methyl signal at δ 2.47 showed a 3-bond correlation to the aromatic carbon signal at 135.6, which was also coupled to



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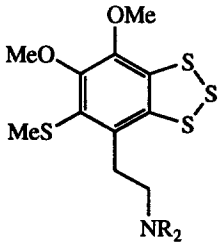

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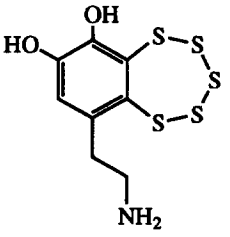

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Table 1. ^{1}H NMR data for compounds 6-9.

H#	6[a]	7[a]	8[b]	9[b]
7	3.77 (m, 1 H)	3.33 (t, 2 H, 7)	3.48 (m, 1 H)	3.08 (t, 2 H, 7.5)
	3.67 (m, 1 H)		3.38 (m, 1 H)	
8	3.06 (m, 2 H)	3.11 (t, 2 H, 7)	2.85 (m, 1 H)	2.83 (t, 2 H, 7.5)
			2.77 (m, 1 H)	
9	3.87 (s, 3 H)	3.89 (s, 3 H)	3.85 (s, 3 H)	3.87 (s, 3 H)
10	3.95 (s, 3 H)	3.87 (s, 3 H)	3.93 (s, 3 H)	3.86 (s, 3 H)
11	2.47 (s, 3 H)	2.38 (s, 3 H)	2.43 (s, 3 H)	2.34 (s, 3 H)
12	2.88 (s, 6 H)	2.91 (s, 6 H)		

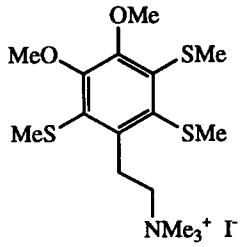
Table 2. ^{13}C NMR data for compounds 6-10.

C#	6[a]	7[a]	8[b]	9[b]	10[c]
1	139.9	137.3	143.0	137.5	132.2
2	141.1	137.2	140.8	136.7	130.0
3	154.2	148.7	153.9	148.3	147.8
4	157.1	154.3	157.1	154.4	148.8
5	135.6	130.7	136.0	131.1	118.1
6	139.9	129.4	140.2	133.1	134.3
7	29.5	30.8	37.7	39.4	33.2
8	56.7	56.0	42.3	41.4	40.5
9	62.0	60.8	62.2	60.9	
10	60.8	60.7	60.9	60.9	
11	19.0	19.2	19.0	19.2	
12	42.6	42.6			

[a] $CDCl_3$ [b] 4:1 $CDCl_3$-CD_3OD [c] DMSO-d_6

the methylene signals at 3.67 and 3.77, indicating that the S-methyl and the methylene group were attached to adjacent aromatic carbon atoms. The methylene proton signals were also coupled to carbon signals at δ 139.9 and 56.7 with the latter signal showing an additional correlation to the N,N-dimethyl signal at 2.88, establishing the Ar-CH_2-CH_2-NMe_2 chain. We deduced that the signal at δ 139.9 must be due to both the C-1 and C-6 signals, one of which is attached to the pentathiepin ring. This assignment automatically places the O-methyl groups at C-3 and C-4 and completes the structural assignment of N,N-dimethyl-5-(methylthio)varacin (**6**).

The trifluoroacetate salt of the corresponding trithiane, 3,4-dimethoxy-6-(2′-N,N-dimethylaminoethyl)-5-(methylthio)benzotrithiane (**7**), was isolated as a pale yellow oil. The high resolution mass spectrum showed a positive molecular ion for $C_{13}H_{20}NO_2S_4$ at *m/z* = 350.0395 $(M+H)^+$. The spectral data of **7** were remarkably similar to those of **6** except that there were absorptions in the UV spectrum at 209 nm (ε 15900), 238 nm (sh, ε 7410) and 277 nm (ε 5960) and the methylene signals in the ^1H NMR spectrum appeared as an AA′BB′ system at δ 3.11 (m, 2 H) and 3.33 (m, 2 H). The structure of **7** was therefore proposed to be the trithiane corresponding to N,N-dimethyl-5-(methylthio)varacin (**6**). This assignment was confirmed by showing that both **6** and **7** could be converted into the same tris(methylthio) ether **11** by reduction with lithium tri-*t*-butoxyaluminum hydride in THF at 25° followed by methylation of the reaction product with methyl iodide. Although it has been demonstrated that pentathiepins can be slowly converted into trithianes and sulfur by heating, we believe that the trithiane **7** is a natural product because the ratio of **6** and **7** did not change during the isolation procedure.



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A thin encrusting orange ascidian of the genus *Lissoclinum* was collected by hand using scuba (-5 to -25m) at Ant Atoll, Pohnpei, and was kept frozen for 9 months. As part of a search for selective inhibitors of PKC, several thousand extracts of marine invertebrates were screened for inhibition of rat brain PKC and, in the case of those extracts that inhibited PKC, for inhibition of PKA. The crude dichloromethane-methanol (1:1) extract of the ascidian selectively inhibited PKC with an IC_{50} of 28.8 μg/ml. A bioassay-guided fractionation resulted in a 2:3 mixture (5.5 mg) of 5-(methylthio)varacin (**8**) and 3,4-dimethoxy-6-(2′-aminoethyl)-5-(methylthio)benzotrithiane (**9**) that could not readily be separated. Since the components of the mixture could be identified by spectroscopic analysis, attempts at further purification were abandoned in favor of preserving material for biological evaluation.

The high resolution FAB mass spectrum of the mixture contained two peaks at *m/z* = 322.0064 $(M+H)^+$ and 385.9518 $(M+H)^+$ due to two compounds of molecular formula $C_{11}H_{16}NO_2S_4$ and $C_{11}H_{16}NO_2S_6$, respectively. The ^1H NMR spectrum confirmed that there was a mixture of two compounds and that the

peak at *m/z* = 322 had not resulted simply from the loss of S_2 from the *m/z* = 386 peak by mass spectral fragmentation. The ^1H and ^{13}C NMR spectra, both of which lacked the N-methyl signals found in compounds **6** and **7**, contained two sets of signals in a 3:2 ratio that could be assigned by interpretation of the HMQC and HMBC experiments as shown in Tables 1 and 2. Comparison of the NMR data with those of compounds **6** and **7** revealed that the minor compound was 5-(methylthio)varacin (**8**) and the major compound was 3,4-dimethoxy-6-(2′-aminoethyl)-5-(methylthio)benzotrithiane (**9**).

A dark brown ascidian of the genus *Eudistoma* was collected by hand using scuba (-15m) at Aru Pass, Pohnpei, and was kept frozen for a year. The crude methanol extract of the lyophilized tunicate was purified by PKC bioassay-guided reversed phase chromatography on C_{18} preparative and HPLC columns to obtain 3,4 desmethylvaracin (**10**) as a trifluoroacetate salt.

The TFA salt of desmethylvaracin (**10**) was obtained as a pale yellow semi-solid. The high resolution FAB mass spectrum showed a peak for $C_8H_{10}NO_2S_5$ at *m/z* = 311.9328 $(M+H)^+$. The low resolution EI mass spectrum showed a loss of NH_3, typical of primary amines, and a deuterium exchange experiment indicated the presence of five exchangeable hydrogens. The IR spectrum contained bands at ca. 3000 (br, -OH and -NH) and 1678 cm^{-1}. The ^{13}C NMR spectrum contained six aromatic carbon signals and two aliphatic carbon signals. The ^1H NMR spectrum contained a single aromatic proton signal at δ 6.79 (s, 1 H) and signals at 3.08 (m, 1 H), 2.97 (m, 1 H), and 2.87 (m, 2 H) that were assigned to the -CH_2-CH_2-NH_2 side chain. In the HMBC experiment, correlations were observed from H-5 (δ 6.79) to C-3 (147.8), C-1 (132.2), and C-7 (33.2) and from H-7 (3.08 and 2.97) to C-1, C-5 (118.1), C-6 (134.3), and C-8 (40.5). The side chain must be situated between the aromatic proton and pentathiepin ring and the remaining two aromatic carbons must therefore bear phenolic hydroxyl groups, corresponding to a 3,4-desmethyl derivative of varacin (**1**).

Within this series of compounds, the mixture of compounds **8** and **9** was the most active inhibitor of protein kinase C (IC_{50} = 0.3 μg/mL). The primary amine **10** showed comparable PKC activity. The dimethylamino compounds **6** and **7** were less active but it is significant that the trithiane **7** was twice as active as the pentathiepin **6**, since it has been informally suggested that the activity of varacin and related compounds might be due to evolution of sulfur. The trimethyl ammonium salt **11** is essentially inactive in the assay. Comparison of the inhibitory activity of **6**, **7**, (**8**+**9**), **10** and **11** against PKC and PKA (Table 3) shows selectivity for inhibition of PKC. The mixture of **8** and **9** was further characterized by determining potential isoform selectivity in assays against purified recombinant human PKC isoforms belonging to the three major classes: PKCα, which requires calcium, phospholipid, and diacylglycerol, IC_{50} = 0.8 μg/mL; PKCε, which requires phospholipid and diacylglycerol, IC_{50} = 2.1 μg/mL; PKCζ, which likely requires diacylglycerol only, IC_{50} = 1.6 μg/mL.[10] Pentathiepin **6** and trithiane **7** were both mildly antimicrobial against *B. subtilis*, *S. aureus* (15mm zone @ 100 μg/disk) and *C. albicans* and showed no useful selectivity in the National Cancer Institute's 60 cell-line panel.

Table 3. Inhibition (IC_{50}, μg/mL) of protein kinase C (PKC) and protein kinase A (PKA).

Compound	PKC	PKA
6	3.0	>50
7	1.3	>50
2:3 mixture of **8**+**9**	0.3	25
10	0.5	NT
11	33.0	>50

EXPERIMENTAL SECTION

General: Ultraviolet and infrared spectra were recorded on Perkin-Elmer Lambda 3B and 1600 series spectrometers, respectively. ^{1}H NMR spectra were recorded on a Varian Unity 500 spectrometer (UCSD) or a Bruker AMX400 spectrometer (SB). ^{13}C NMR spectra were recorded on a Bruker WP-200 SY spectrometer (SIO) or a Bruker AMX400 spectrometer (SB). Mass spectra were measured on a VG ZAB mass spectrometer at the Regional Mass Spectrometry Facility, UC Riverside or on VG 70-VSE or VG ZAB-SE4F mass spectrometers at SB.

Collection and Extraction of *Lissoclinum japonicum*: The specimen of *Lissoclinum japonicum*, a thinly encrusting light brown colonial ascidian (93-182, Paris Museum # MNHN Az Lis A 107) was collected by hand using scuba (-25 m) at Malakal Passage, Palau, in January 1993 and was quickly frozen. The sample (385 g) was chopped into small pieces and extracted with MeOH (3 x 500 mL) at 25°. The combined extracts were filtered and the MeOH was evaporated under reduced pressure to obtain a brown aqueous suspension, which was extracted with EtOAc (4 x 300 mL). The organic extract was dried over anhydrous Na_2SO_4 and the solvent was evaporated to obtain a residue that was partitioned between MeOH (150 mL) and hexane (150 mL). After evaporation of the solvent, the hexane extract was fractionated on a reversed phase C_{18} column using 9:1 MeOH/H_2O as eluant. The UV-active fractions were further purified by chromatography on Sephadex LH-20 using MeOH as eluant and finally by HPLC on a reversed phase C_8 column using 30% aqueous MeOH containing 0.1% TFA as eluant to obtain N,N-dimethyl-5-(methylthio)varacin trifluoroacetate (**6**, 40 mg, 1.04 x 10^{-2} % wet wt.) and 3,4-dimethoxy-6-(2′-N,N-dimethylaminoethyl)-5-(methylthio)benzotrithiane trifluoroacetate (**7**, 30 mg, 7.8 x 10^{-3} % wet wt.).

N,N-Dimethyl-5-(methylthio)varacin trifluoroacetate (6): pale yellow oil; UV (MeOH) 211 nm (ε 16500); IR (film) 2928, 2852, 2768, 1678, 1452, 1381, 1276, 1198, 1130, 1061, 1019 cm^{-1}; ^{1}H NMR ($CDCl_3$) see Table 1; ^{13}C NMR ($CDCl_3$) see Table 2; HRFABMS, obsd. *m/z* = 413.9823 $(M+H)^+$, $C_{13}H_{20}NO_2S_6$ requires *m/z* = 413.9818.

3,4-Dimethoxy-6-(2′-N,N-dimethylaminoethyl)-5-(methylthio)benzotrithiane trifluoroacetate (7): pale yellow oil; UV (MeOH) 209 nm (ε 15900), 238 nm (ε 7410), 277 nm (ε 5960); IR (film) 2965, 2923, 1676, 1457, 1389, 1185, 1061, 961 cm^{-1}; ^{1}H NMR ($CDCl_3$) see Table 1; ^{13}C NMR ($CDCl_3$) see Table 2; HRFABMS, obsd. *m/z* = 350.0395 $(M+H)^+$, $C_{13}H_{20}NO_2S_4$ requires *m/z* = 350.0376.

Reduction and Methylation of 6 (and 7): A 0.5 N solution of lithium tri-*tert*-butoxyaluminum hydride in dry THF (0.1 mL, 30.4 x 10^{-3} mmol) was added to a solution of N,N-dimethyl-5-(methylthio)varacin trifluoroacetate (**6**, 15.7 mg, 3.8 x 10^{-3} mmol) in dry THF (2 mL) and the reaction mixture was stirred under argon at room temperature. After 2 hours, methyl iodide, (5 μL, 0.076 mmol) was added to the stirred reaction mixture. After 1 hour, 10% aqueous NH_4Cl solution (2 mL) was added and the mixture was extracted with CH_2Cl_2 (2 x 3 mL). The organic extract was dried over anhydrous Na_2SO_4 and the solvent evaporated to obtain the tris(methylthio) ether (**11**) as the only product. Reduction and methylation of trithiane **7** also gave the tris(methylthio) ether (**11**).

4,5-dimethoxy-N-trimethyl-2,3,6-tris(methylthio)phenylethylamine iodide (11): ^{1}H NMR ($CDCl_3$) δ 2.45 (s, 3 H, SMe), 2.49 (s, 3 H, SMe), 2.53 (s, 3 H, SMe), 3.36 (m, 2 H, H-8), 3.55 (s, 9 H, NMe), 3.78 (m, 2 H, H-7), 3.88 (s, 3 H, OMe), 3.93 (s, 3 H, OMe); HRFABMS, obsd. *m/z* = 362.1298 $(M)^+$, $C_{16}H_{28}NO_2S_3$ requires *m/z* = 362.1281.

Collection and Extraction of *Lissoclinum* sp.: The thin encrusting orange ascidian, *Lissoclinum* sp. (POH93-032, Paris Museum # MNHN Az Lis A 106) was collected by hand using scuba (-5 to -25 m) at Ant Atoll, Pohnpei, Federated States of Micronesia, in January 1993 and was quickly frozen. The lyophilized sample (36 g) was extracted with 1:1 CH_2Cl_2/MeOH (2 x 500 mL) to obtain a crude organic extract (2.5 g, 6.94% dry wt.), which was active in the PKC assay (IC_{50} = 28.8 μg/mL). The crude extract was dissolved in 9:1 MeOH/H_2O (100 mL) and extracted with hexane (3 x 100 mL). The aqueous methanol fraction was adjusted to 7:3 MeOH/H_2O and was extracted with CH_2Cl_2 (3 x 100 mL) to obtain PKC-active material (325 mg, 0.9 % dry wt.) that was twice chromatographed on Sephadex LH-20 using first MeOH then 1:1 CH_2Cl_2/MeOH as eluants. The active material was then chromatographed twice on preparative silica gel TLC plates (E. Merck, Kieselgel 50, 500 micron layer) using first 8:1.5:0.5 CH_2Cl_2/MeOH/NH_4OH then 8:2:0.5 EtOAc/MeOH/H_2O as mobile phases to obtain a PKC-active fraction that consisted of a 2:3 mixture (5.5 mg, 0.015% dry wt.) of 5-(methylthio)varacin (**8**) and 3,4-dimethoxy-6-(2′-aminoethyl)-5-(methylthio)benzotrithiane (**9**). Attempts to separate the mixture of **8** and **9** were unsuccessful and were abandoned in favor of preserving material for biological evaluation.

2:3 mixture of 8 and 9: UV (MeOH) 271 nm (ε 14700), 240 nm (ε 21000), 211 nm (ε 34800); IR (KBr) 3430, 2800-3000, 1632, 1449, 1117, 1023, 619 cm^{-1}; ^{1}H NMR (400 MHz, 4:1 $CDCl_3$-CD_3OD) see Table 1; ^{13}C NMR (100 MHz, 4:1 $CDCl_3$-CD_3OD) see Table 2; HRFABMS, obsd. 385.9518 $(M+H)^+$ and *m/z* = 322.0064 $(M+H)^+$, $C_{11}H_{16}NO_2S_6$ requires *m/z* = 385.9505 and $C_{11}H_{16}NO_2S_4$ requires *m/z* = 322.0064.

Collection and Extraction of *Eudistoma* sp.: The dark brown ascidian *Eudistoma* sp. (POH93-155) was collected by hand using scuba (-15 m) at Aru Pass, Pohnpei, in January 1993 and was immediately frozen. The lyophilized specimen (170 g) was extracted with MeOH to obtain a crude extract (15 g) a portion of which (4.3 g) was fractionated on a reversed phase C_{18} column using eluants of decreasing polarity from H_2O to CH_3CN. The PKC-active material was eluted with 9:1 CH_3CN/H_2O and was purified by HPLC on

a reversed phase C_{18} column using 7:3 CH_3CN/H_2O containing 0.1% TFA as eluant to obtain the TFA salt of 3,4-desmethylvaracin (**10**, 6.4 mg, 1.3 x 10^{-2}% dry wt.).

3,4-Desmethylvaracin trifluoroacetate (10): pale yellow semi-solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.79 (s, 1 H), 3.08 (m, 1 H), 2.97 (m, 1H), 2.87 (m, 2 H); ^{13}C NMR (100 MHz, DMSO-d_6) see Table 2; HRFABMS, obsd. *m/z* = 311.9328 $(M+H)^+$, $C_8H_9NO_2S_5$ requires *m/z* = 311.9341.

Bioassays against PKC and PKA: PKC, purified from rat brain by published procedures,[12,15] was provided by M. Pullen and P. Nambi (Dept. of Renal Pharmacology, SmithKline Beecham). Recombinant human PKC isoforms α, ε, and ζ were cloned, expressed, and provided by A. Dilella (Dept. of Molecular Genetics, SB) and B. Amegadzie (Dept. of Gene expression Sciences, SB). PKC catalytic activity was assayed by the γ-^{32}P transfer from ATP to glycogen synthase peptide as described by Nishizuka.[12] PKA was purchased from Sigma; its activity was assayed by γ-^{32}P transfer from ATP to histone IIA, according to published procedures.[16]

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