Protein Kinase C Inhibitors: Novel Spirosesquiterpene Aldehydes from a Marine Sponge Aka (=Siphonodictyon) coralliphagum

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PROTEIN KINASE C INHIBITORS: NOVEL SPIROSESQUITERPENE ALDEHYDES FROM A MARINE SPONGE *AKA* (=*SIPHONODICTYON*) *CORALLIPHAGUM*

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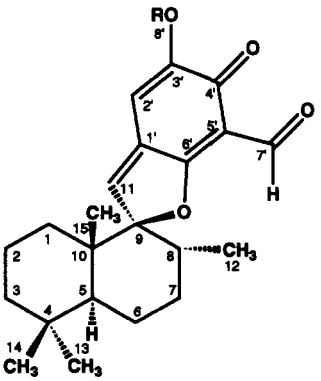
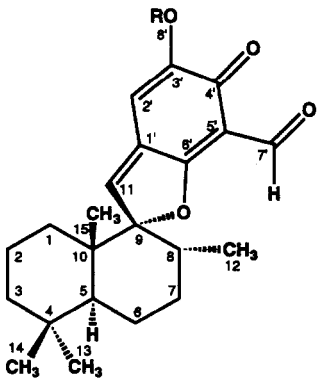
ABSTRACT.—Two novel spirosesquiterpene aldehydes, corallidictyals A [**1**] and B [**2**], were isolated as a mixture from the marine sponge *Aka* (=*Siphonodictyon*) *coralliphagum*, and their structures were determined by detailed spectroscopic methods. These compounds were identified in a screen for inhibitors of protein kinase C.

Protein kinase C (PKC), an important regulator of cell physiology, is a phospholipid-dependent protein phosphorylating enzyme that is activated by a variety of agonist interactions at cell surfaces. In its activated form, it phosphorylates target proteins, which then produce specific physiological responses. Selective inhibitors of PKC are currently being developed as potential therapies for cancer, inflammatory disease, and cardiovascular disease. As part of a mechanism-based screen for novel inhibitors of protein kinase C from a variety of natural sources, an EtOAc extract of a Bahamian sponge *Aka* (=*Siphonodictyon*) *coralliphagum* (Ruetzler, 1971) collected at Little San Salvador Island, was selected for fractionation. The active components were identified as a mixture of two diastereomeric spirosesquiterpene aldehydes, corallidictyals A [**1**] and B [**2**]. This paper describes the isolation, structure determination, and biological activity of these compounds. The relative stereochemistry of each compound is presented; the absolute stereochemistry has not been established.

A number of related spirosesquiterpene compounds isolated from the aspen fungus *Stachybotrys cylindrospora* (1), from the sponge *Siphonodictyon coralliphagum* (2), and from the sponge *Stelospongia conulata* (3) have been reported. These compounds incorporate various sites of oxygenation and various stereochemistries. Some of the known variations of these molecules include dialdehydic, diphenolic, and triphenolic functionalities attached to an aromatic



1 R=H
3 R=CH_3



2 R=H
4 R=CH_3

ring D; methoxylated para-quinones; and various oxygenated substituents on ring A of the drimane moiety.

Bioassay-guided fractionation of the crude EtOAc extract using solvent trituration, and Sephadex LH-20 and reversed-phase cc yielded the active fraction as a yellow amorphous powder. High-resolution deims suggested a molecular formula of $C_{22}H_{28}O_4$. Nmr studies indicated that the active fraction was in reality a 3:7 mixture of two closely related diastereomers, **1** and **2**, which could not be readily separated by normal chromatographic techniques. Each of the two compounds in the mixture could, however, be readily identified by nmr spectroscopy based on the chemical shift differences and integration of the respective signals. The ^{1}H-nmr spectrum indicated the presence of a pair of downfield aldehyde singlets near δ 10.3, a broad exchangeable phenol signal near δ 7.4, two olefinic singlets near δ 7.3 and two near δ 6.4, and an intensely overlapping group of aliphatic protons between δ 2.6 and δ 0.5. Discernible among this cluster of aliphatic proton resonances were a pair of methine multiplets near δ 2.5 and four pairs of methyl signals (three pairs of singlets and one pair of doublets). All of these paired signals appeared in a 3:7 ratio with the less intense peaks corresponding to corallidictyal A [**1**] and the more intense peaks corresponding to corallidictyal B [**2**].

The ^{1}H-nmr chemical shift assignments were based on COSY and nOe data, and they were confirmed by ^{13}C-nmr one- and two-dimensional experiments. The pertinent ^{1}H- and ^{13}C-nmr assignments are presented in Tables 1 and 2, respectively. The nOe results, which are displayed as arrows about the energy minimized structures shown in Figure 1, clearly point to a difference in stereochemistry between compounds **1** and **2** at the spiro center, C-9. All of the other chiral centers retain the same stereochemistry in both **1** and **2**. It is expected that the protons which are near to the spiro center and above the plane of rings A and B would be located further downfield in corallidictyal A [**1**] than in B [**2**] because of their proximity to the ether oxygen. For corallidictyal A [**1**] in which the ether oxygen is above the A/B plane, the axial methyl group CH_3-15 and the axial proton H-8 (which are both located above this plane) resonated 0.1 ppm and 0.2 ppm further downfield, respectively, than for corallidictyal B [**2**]. On the other hand, the axial proton H-5 (which is located below the A/B plane) resonated 0.5 ppm further downfield in corallidictyal B [**2**] in which the ether oxygen is below the A/B plane. CH_3-12 is in an equatorial position and consequently remains at a constant chemical shift in both **1** and **2**.

The ^{13}C-GASPE nmr spectrum of the corallidictyal A [**1**] and B [**2**] mix-

TABLE 1. ^{1}H-Nmr Chemical Shifts (ppm) for Compounds **1–4**.[a]

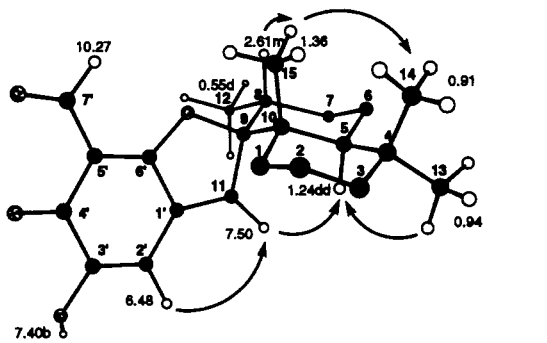
Proton	Compound			
	1	**2**	**3**	**4**
H-7' 1H	10.27 (s)	10.31 (s)	10.28 (s)	10.31 (s)
H-11 ..... 1H	7.50 (s)	7.25 (s)	7.38 (s)	7.13 (s)
H-2' 1H	6.48 (s)	6.43 (s)	6.27 (s)	6.22 (s)
H-8' 3H	7.40 (br s, OH)	7.40 (br s, OH)	3.84 (s, OCH_3)	3.82 (s, OCH_3)
H-8 ..... 1H	2.61 (m)	2.42 (m)	2.61 (m)	2.42 (m)
H-1,-2,-3,-6,-7 .. 10H	2.00–0.80 (m)	1.80–0.80 (m)	2.00–0.80 (m)	2.00–0.80 (m)
H-5 ..... 1H	1.24 (dd, 2.9, 12.0)	1.75 (dd, 2.7, 12.2)	1.24 (dd, 2.9, 12.5)	1.79 (dd, 2.6, 13.2)
CH_3-15 ..... 3H	1.36 (s)	1.28 (s)	1.36 (s)	1.26 (s)
CH_3-13 ..... 3H	0.94 (s)	0.95 (s)	0.94 (s)	0.95 (s)
CH_3-14 ..... 3H	0.91 (s)	0.88 (s)	0.91 (s)	0.87 (s)
CH_3-12 ..... 3H	0.55 (d, 6.6)	0.56 (d, 6.6)	0.56 (d, 6.6)	0.56 (d, 6.6)

[a]Measurements (ppm) performed in $CDCl_3$ at 400 MHz. Multiplicity and coupling constants (J in Hz) in parentheses.

TABLE 2. ^{13}C-Nmr Data (ppm) for Compounds **1** and **2**.[a]

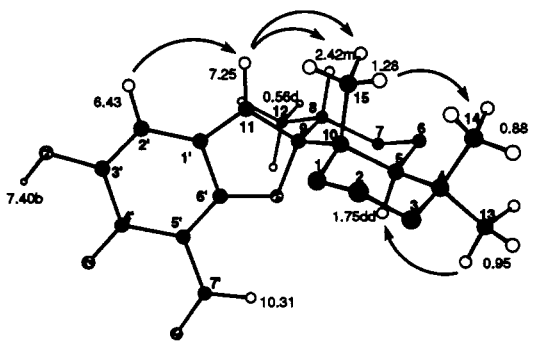
Carbon	Compound	
	1	**2**
C-7′	186.6 (d)	186.1 (d)
C-4′	180.3 (s)	180.0 (s)
C-6′	175.7 (s)	177.0 (s)
C-3′	150.3 (s)	150.3 (s)
C-11	147.8 (d)	149.7 (d)
C-1′	131.7 (s)	130.8 (s)
C-9	113.1 (s)	111.1 (s)
C-5′	107.7 (s)	107.7 (s)
C-2′	98.4 (d)	98.1 (d)
C-5	52.3 (d)	47.3 (d)
C-10	44.8 (s)	44.0 (s)
C-1, -2, -3, -4, -6, -7	41.7–18.2 (d, s)	41.2–18.2 (d, s)
C-8	35.5 (d)	34.2 (d)
C-13	33.5 (q)	33.3 (q)
C-14	21.8 (q)	21.9 (q)
C-15	16.4 (q)	19.3 (q)
C-12	15.5 (q)	15.6 (q)

[a]Measurements (ppm) performed at 100.16 MHz in $CDCl_3$. Multiplicity in parentheses.



Corallidictyal A [**1**]

nOe's



Corallidictyal B [**2**]

FIGURE 1. Energy minimized structure of compounds **1** and **2** with nOes indicated.

ture again indicated the presence of two diastereomers. The pertinent carbon chemical shifts for each compound were assigned by inverse one-bond and multiple-bond correlation techniques and the resulting assignments are presented in Table 2. Consistent with the proton data, the largest differences between corresponding chemical shifts for **1** and **2** ($\Delta\delta$) were found near the spiro center C-9 and near C-5. Two carbonyl resonances were present for each of the compounds near δ186 and δ180 in addition to five quaternary and two protonated carbon resonances observed between δ 177 and δ 98. The spiro carbon, C-9, resonated at δ 113 in corallidictyal A [**1**] and at δ 111 in corallidictyal B [**2**]. Thirteen pairs of aliphatic carbons were observed ranging from δ 53 to δ 15.

HMQC and HMBC nmr correlation data provided evidence supporting these assignments. In particular the HMBC data established the structure and assignments about the C-9 spiro center and the fused quinoid ring. A series of multiple-bond correlations between the C-9 spiro carbon of corallidictyal A [**1**] resonating at δ 113.1 and H-11 at δ 7.50, CH_3-15 at δ 1.36, and CH_3-12 at δ 0.55, were useful in establishing the arrangement of substituents near the spiro carbon. Another set of long-range correlations between H-2′ at δ 6.48 and C-4′ at δ 180.3, C-6′ at δ 175.7, and C-11 at δ 147.8 established the assignments about the quinoid ring in conjunction with correlations between the aldehyde proton at δ 10.27 and C-5′ at δ 107.7 and C-6′ at δ 175.7. Totally analogous correlations were observed for corallidictyal B [**2**].

Heteronuclear correlations again proved useful in assigning the C-5 ring-junction methine carbon as well as the *gem*-dimethyl CH_3-13 and CH_3-14 groups. The CH_3-15 protons near δ 1.36 correlated to the methine carbon C-5 near δ 52.3 as did the *gem*-dimethyl protons CH_3-13 near δ 0.94 and CH_3-14 near δ 0.91.

It is possible that compounds **1** and **2** were not separable because they are interchanging in solution by virtue of common resonance forms resulting from the loss of the phenolic proton. The opening and arbitrary reclosing of the spiro-ring could lead to a mixture of stereochemistries at C-9. To determine if this type of interchange was indeed occurring, a saturation transfer experiment was carried out. The H-11 (δ 7.50) resonance of **1** was saturated for 10 sec and then the proton spectrum was observed in a nOe difference fashion. The adjacent H-2′ singlet at δ 6.48 showed an enhancement, but no saturation transfer was observed for the H-11 (δ 7.25) resonance of **2**. Saturation of the H-11 signal in **2** showed the expected enhancement of the H-2′ signal with no saturation transfer to **1**.

The mixture of **1** and **2** was then heated to 100° in DMSO-d_6. There was no change in their 3:7 ratio suggesting that corallidictyals A and B do not interconvert on a brief time-frame.

Addition of NaOD to the mixture, which generates the phenolic anion, did not alter the ratio of **1** and **2**. This suggests that corallidictyals A and B are two unique compounds which are very difficult to separate. Further attempts at chromatography were abandoned in favor of using the remaining material in additional biological testing.

The chemical shifts taken from the ^{1}H-nmr spectra of the chromatographically separable methyl derivatives, methylcorallidictyals A [**3**] and B [**4**], are presented in Table 1. Similar nOe and COSY data (plus an additional nOe enhancement between H-2′ and OCH_3-8′) indicate that these compounds are totally analogous to corallidictyals A [**1**] and B [**2**] differing only in the presence of an additional methyl group at δ 3.8 in place of the hydroxyl proton. Cd spectra of these two methyl derivatives gave opposite molar ellipticities as expected.

Activity of the corallidictyal mixture

(A and B) was followed by assaying the inhibition of protein kinase C activity by the various fractions. Protein kinase C catalyses the transfer of γ-[^{32}P] from radiolabeled ATP to a peptide substrate; inhibition of this activity was assessed as a quantitative reduction in ^{32}P transferred. The IC_{50} (concentration producing a 50% effect) for inhibition of protein kinase C by corallidictyal was 28 μM while the corresponding methyl ethers were inactive; another serine-threonine protein kinase, cAMP-dependent protein kinase, was not inhibited by 300 μM corallidictyal, consistent with at least 10-fold selectivity for the inhibition of protein kinase C with respect to the other kinase. Using four purified recombinant human isoforms of protein kinase C (α, ε, η, and ζ) it was possible to show that the inhibitor was selective for inhibition of the α isoform (IC_{50}=30 μM) with regard to ε, η, and ζ (IC_{50}s of 89, >300, and >300 μM, respectively). In addition, the corallidictyal mixture (A and B) inhibited the growth of cultured Vero (African green monkey kidney) cells with IC_{50}=1 μM after continuous exposure (72 h).

In spite of the selectivity displayed by the corallidictyals amongst the different isoforms of protein kinase C, these structurally interesting spirosesquiterpenes will not be further developed, as a number of more potent marine natural product inhibitors of PKC have emerged from the same ongoing study.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Ir spectra were recorded on a Nicolet Model 29 DXB Ftir spectrometer and uv spectra with a Beckman DU7 spectrophotometer. Cd spectra were recorded on a Jasco J-500C spectrometer, using MeOH as solvent. For nmr analysis, approximately 5 mg of each sample were dissolved in 0.7 ml of $CDCl_3$ (MSD, 99.96 atom % D). The solvent peak served as a secondary reference to TMS (δ 0) for all ^{1}H- and ^{13}C-nmr spectra. The ^{1}H- and ^{13}C-nmr data were collected using a Bruker AMX400 equipped with an inverse detection probe which was operated at 25°. The proton data included a survey spectrum, COSY, and nOe difference data. The raw nOe spectra were collected as a series in which each individual proton resonance, including an off-resonance comparison frequency, was selectively saturated with low RF power for seven seconds prior to acquisition. For each spectrum, 16 scans were averaged and then the process was repeated until sufficient signal was acquired. Following Fourier transformation, difference spectra were generated by subtracting the off-resonance comparison spectrum from each irradiated spectrum. The carbon data include a GASPE spectrum and inverse one-bond and multiple-bond correlation spectra. All of the spectra were collected by means of standard Bruker automation programs. Full-scan hrdeims were obtained by magnetic scanning on a Fisons VG 70-VSE mass spectrometer at a resolution, m/Δm, of 5000 vs. PFK reference. Modeling was performed using Chem 3D by Cambridge Scientific Computing Inc.

ANIMAL MATERIAL.—The sample was collected by Dr. Brad Carté on June 23, 1988, at a depth of 90 ft at Little San Salvador Island, Bahamas. This bright yellow organism was found burrowing into living coral heads. Ms. Mary Kay Harper identified the sponge as *Aka* (=*Siphonodictyon*) *coralliphagum* (Ruetzler, 1971) (class Demospongiae, order Haplosclerida, family Adociidae). A voucher specimen has been deposited in the Scripps Institution of Oceanography Benthic Invertebrate Collection #P1141.

BIOASSAYS.—Reactions took place in sterile 96-well microtiter plates; total reaction volumes were 50 μl. To determine protein kinase C inhibition, 5 μl of stock solution of test material (in DMSO) were added to 25 μl of reaction buffer containing 1.8 mM EGTA, 2.2 mM $CaCl_2$, 0.2 mM glycogen synthase peptide (Bachem Bioscience, Inc.), 80 μg/ml L-α-phosphatidyl-L-serine, 2 μg/ml 1,3-diolein, 20 mM $MgCl_2$, and 20 mM Tris, pH 7.5 (4). Then 10 μl (10 μg) of protein kinase C (3.3×10^3 pmol/min/mg protein, prepared from frozen rat brain tissue in the Department of Pharmacology, or, in the case of the recombinant human enzymes, cloned and expressed in the Departments of Gene Expression Sciences and Molecular Genetics, SmithKline Beecham Pharmaceuticals) were added. Reactions were initiated by the addition of 10 μl of γ-[^{32}P]-ATP (adenosine-5′-triphosphate, tetra[triethylammonium] salt), specific activity 6000 Ci/mmol (DuPont-New England Nuclear). The reaction mixtures were incubated at 37° for 15 min, and then the mixtures were spotted onto squares of P-81 protein-adsorbing paper (Whatman). The filters were washed once with 0.5% phosphoric acid and twice with distilled H_2O, and dried with Me_2CO. ^{32}P radioactivity bound to protein and adsorbed to the filters was determined by liquid scintillation

spectrometry. Inhibition of protein kinase C activity was determined by comparing radioactivity bound to the filters in the presence and in the absence of inhibitor.

Inhibition of the growth of Vero cells was determined spectrophotometrically. Altogether, 2×10^3 cells were inoculated into wells of a 96-well microtiter plate and incubated at 37° in a humidified, 5% CO_2 incubator overnight in growth medium (Eagle's minimal essential medium containing 10% fetal bovine serum and antibiotics) to permit attachment. Various concentrations of compound were then added to the wells, and the cells were incubated in growth medium with or without compound for 72 h. Controls were incubated with 0.5% DMSO, the compound vehicle. After 72 h, the growth medium was removed and the cells were stained for 90 min with XTT [(2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt), Sigma]. Numbers of viable cells were calculated from absorbance values at 450 nm.

EXTRACTION AND ISOLATION.—The sample was frozen on collection and lyophilized to yield 141 g of dry mass, which was extracted twice with EtOAc, followed by removal of the solvent *in vacuo*. The crude extract (4.2 g) was triturated with CH_2Cl_2-MeOH (1:1) and the active soluble portion (2 g) upon evaporation to dryness was subjected to Sephadex LH-20 cc using CH_2Cl_2/MeOH as eluent. The active fraction from the LH-20 column was further purified using reversed-phase medium pressure cc which afforded a mixture of **1** and **2** as a yellow powder (164 mg). Attempts to further separate the two isomers using normal-phase and reversed-phase hplc (some of which were RP-18 and RP-8 solid supports with either H_2O or TEAP buffer in combination with MeOH or CH_3CN as modifier) were unsuccessful.

Corallidictyals A [**1**] *and B* [**2**].—Uv (MeOH) λ max 322 (ε 16,000), 266 (ε 10,400), 209 (ε 5100) nm; ir (KBr) ν max 3328, 3081, 1694, 1643, 1618, 1600, 1583, 1390 cm^{-1}; ^1H nmr, see Table 1; ^{13}C nmr, see Table 2; hrdeims *m/z* 356.1979 ($C_{22}H_{28}O_4$, Δ 0.8 mDa).

Preparation of **3** and **4**.—Methylcorallidictyals A [**3**] and B [**4**] were prepared by treating a solution of **1** and **2** (30 mg) in 10 ml of dry Me_2CO with 3 ml of CH_3I and 50 mg of K_2CO_3 and refluxing for 16 h. The cooled reaction mixture was poured onto ice, acidified with 5 N HCl, and the products were extracted with 2×20 ml of EtOAc. The crude product was purified by Si gel prep. tlc using EtOAc-hexane (3:2) as eluent to obtain 4 mg of **3** and 6 mg of **4**.

Methylcorallidictyal A [**3**].—Uv (MeOH) λ max 321 (ε 20,100), 261 (ε 10,900), 197 (ε 15,300) nm; cd (MeOH) λ max [θ] 327 (3,400), 262 (15,400), 234 (−10,400) nm; ir (KBr) ν max 3100, 1740, 1694, 1623, 1601, 1572, 1458 cm^{-1}; ^1H nmr, see Table 1; hrdeims *m/z* M^+ 370.2140 (70, $C_{23}H_{30}O_4$, Δ 0.4 mDa), 245.0827 (9, $C_{14}H_{13}O_4$, Δ −1.3), 232.0744 (63, $C_{13}H_{12}O_4$, Δ −0.8 mDa), 219.0661 (100, $C_{12}H_{11}O_4$, Δ −0.4 mDa), 181.0503 (9, $C_9H_9O_4$, Δ −0.2 mDa), 123.1177 (11, C_9H_{15}, Δ −0.3 mDa).

Methylcorallidictyal B [**4**].—Uv (MeOH) λ max 321 (ε 22,100), 260 (ε 10,900), 197 (ε 14,400) nm; cd (MeOH) λ max [θ] 322 (−16,100), 263 (−15,800), 237 (11,900) nm; ir (KBr) ν max 3100, 1745, 1692, 1625, 1573, 1458 cm^{-1}; ^1H nmr, see Table 1; hrdeims *m/z* M^+ 370.2147 (79, $C_{23}H_{30}O_4$, Δ −0.3 mDa), 245.0819 (9, $C_{14}H_{13}O_4$, Δ −0.5), 232.0738 (62, $C_{13}H_{12}O_4$, Δ −0.2 mDa), 219.0661 (100, $C_{12}H_{11}O_4$, Δ −0.4 mDa), 181.0502 (9, $C_9H_9O_4$, Δ −0.7 mDa), 123.1167 (8, C_9H_{15}, Δ 0.7 mDa).

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