A New Cacospongionolide Inhibitor of Human Secretory Phospholipase A_2 from the Tyrrhenian Sponge *Fasciospongia cavernosa* and Absolute Configuration of Cacospongionolides

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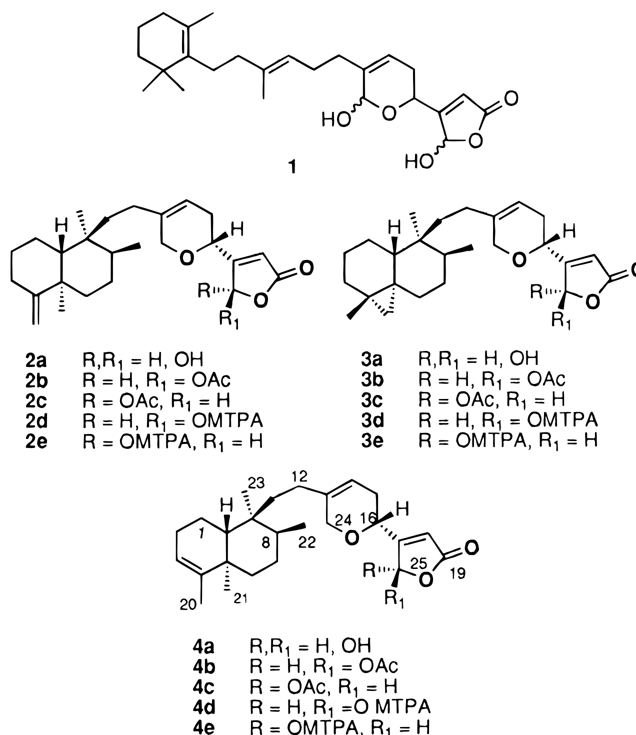
A new inhibitor of human secretory phospholipase A_2 (PLA_2), cacospongionolide E (**4a**), has been isolated from the Tyrrhenian sponge *Fasciospongia cavernosa*. The structure was proposed on the basis of spectroscopic data and by chemical transformations. The absolute configuration of cacospongionolides **2a**−**4a** was established using the modified Mosher's method. Cacospongionolide E was the most potent inhibitor toward human synovial PLA_2, showing higher potency than the reference compound manoalide and exerting no signs of toxicity on human neutrophils. It showed high activity in the *Artemia salina* bioassay and moderate toxicity in the fish (*Gambusia affinis*) lethality assay.

In recent years, marine natural products have proved to be a potential source of antiinflammatory drugs.[2] Some contain a 1,4-hydroquinone/quinone[3] moiety, for example, avarol[4] or an *ortho*-quinone;[2] others have a tetronic acid moiety, such as ircinin;[5] still others are dialdehydes such as scalaradial,[6] or γ-hydroxybutenolides such as manoalide (**1**). This last compound was the first sesterterpene to be reported from a *Luffariella* sp.[7] and has been extensively investigated as a potent inhibitor of phospholipase A_2 (PLA_2).[8] Subsequently many related metabolites from *Luffariella* sp. were reported.[9] Among the manoalide congeners, particularly interesting is cacospongionolide B (**2a**),[10] which exhibits specific inhibition of human PLA_2[11,12] and is more stable than manoalide.

Our group has recently investigated the chemistry of a number of specimens of *Fasciospongia cavernosa* Schmidt (family Thorectidae) collected in the northern Adriatic, to provide sufficient cacospongionolide B (**2a**) and has reported the isolation and structure elucidation, including the relative stereochemistry, of novel related metabolites.[13] The problem of the absolute configuration, however, remained unsolved. We have recently reported the absolute configuration of cacospongionolide B (**2a**) and cacospongionolide (**3a**),[14] the first compound of this group isolated from *F. cavernosa*, by means of CD studies.[15] We then undertook an extensive collection of *F. cavernosa* from the Tyrrhenian Sea. We were surprised to find that a small number of samples, collected in the bay of Naples, contained a new metabolite, named cacospongionolide E (**4a**), together with cacospongionolide B. We report here the isolation, structure determination, and some biological activities of the new compound. Furthermore, the absolute configuration of cacospongionolides **2a**−**4a** is also reported.



Results and Discussion

The Et_2O-soluble fraction of the Me_2CO extract of *F. cavernosa* was chromatographed on Si gel to give cacospongionolide B (**2a**) and a mixture of two isomers that was separated by reversed-phase HPLC to give **2a** and a new related compound, named cacospongionolide E (**4a**, 0.046% dry wt).

Cacospongionolide E (**4a**) had $[\alpha]_D$ −85.4° and a molecular formula $C_{25}H_{36}O_4$ by HRMS of the parent ion, and, thus, it was isomeric with cacospongionolide B. The UV absorption at 223 nm and IR bands at 3380, 1785, and 1760 cm^{-1} were characteristic of a γ-hydroxy-

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Table 1. NMR Spectral Data of **4a** in $CDCl_3$ Solution[a]

carbon no.	^{13}C	^{1}H	HMBC (J_{C-H} = 10 Hz)
1	20.0 t	1.05 m, 0.90 m	5.33 (H-3), 1.40 (H-10)
2	25.8 t	1.98 m	5.33 (H-3), 1.40 (H-10)
3	122.4 d	5.33 m	1.98 (H-2), 1.63 (H-20)
4	142.0 s		1.98 (H-2), 1.63 (H-20), 1.13 (H-21)
5	38.6 s		5.33 (H-3), 1.63 (H-20), 1.13 (H-21)
6	32.2 t	1.58 m, 1.40 m	1.13 (H-21)
7	27.2 t	1.48 m, 1.32 m	0.84 (H-22)
8	37.4 d	1.55 m	1.00 (H-23), 0.84 (H-22)
9	38.7 s		1.85−1.20 (H-11), 1.00 (H-23), 0.84 (H-22)
10	44.6 d	1.40 m	1.98 (H-2), 1.13 (H-21), 1.00 (H-23)
11	35.8 t	1.85 m, 1.20 m	1.00 (H-23)
12	26.7 t	1.95 m, 1.84 m	5.56 (H-14)
13	138.4 s		4.20 (H-24), 1.95−1.84 (H-12)
14	116.5 d	5.56 br s	4.20 (H-24), 1.95−1.84 (H-12)
15	29.4[b] t	2.26[b] m	5.56 (H-14)
16	69.5[b] d	4.40[b]	
17	167.3[b] s		
18	117.8[b] d	6.06[b]	6.16 (H-25)
19	170.9 s		
20	19.3 q	1.63 s	5.33 (H-3)
21	27.7 q	1.13 s	
22	15.4 q	0.84 d (7.0)	1.48−1.32 (H-7)
23	26.2 q	1.00 s	1.85−1.20 (H-11)
24	68.5 t	4.20 ABq (15.5)	5.56 (H-14), 1.95−1.84 (H-12)
25	97.3[b] d	6.16[b]	

[a] Chemical shifts are referred to TMS. Multiplicities are indicated by usual symbols. Coupling constants (Hz) are in parentheses. [b] Broad signal due to the presence of a mixture of epimers at C-25.

butenolide moiety. The analysis of its ^{1}H and ^{13}C NMR spectra showed that **4a** is closely related to the cacospongionolide B **2**.[10] As already observed for **2a**, the NMR spectra of **4a** are highly solvent dependent, and the interpretation of the signals related to the polar moiety (the hetereocyclic region) of the molecule is difficult.

The ^{1}H and ^{13}C NMR spectra of both acetates **4b** and **4c**, obtained by treatment of **4a** with Ac_2O in pyridine at room temperature, showed a single set of sharp resonances for the heterocyclic region (C-12 to C-19) of cacospongionolide E. The chemical shifts were in excellent agreement with those of the corresponding resonances observed for cacospongionolide B acetates **2b** and **2c**,[10] defining the structure of the C-12 to C-19 hetereocyclic region as shown.

The remaining signals in the ^{1}H and ^{13}C NMR spectrum were due to one secondary and two tertiary methyl groups [δ 0.84 (d, J = 7.0 Hz), 1.00, 1.13; 15.4, 26.0, 27.7] assigned to Me-8, Me-9, and Me-5, respectively; a trisubstituted double bond [δ 5.33; 122.4 (d), 142.0 (s)]; and an olefinic methyl group (δ 1.63; 19.3), which was assigned to Me-4. The COSY-45 spectrum indicated that the olefinic proton at δ 5.33 (H-3) was long-range coupled to the olefinic methyl and to the methylene at δ 1.98 (H-2), which, in turn, was coupled to nonequivalent methylene H-1 (δ 1.05, 0.90). The latter two protons were coupled to a methine proton at δ 1.40 (H-10). The remaining COSY data allowed generation of the spin system delineated by H-6/H-7/H-8/H-22 and H-11/H-12. HMBC correlations observed between the H-23 methyl protons (δ 1.00) and the carbons observed at δ 44.6 (C-10), 38.7 (C-9), 37.4 (C-8), and 35.8 (C-11) defined the connection between the heterocyclic region and the rigid part of molecule. Other HMBC correlations reported in Table 1, and the biosynthetic context of this family of compounds allowed us to propose the structure **4a** without stereochemical implications.

The relative stereochemistry of the carbobicyclic part of **4a** was deduced by a NOESY spectrum, which exhibited the presence of NOEs indicating that the Me-5 (δ 1.13) and Me-9 (δ 1.00) are oriented on the same side (α) of the molecule, while H-10 has the same orientation (β) as Me-8. The stereochemistry at C-16 was deduced from the magnitude of the coupling constants of H-16 in the ^{1}H NMR spectrum of **4b**. Because it exhibits coupling constants of 10.5, 4.2, and 1.5 Hz (the last attributable to the long-range coupling with H-18 and/or H-25), only an axial position of H-16 is in accordance with the Karplus dihedral-angles relationship.

The new compound **4a** is closely related structurally, including the relative stereochemistry, to both cacospongionolide B (**2a**) and cacospongionolide (**3a**). The difference observed in the ^{1}H NMR chemical shifts of the carbinyl proton (H-25) of the two diastereoisomeric acetates of the cacospongionolides and the possibility to recognize the relative configuration of each acetate allowed us to determine the absolute stereochemistry of cacospongionolides (**2a**−**4a**) by means of the modified Mosher's method.[16,17]

The relative configuration at the chiral centers (C-4*R**, C-5*R**, C-8*R**, C-9*R**, C-10*S**, C-16*S**, C-25*S**) of the major acetate of cacospongionolide (**3b**) was established by X-ray analysis.[18] The carbinyl proton (H-25) of each major acetate (**2b**−**4b**) of cacospongionolides shows a chemical shift at δ 6.95, while the H-25 of each minor acetate derivative (**2c**−**4c**), having the opposite relative stereochemistry at C-25, was deshielded at δ 7.05.

Each cacospongionolide (**2a**−**4a**) was treated with an excess of (*R*)- and (*S*)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride (MTPA-Cl) to yield pairs of (*S*)- and (*R*)-MTPA esters (**2d**−**4d**, **2e**−**4e**), that were separated by Si gel chromatography. The (*S*)-configuration at C-25 of the MTPA esters **2d**−**4d** and the (*R*)-configuration at C-25 of MTPA esters **2e**−**4e**, respectively, were easily deduced by the $\Delta\delta$ values in their

Table 2. Effect of Cacospongionolides on Different $sPLA_2$ Activities[a]

compound	*N. naja* venom %I (10 μM)	pancreas %I (10 μM)	IC_{50} (μM)	human synovial %I (10 μM)	IC_{50} (μM)	RAP + zymosan %I (10 μM)	IC_{50} (μM)	bee venom %I(10 μM)	IC_{50} (μM)
cacospongionolide B (**2a**)	0.0 ± 0.0	64.2 ± 2.1^b	4.0	86.7 ± 2.5^b	4.3	36.9 ± 1.4^b	N.D.	35.4 ± 1.2^b	N.D.
cacospongionolide (**3a**)	3.5 ± 1.6	14.4 ± 1.2		90.7 ± 1.3^b	3.0	21.8 ± 3.0		96.3 ± 0.6^b	2.3
cacospongionolide E (**4a**)	0.0 ± 0.0	5.3 ± 4.4		96.7 ± 0.4^b	1.4	65.1 ± 4.2^b	7.8	94.8 ± 1.1^b	2.8
manoalide (**1**)	17.0 ± 1.7^c	32.3 ± 2.7^b	N.D.	93.2 ± 0.2^b	3.9	38.4 ± 0.5^b	N.D.	62.5 ± 3.8^b	7.5

[a] IC_{50} values were determined for those compounds that reach 50% inhibition at 10 μM. Mean $\pm$ S.E.M. ($n = 6$). N. D. = not determined. [b] $p < 0.01$. [c] $p < 0.05$.

respective structures. Because the C-25*S* configuration of the MTPA esters of the cacospongionolides is related to the C-25*R* configuration of the corresponding major acetate, we can establishe the absolute configuration of all the cacospongionolides. The structure of cacospongionolide B is that shown in **2a**: 5*R*, 8*S*, 9*S*, 10*R*, 16*R*; cacospongionolide (**3a**) is 4*S*, 5*S*, 8*S*, 9*S*, 10*R*, 16*R*, and cacospongionolide E (**4a**) is 5*R*, 8*S*, 9*S*, 10*R*, 16*R*. These results are in accord with those obtained on the basis of CD spectra.[15]

This series of compounds was tested for their inhibitory effects on secretory PLA_2 ($sPLA_2$) belonging to the groups I (*Naja naja* venom and porcine pancreatic enzymes), II (human synovial recombinant and rat airpouch secretory enzymes), and III (bee venom enzyme). All the cacospongionolides preferentially inhibited bee venom and human synovial PLA_2 in the micrometer range (Table 2). Cacospongionolide E, however, was the most potent inhibitor toward human synovial PLA_2, showing higher potency than the reference compound manoalide. As has been suggested,[8,19] our results confirm that the pyranofuranone part interacts with PLA_2 enzymes but that the hydrophobic region of the molecule, which can be partially linear (manoalide) or cyclic (cacospongionolides) may facilitate this interaction. None of the compounds tested showed cytotoxicity effects on human neutrophils in the micrometer range (data not shown).

Cacospongionolide E showed high cytotoxicity (LC_{50} 1.29 μg/mL) in the *Artemia salina* bioassay[20,21] and a moderate toxicity (LC_{50} 1.01 μg/mL) in the fish lethality assay.[22]

Experimental Section

General Experimental Procedures. General experimental procedures were as previously reported.[10] For the biological assays, the results are presented as mean $\pm$ S. E. M. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations ($n = 6$). The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

Chemicals and Enzymes. Human synovial recombinant PLA_2 was kindly provided by Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK. [9,10-^{3}H]Oleic acid was purchased from Du Pont (Itisa, Madrid, Spain). The rest of the reagents were from Sigma Chemical Company (St. Louis, MO). *E. coli* strain CECT 101 was a gift from Prof. Uruburu, Department of Microbiology, University of Valencia, Spain.

Biological Material. *Fasciospongia cavernosa* (order Dictyoceratida; family Thorectidae) collected in June 1996, in the bay of Naples (Italy) at a depth of 8 m, was frozen at −20° until extracted. It was identified by Prof. R. Pronzato of the Istituto di Zoologia dell'Università di Genova, Italy. A voucher specimen is maintained in the Arco Felice institute collection (voucher no. S6C/96/B).

Extraction and Isolation of Cacospongionolide E. The frozen sponge (130 g dry wt after extraction) was extracted with Me_2CO and, after elimination of the solvent in vacuo, the aqueous residue was extracted with Et_2O and then with *n*-BuOH. The Et_2O extract was evaporated in vacuo to obtain a brown oil (6.1 g) that was applied on a column of Si gel. The column was eluted with a solvent gradient system from petroleum ether (40−70 °C) to Et_2O. Fractions, eluted with petroleum ether−Et_2O (7:3), exhibiting a similar TLC profile were combined to obtain cacospongionolide B and a mixture of two isomers that was separated by preparative HPLC (Spherisorb S5ODS2; CH_3CN; flow 3 mL/min) to give cacospongionolide B (**2a**, 500 mg, total amount), which crystallized from MeOH, and cacospongionolide E (**4a**, 60 mg) as an amorphous solid.

Cacospongionolide B (2a): mp 116−118 °C; $[\alpha]_D$ +28.3° (*c* 2.5, $CHCl_3$); UV, IR, MS, and ^{1}H and ^{13}C NMR data in agreement with those of authentic sample.

Cacospongionolide E (4a): $[\alpha]_D$ −85.4° (*c* 0.2, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 223 (3.64) nm; IR ($CHCl_3$) ν_{max} 3380 (br), 1785, 1760 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), Table 1. Cross peaks were observed in a NOESY spectrum between the following signals δ 1.13−1.00 (H_3-21, H_3-23) and 1.40−0.84 (H-10, H_3-22), the ^{1}H NOESY spectrum was recorded at 500 MHz; only cross peaks not sensitive to strong filtering are reported; EIMS (70 eV) *m*/*z* 400 $[M]^+$ (8), 382 $[M - H_2O]^+$ (12), 367 (4), 208 (22), 205 (12), 195 (25), 192 (70), 191 (90), 190 (20), 189 (100), 177 (15); HREIMS *m*/*z* 400.2638 (calcd for $C_{25}H_{36}O_4$, 400.2635).

Acetylation of Cacospongionolide E (4a). A solution of cacospongionolide E (**4a**, 25 mg) in pyridine (3 mL) and Ac_2O (0.5 mL) was kept at room temperature overnight. The excess reagents were removed in vacuo, and the residue was partioned between H_2O and Et_2O. The ether extracts were dried over anhydrous Na_2SO_4, and the solvent was evaporated to obtain a mixture of acetates **4b** and **4c** (24 mg), which were separated by Si gel column, petroleum ether−Et_2O (3:2) as eluent, to obtain acetate **4b** (14 mg) and acetate **4c** (9 mg).

Acetate 4b: amorphous solid; UV(MeOH) λ_{max} (log ϵ) 207 (3.87) nm; IR ($CHCl_3$) ν_{max} 1787, 1748 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.95 (1H, br s, H-25), 6.17 (1H, br s, H-18), 5.54 (1H, br d, $J = 3.8$ Hz, H-14), 5.33 (1H, H-3), 4.34 (1H, ddd, $J = 10.5$, 4.2, 1.5 Hz, H-16), 4.18 (2H, ABq, $J = 15.7$, H-24), 2.17 (3H, s, COCH_3), 1.64 (3H, br s, H-20), 1.13 (3H, s, H-21), 1.01 (3H, s, H-23), 0.84 (3H, d, $J = 6.9$ Hz, H-22); ^{13}C NMR ($CDCl_3$) δ 169.3 (s, *C*OCH_3), 168.8 (s, C-19), 166.3 (s, C-17), 142.0 (s, C-4),

138.6 (s, C-13), 122.4 (d, C-3), 117.9 (d, C-18), 115.5 (d, C-14), 92.2 (d, C-25), 69.5 (d, C-16), 68.4 (t, C-24), 44.6 (d, C-10), 38.7 (s, C-9), 38.6 (s, C-5), 37.4 (d, C-8), 35.8 (t, C-11), 32.3 (t, C-6), 29.7 (t, C-15), 27.4 (q, C-21), 27.2 (t, C-7), 26.7 (t, C-12), 26.2 (q, C-23), 25.8 (t, C-2), 20.7 (q, CO*C*H$_3$), 20.0 (t, C-1), 19.3 (q, C-20), 15.4 (q, C-22); EIMS (70 eV) *m/z* 442 [M]$^+$ (8), 382 [M − HAc]$^+$ (15), 205 (17), 192 (100), 191 (85), 189 (85), 177 (25).

Acetate 4c: oil; UV(MeOH) λ_{max} (log ϵ) 208 (3.86) nm; IR (CHCl$_3$) ν_{max} 1785, 1750 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 7.05 (1H), 6.09 (1H), 5.56 (1H, br d, *J* = 3.8 Hz), 5.33 (1H), 4.31 (1H, br dd, *J* = 10.5, 4.2 Hz), 4.12 (2H, ABq, *J* = 15.7), 2.17 (3H, s), 1.64 (3H, br s), 1.13 (3H, s), 1.01 (3H, s), 0.84 (3H, d, *J* = 7.1 Hz); ^{13}C NMR (CDCl$_3$) δ 169.5 (s, *C*OCH$_3$), 168.3 (s), 166.1 (s), 142.0 (s), 138.4 (s), 122.4 (d), 118.4 (d), 115.5 (d), 92.8 (d), 68.5 (d), 68.3 (t), 44.6 (d), 38.7 (s), 38.6 (s), 37.4 (d), 35.8 (t), 32.3 (t), 29.7 (t), 27.6 (q), 27.2 (t), 26.7 (t), 26.2 (q), 25.8 (t), 20.7 (q), 20.0 (t), 19.3 (q), 15.4 (q); EIMS (70 eV) *m/z* 442 [M]$^+$ (3), 382 [M − HAc]$^+$ (22), 205 (17), 192 (100), 191 (75), 189 (75), 177 (32).

General Procedures for the Preparation of MTPA Esters. (*R*)- or (*S*)-MTPA-Cl (Aldrich) (20 μL) was added to a solution of starting cacospongionolides **2a**−**4a** (usually 8−15 mg) in dry pyridine (0.5 mL), and the resulting mixture was kept at room temperature for 2 h. After the removal of the solvent, under vacuum, the residue was chromatographed on Si gel column eluted with petroleum ether−Et$_2$O (3:2) to give the two C-25 diastereoisomer Mosher esters, paying attention that (*R*)-MTPA-Cl gives (*S*)-MTPA ester and vice versa. Only chemical shifts of heterocyclic region are reported because all other protons are remote from the MTPA group, so that their $\Delta\delta$ values are zero, and their chemical shifts were reported earlier.[10,14]

(*R*)-MTPA Esters 2d, 2e. Cacospongionolide B (**2a**) (10 mg) was esterified according to the general procedure to give (*R*)-MTPA ester **2d** (5 mg) and (*R*)-MTPA ester **2e** (3 mg).

(*R*)-MTPA esters 2d: ^{1}H NMR (CDCl$_3$) δ 7.10 (1H, br s, H-25), 6.15 (1H, br s, H-18), 5.55 (1H, br d, *J* = 3.8 Hz, H-14), 4.43 (1H, ddd, *J* = 10.5, 4.2, 1.5 Hz, H-16), 4.12 (2H, ABq, *J* = 15.7 Hz, H-24).

(*R*)-MTPA esters 2e: ^{1}H NMR (CDCl$_3$) δ 7.24 (1H, br s, H-25), 6.00 (1H, br s, H-18), 5.38 (1H, br d, *J* = 3.8 Hz, H-14), 3.95 (1H, br dd, *J* = 10.5, 4.2 Hz, H-16), 3.87 (2H, br s, H-24).

(*S*)-MTPA Esters 2d, 2e. Cacospongionolide B (**2a**) (12 mg) was esterified according to the general procedure to give (*S*)-MTPA ester **2d** (7 mg) and (*S*)-MTPA ester **2e** (3 mg).

(*S*)-MTPA esters 2d: ^{1}H NMR (CDCl$_3$) δ 7.10 (1H, br s, H-25), 6.15 (1H, br s, H-18), 5.38 (1H, br d, *J* = 3.8 Hz, H-14), 4.05 (1H, dd, *J* = 10.5, 4.2 Hz, H-16), 4.00 (2H, br s, H-24).

(*S*)-MTPA esters 2e: ^{1}H NMR (CDCl$_3$) δ 7.24 (1H, br s, H-25), 6.00 (1H, br s, H-18), 5.55 (1H, br d, *J* = 3.8 Hz, H-14), 4.30 (1H, br dd, *J* = 10.5, 4.2 Hz, H-16), 4.08 (2H, ABq, *J* = 15.7 Hz, H-24).

(*R*)-MTPA Esters 3d, 3e. Cacospongionolide (**3a**) (available from previous studies[14]) (9 mg) was esterified according to the general procedure to give (*R*)-MTPA ester **3d** (4 mg) and (*R*)-MTPA ester **3e** (3 mg).

(*R*)-MTPA esters 3d: ^{1}H NMR (CDCl$_3$) δ 7.10 (1H, br s, H-25), 6.15 (1H, br s, H-18), 5.55 (1H, br d, *J* = 3.8 Hz, H-14), 4.43 (1H, ddd, *J* = 10.5, 4.2, 1.5 Hz, H-16), 4.11 (2H, ABq, *J* = 15.7 Hz, H-24).

(*R*)-MTPA esters 3e: ^{1}H NMR (CDCl$_3$) δ 7.24 (1H, br s, H-25), 6.00 (1H, br s, H-18), 5.38 (1H, br d, *J* = 3.8 Hz, H-14), 3.95 (1H, br dd, *J* = 10.5, 4.2 Hz, H-16), 3.86 (2H, br s, H-24).

(*S*)-MTPA Esters 3d, 3e. Cacospongionolide (**3a**) (12 mg) was esterified according to the general procedure to give (*S*)-MTPA ester **3d** (6.5 mg) and (*S*)-MTPA ester **3e** (3 mg).

(*S*)-MTPA esters 3d: ^{1}H NMR (CDCl$_3$) δ 7.10 (1H, br s, H-25), 6.15 (1H, br s, H-18), 5.37 (1H, br d, *J* = 3.8 Hz, H-14), 4.05 (1H, dd, *J* = 10.5, 4.2 Hz, H-16), 4.00 (2H, br s, H-24).

(*S*)-MTPA esters 3e: ^{1}H NMR (CDCl$_3$) δ 7.24 (1H, br s, H-25), 6.00 (1H, br s, H-18), 5.55 (1H, br d, *J* = 3.8 Hz, H-14), 4.31 (1H, br dd, *J* = 10.5, 4.2 Hz, H-16), 4.10 (2H, ABq, *J* = 15.7 Hz, H-24).

(*R*)-MTPA Esters 4d, 4e. Cacospongionolide E (**4a**) (8 mg) was esterified according to the general procedure to give (*R*)-MTPA ester **4d** (3.5 mg) and (*R*)-MTPA ester **4e** (2.5 mg).

(*R*)-MTPA esters 4d: ^{1}H NMR (CDCl$_3$) δ 7.10 (1H, br s, H-25), 6.15 (1H, br s, H-18), 5.55 (1H, br d, *J* = 3.8 Hz, H-14), 4.44 (1H, ddd, *J* = 10.5, 4.2, 1.5 Hz, H-16), 4.10 (2H, ABq, *J* = 15.7 Hz, H-24).

(*R*)-MTPA esters 4e: ^{1}H NMR (CDCl$_3$) δ 7.24 (1H, br s, H-25), 6.00 (1H, br s, H-18), 5.38 (1H, br d, *J* = 3.8 Hz, H-14), 3.95 (1H, br dd, *J* = 10.5, 4.2 Hz, H-16), 3.88 (2H, br s, H-24).

(*S*)-MTPA Esters 4d, 4e. Cacospongionolide E (**4a**) (8 mg) was esterified according to the general procedure to give (*S*)-MTPA ester **4d** (4.5 mg) and (*S*)-MTPA ester **4e** (1.5 mg).

(*S*)-MTPA esters 4d: ^{1}H NMR (CDCl$_3$) δ 7.10 (1H, br s, H-25), 6.15 (1H, br s, H-18), 5.39 (1H, br d, *J* = 3.8 Hz, H-14), 4.05 (1H, dd, *J* = 10.5, 4.2 Hz, H-16), 4.00 (2H, br s, H-24).

(*S*)-MTPA esters 4e: ^{1}H NMR (CDCl$_3$) δ 7.24 (1H, br s, H-25), 6.00 (1H, br s, H-18), 5.55 (1H, br d, *J* = 3.8 Hz, H-14), 4.31 (1H, br dd, *J* = 10.5, 4.2 Hz, H-16), 4.09 (2H, ABq, *J* = 15.7 Hz, H-24).

Determination of PLA$_2$ Activity. sPLA$_2$ was assayed by using a modification of the method of Franson et al.[23] *E. coli* strain CECT 101 was seeded in medium containing 1% tryptone, 0.5% NaCl, and 0.6% NaH$_2$PO$_4$, pH 5.0, and grown for 6−8 h at 37 °C in the presence of 5 μCi/mL [^{3}H]oleic acid (specific activity 10 Ci/mmol). After centrifugation at 2500 × *g* for 10 min, the cells were washed in buffer (0.7 M Tris−HCl, 10 mM CaCl$_2$, 0.1% bovine serum albumin, BSA, pH 8.0), resuspended in saline, and autoclaved for 30−45 min. At least 95% of the radioactivity was incorporated into phospholipids. *Naja naja* venom enzyme, porcine pancreatic enzyme, human recombinant synovial enzyme, and bee venom enzyme were diluted in 10 μL of 100 mM Tris-HCl, 1 mM CaCl$_2$ buffer, pH 7.5. Supernatants (10 μL) of exudates from zymosan-injected rat air pouch[24] were also used as a source of sPLA$_2$. Enzymes were preincubated at 37 °C for 5 min with 2.5 μL of test compound solution or its vehicle in a final volume of 250 μL. Incubation proceeded for 15 min in the presence of 10 μL of autoclaved oleate-labeled membranes and was

terminated by addition of 100 μL ice-cold solution of 0.25% BSA in saline to a final concentration of 0.07% w/v. After centrifugation at 2500 $\times$ *g* for 10 min at 4 °C, the radiactivity in the supernatants was determined by liquid scintillation counting.

Cytotoxicity Assays. The mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan[25] was used to assess the possible cytotoxic effects of test compounds on human neutrophils.

Brine Shrimp Lethality Assay. A brine shrimp (*Artemia salina)* lethality assay, performed as previously described,[20,21] gave LC_{50} 1.29 μg/mL (1.94/0.82, 95% confidence limits).

Fish Lethality Assay. A fish lethality assay using *Gambusia affinis* performed as previously described[22] gave LC_{50} 1.01 μg/mL (3.59/0.30, 95% confidence limits).

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