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Coscinolactams A and B: new nitrogen-containing sesterterpenoids from the marine sponge *Coscinoderma mathewsi* exerting anti-inflammatory properties

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ABSTRACT

Investigation of the marine sponge *Coscinoderma mathewsi* led to the isolation of two novel nitrogen-containing cheilanthane sesterterpenoids, coscinolactams A and B, together with known suvanine. The structures were elucidated by extensive spectroscopic measurements including NOE experiments to deduce the stereochemistry. The natural compounds, as well as a semisynthetic derivative, showed moderate anti-inflammatory activity measured as their capability to inhibit PGE_2 and NO production. The suvanine aldehyde derivative **4** inhibited inducible nitric oxide protein expression with an IC_{50} value of 7.3 μM.

1. Introduction

Marine sponges of the *Coscinoderma* genus have been a source of sulfated terpenoids often characterized by unusual biological effects. As examples halisulfates 1[1] and 7[2] were isolated from a Micronesia collection of the sponge. Halisulfate 7 is an inhibitor of the catalytic subunits of the mammalian Ser/Thr protein phosphatases calcineurin, PP-1, and PP-2A,[3] whereas halisulfate 1 is a potent isocitrate lyase inhibitor.[4] Coscinosulfate, a sesquiterpene sulfate from the New Caledonian sponge *Coscinoderma mathewsi*, displays significant inhibitory activity towards CDC25A phosphatase.[5] The tricyclic sesterterpene suvanine[6] is a thrombin and trypsin inhibitor[1] and shows moderate antimycobacterial activity.[7]

In the course of a project directed to the chemical investigation and evaluation of the marine invertebrates of South Pacific we had the opportunity to study the sponge *C. mathewsi* Lendenfield (order Dictyoceratida, family Spongiidae) collected in Solomon Islands, whose crude ethanolic extract exhibited an anti-PLA_2 activity (72% inhibition at 400 μg/mL). The purification of the polar extracts afforded two new nitrogen-containing cheilanthane sesterterpenoids, coscinolactams A (**1**) and B (**2**), together with the known suvanine (**3**) (Fig. 1). Herein we report the isolation and structure elucidation of these compounds, as well as the preparation of two suvanine semisynthetic derivatives **4** and **5**. The in vitro pharmacological evaluation of **1** and **3**–**5** on the inhibition of four different secretary PLA_{2s} ($sPLA_2$), belonging to groups I (*Naja naja* venom and porcine pancreatic enzymes), II (human synovial recombinant enzyme) and III (bee venom enzyme), as well as on NO and PGE_2 production from macrophage line RAW 264.7, showed that the suvanine aldehyde derivative **4** exerted an interesting anti-inflammatory profile mainly through the inhibition of inducible nitric oxide expression.

2. Results and discussion

The lyophilized sponge (322 g) was extracted with MeOH, and the combined extracts were fractionated according to the Kupchan partitioning procedure.[8] The bioactive chloroform extract was purified by DCCC ($CHCl_3$/MeOH/H_2O ascending mode), followed by reverse-phase HPLC (MeOH aqueous 65%) to give suvanine (**3**).

The more polar coscinolactams A (**1**) and B (**2**) were obtained from the butanol extract chromatographed by DCCC and reverse-phase HPLC.

Coscinolactam A (**1**) was obtained as amorphous solid, $[\alpha]_D$ +25.7. The molecular formula was determined to be $C_{27}H_{41}NO_7S$ by HRESIMS (*m*/*z* 522.2547 $[M-H]^-$). The gross structure of **1** was deduced from detailed analysis of the ^1H and ^{13}C NMR spectroscopic data (Table 1) aided by 2D NMR experiments (^1H–^1H COSY, TOCSY, HSQC and HMBC). The NMR spectroscopic data showed the signals for two acyl carbons at δ 176.3 and 173.7, two trisubstituted

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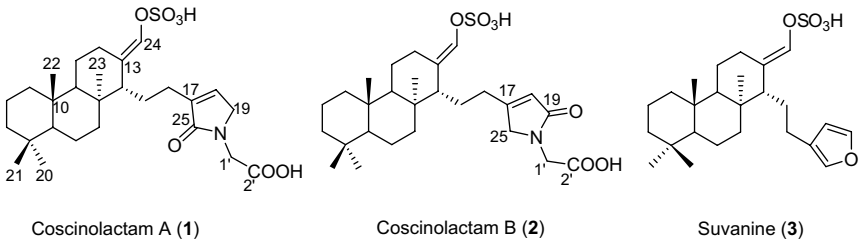


Figure 1. Cheilanthane sesterterpenoids isolated from the marine sponge *C. mathewsi*.

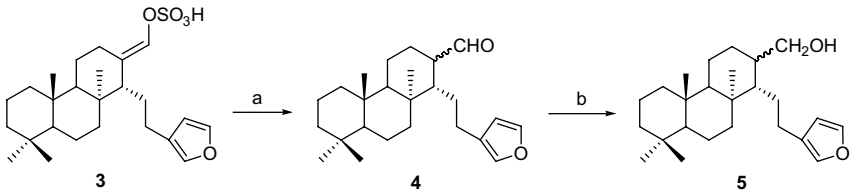
double bonds [δ_C 125.2 (s), 140.5(s), 133.9 (d), 137.8 (d); δ_H 6.89 (1H, s) and 6.36 (1H, s)] and two diastereotopic methylenes linked to a nitrogen atom [δ_H 4.00 (1H, d, J=15.9 Hz)–4.12 (1H, d, J=15.9 Hz), δ_C 52.8 and δ_H 3.89 (1H, d, J=16.9 Hz)–4.15 (1H, d, J=16.9 Hz), δ_C 46.8]. The remaining signals are at higher field and correspond to four methyls on quaternary carbons, nine methylenes, three methines and three quaternary carbons. Comparison with the NMR spectroscopic data of suvanine clearly indicated that **1** possesses the same tricyclic spongiane-like framework of suvanine. The enol sulfate functionality at C-13, characteristic of suvanine, was also present, as evidenced by ^{13}C NMR spectroscopic data and by the presence of the diagnostic ^{1}H NMR signal at δ 6.36 relative to H-24. COSY and HMBC data revealed the linkage of the C-14 to the methylene at C-15 (δ_H 1.47, 1.91, δ_C 23.6) and the allylic methylene C-16 (δ_H 2.22, 2.51, δ_C 25.9). HMBC correlations of H-18 to C-17 (δ_C 140.5), C-19 (δ_C 52.8) and C-25 (δ_C 173.7) and chemical shift of C-19 (δ_C 52.8) revealed the presence of an α,β-unsaturated γ-lactam ring (carbon atoms in **1**: C-17 to C-19, C-25 and N-25). The linkage between C-16 and C-17 was inferred by key HMBC correlation H-18 to C-16, and H_2-16 to C-25. The remaining set of resonances, a methylene (δ_H 3.89 and 4.15, δ_C 46.8) and an acyl carbonyl (δ_C 176.3), was easily assigned to a glycine residue (C-1′ to C2′). The linkage of this latter unit to N-25 was evidenced by HMBC correlations H_2-1′ to C-19 and C-25. Therefore the structure of coscinolactam A was defined as **1**. The relative stereochemistry of the tricyclic system was determined by NOESY correlations. In particular, intense NOESY cross-peaks between CH_3-22 and CH_3-21, H-12 and H-14 suggested a tricyclic system with an AB trans, BC cis stereochemistry. The stereochemistry of the exocyclic double bond was established as *E* on the basis of the observed NOE contact between H-24 (δ_H 6.36) and one of the protons belonging to diastereotopic methylene at C-15 (δ_H 1.47). The good match with the ^{1}H and ^{13}C NMR resonances of coscinolactam A with the corresponding resonances of the tricyclic portion of suvanine indirectly confirmed the proposed structure.

Coscinolactam B (**2**) was isomeric with coscinolactam A (*m*/*z* 522.2509 [M−H]$^-$). ^{1}H and ^{13}C NMR spectra of **2** were very similar to those of **1** except for the signals relative to the α,β-unsaturated γ-lactam ring. In particular the upfield shift of the olefinic proton (δ_H 5.85 in **2** vs 6.89 in **1**) and the downfield shift of the γ-carbon (δ_C 56.3 in **2** vs 52.8 in **1**) suggested the presence of a β-substituted α,β-unsaturated γ-lactam ring. The linkage of the β-carbon of the lactam ring to C-16 was also confirmed by the diagnostic HMBC correlation between H_2-16 and C-25.

Table 1
^{1}H and ^{13}C NMR spectroscopic data (700 MHz, CD_3OD) for coscinolactams A (**1**) and B (**2**)

	1			**2**		
	δ_H[a]	δ_C	HMBC	δ_H[a]	δ_C	HMBC
1	1.86 ovl, 0.87 dd (14.0, 7.4)	43.2		1.86 ovl, 0.87	43.1	
2	1.63 ovl, 1.40 ovl	19.7		1.62 ovl, 1.40 ovl	19.6	
3	1.40 ovl, 1.16 m	43.0		1.40 ovl, 1.16 m	43.0	
4	—	34.3		—	34.0	
5	1.06 dd (9.0, 4.4)	54.2	C1, C4, C6, C10, C21,	1.08 dd (11.2, 4.6)	54.1	C1, C4, C6, C21
6	1.54 m, 1.33 ovl	19.3		1.60 ovl, 1.34 ovl	19.3	
7	1.90 ovl, 1.33 ovl	36.3	C5, C6, C8, C9, C23	1.90 ovl, 1.37 ovl	36.1	
8	—	39.9		—	39.7	
9	0.91 t (7.6)	58.6	C1, C8, C11, C12, C22, C23	0.92 t (6.0)	58.3	C1, C8, C11, C12, C22, C23
10	—	40.2		—	40.1	
11	1.70 m, 1.61 ovl	20.9		1.71 m, 1.62 ovl	20.9	
12	2.60 dt (15.9, 6.1), 2.18 m	25.4	C9, C11, C13, C14, C24	2.59 m, 2.18 dt (16.2, 8.5)	25.2	C9, C11, C13, C14, C24
13	—	125.2		—	124.9	
14	2.35 d (10.4)	43.9	C8, C13, C15, C16, C23, C24	2.33 d (10.4)	43.9	C8, C13, C15, C16, C23, C24
15	1.91 ovl, 1.47 m	23.6		1.89 ovl, 1.57 ovl	23.7	
16	2.51 m, 2.22 m	25.9	C14, C15, C17, C18, C25	2.62 m, 2.36 dt (16.1, 8.1)	29.1	C14, C15, C17, C18, C25
17	—	140.5		—	164.2	
18	6.89 s	137.8	C16, C17, C19, C25	5.85 s	121.3	C16, C17, C19, C25
19	4.12 d (15.9), 4.00 d (15.9)	52.8	C17, C18	—	174.0	
20	0.86 s	33.7	C3, C4, C5, C21	0.87 s	33.8	C3, C4, C5, C21
21	0.89 s	22.2	C3, C4, C5, C20	0.89 s	22.1	C3, C4, C5, C20
22	1.05 s	18.5	C1, C5, C9, C10	1.05 s	18.5	C1, C5, C9, C10
23	0.84 s	26.5	C7, C8, C9, C14	0.86 s	26.5	C7, C8, C9, C14
24	6.36 s	133.9	C12, C13, C14	6.27 s	133.3	C12, C13, C14
25	—	173.7		4.18 d (16.8), 4.20 d (16.8)	56.3	C17, C18, C19,
1′	4.15 d (16.9), 3.89 d (16.9)	46.8	C19, C25, C2′	3.82 d (17.1), 3.84 d (17.1)	39.5	C19, C25
2′	—	176.3		—	n.o	

Ovl: overlapped.
[a] Coupling constants are in parentheses and given in hertz. ^{1}H and ^{13}C assignments aided by COSY, TOCSY, HSQC and HMBC experiments.



Scheme 1. (a) dioxane/pyridine, 180 °C; (b) $NaBH_4$, MeOH, rt.

Table 2
Inhibitory activity (% I) of compounds **1** and **3**–**5** at 10 μM on different $sPLA_2$ belonging to the groups IIA, IA (*Naja naja* venom), IB (porcine pancreatic enzyme) and III (bee venom enzyme)

Compound (10 μM)	GroupIIA-$sPLA_2$, % inhibition	GroupIA-$sPLA_2$, % inhibition	GroupIB-$sPLA_2$, % inhibition	GroupIII-$sPLA_2$, % inhibition
1	6.7±1.6	0.0±0.0	0.0±0.0	0.0±0.0
3	32.8±1.3**	0.0±0.0	0.0±0.0	29.4±3.0**
4	10.6±5.3	0.0±0.0	0.0±0.0	0.0±0.0
5	7.6±4.0	0.0±0.0	0.0±0.0	0.0±0.0
LY311727	96.3±1.7**	7.9±5.6	36.9±11.0**	2.4±1.8

Results show mean±S.E.M. (n=6). Statistical significances: **$p<0.01$, with respect to the corresponding enzyme control group (IIA $sPLA_2$=12,129±384 cpm; IA $sPLA_2$=10,973±350 cpm; IB $sPLA_2$=8008±47 cpm; III $sPLA_2$=14,854±1054 cpm). Enzyme control group contains the vehicle (ethanol 1%). LY311727 used as control.

Whereas lactam systems are quite unusual in natural products, a similar system consisting of an aminoacyl-derived unit fused with a terpenoid system was found in echinophyllins A[9] and D[10] clerodane diterpenoids from the Brazilian medicinal plant *Echinodorus macrophyllus*. ^1H and ^{13}C NMR spectroscopic data of coscinolactams A and B match very well with those of echinophyllins A and D relative to the isomeric γ-lactam ring subunits. A glycine-derived lactam system was found in spongolactam C, a diterpene derivative isolated from the Okinawan marine sponge *Spongia* sp.[11]

Table 3
Inhibitory activity of compounds **1** and **3**–**5** at 10 μM on the production of PGE_2 and NO in LPS-stimulated RAW 264.7 cells

Compound (10 μM)	PGE_2 % inhibition	NO % inhibition
1	48.5±3.0**	31.8±4.0**
3	41.6±4.6**	21.0±5.7
4	49.2±4.1**	60.0±8.1**
5	14.9±2.1**	31.9±2.8**
Dexamethasone (1 μM)	78.7±5.4**	96.6±1.4**

Results show mean±SEM (n=6). Statistical significances: **$p<0.01$, with respect to the LPS-stimulated control group (contains the vehicle ethanol 1%). PGE_2 (non-stimulated cells=0.6±0.2 ng/mL; LPS-stimulated cells=16.0±1.6 ng/mL). Nitrite (non-stimulated cells=48.8±3.6 ng/mL; LPS-stimulated cells=414.1±16.4 ng/mL). Dexamethasone used as control.

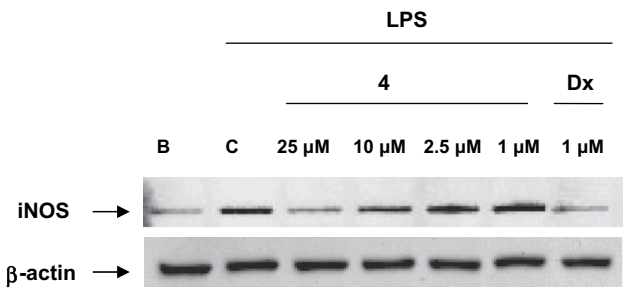


Figure 2. Effect of compound **4** on iNOS expression in LPS-stimulated RAW 264.7 cells. The figure is representative of two similar experiments; B: normal cells; C: LPS-stimulated cells. Dx=Dexamethasone.

Biogenetically the isomeric coscinolactams A and B may be derived from suvanine, through oxidation to isomeric γ-hydroxybutenolide derivatives, condensation with glycine and reduction to γ-lactam, which has been chemically explored in the semisynthesis of spongolactam C from the corresponding furanoterpene.[11]

To investigate the role of the sulfate group on the pharmacological profile of these natural compounds, major suvanine was subjected to hydrolytic and reductive transformations depicted in Scheme 1.

Compounds **1** and **3**–**5** were tested against four different secretory PLA_2 (belonging to groups IA, IB, IIB and III) (Table 2). LY311727, a well known inhibitor of group IIA $sPLA_2$, was used as a reference tool.[12] Despite the good activity previously exhibited by the crude extract, only suvanine showed a moderate activity against human synovial $sPLA_2$-IIA and bee venom $sPLA_2$-III. In addition, compounds **1** and **3**–**5** were devoid of significant cytotoxic effects on RAW 264.7 at concentrations up to 10 μM, as assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (data not shown). Their ability to inhibit PGE_2 and NO production on RAW 264.7 cells stimulated with LPS was also investigated (Table 3). Interestingly, the suvanine aldehyde derivative **4** showed an improved capability to inhibit NO production and to a lesser extent PGE_2, whereas the suvanine alcoholic derivative **5** was proved to be almost inactive in all experiments. Compound **4** inhibited dose-dependently the production of NO, showing an IC_{50} value of 7.3±2.3 μM. The NO reduction was the consequence of the inhibition of the expression of inducible NO synthase (Fig. 2), exactly as the dexamethasone control drug did. This suvanine aldehyde derivative **4**, which can mainly reduce NO and PGE_2 production by affecting the expression of the inducible NO synthase enzyme, could be an interesting strategy to obtain promising anti-inflammatory agents.

3. Experimental

3.1. General experimental procedures

Specific rotations were measured on a Perkin–Elmer 243 B polarimeter. High-resolution ESI-MS spectra were performed with a Micromass QTOF Micromass spectrometer. ESI-MS experiments were performed on an Applied Biosystem API 2000 triple-quadrupole mass spectrometer. NMR spectra were obtained on Varian Inova 500 and Varian Inova 700 NMR spectrometers (^1H at 500 and 700 MHz, ^{13}C at 125 and 175 MHz, respectively) equipped with a Sun hardware, δ (ppm), J in Hz, spectra referred to CD_3OD as internal standards (δ_H=3.31). HPLC was performed using a Waters Model 510 pump equipped with Waters Rheodine injector and a differential refractometer, model 401.

All reagents were commercially obtained (Aldrich, Fluka) at the highest commercial quality and used without further purification except where noted. All reactions were monitored by TLC on silica gel plates (Macherey–Nagel).

3.2. Sponge material and separation of individual sesterterpenoids

C. mathewsi Lendenfield (order Dictyoceratida, family Spongiidae) was collected on the barrier reef of Vangunu Island, Solomon Islands, in July 2004. The samples were frozen immediately after collection and lyophilized to yield 322 g of dry mass. The sponge was identified by Dr. John Hooper, Queensland Museum, Brisbane, Australia, where a voucher specimen is deposited under the accessing number G322695.

The lyophilized material (322 g) was extracted with methanol (3×3 L) at room temperature and the crude methanolic extract (72.6 g) was subjected to a modified Kupchan's partitioning procedure as follows. The methanol extract was dissolved in a mixture of MeOH/H_2O containing 10% H_2O and partitioned against *n*-hexane. The water content (% v/v) of the MeOH extract was adjusted to 30% and partitioned against $CHCl_3$. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH. The hexane, chloroform and butanol extracts were tested against PLA_2 at 400 mg/mL dose showing 94, 93 and 59% inhibition, respectively.

The *n*-BuOH extract (4 g) was chromatographed in four runs by DCCC ($CHCl_3$/MeOH/H_2O, 7:13:8, ascending mode) and fractions of 6 mL were collected and combined on the basis of their similar TLC retention factors.

Fraction 7 (28.4 mg) was purified by HPLC on a Nucleodur C18 column (3 μm, 150×4.60 mm, flow rate 1.0 mL/min) with 45% MeOH/H_2O as eluent to give 3.1 mg of coscinolactam A (**1**) (t_R=10 min) and 1.9 mg of coscinolactam B (**2**) (t_R=11 min).

The chloroform-soluble material (1.5 g), mainly containing suvanine, was chromatographed by DCCC using $CHCl_3$/MeOH/H_2O (7:13:8) in the ascending mode (the lower phase was the stationary phase); the flow rate was 18 mL/h; 6 mL fractions were collected and combined on the basis of their similar TLC retention factors.

Fraction 2 (262 mg) was purified by HPLC on a μ-Bondapack C18 column (10 μm, 300×7.8 mm, flow rate 4.0 mL/min) with 65% MeOH/H_2O as eluent to give 240 mg of suvanine (t_R=8 min).

3.3. Characteristic data for each compound

Coscinolactam A: white amorphous solid; $[\alpha]_D^{25}$ +25.7 (*c* 0.07, methanol); ^1H and ^{13}C NMR data in CD_3OD given in Table 1; ESI-MS: *m*/*z* 522.3 $[M-H]^-$. HRMS (ESI): calcd for $C_{27}H_{40}NO_7S$: 522.2525; found 522.2547 $[M-H]^-$.

Coscinolactam B: white amorphous solid; $[\alpha]_D^{25}$ +8.57 (*c* 0.07, methanol); ^1H and ^{13}C NMR data in CD_3OD given in Table 1; ESI-MS: *m*/*z* 522.3 $[M-H]^-$. HRMS (ESI): calcd for $C_{27}H_{40}NO_7S$: 522.2525; found 522.2509 $[M-H]^-$.

Suvanine: white amorphous solid; $[\alpha]_D^{25}$ +12.2 (*c* 0.4, methanol); ESI-MS: *m*/*z* 522.3 $[M-H]^-$. HRMS (ESI): calcd for $C_{25}H_{37}O_5S$: 449.2362; found 449.2387 $[M-H]^-$.

3.4. Synthetic procedures

3.4.1. Aldehyde *4*

A solution of suvanine **3** (15 mg, 0.033 mmol) in pyridine (0.5 mL) and dioxane (0.5 mL) was heated at 150 °C for 2.5 h in a stoppered reaction vial. After the solution was cooled, the mixture was evaporated to dryness and then purified by HPLC on a μ-Bondapak C18 column (30 cm×3.9 mm i.d.) with MeOH/H_2O 80:20, to give aldehyde **4** as a 9:1 mixture of two diastereoisomers (9.2 mg, 80%). ESI-MS: 371.30 $[M+H]^+$; selected ^1H NMR (500 MHz, $CDCl_3$) for major diastereoisomer: 9.48 (1H, s, –CHO), 7.39 (1H, s), 7.28 (1H, s), 6.33 (CH-18, s), 1.04 (3H, s), 0.87 (3H, s), 0.86 (3H, s), 0.84 (3H, s).

3.4.2. Alcohol *5*

$NaBH_4$ was added in one portion to a stirred solution of **4** (5.2 mg, 0.014 mmol) in MeOH at room temperature. The mixture was stirred for 1 h and then was concentrated in vacuo and then diluted with NH_4Cl and ethyl acetate. The aqueous phase was extracted with ethyl acetate and then the combined organic extracts were washed with brine, dried (Na_2SO_4) and concentrated in vacuo to yield alcohol **5** as a 9:1 mixture of two diastereoisomers (4.7 mg, 90%). ESI-MS: 373.40 $[M+H]^+$; selected ^1H NMR (500 MHz, $CDCl_3$) for major diastereoisomer: 7.39 (1H, s), 7.28 (1H, s), 6.33 (1H, s), 3.71 (d, *J*=11.3 Hz, H-24), 3.43 (dd, *J*=11.3, 4.5 Hz, H-24), 1.13 (3H, s), 0.90 (3H, s), 0.86 (6H, s).

3.5. Anti-inflammatory assays

3.5.1. Materials

[5,6,8,11,12,14,15(*n*)-^3H] PGE_2 and [9,10-^3H]oleic acid were purchased from Amersham Biosciences (Barcelona, Spain). Inducible NO synthase specific polyclonal antiserum was purchased from Cayman Chem. (MI, USA). The peroxidase-conjugated goat anti-rabbit Immunoglobulin G (IgG) was purchased from Dako (Copenhagen, Denmark). The rest of reagents was from Sigma (MO, USA).

3.5.2. Assay of $sPLA_2$

$sPLA_2$ activity was assayed using [^3H]-oleate labelled membranes of *Escherichia coli*, following a modification of the method of Franson et al.[13,14] *E. coli* strain CECT 101 was grown for 6–8 h at 37 °C in the presence of 5 μCi/mL [^3H]-oleic acid (specific activity 10 Ci/mmol) until the end of the logarithmic phase. After centrifugation at 1800*g* for 10 min at 4 °C, the membranes were washed, resuspended in PBS and autoclaved for 30–45 min. At least 95% of the radioactivity was incorporated into the phospholipid fraction. *Naja naja* venom (Group IA $sPLA_2$), porcine pancreatic (Group IB $sPLA_2$), human recombinant synovial (Group IIA $sPLA_2$), and bee venom (Group III $sPLA_2$) enzymes were used as sources of $sPLA_2$. Enzymes were diluted in 10 μL of 100 mM Tris–HCl, 1 mM $CaCl_2$ buffer pH 7.5 and preincubated at 37 °C for 5 min with test compound or vehicle in a final volume of 250 μL. Incubation proceeded for 15 min in the presence of 20 μL of [^3H]oleic-*E. coli* 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×*g* for 10 min at 4 °C, the radioactivity in the supernatants was determined by liquid scintillation counting.

3.5.3. Culture of murine macrophage RAW 264.7

The mouse macrophage cell line RAW 264.7 (Cell Collection, Department of Animal Cell Culture, C.S.I.C., Madrid, Spain) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% foetal bovine serum. Cultures were maintained at 37 °C in 5% CO_2 (air: CO_2, 95:5) humidified incubator. Cells were resuspended at a concentration of 1.5×10^6 cells/mL.

3.5.4. Nitric oxide and PGE_2 production in RAW 264.7 macrophages

RAW 264.7 macrophages (1.5×10^6 cells/mL) were co-incubated in 96-well culture plate (200 μL) with 1 μg/mL of *E. coli* [serotype 0111:B4] lipopolysaccharide (LPS) at 37 °C for 20 h in the presence of test compounds or vehicle. Nitrite concentration as reflection of NO release was assayed fluorometrically.[15] PGE_2 levels were determined in culture supernatants by radioimmunoassay.[16] The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan[17] was used to assess the possible cytotoxic effects of compounds (100% viability=0.533±0.010 at 492 nm).

3.5.5. Western blot assay of iNOS

Cellular lysates from RAW 264.7 (murine macrophages 1.5×10^6 cell/mL) incubated for 18 h with LPS (1 μg/mL) were obtained with lysis buffer A (10 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol, 5 mM NaF, 1 mM Na_3VO_4, 10 mM Na_2MoO_4, 1 μg/mL leupeptin, 0.1 μg/mL aprotinin and 0.5 mM phenylmethyl sulfonyl fluoride). Following centrifugation (10,000×*g*/15 min/4 °C), supernatant protein was determined by the Bradford method[18] using bovine serum albumin (BSA) as standard. iNOS protein expression was studied in the total fraction. Equal amounts of protein (25 μg) were loaded on 15% SDS-PAGE and transferred onto polyvinylidene difluoride membranes for 90 min at 125 mA. Membranes were blocked in PBS (0.02 M pH 7.0)–Tween 20 (0.1%), containing 3% w/v unfatted milk and incubated with specific polyclonal antibody against iNOS (1/1000). Finally, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1/10,000). The immunoreactive bands were visualized using an enhanced chemiluminescence system (Amersham Biosciences, Barcelona, Spain).

3.5.6. Statistical analysis

The results are presented as mean±SEM, which represents the number of experiments. 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.[19]

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