Pergamon

Tetrahedron, Vol. 53, No. 21, pp. 7305-7310, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00406-7

Structure and Absolute Stereochemistry of the New Cyclolinteinol and Cyclolinteinol Acetate, Macrophage Activation Modulators

Alfonso Carotenuto[a], Ernesto Fattorusso[a]*, Virginia Lanzotti[b], Silvana Magno[a], Rosa Carnuccio[c], Fulvio D'Acquisto[c]

[a]Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli Federico II, Via D. Montesano 49, I-80131 Napoli, Italy

[b]Dipartimento di Scienze e Tecnologie Agroalimentari, Ambientali e Microbiologiche, Università degli Studi del Molise, Via Tiberio 21, I-86100 Campobasso, Italy

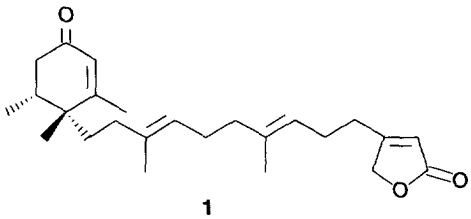
[c]Dipartimento di Farmacologia Sperimentale, Università degli Studi di Napoli Federico II, Via D. Montesano 49, I-80131 Napoli, Italy.

Abstract: Two novel sesterterpenes, cyclolinteinol (**5**) and cyclolinteinol acetate (**6**), have been isolated from the Caribbean sponge *Cacospongia* cf. *linteiformis*. Their structures and absolute stereochemistry were determined using spectroscopic and chemical methods. The new compounds (**5, 6**) and the related compounds **1-3**, previously isolated from the sponge, strongly inhibited NO production by LPS-stimulated murine macrophages J774. © 1997 Elsevier Science Ltd.

Our recent investigation of the metabolites of the Caribbean sponge *Cacospongia* cf. *linteiformis* has uncovered a number of novel sesterterpenes, most of them characterized by unprecedented bicyclic, tetracyclic and pentacyclic skeletons[1-5]. Some of these compounds could play a role as natural feeding deterrents since they showed antifeedant activity against *Carassius auratus* as well as ichthyotoxicity to *Gambusia affinis*. Further investigation on the sponge's secondary metabolites resulted in the isolation of the two novel sesterterpenes **5** and **6.** These compounds were shown to inhibit the induction of nitric oxide synthase in cultured macrophages J774 stimulated with LPS (lipopolysaccharide). It is well known that macrophages play a significant role in the host defense mechanism. Activated macrophages secrete a variety of cytostatic/cytotoxic factors affecting the growth of tumor cells and microorganisms, including NO[6]. NO is produced enzymatically by a family of enzymes named NO synthases inhibited by L-arginine analogues such as L-NMMA. The inducible NO synthase isoform is expressed to significant levels in macrophages following immunologic stimuli[7]. The NO synthesized by this enzyme is an important cytotoxic effector molecule[8] and it has been shown to be necessary to account for the macrophages microbiocidal activity[9].

The inhibitory effect on NO production by cyclolinteinol (**5**) and cyclolinteinol acetate (**6**) prompted us to investigate the effect of the other structurally related constituents **1-4** of *Cacospongia* cf. *linteiformis*[1-3].

A samples of *Cacospongia* cf. *linteiformis* was collected by hand using scuba (-9 m) along the coast of Grand Bahama Island (Bahamas). The EtOAc-soluble fraction was purified using direct and reverse phase chromatography to obtain cyclolinteinol (**5**) and cyclolinteinol acetate (**6**).



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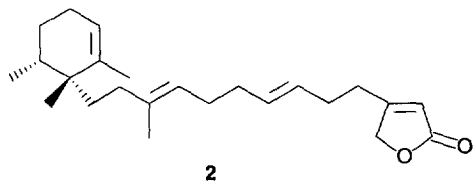

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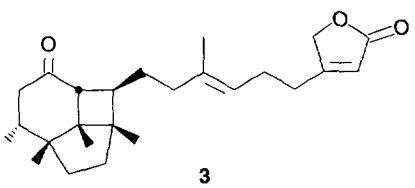

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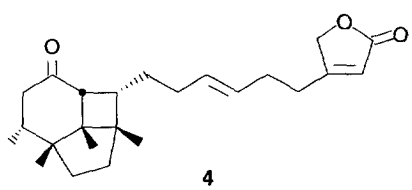

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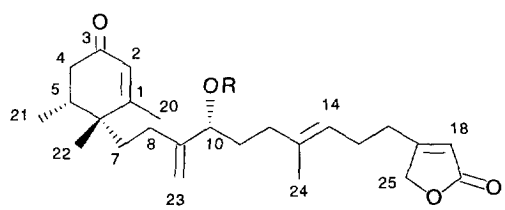

5 R= H

6 R= $COOCH_3$

7 R= *R*-MTPA

8 R= *S*-MTPA

Cyclolinteinol (**5**), isolated as a colorless oil, had the composition $C_{25}H_{40}O_4$ (HREIMS). A diagnostic peak in the EIMS at m/z 382 (M - H_2O) suggested the presence of an OH group. In addition, further evidence for the existence of an OH group in the structure of **5** came from the IR band at ν_{max} 3582, while absorptions at ν_{max} 1782 and 1749 cm^{-1} indicated the presence of the same α,β-unsaturated γ-lactone functionality already found in **1-4**. This was confirmed by ^1H [δ 5.85 (1H, m, H-18), 4.73 (2H, d, J = 1.7 Hz, H_2-25)] and ^{13}C [δ 170.1 (s, C-17), 115.6 (d, C-18), 173.6 (s, C-19), 73.1 (t, C-25)] NMR spectra. Inspection of ^1H and ^{13}C NMR data (Table I) revealed that **5** was closely related to cyclolinteinone (**1**). In particular the ^1H NMR spectrum contains distinct signals due to three methyls at δ 1.03 (d, J = 6.7 Hz, H_3-21), 1.23 (s, H_3-22), 1.95 (d, J = 1.0 Hz, H_3-20) and one vinyl proton at δ 5.88 (d, J = 1.0 Hz, H-2) indicative of the same trimethyl substituted cyclohexenone ring as in **1**. The ^{13}C NMR spectrum (Table I) confirmed this structural feature containing signals of a α,β-unsaturated β,β-dialkylketone [δ 167.9 (s, C-1), 127.3 (d, C-2), 198.7 (s, C-3)]. The most evident differences when comparing the NMR spectra of **5** with those of **1** regarded the presence of signals attributable to an exomethylene [^1H : δ 4.87 (bs, Ha-23) and 5.03 (bs, Hb-10); ^{13}C : δ 110.2 (t, C-23)] and signals due to an allylic oxymethine [^1H : δ 4.04 (m, H-10); ^{13}C : δ 75.2 (d, C-10)]. Consideration of the characteristics of this ^1H NMR spectrum and ^1H-^1H connectivities observed in 2D COSY and HOHAHA experiments established the presence of the fragment A (C-18, C-17, C-25, C-16, C-15, C-14, C-13, C-24). Further sets of COSY-derived ^1H-^1H connectivities were consistent with the fragments B (C-12, C-11, C-10), C (C-23, C-9, C-8, C-7), D (C-4, C-5, C-21) and E (C-2, C-1, C-20). Four bond couplings of H_2-16/H-18 and H_2-16/H_2-25 (segment A), H-14/H_3-24 (segment A), H-23b/H_2-8 and H-23a/H_2-8 (segment C), H-2/H_3-20 (segment E) were particularly useful to define the above segments. Unambiguous assignment of the carbon atom resonances belonging to the A-E segments was established by using a 2D HMQC NMR

experiment (see Table I). Interproton contact in a ROESY spectrum of **5** between H-5/H_3-22 and H_3-21/H_2-7 determined the relative stereochemistry at the chiral centers C-5 and C-6 as that of **1**.

Table I. ^1H and ^{13}C NMR data ($CDCl_3$) for compounds **5-6**

	5		**6**	
Pos.	δ_C (mult.)	δ_H (mult., *J* [Hz])	δ_C (mult.)	δ_H (mult., *J* [Hz])
1	167.9 (C)		167.8 (C)	
2	127.3 (CH)	5.88 (d, 1.0)	127.3 (CH)	5.89 (d, 1.0)
3	198.7 (C)		198.8 (C)	
4α	42.6 (CH_2)	2.49 (dd, 5.1, 17.7)	42.6 (CH_2)	2.50 (dd, 5.1, 17.7)
β		2.30 (dd, 9.6, 17.7)		2.29 (dd, 9.6, 17.7)
5	38.0 (CH)	2.14[a]	37.9 (CH)	2.13[a]
6	41.4 (C)		41.3 (C)	
7a	34.3 (CH_2)	1.73 (dt, 5.1, 13.2, 13.2)	34.0 (CH_2)	1.73 (dt, 5.1, 13.2, 13.2)
b		1.64[a]		1.59[a]
8	25.8 (CH_2)	2.08[a]	26.6 (CH_2)	2.00[a]
9	151.7 (C)		147.5 (C)	
10	75.2 (CH)	4.04 (m)	76.5 (CH)	5.12 (dd, 6.0, 7.5)
11	33.6 (CH_2)	1.60[a]	31.4 (CH_2)	1.78-1.68[a]
12	35.7 (C)	2.06-2.00[a]	35.3 (C)	2.00[a]
13	137.1 (C)		136.2 (C)	
14	122.3 (CH)	5.13 (dt, 1.0, 7.0, 7.0)	122.7 (CH)	5.09 (bt, 7.1, 7.1)
15	25.6 (CH_2)	2.31 (bquart., 7.0, 7.0, 7.0)	25.5 (CH_2)	2.31 (bquart., 7.1, 7.1, 7.1)
16	28.6 (CH_2)	2.46 (bt, 7.0, 7.0)	28.5 (CH_2)	2.45 (bt, 7.1, 7.1)
17	170.1 (C)		170.1 (C)	
18	115.6 (CH)	5.85 (m)	115.6 (CH)	5.85 (m)
19	173.6 (C)		173.6 (C)	
20	21.3 (CH_3)	1.95 (d, 1.0)	21.2 (CH_3)	1.94 (d, 1.0)
21	16.2 (CH_3)	1.03 (d, 6.7)	16.1 (CH_3)	1.03 (d, 6.8)
22	24.2 (CH_3)	1.23 (s)	24.2 (CH_3)	1.23 (s)
23a	110.2 (CH_2)	4.87 (bs)	111.9 (CH_2)	4.91 (bs)
b		5.03 (bs)		5.03 (bs)
24	15.9 (CH_3)	1.63 (bs)	15.9 (CH_3)	1.62 (bs)
25	73.1 (CH_2)	4.73 (d, 1.7)	69.4 (CH_2)	4.73 (d. 1.4)
26			170.2 (C)	
27			21.2 (CH_3)	2.05 (s)

[a] submerged by other signal

The absolute stereochemistry of the cyclohexenone ring was established by comparing the CD spectrum of **5** with that of cyclolinteinone (**1**). Both **1** and **5** showed positive Cotton effect with $[\theta]_{244} = +557$ and $[\theta]_{240.5} = +513$, respectively, thus determining an identical stereochemistry around the enone functionality in **1** and **5**. The absolute stereochemistry at C-10 of **5** was determined using the advanced Mosher's method[10]. The hydroxyl group of **5** was converted into both the *S*- and *R*-MTPA esters **7** and **8**, respectively, each of which was a single diastereoisomer by ^1H NMR spectroscopy, showing the enantiomeric purity of **5**; comparison of the ^1H NMR data for the MTPA esters (Table II) indicated the 10*R* absolute stereochemistry. Thus, the absolute stereochemistry of cyclolinteinol (**5**) is 5*R*, 6*R*, 10*R*.

Table II. ^{1}H NMR data for the *S*- and *R*-MTPA esters of compound **5**.

Pos.	δ_S (ppm)	δ_R (ppm)	$\Delta\delta_{S-R}$ (ppm)
H_3-20	1.87	1.88	- 0.01
H_3-22	1.14	1.18	- 0.04
H_a-23	5.02	5.09	- 0.07
H_b-23	4.93	4.98	- 0.05
H_2-11	1.81	1.76	+ 0.05
H_3-24	1.60	1.56	+ 0.04
H-14	5.06	5.03	+ 0.03
H_2-15	2.30	2.28	+ 0.02

Cyclolinteinol acetate (**6**), obtained as a colorless oil, had the molecular formula $C_{27}H_{38}O_5$ (HRMS and ^{13}C NMR data). A diagnostic peak at m/z 382 (M - AcOH) in the EI MS indicated the presence of one acetoxyl group. This functionality was also deduced from an IR band at 1745 cm^{-1}, a sharp three-proton singlet at δ 2.05 (H_3-27) in ^{1}H NMR spectrum, and a singlet at δ 170.2 (C-26) and a quartet at δ 21.2 (C-27) in the ^{13}C NMR spectrum. The IR spectrum of **6** also showed the same functionalities found for compound **5** (ν_{max} 1782, 1747 cm^{-1} for α,β-unsaturated γ-lactone and 1715 cm^{-1} for ketone). Also ^{1}H and ^{13}C NMR spectra of **6** were similar to those of **5**. An accurate comparison of ^{1}H and ^{13}C NMR data of **6** with those of **5** (see Table I) revealed that the most significant variations were found for the atoms surrounding the chiral center C-10. In particular, a significant change was observed for the H-10 proton signal of **6** that was downfield shifted (δ 5.12 in **6** vs. δ 4.04 in **5**). The foregoing data along with the downfield shift of the C-10 signal in the ^{13}C NMR spectrum of **6** (δ 76.5 in **6** vs. δ 75.2 in **5**) strongly suggested that the difference between these two compounds was the replacing of the hydroxyl at C-10 in **5** with an acetoxyl group in **6**. Two dimensional COSY, HMQC and HMBC correlations of **6**, which permitted assignation of all the protons and carbon resonances (see Table I), fully confirmed this hypothesis. Conclusive proof was obtained by acetylation of cyclolinteinol (**5**) with Ac_2O/Py which yielded cyclolinteinol acetate (**6**) identical in all respects, including optical rotation, to an authentic specimen. This result also interrelated **5** and **6** at the chiral centers C-5, C-6 and C-10.

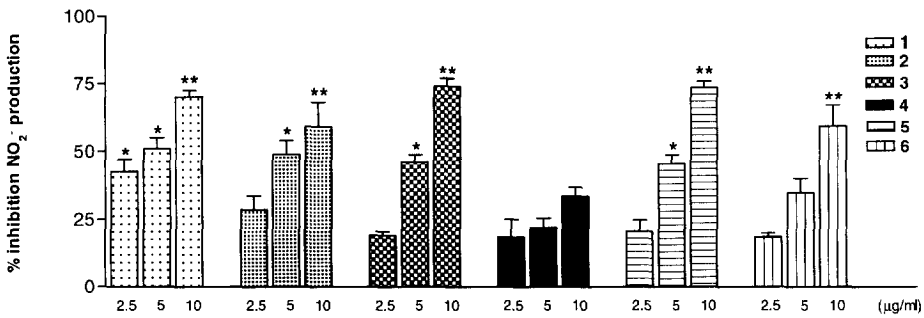

Fig. 1. Effect of compounds **1**, **2**, **3**, **4**, **5**, and **6** at various concentrations on nitrite generation in J774 macrophages stimulated with LPS (0.1 μg/ml). Each column represents the mean ± S.E.M. of 3-4 experiments in triplicate. *P < 0.05; **P < 0.01.

The effect of related compounds **1-6** on NO production by LPS-stimulated murine macrophages J774 has been investigated. The production of NO_2^- by unstimulated cells was undetectable (<1.0 nmol/10^6 cells), whereas the cells stimulated with LPS (0.1 μg/ml) produced significant amounts of NO_2^- (32.7±3.1 nmol/10^6 cells; n=4). Cyclolinteinone (**1**) and its analogues **2**, **5** and **6** (5-10 μg/ml), added to the cells 0.5 h before activation with LPS (0.1 μg/ml), inhibited significantly and in a concentration-dependent fashion the NO_2^- production (Fig. I). Lintenone (**3**) exhibited the same activity, while its epimer (**4**) showed no significant inhibition.

These compounds were not able to inhibit NO production when added after LPS challenge (Fig. II), suggesting that they inhibited the induction but not the activity of the inducible NO synthase, similarly to dexamethasone (Dex) a known inhibitor of NO synthase induction[11]. Conversely, L-NMMA (N^G-monomethyl-L-arginine), an inhibitor of NO synthase activity, inhibited NO production both when added 0.5h before and 12h after LPS challenge (Fig. II).

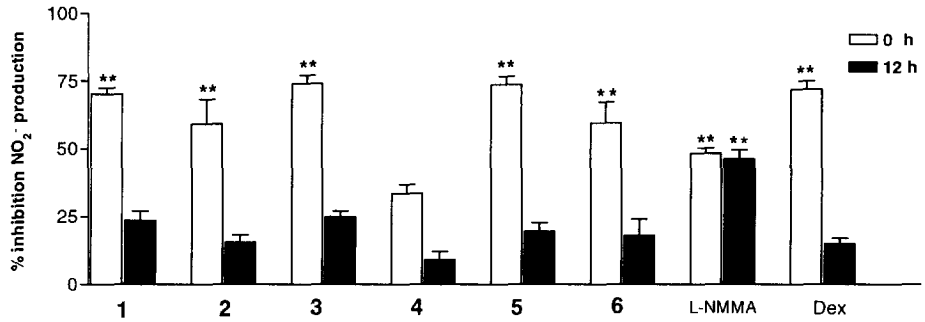

Fig. II. Effect of compounds **1**, **2**, **3**, **4**, **5**, and **6** (10 μg/ml), L-NMMA (30 μM), and Dex (1 μM) on nitrite generation by J774 macrophages stimulated with LPS (0.1 μg/ml). Each compound was added to the cells 0.5 h before or 12 h after LPS challenge. Each column represents the mean ± S.E.M. of 3-4 experiments in triplicate. *P < 0.05; **P < 0.01.

Experimental Section

General methods. HREIMS were obtained by electron impact at 55eV on a VG Prospec Fisons mass spectrometer. Optical rotations were determined on a Perkin Elmer 192 polarimeter. All NMR spectra were recorded on a Bruker AMX-500 spectrometer in $CDCl_3$ solution. ^1H and ^{13}C chemical shifts were referenced to the residual solvent signals. ^{13}C resonances' multiplicities were determined by DEPT experiments. ^1H connectivities were determined by using COSY and HOHAHA[12] experiments; ^1H-^{13}C connectivities were determined with 2D HMQC experiments[13], interpulse delays were adjusted for an average $^1J_{CH}$ of 125 Hz. Two and three bond heteronuclear ^1H-^{13}C connectivities were determined with 2D HMBC experiments[14], optimized for $^{2-3}J_{CH}$ of 8 Hz. Nuclear Overhauser effect (nOe) measurements were performed by 2D ROESY experiments. Medium-pressure liquid chromatography (MPLC) was performed on a Buchi 861 apparatus using a SiO_2 (230-400 mesh) and RP-8 columns. High-performance liquid chromatography (HPLC) separations were performed on a Varian apparatus equipped with an RI-3 refractive index detector. Hibar LiChrospher SiO_2 columns were used.

Extraction and isolation. A sample of *C.* cf. *linteiformis* (103 g, dry weight after extraction) was collected in the Caribbean area, along the coast of Grand Bahama Island (Bahamas) at 9 m depth. A reference specimen has been deposited at the Istituto di Zoologia, Universita' di Genova, Italy. The sponge was frozen and stored until extraction with MeOH/toluene (3:1). The EtOAc-soluble part of the extracts (23 g) was purified by MPLC over silica gel column using sequential mixtures of increasing polarities from petroleum ether to

EtOAc as eluants. Fractions eluted with EtOAc, purified by HPLC on a Hibar LiChrospher Si60 column with mobile phase *n*-hexane/EtOAc (6:4) gave pure compounds **6** (40 mg) and **5** (20 mg).

Cyclolinteinol (5) : $[\alpha]^{25}_{D}$ = +63° (c 0.03, $CHCl_3$); ^1H and ^{13}C NMR spectra see Table I; HREIMS (70 eV) obsd m/z 400.2609, $C_{25}H_{36}O_4$, calcd m/z. 400.2604.

Cyclolinteinol acetate (6) : $[\alpha]^{25}_{D}$ = +61° (c 0.003, $CHCl_3$); ^1H NMR and ^{13}C NMR spectra see Table 1; HREIMS (70 eV) obsd m/z 442.2715, $C_{27}H_{38}O_5$, calcd m/z 442.2709.

Synthesis of the R- and S-MTPA esters of alcohol 5 (7 and 8). To compound **5** (1 mg) in 100 µl of anhydrous pyridine, 5 µl of *S*-MTPA [α-methoxy-α-(trifluoromethyl) phenylacetyl] chloride were added and the mixture was then let stand for 2h at room temp. Unchanged chloride was decomposed by addition of MeOH. Evaporation of the reaction mixture and purification on HPLC (*n*-hexane/EtOAc 1:1) afforded 0.7 mg of *R*-MTPA ester **7**. The use of *R*-MTPA choride in the procedure led to 0.5 mg of the *S*-MTPA ester **8**.

Cell culture : Murine macrophages J774 were grown in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% foetal calf serum, 2 mm L-glutamate, 25 mm HEAPS, 100 U/ml penicillin and 100 µg/ml streptomycin. For all experiments J774 cells were plated in 24 well culture plates at a density of $2.5x10^6$ cells/well and allowed to adhere for 2h at 37°C in 5% CO_2/95% air. The cells were treated with compounds **1-6** 0.5h before (Fig. I) activation with 0.1 µg/ml LPS (lipopolysaccharide, Salmonella typhosa). In parallel experiments compounds **1-6**, L-NMMA (30 µmol), and Dex (1 µmol) were incubated 12h after (Fig. II) activation with 0.1 µg/ml LPS. The cell viability was shown by trypan blue exclusion to be >95%.

Determination of NO : Production of NO was assayed 24h after LPS challenge measuring the amount of NO_2^- in the culture medium by the Griess reaction[11]. Results were expressed as nmol of NO_2^- released by 10^6 cells (Fig. I and II).

Statistical : Comparison were made by the unpaired two-tailed Student's t-test. The level of statistically significant difference was defined as $P<0.05$.

Acknowledgements

This work was sponsored by CNR (95 1304 CT03) and by MURST (40 and 60%), Rome, Italy. We thank Prof. W. Fenical for giving us the opportunity to participate in an expedition to the Caribbean Sea, during which the sponge was collected. We thank Mr. G. Scognamiglio (Istituto MIB del CNR, Arco Felice, Napoli) for the CD spectra.

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(*Received in UK* 19 *March* 1997; *accepted* 7 *April* 1997)