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Paraliane and pepluane diterpenes as anti-inflammatory agents: First insights in structure–activity relationships

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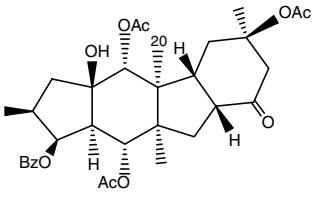
Abstract—Two new diterpenes, named paralianone (**2**) and pepluene (**3**), based, respectively, on rare paraliane and pepluane skeletons, have been isolated from *Euphorbia paralias*, together with two known analogues (**4** and **5**), and their stereostructure determined by spectroscopic methods. The isolated compounds were tested as anti-inflammatory agents in vitro for evaluating their ability to inhibit the nitric oxide production in LPS-stimulated J774 murine macrophages. Compound **4** showed the highest anti-inflammatory activity comparable to those recently discovered for pepluanone (**1**). The data obtained provided first insights towards the structure–activity relationship of this class of compounds highlighting the key role of D-ring structure.

Plants of the genus *Euphorbia* are prolific producers of diterpenes of great biomedical interest. Among them, from petty spurge, *Euphorbia peplus* L., we have recently discovered pepluanone (**1**),[1] a unique metabolite based on a rare pepluane skeleton. This compound has been demonstrated to possess high anti-inflammatory property in vivo since it was able to inhibit the carrageenin-induced rat paw oedema, an experimental model of acute inflammation, with an activity comparable to that of the reference drug dexamethasone. Furthermore, in vitro assays of pepluanone on LPS-stimulated J774 murine macrophages showed that it reduced the production of NO, PGE_2 and TNF-α by reducing the expression of iNOS, COX-2 and TNF-α mRNA, respectively. The inhibitory effect on the expression of these genes involved in inflammatory response has been correlated to the inhibition of 'nuclear factor-κB' (NF-κB) activation. Activated NF-κB results in the phosphorylation, ubiquitination and proteasome-mediated degradation of IκB proteins, followed by the translocation of NF-κB to the nucleus, and induction of gene transcription through the binding to the *cis*-acting κB element.[2] Thus, because of the important role of the transcription factor NF-κB especially in onset of inflammation, it is intuitive that NF-κB has become a candidate target for new anti-inflammatory treatments.



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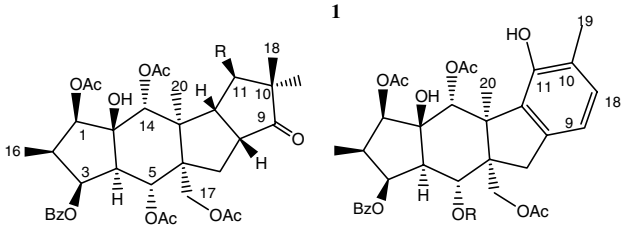

2 R = OH
4 R = H

3 R = H
5 R = Ac

As part of our investigation on Mediterranean spurges,[3–7] we have analyzed samples of sea spurge, *Euphorbia paralias*, which has been reported in traditional medicine to treat illness with inflammation and as a purgative.[8]

Keywords: Euphorbiae; Natural products; Diterpenes; Paraliane; Pepluane; Anti-inflammatory activity; Nitric oxide; SAR studies.
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From its active extract we isolated and identified two new metabolites, named paralianone (**2**) and pepluene (**3**), along with two known diterpene compounds (**4** and **5**), previously isolated from *E. segetalis*.[9] The structure elucidation of the new compounds has been obtained by extensive NMR studies. The close structural analogy of compounds **2**–**5** with pepluanone (**1**) prompted us to test them for evaluating their anti-inflammatory properties.

Thus, all the isolated compounds were able to inhibit the production of NO_2^- in LPS-stimulated J774 macrophages by iNOS which has been implicated in the pathogenesis of the inflammatory response.[10–12] In particular, one of the isolated metabolites, compound **4**, showed the highest activity comparable to that found for pepluanone. The results showed the existence of definite structure–activity relationships, suggesting the importance of the D-ring structure for the activity (Fig. 1) and its possible involvement in the inhibition of NF-κB activation.

The EtOAc extract of *E. paralias*, collected in Cala Mineo, Favignana (Egadi Islands, Sicilia, Italy) on August 2004, was subjected to successive MPLC and HPLC separations on silica gel column (hexane-EtOAc, gradient) to afford compounds **2**–**5**.

Compound **2**, named paralianone (yield 1.6 mg), isolated as colourless amorphous solid with an $[\alpha]_D^{25}$ +34.4° ($c = 0.1$, $CHCl_3$), had a molecular weight of 672.2782 (HRFABMS), corresponding to the molecular formula $C_{35}H_{44}O_{13}$ [*m/z* 673.2858 $[M+H]^+$; calculated for $C_{35}H_{45}O_{13}$ *m/z* 673.2861]. The ^1H NMR and ^{13}C NMR spectra (Table 1) indicated the presence of four acetates [methyl singlets at δ_H 1.95, 2.07, 2.14 and 2.15, respectively; carbonyl resonances at δ_C 170.46, 169.72, 169.05 and 170.46, respectively] and one benzoate [aromatic protons at δ_H 7.47, 7.60 (both t, 2H) and 8.05 (1H, d, $J = 7.7$ Hz); carbonyl resonance at δ_C 166.12]. The ^{13}C NMR spectrum (Table 1) contained a carbonyl carbon (δ 210.54) indicating the presence of a ketone functionality. At this point, taking into account the number of unsaturations implied by the molecular formula and those due to the above ester and ketone groups, it appeared clear that compound **2** skeleton must be tetracyclic. Furthermore, a singlet at δ 3.04 and a doublet at δ 2.10 ($J = 2.0$ Hz) in the ^1H NMR spectrum of **2**, both exchangeable with D_2O, gave indications for the presence of two hydroxyl groups, one tertiary and one secondary, respectively.

Application of ^1H–^1H COSY and HSQC experiments allowed us to sequence the multiplets of the core diterpene structure into two spin systems. The first included six carbons: C-1/C2 (C16)/C-3/C-4/C-5, while the second comprised four carbons: C-7/C-8/C-12/C-11. Five isolated protonated carbons [one methine (C-14), one methylene (C-17) and three methyls (C-18, C-19, and C-20)] were also present (see Table 1). Extensive study of the $^{2,3}J_{C-H}$ correlations, inferred from the HMBC spectrum (Fig. 2), allowed to build up the skeleton and also indicated the acylation pattern of the compound by the observation of diagnostic $^3J_{C-H}$ couplings between oxymethine protons and the related carbonyl ester carbons (see Fig. 2). The relative stereochemistry of the A/B/C part structure of **1** was deduced from the similarity of its spectral features with those reported for paraliane model compound[9] and from a series of NOE correlations observed in a ROESY spectrum (Fig. 3).

The stereochemistry of the D-ring was evidenced by interactions between H-11α with H_3-20 and H_3-19, and between H_3-18 with H-8 and H-12 (Fig. 3) indicating the stereochemistry at C-11 and confirming the *cis* C/D junction. All the above data pointed to the structure of paralianone as depicted in formula with a tetracyclic skeleton constituted by 1 six- and 3 five-membered rings. This skeleton, named paraliane, is very rare and it was only found in diterpenes of *E. paralias* and *E. segetalis*.[9,13,14]

The paraliane diterpene **4** (94.0 mg) and the pepluane diterpene **5** (28.1 mg), two major constituents of *E. paralias*, have been already isolated from *E. segetalis*.[9] In particular, the NMR spectra of compound **5** were particularly useful for the structural elucidation of compound **3**.

Compound **3**, named pepluene (yield 5.4 mg), isolated as a colourless amorphous solid, $[\alpha]_D^{25}$ +25.7° ($c = 0.1$, $CHCl_3$), had a molecular weight of 610.2414 (HRFABMS), corresponding to the molecular formula $C_{33}H_{38}O_{11}$ [found *m/z* 611.2480 $[M+H]^+$; calculated for $C_{33}H_{39}O_{11}$ *m/z* 611.2493]. Both MS and NMR data in comparison with those obtained for compound **5** indicated the structure of **3** as the 5-deacetyl analogue of **5**. Indeed, the ^1H NMR spectrum of **3** showed the absence of the characteristic 5-acetyl singlet (Table 1) replaced by an exchangeable doublet at δ 3.58 ($J = 2.0$ Hz) attributed to 5-OH group by HMBC (5-OH/C-5). Accordingly, the doublet of H-5 was upfield shifted (δ 3.83 in **3** vs δ 5.80 in **5**). Detailed 2D NMR analyses confirmed the proposed structure for compound **3**. Taking into account the absolute stereochemistry of pepluanone, unambiguously determined by chemical methods,[1] and those of the other paralianes and pepluanes isolated to date,[9,13,14] determined by X-ray data, the absolute configuration depicted in formula has been assumed for compounds **2** and **3**.

The close analogy in the chemical structures of the isolated compounds (**2**–**5**) with pepluanone (**1**) prompted us to test them for evaluating the presence of anti-inflammatory property. To investigate the potential anti-inflammatory activity of the isolated compounds we evaluated their ability to inhibit nitric oxide production in LPS-stimulated J774 macrophages measuring the production of NO_2^- (stable metabolite of NO) as a parameter of macrophage activation. Nitric oxide (NO), a short-lived mediator, is synthesized by a family of enzymes termed NO-synthase (NOS).[15] Two types of NOS are recognised: constitutive isoforms (endothelial NOS and neuronal NOS) and an

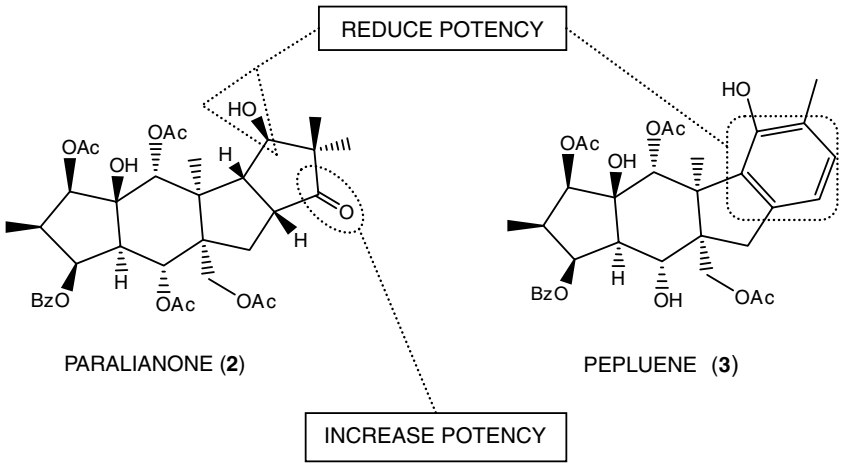

Figure 1. Key pharmacophoric elements for the anti-inflammatory activity.

Table 1. ^{1}H and ^{13}C NMR data of paralianone (**2**) and pepluene (**3**)[a]

Position	**2**		**3**	
	δ_H (int., mult., J in Hz)	δ_C (mult.)	δ_H (int., mult., J in Hz)	δ_C (mult.)
1	5.09 (d, 10.0)	74.44 d	5.10 (d, 10.0)	75.58 d
2	2.90 (ddq, 10.0, 7.6, 7.0)	38.01 d	2.83 (ddq, 10.0, 7.6, 7.0)	38.27 d
3	5.83 (dd, 7.0, 5.8)	72.68 d	5.57 (dd, 7.0, 5.8)	70.95 d
4	2.61 (dd, 5.8, 12.1)	43.02 d	2.56 (dd, 5.8, 11.3)	42.39 d
5	5.80 (d, 12.1)	67.42 d	3.83 (d, 11.3)	69.25 d
6		56.06 s		50.17 s
7a	1.69 m	29.25 t	3.10 (d, 15.6)	31.28 t
7b	1.69 m		2.68 (d, 15.6)	
8	3.61 (ddd, 7.0, 9.4, 13.2)	45.84 d		123.38 s
9		210.54 s	6.79 (d, 7.7)	114.91 d
10		45.97 s		122.47 s
11	4.07 (d, 5.5)	77.21 d		151.31 s
12	4.18 (dd, 13.2, 5.5)	51.85 d		148.81 s
13		53.69 s		55.37 s
14	5.12 s	73.22 d	5.59 s	73.15 d
15		82.09 s		81.99 d
16	0.88 (d, 7.6)	10.22 q	0.98 (d, 7.6)	10.32 q
17	4.29 (d, 11.8)	63.19 t	4.41 (d, 11.5)	63.92 t
17′	4.38 (d, 11.8)		4.58 (d, 11.5)	
18	1.09 s	20.50 q	6.99 (d, 7.7)	130.23 d
19	1.18 s	20.00 q	2.18 s	15.75 q
20	0.83 s	17.58 q	1.05 s	24.21 q
5-OH			3.58 s	
11-OH	2.10 (d, 2.0)		3.97 s	
15-OH	3.05 s		2.31 s	
1-OAc	2.15 s	20.79 q	2.20 s	20.85 q
		170.46 s		172.21 s
5-OAc	1.95 s	20.75 q		
		170.46 s		
14-OAc	2.14 s	20.69 q	2.19 s	20.55 q
		169.05 s		170.02 s
17-OAc	2.07 s	21.21q	2.18 s	20.87 q
		169.72 s		169.80 s
3-OBz		166.12 s		166.11 s
1		129.47 s		129.15 s
2,6	8.05 (d, 7.7)	129.73 d	7.97 (d, 7.7)	129.25 d
3,5	7.47 t	128.47 d	7.41 t	128.79 d
4	7.60 t	133.22 d	7.57 t	133.58 d

[a] ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ (δ_H 7.26, δ_C 77.0) on a Varian Unity Inova spectrometer at 500.13 and 125.77 MHz, respectively.

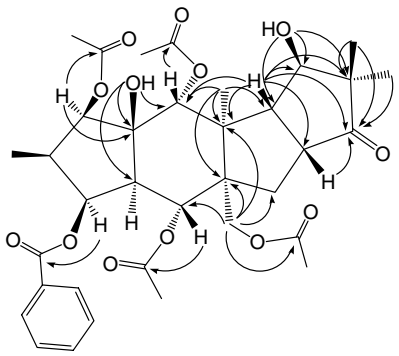

Figure 2. Selected HMBC correlations exhibited by paralianone (**2**).

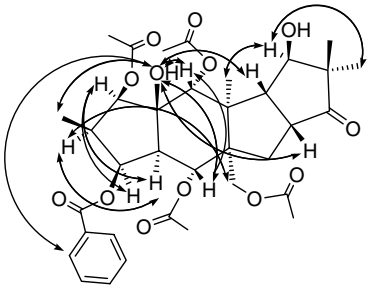

Figure 3. Selected ROESY correlations exhibited by paralianone (**2**).

inducible isoform for which mRNA translation and protein synthesis are required.[15] Inducible NOS (iNOS) is regulated by inflammatory mediators (LPS, cytokines),[16,17] and the excessive production of NO by iNOS has been implicated in the pathogenesis of the inflammatory response.[12–14] In fact, when NO production is impaired as occurs when the vascular endothelium becomes damaged or dysfunctional, an increased inflammation and tissue damage mediated by reactive oxygen species such as superoxide anion and hydroxyl radical can result.

In preliminary experiments[18] we established that cell viability (>95%) was not affected by any of compounds used (up to 10 μM, data not shown). Unstimulated J774 cells generated undetectable (<5 nmol/ml) amounts of NO_2^-. Stimulation of the cells with LPS (100 ng/ml) produced a release of NO_2^- (11.3 ± 0.1 nmol/ml). When the cells were stimulated with LPS in presence of pepluanone (**1**), pepluene (**3**), compound **4** and compound **5** (0.1–10 μM) a remarkable and concentration-related inhibition of NO_2^- generation was observed (Fig. 4). In contrast, paralianone (**2**) inhibited NO_2^- production only at highest dose used (Fig. 4). Among the tested metabolites, compound **4** showed the highest anti-inflammatory activity comparable to that recently discovered for pepluanone (**1**).

By comparing the different anti-inflammatory activity found for the tested compounds in terms of chemical structure, interesting structure–activity relationships can be deduced. First of all, comparison of the activity of paralianes (**2**, **4**) and pepluanes (**3**, **5**) shows the positive effect of a cyclopentanone D-ring (Fig. 1) confirming the key role of a carbonyl on D-ring found for pepluanone.[1] Moreover, comparison of the activity of paralianone (**2**) and compound **4**, the only structural difference being confined to an additional hydroxyl group at C-11 in the former, evidenced a collapse of the anti-inflammatory activity in paralianone which indicated a clear detrimental effect of an hydroxyl group at this position (Fig. 1). A negative effect has also been found for an hydroxyl group at C-5 instead of an acetyl (compounds **3** and **5**, respectively) thus indicating that substitution at this position could also modulate the activity. On the contrary, it seems that the presence of an acetoxyl function on D-ring, as found in pepluanone, does not reduce the activity.

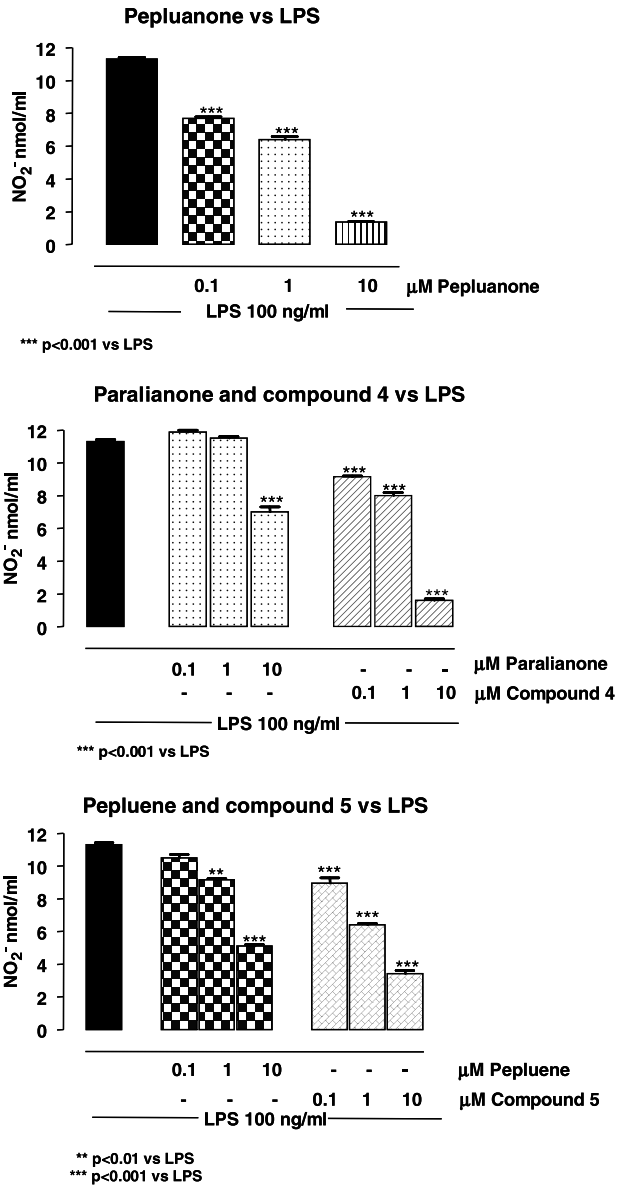

Figure 4. Effect of the compounds **1**–**5** on NO_2^- production by J774 macrophages.

In conclusion, our experiments have demonstrated that paraliane and pepluane diterpenes can be conceived as

new classes of promising anti-inflammatory agents, being able to inhibit NO generation in LPS-stimulated J774 macrophages. In addition, the isolation of several structurally related analogues has allowed us to demonstrate the crucial role of a carbonyl on D-ring and negative effects when D-ring is hydroxylated or aromatic (Fig. 1). Within the set of compounds investigated, the powerful inhibition in the iNOS activation of compound **4** qualifies this compound as promising lead for the control of inflammatory and immune reactions and validates the biological potential of paraliane and pepluane diterpenes.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.05.072. ^{1}H NMR spectra of compounds **2**–**5**.

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18. *Additional data.* The murine monocyte/macrophages cell line J774 was from ECACC. J774 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Biowhittaker) and cultured at 37 °C in humified 5% CO_2/95% air. The cells were plated in 24-well culture plates (Falcon) at a density of 2.5×10^6 cells/ml/well and allowed to adhere for 2 h. Thereafter the medium was replaced with fresh medium and cells were activated by lipopolysaccharide (LPS 100 ng/ml) from *Escherichia coli* (Fluka, serotype 0111:B4). Thirty minutes before LPS test compounds were added to cells at various concentration ranging from 0.1 up to 10 μM. After 24 h culture medium was removed, centrifuged and the supernatant was used for the determination of nitrite (NO_2^-) production. Cell viability (>95%) was determined with the MTT assay.[19] NO_2^- levels in culture media from J774 macrophages were measured 24 h after LPS or compounds challenge with the Griess reaction as previously described.[20] Results are expressed as nmol/ml of NO_2^- and represent the means ± SEM of *n* experiments runned in triplicates.
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