Journal of
Medicinal Chemistry

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J. Med. Chem., **2005**, 48 (22), 7055-7062• DOI: 10.1021/jm050321r • Publication Date (Web): 12 October 2005

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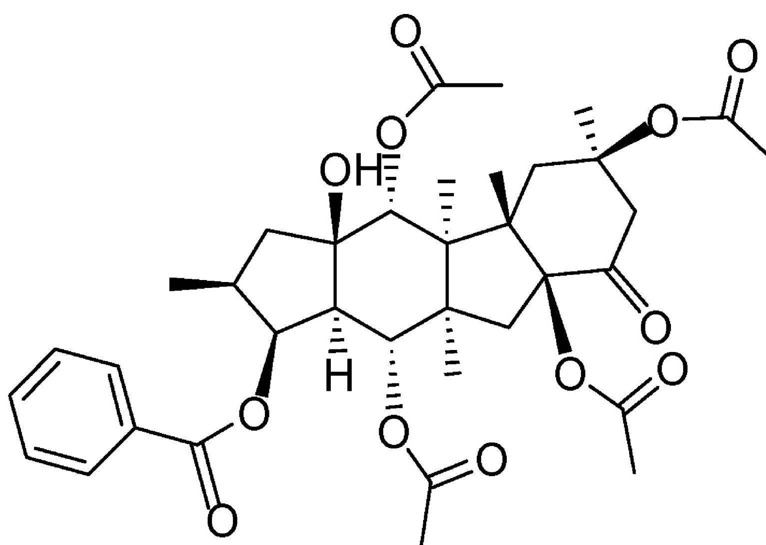

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Discovery and Biological Evaluation of the Novel Naturally Occurring Diterpene Pepluanone as Antiinflammatory Agent

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Received April 8, 2005

From the whole plant of *Euphorbia peplus* L., a new diterpene based on a rare pepluane skeleton, named pepluanone (**1**), was isolated together with a known pepluane diterpene (**2**). The stereostructure of pepluanone was determined on the basis of an extensive NMR study, MS data, and chemical reaction. The ability of these compounds to act as antiinflammatory agents has been evaluated for the first time by in vivo tests on carrageenin-induced rat paw edema, an experimental model of acute inflammation. Comparison of the bioactivity of pepluanone and compound **2** in terms of chemical structure, evidenced the high efficiency of pepluanone and the absence of appreciable activity for compound **2**, thus giving a first insight into the structure−activity relationship. Further in vitro experiments performed on pepluanone let us hypothesize that its activity could be explained in reducing the production of nitric oxide, prostaglandin E_2, and TNF-α by inhibiting the expression of inducible nitric oxide synthase, cyclooxygenase-2, and TNF-α mRNA through the down-regulation of NF-κB binding activity.

Introduction

Is well-known that the pathogenesis of inflammatory response involves the sequential activation of signaling molecules, among which prostaglandins (PGs) and nitric oxide (NO) are well-known proinflammatory key mediators, generated by the inducible isoforms of cyclooxygenase (COX-2) and NO synthase (iNOS).[1−4] Furthermore, tumor necrosis factor (TNF-α) is a potent proinflammatory cytokine that plays an important role in immunity and inflammation.[5]

The expression of several genes involved in inflammatory response, including iNOS, COX-2, and TNF-α, is regulated at the transcriptional level by the inducible transcription factor "nuclear factor-κB" (NF-κB).[6]

NF-κB represents a group of structurally related and evolutionarily conserved proteins that includes five members in mammals: p65 (RelA), c-Rel, RelB, p50 (and its precursor p105), and p52 (and its precursor p100).[6] Most members of this family can homodimerize, as well as heterodimerize, with one to each other, although the most prevalent form of NF-κB is a heterodimer consisting of the p50 and p65 subunits. In unstimulated cells, NF-κB proteins are localized in cytoplasm, through their association with members of a family of inhibitory proteins known as IκBs. NF-κB is activated in response to various stimuli, including bacterial lipopolysaccharide (LPS), cytokines, and viral proteins.[7]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.[6]

As part of our investigation on Mediterranean spurges,[8−11] we have now analyzed Mediterranean samples of petty spurge, *Euphorbia peplus*, which has been reported in traditional medicine to treat illness with inflammation.[12] From its active extract we isolated and identified a new molecule, named pepluanone (**1**), the structure of which was elucidated by extensive NMR techniques and chemical methods, together with a known pepluane diterpene (**2**).[13,14] Pepluanone is based on a rare pepluane skeleton and represents the major metabolite of the diterpene fraction, which also contains a number of diterpenes based on a jatrophane skeleton that have been recently shown to act as modulators of multidrug resistance.[10] In addition, we have obtained evidence that pepluanone possesses antiinflammatory effect in vivo, since it was able to inhibit the carrageenin-induced rat paw edema, an experimental model of acute inflammation. In vivo antiinflammatory tests performed on compound **2** evidenced the absence of appreciable activity, thus giving a first insight into the structure−activity relationship. Furthermore, to investigate the mechanism of the antiinflammatory activity, the effect of pepluanone on LPS-stimulated J774 murine macrophages has been examined. In vitro results showed that pepluanone reduced the production of NO, PGE_2, and TNF-α by reducing the expression of iNOS, COX-2, and TNF-α mRNA, and these inhibitory effects have been correlated to the inhibition of NF-κB activation.

Results

Isolation and Structural Elucidation of Pepluanone (1). The EtOAc extract of *E. peplus*, collected in the vulcanic area of Vesuvio (Campania, Italy) on March

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10.1021/jm050321r CCC: $30.25 © 2005 American Chemical Society
Published on Web 10/12/2005

Table 1. ^{1}H NMR, ^{13}C NMR, NOE, and HMBC Data of Pepluanone (**1**) [500 MHz, $CDCl_3$; δ (ppm) and multiplicities (J in Hz)]

position	^{1}H	^{13}C	NOE	HMBC
1α	2.07 dd[a]	44.31 t	2	2, 3 14, 15, 16
1β	1.48 dd (14.2, 5.1)		15-OH	2, 3, 4, 15, 16
2	2.56 m[a]	35.53 d	1α, 3, 16	16
3	5.77 m	75.94 d	2, 4	1, 4, 15, CO_{OBz}
4	2.38 dd (12.0, 4.3)	48.02d	3, 7α, 17	5, 6, 15
5	5.85 d (12.0)	69.22 d	7β, 12, 14, 15-OH	3, 4, 7, 15, 17, CO_{5-OAc}
6		48.25 s		
7α	1.74 d (16.0)	38.23 t	4, 17	5, 6, 8, 9, 17
7β	2.55 d (16.0)		5, 12	5, 6, 8, 12, 13
8		87.72 s		
9		205.84 s		
10		82.38 s		
11α	1.16 dd (13.0, 6.0)	32.04 t		12, 13
11β	2.47 dd (13.0,6.0)		14	8, 18, 10, 12, 19
12	4.56 dd (13.0, 6.0)	48.48 d	5, 7β, 14, 15-OH, 8-OAc	9, 10, 11, 13, 14, 20
13		51.82 s		
14	4.93 s	72.67 d	5, 11β, 12, 15-OH	1, 4, 6, 12, 13, 15, CO_{14-OAc}
15		72.59 s		
16	1.07 d (7.3)	16.03 q	15-OH, 2	1, 2, 3
17	1.09 s	16.05 q	4, 7α, 20	5, 6, 7, 14
18α	2.31 d (16.5)	48.56 t	19	8, 9, 19
18β	3.27 d (16.5)			11, 12
19	1.59 s	27.65 q	18α	10, 11, 12
20	0.65 s	16.05 q	17	13, 14, 15
15-OH	3.06 s		1β, 5, 12, 14, 16, OBz 2,6	1,4, 14, 15
5-OAc		171.11 s	5, 3	
	1.85 s	20.55 q		CO_{5-OAc}
8-OAc		169.58 s	18	
	2.14 s	22.08 q		CO_{8-OAc}
10-OAc		169.81 s	12, OBz 2,6, OBz 3,5	
	1.94 s	20.60 q		CO_{10-OAc}
14-OAc		169.34 s		
	2.08 s	21.96 q		CO_{14-OAc}
OBz		165.48 s		
1		130.13 s		
2,6	7.97 d (7.4)	129.53 d	15-OH, 8-OAc	CO_{OBz}
3,5	7.37 t	128.12 d	8-OAc	
4	7.54 d (7.4)	133.07 d		

[a] Overlapped by other signals.

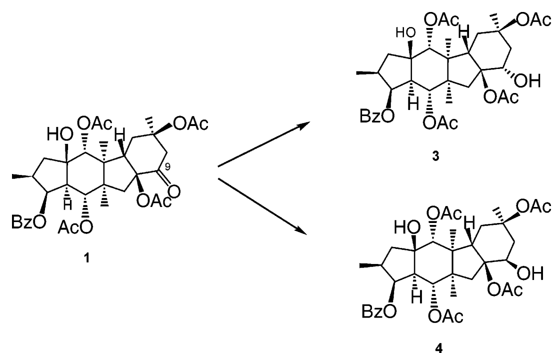
2002 was subjected to successive MPLC and HPLC chromatographic separations on silica gel column (hexanes−EtOAc, gradient) to afford pepluanone and the known compound **2**, whose structure has been identified by comparison of its NMR and MS data with those reported in the literature.[13,14]

Pepluanone (**1**) had a molecular weight of 657.2894 (HRFABMS), corresponding to the molecular formula $C_{35}H_{44}O_{12}$. The ^{1}H NMR and ^{13}C NMR spectra (Table 1) indicated the presence of four acetates (singlets at δ_H 1.85, 1.94, 2.08, and 2,14; δ_C 171.11, 169.81, 169.34, and 169.58, respectively) and one benzoate [δ_H 7.37 (t), 7.54 and 7.97 (both d, J = 7.4 Hz); δ_C 165.48). These assignments were confirmed by heteronuclear single quantum correlation (HSQC) spectrum, which associated the above proton resonances to the signals of the directly attached carbon atoms, and by heteronuclear multiple-bond correlation (HMBC) spectrum, which connected ester carbonyls to the directly attached methyl and phenyl groups. The ^{13}C NMR spectrum (Table 1) contained only a further sp^2 carbon atom (δ 205.84), which indicated the presence of a ketone carbonyl group. 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 was evident that the pepluanone skeleton must be tetracyclic. Furthermore, a singlet at δ 3.06 in the ^{1}H NMR spectrum of **1**, exchangeable with D_2O, gave indication for the presence of a tertiary hydroxyl group.

Application of ^{1}H−^{1}H correlation spectroscopy (COSY) and HSQC experiments allowed us to sequence the multiplets of the core diterpene structure into two spin systems of six [C-1/C2 (C16)/C-3/C-4/C-5] and two (C-11/C-12) carbon atoms. Six isolated protonated carbons [one methine (C-14), two methylenes (C-7 and C-18) and three methyls (C-17, C-19 and C-20)] were also evidenced from the spectra. Extensive study of the $^{2,3}J_{C-H}$ correlations, inferred from the HMBC spectrum, showed in Table 1, allowed the connection of the two above deduced moieties and methyls 17, 19, and 20 through the unprotonated carbons, C-6, C-8, C-10, C-13, and C-15. In particular, the following HMBC cross-peaks were particularly diagnostic to assemble the A/B and B/C rings: 15-OH with C-15, C-4 and C-14; H-1α and H-1β with C-15; H_3-20 with C-13, C-6, C-12 and C-14; and H_3-17 with C-6, C-13, C-5, and C-7. The C/D ring substructure was obtained by the following HMBC cross-peaks: H-12 with C-11, C-13, C-20, C-8, C-10, and C-14. A further cross-peak between H-12 and the ketone carbon atom suggested the position 9 as site of the carbonyl. This was confirmed by the cross-peaks of this carbon with H-18α, H-18β, and H-7α and by the downfield shift of protons linked at C-18, indicative of a deshielding effect of the adjacent carbonyl.

The HMBC spectrum 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. Thus, the benzoate was

Scheme 1. Reduction of Pepluanone (**1**) with $NaBH_4$ in MeOH To Afford the Pepluane Diterpene Epimers (**3** and **4**)[a]



[a] In the chemical structures, Bz = benzoyl and Ac = acetyl.

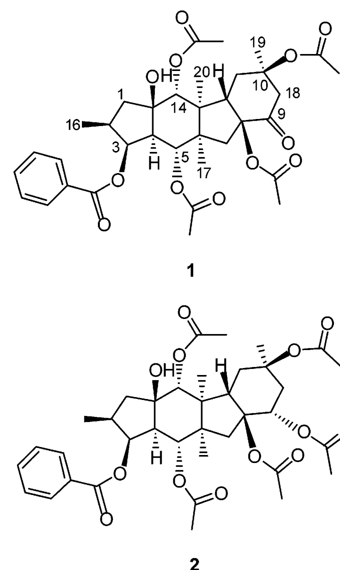


Figure 1. Chemical structure of pepluanone (**1**) and compound **2**.

located at C-3 and the acetates at C-5 and C-14. The remaining acetates were placed at the remaining quaternary carbons C-8 and C-10.

The relative stereochemistry of **1** was obtained from a series of NOE correlations observed in a ROESY spectrum (Table 1). The interactions of 15-OH with H-1β, H_3-16, and the ortho benzoyl protons, as well as H-2 with H-4, indicated a trans-fused A/B junction. Correlations of H-7β with H-12 and H-5 and of H-12 with H-14 required a B/C cis junction.

The α-positions of the oxygenated functionalities at C-14 and C-5 came from the observation of NMR correlations between H-14 and H-11β and between H-5 and 15-OH. This last proton was also mutually coupled with H-12, thus defining the β stereochemistry of H-5. The NOE effects between H-12 and 8-OAc and between 8-OAc with the ortho and meta benzoyl protons determined a cis stereochemistry of C/D rings. Finally, an interaction between H-18α and H_3-19 indicated the relative stereochemistry at C-10.

To determine the absolute configuration of pepluanone (**1**), we have performed its chemical reduction, which afforded the known pepluane diterpene **3** (Scheme 1), previously isolated by Hohmann et al.[15] Thus, pepluanone, solubilized in MeOH, was treated with $NaBH_4$ at 50 °C (see Materials and Methods), affording a mixture of alcohol epimers at C-9 (**3** and **4**, Scheme 1) that were separated by HPLC. The α epimer (**3**), as determined by $[\alpha]^{25}_D$ and spectra comparison, was identical to the known compound, thus determining the absolute configuration of **1**.

Effect of Pepluanone and Compound 2 on Paw Edema. The antiinflammatory effects of pepluanone and compound **2** were evaluated in an in vivo study by testing their ability to inhibit carrageenin-induced rat paw edema, an experimental model of acute inflammation. Thus, carrageenin injection caused a time-dependent increase of paw volume with a peak occurring at 4 h (Figure 2a). Treated rats received pepluanone at 0.013, 0.13, and 1.3 mg/kg ip 1 h before carrageenin injection. Pepluanone at a dose of 0.013 mg/kg was ineffective, whereas the inflammatory reaction was inhibited when higher doses were used. At the time of maximum foot increase, pepluanone at 0.13 and 1.3 mg/kg dose-dependently and significantly reduced carrageenin-induced edema by 40% ($p < 0.01$; $n = 8$) and 60% ($p < 0.01$; $n = 9$). In contrast, compound **2** at the higher dose used (1.3 mg/kg) did not significantly modify the time course of edema formation (Figure 2b). Dexamethasone (0.1 mg/kg/ip), used as reference drug, inhibited the edema by 50% ($p < 0.01$; $n = 8$). In adrenalectomized animals, the inflammatory reaction induced by carrageenin, compared with control rats (not adrenalectomized), was significantly enhanced (Figure 2c), whereas in sham-operated rats, it was superimposable on that observed in normal rats (data not shown). Pepluanone at 0.13 and 1.3 mg/kg inhibited carrageenin-induced edema by 37% ($p < 0.01$; $n = 10$) and 63% ($p < 0.01$; $n = 9$), while at 0.013 mg/kg pepluanone was ineffective (Figure 2c).

Effect of Pepluanone on NO_2^-, PGE_2, and TNF-α Production by J774 Macrophages. In preliminary experiments, we established that pepluanone alone did not induce NO_2^-, PGE_2, and TNF-α, production (Figure 3a–c). Stimulation of J774 macrophages with LPS for 24 h (10 ng/mL) produced a substantial and significant release of both NO_2^- (12.1 ± 0.6 nmol/mL; $n = 3$) and PGE_2 (1960 ± 140 pg/mL, $n = 3$) compared to that observed in unstimulated cells (Figure 3a,b). When J774 macrophages were stimulated with LPS in the presence of pepluanone (0.1–100 μM), a concentration-related inhibition of both NO_2^- and PGE_2 production was observed (Figure 3a,b). Pepluanone at 0.1 μM was ineffective, while at 1, 10, and 100 μM significantly ($p < 0.01$ or $p < 0.001$, $n = 3-4$) inhibited both NO_2^- release (by 46%, 60%, and 98%, respectively) and PGE_2 production (by 16%, 23%, and 52%, respectively). Dexamethasone (1 μM), used as reference drug, inhibited both NO_2^- release and PGE_2 production by about 50% ($p < 0.001$; $n = 3$).

Unstimulated J774 macrophages generated undetectable (<15pg/mL) amounts of TNF-α. Stimulation of these cells with LPS for 18 h (10 ng/mL) produced an increase of TNF-α production (15.8 ± 1.3 ng/mL). When J774 macrophages were stimulated with the same amounts of LPS in the presence of pepluanone (0.1–100 μM), a concentration-dependent inhibition of TNF-α production was observed (Figure 3c). Thus, pepluanone, which was ineffective at 0.1 μM, significantly inhibited

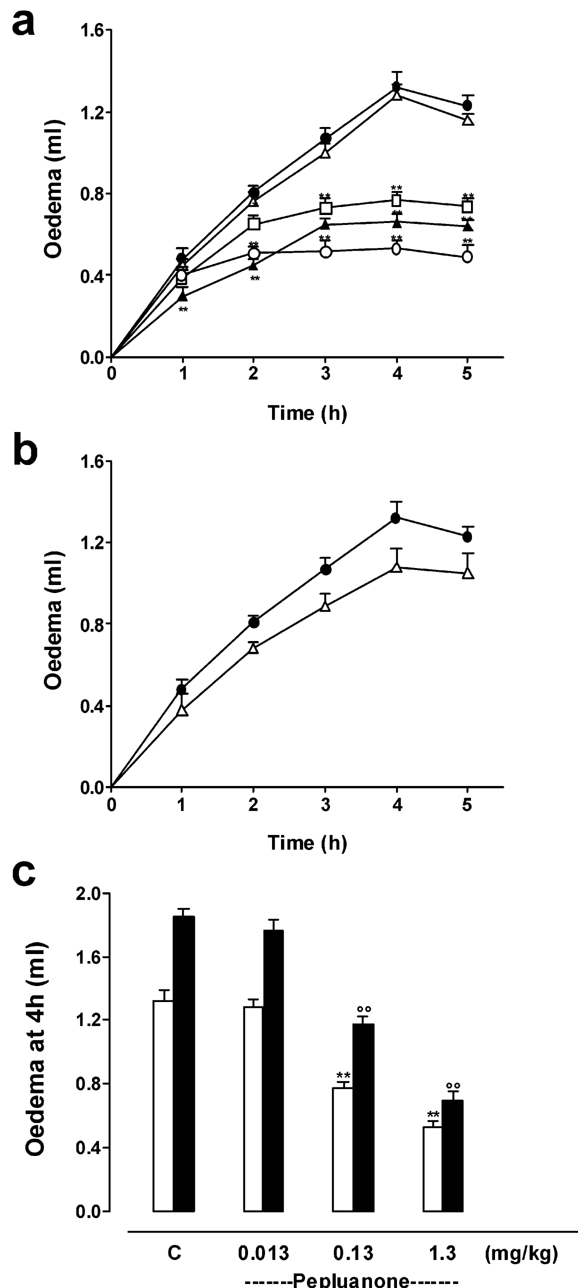


Figure 2. Effect of pepluanone and compound **2** on carrageenin-induced rat paw edema. (a) Effect of 0.013 mg/kg (△), 0.13 mg/kg (□), and 1.3 mg/kg (○) of pepluanone on carrageenin-induced edema in rat. The edema induced in dexamethasone-treated rats (0.1 mg/kg) is shown by solid triangles. Both pepluanone and dexamethasone were administered ip 1 h before carrageenin injection. The edema induced in control animals is shown by solid circles. The results are expressed as mean ± SEM, where $n = 8-10$ rats. $^{**}p < 0.01$, vs control group. (b) Effect of 1.3 mg/kg (Δ) of compound **2** on carrageenin-induced edema in rat. The edema induced in control animals is shown by solid circles. The results are expressed as mean ± SEM, where $n = 7-9$ rats. (c) Effect of pepluanone on paw edema induced by carrageenin in normal (open bars) or adrenelectomized rats (solid bars). The results are expressed as mean ± SEM (where $n = 8-10$ rats) of the edema occurring at 4 h. C = carrageenin alone. $^{**}p < 0.01$ vs normal rats; $^{\circ\circ}p < 0.01$ vs adrenelctomized rats.

TNF-α release at 1, 10 and 100 μM by 26%, 35% and 73% ($p < 0.001$, $n = 3-4$), respectively. Dexamethasone (1 μM) inhibited TNF-α release by about 70% ($p < 0.001$; $n = 3$).

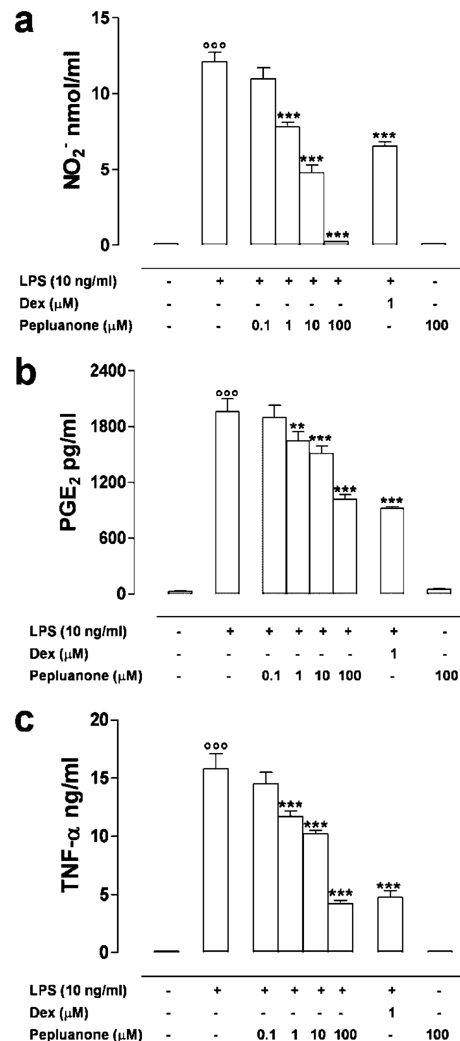


Figure 3. Effect of pepluanone on NO_2^-, PGE_2, and TNF-α production by J774 macrophages. Effect of different concentrations of pepluanone on the production of NO_2^- (a), PGE_2 (b), and TNF-α (c) by J774 macrophages stimulated with LPS (10 ng/mL). Each column represents the mean ± SEM, where $n = 3-4$ experiments run in triplicate. Dex = dexamethasone. $^{\circ\circ\circ}p < 0.001$ vs ubstimulated cells; $^{**}p < 0.01$, $^{***}p < 0.001$ vs control (LPS alone).

Effect of Pepluanone on iNOS, COX-2 and TNF-α mRNA Expression Levels by J774 Macrophages. As shown in Figure 4 (upper panel) stimulation of J774 macrophages with LPS (10 ng/mL) for 4 h caused a significant increase of iNOS, COX-2, and TNF-α mRNA expression levels compared to unstimulated cells, but increases were prevented by pepluanone (10 μM). As demonstrated by the relative densitometric analysis, the mRNA levels of iNOS, COX-2, and TNF-α, normalized to expression levels of housekeeping gene β-actin, were significantly inhibited ($p < 0.001$ or $p < 0.01$; $n = 3$) by 87%, 29%, and 45%, respectively (Figure 4, lower panel). Pepluanone alone induced a very slight increase of iNOS, COX-2, and TNF-α mRNA expression not sufficient to induce the production of their respective metabolites.

Effect of Pepluanone on NF-κB/DNA Binding Activity. A low level of NF-κB/DNA binding activity was detected in nuclear protein extracts from unstimulated J774 macrophages. Conversely, a retarded band was clearly shown in LPS-stimulated J774 cells (Figure

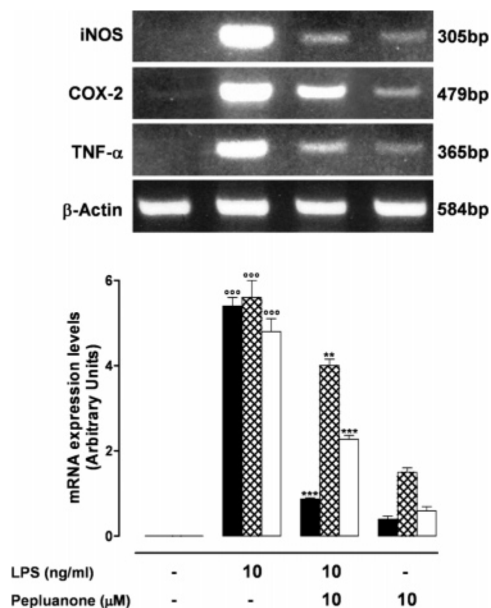


Figure 4. Effect of pepluanone on iNOS, COX-2, and TNF-α mRNA expression levels in J774 macrophages. (Upper panel) PCR amplification of reverse-transcribed mRNA was performed on total RNA isolated from cells harvested 4 h after LPS challenge. Parallel amplification of mouse housekeeping gene β-actin was performed as an internal control. Data are representative of three separate experiments. (Lower panel) Densitometric analysis of iNOS (solid bars), COX-2 (cross-hatched bars), and TNF-α (open bars) mRNA expression levels. Data are expressed as mean ± SEM of three experiments run in triplicate.

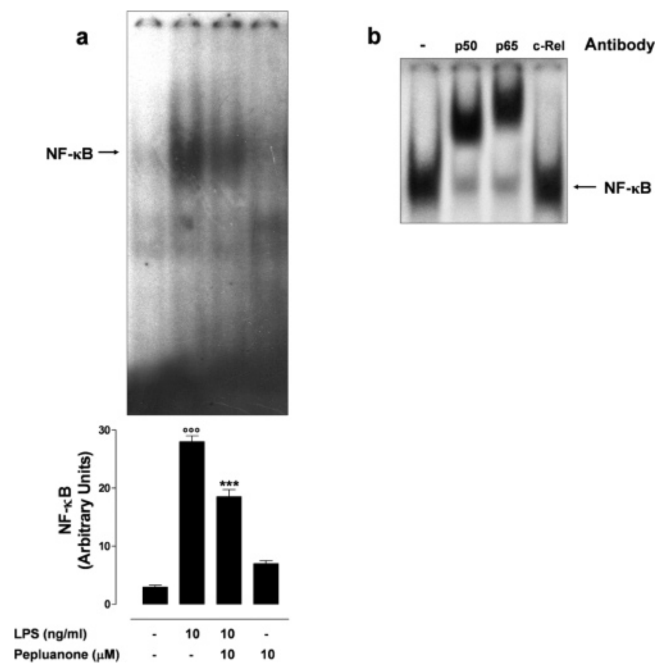


Figure 5. Effect of pepluanone on NF-κB/DNA binding activity in J774 macrophages. (a) EMSA was performed on nuclear extracts from cells harvested 2 h after LPS challenge (upper panel). Data are representative of three separate experiments. Densitometric analysis of NF-κB/DNA binding activity is shown in the lower panel. Data are expressed as mean ± SEM of three experiments. °°°$p < 0.001$ vs unstimulated cells; ***$p < 0.001$ vs control (LPS alone). (b) Supershift experiments in which nuclear extracts were incubated with antibodies against p50, p65, or c-Rel 30 min before incubation with the radiolabeled NF-κB probe.

5a, upper panel). As shown by densitometric analysis, pepluanone (10 μM) inhibited NF-κB/DNA binding activity by 33% ($p < 0.001$, $n = 3$) (Figure 5a, lower panel). The composition of the NF-κB complex activated by LPS was determined by supershift experiments by using specific antibodies against p50, p65, and c-Rel subunits of the NF-κB complex (Figure 5b). Addition of either anti-p50 or anti-p65, but not anti-c-Rel, caused both retardation of mobility and decrease in intensity of NF-κB band.

Discussion

In this study we have described the identification of a new diterpene, named pepluanone (**1**), that we have isolated from *E. peplus*, based on a rare pepluane skeleton characterized by a fused tetracyclic core, substituted with oxygenated functionalities, such as hydroxyl, benzoate, and acetates. The stereostructure elucidation of pepluanone has been obtained through a combination of extensive HRMS, 2D NMR, and chemical methods. The pepluane skeleton is very rare and found previously only in three other diterpenes, all isolated from the same plant, from which the name of the skeleton was taken.[13−15] Only one of the already described pepluane diterpenes, compound **2**,[13,14] has been also isolated in the plant material analyzed. The observed different composition of pepluane diterpenes in the same plant species between this and previous work[14,15] is likely related to the different geographical origin and environmental conditions of the samples (Southern Italy and Hungary, respectively).

Furthermore, in this study we demonstrated, by using a rat model of acute inflammation, that pepluanone, but not compound **2**, was able to reduce inflammatory reaction. Previous studies have shown that carrageenin-induced edema is characterized by an early phase (1−2 h) brought about by the release of histamine, 5-hydroxytryptamine, and bradykinin,[16] followed by a late phase (3−4 h), which is inhibited by nonsteroidal antiinflammatory drugs, sustained by the release of PGs and NO following the induction of COX-2 and iNOS protein expression in activated leukocytes that infiltrated into the carrageenin-injected rat paw.[1,3,4,17] Rat carrageenin-induced hind paw edema is controlled by endogenous steroid because, in adrenolectomized rats, the inflammatory reaction was much greater compared with that occurring in either normal or sham-operated animals. The doses of pepluanone (0.13 and 1.3 mg/kg) that significantly reduced edema formation in normal rats exhibited an identical inhibitory effect in adrenolectomized rats, showing that the antiinflammatory activity of pepluanone was not dependent on the stimulation of endogenous corticoid production. In contrast, compound **2** at the higher dose used (1.3 mg/kg) did not significantly modify the time course of edema formation. Comparison of the in vivo activity of pepluanone and compound **2** (Figure 2, parts a and b, respectively) evidenced a key role of a carbonyl at C-9 for antiinflammatory activity. In fact, the only structural differences between the two compounds is confined to the functionality at C-9, a ketone in pepluanone and an acetoxyl in compound **2**.

To investigate the mode of action of pepluanone as an antiinflammatory agent, the effect of this molecule

on LPS-induced inflammatory responses was investigated in a J774 murine macrophage cell line. Our results show that pepluanone inhibited in a concentration-dependent manner, without notable cytotoxicity, both LPS-induced NO and PGE_2 productions. In addition, RT-PCR analysis demonstrated that the low levels of NO and PGE_2 measured occurred through the inhibition of iNOS and COX-2 gene expression. Pepluanone was also able to inhibit TNF-α mRNA expression and consequently to reduce the release of this cytokine, which is involved in the propagation of local or systemic inflammatory processes.[7] Furthermore, our results indicate that the mechanism by which pepluanone inhibits iNOS, COX-2, and TNF-α mRNA involves the suppression of NF-κB activation. This is in agreement with previous findings showing that NF-κB response elements are present on the promoters of iNOS, COX-2, and TNF-α genes.[18−20] Interestingly, much evidence has demonstrated that NF-κB plays a crucial role in inflammatory processes and that many of the clinically important antiinflammatory drugs, including salicylates[21] and glucocorticoids,[22,23] share the ability to inhibit NF-κB activation and therefore a large variety of inflammatory genes.[6]

In summary, our results show that pepluanone has antiinflammatory effect due to its ability to inhibit LPS-induced NO, PGE_2, and TNF-α release via gene expression, and this inhibition depends by the blocking of NF-κB activation as demonstrated in J774 macrophages. In addition, comparison of in vivo antiinflammatory activity exhibited by pepluanone and compound **2** has allowed us to propose a first insight into the structure−activity relationship, highlighting the importance of a carbonyl function at C-9. Although a number of important issues, such as safety and side effects, have not been addressed in this study, our results open new perspectives in the design and development of a new nonsteroidal approach for antiinflammatory drugs.

Experimental Section

General Experimental Procedures. Low- and high-resolution FAB mass spectra (glycerol matrix) were measured on a Prospec Fisons mass spectrometer. Optical rotations were determined on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and 10-cm microcell. ^1H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker AMX-500 spectrometer. Chemical shifts were referred to the residual solvent signal ($CDCl_3$: δ_H, 7.26; δ_C, 77.0). The multiplicities of ^{13}C NMR resonances were determined by DEPT experiments. ^1H connectivities were determined by using COSY experiments; one-bond heteronuclear $^1H-^{13}C$ connectivities were determined with 2D HSQC pulse sequence with an interpulse delay set for $^1J_{CH}$ of 130 Hz. Two- and three-bond heteronuclear $^1H-^{13}C$ connectivities were determined with 2D NMR HMBC experiments, optimized for $^{2-3}J_{CH}$ of 8 Hz. Measurement of spatial coupling was obtained through 2D ROESY experiments. Medium-pressure liquid chromatography (MPLC) was performed on a Büchi 861 apparatus using silica gel (230−400 mesh) as the stationary phase. HPLC in isocratic mode was performed on a Varian apparatus equipped with an RI-3 refractive index detector.

Plant Material. Samples of *E. peplus* L. were collected in the vulcanic area of Vesuvio (Campania, Italy) in March 2002. A voucher specimen is kept at the Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, University of Naples “Federico II”.

Extraction and Isolation. Fresh whole plants (2.097 kg, fresh plant), including latex and roots, were extracted five times with 7 L of EtOAc during 15 days at room temperature. The EtOAc extract (81.9 g) was partitioned between H_2O and EtOAc, to remove hydrophilic and gummy compounds, and then the sole apolar fraction (35.5 g) was chromatographed by MPLC on silica gel column (230−400 mesh) using a gradient system from hexane 100% to EtOAc 100%.

The fraction that eluted with hexane:EtOAc 1:1 (592.5 mg) was successively purified on a Lichrocart Si60 (10 μm) HPLC column, using hexane:EtOAc 65:35 as mobile phase at a flow rate of 2.5 mL/min, and afforded the new pepluanone (**1**, 38.3 mg, $t_R = 36.0$ min), together with the known compound **2** (17.7 mg, $t_R = 46.0$ min).

Pepluanone (1): yield 38.3 mg; colorless amorphous solid; $[\alpha]^{25}_D$ +8.2° (c = 0.1, $CHCl_3$); HRFABMS (positive ion) found m/z 657.2894 $[M + H]^+$, calculated for $C_{35}H_{44}O_{12}$ m/z 656.2820; ^1H and ^{13}C NMR ($CDCl_3$) data, see Table 1.

Purity Criteria for Target Compound. The degree of purity of tested compounds was over 95% as indicate by the appearance of a single peak using two different HPLC eluent systems. Retention times (t_R) are expressed in minutes.

Pepluanone (**1**): hexane/EtOAc 65:35, $t_R = 36.0$ min; CH_2Cl_2/EtOAc 7:3, $t_R = 26.9$ min.

Compound **2**: hexane/EtOAc 65:35, $t_R = 46.0$ min; CH_2Cl_2/EtOAc 7:3, $t_R = 34.1$ min.

Reduction of Pepluanone (1) to Compounds 3 and 4. To a stirred solution of pepluanone (6.2 mg) in methanol (3 mL) was added excess of $NaBH_4$ (0.8 mg) portionwise during ca. 5 min, and the reaction mixture was kept in oil bath at 50 °C with stirring for 3 h. After 2 h, the reaction was monitored by TLC (hexane:EtOAc 65:35). After being stopped, the reaction was worked up by addition of saturated aqueous NH_4Cl (9 mL) and EtOAc (27 mL). The organic phase was washed with brine, dried (Na_2SO_4), and evaporated, to give a semisolid residue. Trituration with ether afforded 5 mg of **3** and **4**. HPLC separation with hexane/EtOAc 65:35, on a Lichrospher Si60 (5 μm) column at a flow rate of 1 mL/min, afforded pure compounds **3** (2.1 mg, $t_R = 20$ min) and **4** (1.9 mg, $t_R = 22$ min).

Animals. Male Wistar rats (Harlan), weighing 140−160 g, were used in all experiments. Animals were provided with food and water ad libitum. The light cycle was automatically controlled (on 07 h 00 min, off 19 h 00 min) and the room temperature thermostatically regulated to 22 ± 1 °C. Prior to the experiments, animals were housed in these conditions for 3−4 days to become acclimatized. Some rats were adrenalectomized or sham-operated under isofluorane anaesthesia and used 4−5 days after the surgical procedure. Adrenalectomized animals received isotonic saline as drinking water until use. Animal care was in accordance with Italian and European regulations on protection of animals used for experimental and other scientific purposes.

Paw Edema. Paw edema was induced by subplantar injection into the right hind paw of 0.1 mL sterile saline containing 1% λ-carrageenin (control group). Pepluanone or compound **2** (0.013, 0.13, and 1.3 mg/kg) was administered intraperitoneally (ip) 1 h before carrageenin injection. In some experiments, pepluanone was given by the same route to adrenalectomized or sham-operated rats. The volume of the paw was measured by a plethysmometer (Basile) immediately after the injection as previously described.[24] Subsequent readings of the same paw were carried out at 1 h intervals up to 5 h and compared to the initial readings. The increase in paw volume was taken as edema volume. Carrageenin-induced paw edema was also induced in some rats treated with dexamethasone sodium phosphate (0.1 mg/kg) used as reference drug and given ip 1 h prior to the phlogogenic agent.

Cell Culture. The murine monocyte/macrophage cell line J774 was from the European Collection of Animal Cell Cultures (Salisbury, U.K.). J774 cells were grown in Dulbecco’s modified Eagle’s medium and cultured at 37 °C in humidified 5% CO_2/95% air. Culture medium was supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 mM sodium pyruvate. Dexamethasone sodium phosphate (1 μM; Sigma)

has been used as reference drug. In preliminary experiments, we established that cell viability (>95%) was not affected by pepluanone challenge (data not shown).

Preparation of Nuclear Extracts. J774 cells were seeded in 6-well plates (Falcon) at a density of 3×10^6/well, where they were allowed to adhere for 2 h. Thereafter, these cells were treated with pepluanone (10 μM), and 30 min after treatment they were stimulated with 10 ng/mL LPS from *Escherichia coli* (Fluka) for 2 h. All the extraction procedures were performed on ice with ice-cold reagents. Cells were washed twice with ice-cold PBS and centrifuged at 1000*g* for 10 min at 4 °C. The cell pellet was resuspended in one packed cell volume of lysis buffer and incubated on ice for 5 min with occasional vortexing. After centrifugation at 11 000*g* at 4 °C for 5 min, 1 cell pellet volume of extraction buffer was added to the nuclear pellet and the mixture incubated on ice for 15 min with occasional vortexing. Nuclear proteins were isolated by centrifugation at 11 000*g* for 15 min, and the supernatant was aliquoted and stored at −80 °C. Protein concentration was determined by the Bio-Rad protein assay kit.

Electrophoretic Mobility-Shift Assay (EMSA). Double-stranded oligonucleotides containing the NF-κB recognition sequence (5′-GAT CGA GGG GAC TTT CCC TAG-3′) were end labeled with γ-[^{32}P]ATP, and a gel mobility assay was performed as described.[25] Aliquots of nuclear extracts (10 μg of protein for each sample) were incubated for 30 min at room temperature with radiolabeled oligonucleotides ($2.5-5.0 \times 10^4$ cpm) in 20 μL of reaction buffer containing 2 μg of poly dI-dC, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mg/ mL bovine serum albumin, 10% v/v glycerol. In the supershift assay, antibodies reactive to p50, p65, or c-Rel subunits of NF-κB complex were added to the reaction mixture 30 min before the addition of radiolabeled probes. Protein−nucleic acid complexes were resolved by electrophoresis on 4% nondenaturing polyacrylamide gel in 0.5× Tris-borate/EDTA at 150 V for 2 h at 4 °C. The gel was dried and autoradiographed with an intensifying screen at −80 °C for 20 h. Subsequently, the relative bands were quantified by densitometric scanning of the X-ray films with a GS-700 imaging densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM).

RT-PCR of iNOS, COX-2, and TNF-α. J774 cells were seeded in 6-well plates (Falcon) at a density of 3×10^6/well, where they were allowed to adhere for 2 h. Thereafter, the cells were treated with pepluanone (10 μM) and, 30 min after treatment, stimulated with 10 ng/mL LPS from *E. coli* (Fluka) for 4 h. Total RNA was isolated from the cell using TRIzol (Invitrogen). Briefly, the cells were washed twice with ice-cold PBS, and then 1 mL of TRIzol reagent was added to each well. The cells were collect after scraping, transferred to a microcentrifuge tube, and homogenated by passing 5−10 times in a 20 gauge needle fitted onto a 3-mL syringe. Two hundred milliliters of chloroform was added and the tube was shaken for 15 s, followed by centrifugation at 10 000*g* for 15 min. The aqueous phase was transferred to a new microcentrifuge tube, and the total RNA was precipitated using 0.5 mL of isopropyl alcohol. RNA was allowed to precipitate at room temperature for 10 min and the tube centrifuged at 10 000*g* for 10 min. The supernatant was removed, and the RNA pellet was washed with 1 mL of 70% ethanol followed by centrifugation at 10 000*g* for 5 min. The RNA pellet was air-dried for 5 min, resuspended in diethyl pyrocarbonate-treated water, and then heated at 55 °C for 15 min. The final amount of RNA was determined by absorbance at 260 nm. Five micrograms of total RNA was reverse-transcribed into cDNA by using oligo (dT)12−18 primer (Invitrogen) and Superscript II reverse transcriptase (Invitrogen). One microliter of cDNA was amplified by PCR using Taq polymerase (Invitrogen) according to the manufacturer's instructions. The primers were as follows: i-NOS, sense 5′-TGGGAATGGAGACTGTCCCAG-3′, antisense 5′-GGGATCT-GAATGTGATGTTTG-3′; TNF-α, sense 5′-GTTCTATGGCCCA-GACCCTCACA-3′, antisense 5′-TACCAGGGTTTGAGCTCAGC-3′; COX-2, sense 5′-CCGGGTTGCTGGGGGAAGA-3′, antisense 5′-GTGGCTGTTTTGGTAGGCTGTGGA-3′; β-actin, sense 5′-ATGAAGATCCTGACCGAGCGT-3′, antisense 5′-AACGCAGCT-CAGTAACAGTCCG-3′. The amplified fragments were 305bp, 365bp, 479bp, and 584bp, respectively. The PCR reaction was performed under the following conditions: a first cycle of denaturation at 94 °C for 1 min 40 s and then 25 cycles of denaturation at 94 °C for 40 s, annealing at 56 °C for 40 s, extension at 72 °C for 1 min, and 1 additional cycle of extension at 72 °C for 8 min. The PCR products were run on a 1% agarose gel and visualized by ethidium bromide staining.

TNF-α, NO_2^-, and PGE_2 Assays. J774 cells were plated onto 24-well culture plates (Falcon) at a density of 2.5×10^5/mL per well, where they were allowed to adhere for 2 h. Thereafter, these cells were treated with various concentrations of pepluanone (0.1−100 μM). Thirty minutes after treatments, J774 cells were stimulated with 10 ng/mL LPS from *E. coli* (Fluka). TNF-α levels in the cell culture medium were assayed 18 h after LPS stimulation by using a commercially available human cytokine enzyme-linked immunosorbent assay test kit (R&D System) according to the manufacturer's instructions, and the results are expressed as ng/mL. The amount of NO_2^-, stable metabolites of nitric oxide, present in culture media from cells were measured 24 h after LPS stimulation with the Griess reaction as previously described,[26] and results are expressed as nmol/mL. The accumulation of PGE_2 in the cell culture medium was measured, without prior extraction or purification, 24 h after LPS stimulation by EIA according to the manufacturer's instruction (Cayman) and results are expressed as pg/mL of PGE_2.

Statistical Analysis. Values are expressed as the mean ± SEM of *n* animals for in vivo experiments and *n* experiments run in triplicate for in vitro experiments. Comparisons were calculated by one-way analysis of variance (ANOVA) and Bonferroni-corrected *p* value for multiple comparisons. The level of statistically significant difference was defined as $p < 0.05$.

Acknowledgment. This work was supported by Italian MIUR (Progetto Sostanze Naturali ed Analoghi Sintetici ad Attività Antitumorale) and completed by FIRB 2003-RBNE03B8K (Progetto Riconoscimento Molecolare nelle Interazioni Proteina-Ligando) support. We thank Dr. Riccardo Motti, University of Naples Federico II, for collecting and identifying the plant material. Mass and NMR spectra were recorded at the "Centro Interdipartimentale di Analisi Strumentale" of the University of Naples "Federico II".

Supporting Information Available: ^{1}H and ^{13}C NMR, and 2D COSY, HSQC, HMBC, and ROESY NMR spectra of pepluanone. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM050321R