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Parthenolide and Its Photochemically Synthesized 1(10)*Z* Isomer: Chemical Reactivity and Structure–Activity Relationship Studies in Human Leucocyte Chemotaxis

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Abstract—The present study has achieved the photochemical conversion of a germacrolide into a melampolide. The investigation on their chemical properties allowed us to evaluate the minimum interatomic distance needed for transannular bridging of C_{10} ring in germacrolides and to explain the regiochemical selectivity of electrophilic cyclizations. The antiinflammatory activity of parthenolide and its semisynthetic derivatives was evaluated by in vitro chemotaxis assay with human neutrophiles. These structure–activity relationship studies have led to hypothesize a new pharmacophore and have provided useful information for computationally designed drugs.

Introduction

Parthenolide **1** (Fig. 1) has been revealed to be the major active principle of European feverfew (Tanacetum parthenium) on account of numerous and diverse assays. Despite the wide spectrum of biological properties that **1** exhibits and the intense pharmacological research,[1] the interrogatives about its mechanism of action and the relationship between chemical structure and biological activity are still left unsolved.

The exomethylene lactone functionality was immediately deemed to have fundamental importance because of its exceptional reactivity with nucleophilic groups.[2]

Later, several sesquiterpene lactones (SL), tested as witchweed germination stimulants, displayed that the exomethylene lactone moiety did not have an essential role but the spatial arrangement of the terpenoid skeleton was more important than the presence of any specific functional group in the molecule.[3] For instance, **1** and its 11,13-dihydro derivative **2** stimulated witchweed germination at a comparable percentage and to a lesser extent than the eudesmanolides santamarin and reynosin; these four compounds share a similar spatial arrangement but differ as to the chemical functionalities.

A subsequent investigation on the influence of chemical functionalities and molecular conformations strengthened

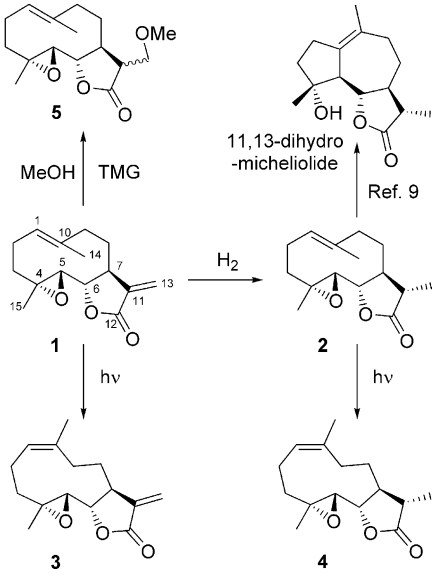

Figure 1. Chemical transformations of **1**.

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this conclusion.[4] However three different skeletons were selected so that no reasonable generalization emerged from these biological assays.

Extremely interesting studies on the acid catalyzed rearrangements of 4,5-epoxygermacrolides were eventually published (Fig. 2a and b);[5] the authors suggested a new pathway in their mechanism of biological action. In fact the facile transannular cyclization to *cis*-guaianes furnishes a carbocation at C-10 which becomes a second alkylating site for nucleophilic groups in addition to the exomethylene lactone functionality.

This is an appealing hypothesis that has been supported by results on the capability of many SL at inhibiting the transcription factor NFkB.[6] Despite several discrepancies, the authors pointed out that the most active SL contain two electrophilic sites: the exomethylene lactone and an enone functionality. Parthenolide was considered to be an exception being a highly active though monofunctional compound. Notably, the most powerful group comprises compounds of different skeletal types: guaianolides, pseudoguaianolide, germacrolides, melampolides, heliangolides, and 4,5-dihydrogermacranolides.

Our impression is that metabolites have been chosen for bioassays till now on the basis of their availability and that a previous protocol ought to be revised.[4] In order to better understand the mode of action we believe that SL should be selected on the basis of the following criteria: (i) test a compound with an exomethylene lactone functionality and its 11,13-dihydro analogue; (ii) examine the most stable molecular conformations and, if necessary, compare the influence of slight modifications from the adopted spatial arrangement; (iii) study their chemical properties and the way they could be modified by the introduction of new functional groups.

We decided to verify the effectiveness of such research procedure and in this study we have chosen parthenolide **1**, an SL the pharmacologists are chiefly concerned with, and the chemotaxis index bioassay.

It is known that injured vascular endothelium shows an increased release of chemokines, that attract neutrophils to sites of injury; interleukin-8 is among these and is known to be the strongest chemoattractant for these leukocytes.[7] Neutrophils migrate into the vascular intima and lead to further destruction of endothelium by enhanced production and release of superoxide anions.[8] Activation and migration of neutrophils play an important role in the development of tissue injury and is a hallmark of every inflammatory process.

Chemistry

The 11,13-dihydroparthenolide **2** was easily obtained through catalytic hydrogenation (Fig. 1) whereas a derivative suitable for comparison with the (*E*)/(*Z*) stereochemistry was identified in the geometrical isomer 1(10)-(*Z*)-parthenolide **3**. We attempted to carry out the 1(10)-(*E*) to 1(10)-(*Z*) photoisomerization: parthenolide and its 11,13-dihydro analogue **2** were exposed to 254 nm UV light in benzene for 20 h at rt furnishing the respective *cis*-geometric isomers in 76% (**3**) and 100% (**4**) yield (Fig. 1). The (*Z*)-configuration at the C(1)–C(10) double bond in both **3** and **4** was proven by the typical downfield shift of C(14) and by NOE enhancement between H(1) and 3H(14). These represent the first syntheses of 1(10)-(*Z*)-parthenolide and its 11,13-dihydro analogue and their full spectroscopic characterizations. It has been reported that UV irradiation of dihydroparthenolide **2** in benzene gave

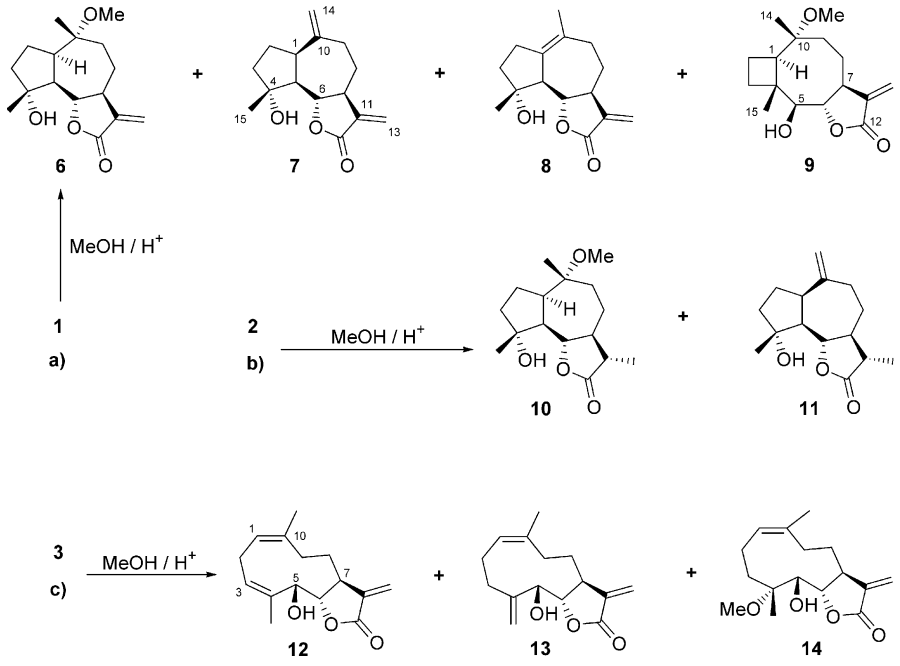

Figure 2. Reactions of **1**, **2** and **3** with pTSA / MeOH.

11,13-dihydromicheliolide (Fig. 1),[9] so we looked for the reasons of such a diverse outcome. Since Govindachari et al. purified the product by sublimation at 110 °C, we decided to examine thermal as well as acid-base chemical properties of **1**.

The parthenolide was warmed in toluene at 110 °C under N_2 for 7 h and gave decomposition to unknown products in only 2%, whereas stirring in MeOH/TMG (tetramethyl-guanidine) led exclusively to the Michael type product **5** by addition of methanol to the 11,13-double bond (Fig. 1). The structure follows from the observed parent peak at $m/z = 280$, the disappearance of the olefinic protons of the exomethylene moiety and the appearance of a singlet signal at 3.38 ppm, in accordance with a methoxy substituent at C(13). Parthenolide **1** was dissolved in MeOH/H^+ at rt and resulted in the formation of compounds shown in Figure 2a. The reaction mixture was purified by repeated HPLC and the products were characterized by MS and NMR spectroscopy. Compound **6** (a bicyclo[5.3.0]-decane skeleton) exhibited a parent peak at $m/z = 280$, the ^1H and ^{13}C NMR spectra were compatible with a guaianolide structure bearing hydroxy and methoxy groups at quaternary carbons. Compound **7** and **8** were identified by comparison of their spectral data (^1H, ^{13}C NMR and MS) with reported values.[10] The four membered ring of compound **9** (a bicyclo[6.2.0]decane skeleton) was established by COSY maps and COLOC correlations of H(1) with C(2), C(4), C(5), C(10) and of 3H(15) with C(1), C(3), C(5). The stereochemistry of the ring fusion was unambiguously established as *trans*, due to NOE enhanced signals of: $H_\beta(2)$ on irradiation at 3H(14), $H_\beta(2)$ and H(6) on irradiation at 3H(15), H(1) on irradiation at the methoxy protons.

Our experiments prove that the 11,13-dihydromicheliolide (Fig. 1) can only be obtained through acid catalyzed rearrangement, it is therefore necessary to suppose that either the solvent or the glassware the CIBA researchers used, contained acidic impurities. It should be added that nucleophiles bound to C(13) or C(10) in base or acid conditions respectively.

Compounds **10** and **11** (Fig. 2b) were generated from 11,13-dihydroparthenolide **2** and identified by spectral data. The set of products obtained by treatment of both **1** and **2** with H^+/MeOH parallels the results from a study on the lipiferolide.[11] The measure of relative concentrations for **6** and **10** allowed us to establish that their rate of formation are comparable and only slightly affected by the exomethylene lactone functionality (Fig. 3). Compound **3** was also dissolved in H^+/MeOH but it was consumed in three weeks; the reaction mixture was purified by reversed phase HPLC and three main products **12**, **13** and **14** were isolated: they all derived from exclusive opening of the epoxide ring without any transannular cyclization (Fig. 2c). All three products were seen to possess the (*Z*)-configuration at C(1)–C(10), as was confirmed by one-dimensional NOE difference spectroscopy.

In compound **12** two olefinic protons were found at 5.26 and 5.51 ppm and the connectivity H(14)–H(1)–H(2)–H(3)–H(15) was established by a COSY experiment. Compound **13** showed two exomethylenic protons at C(15) instead of the methyl signal of the starting compound. Both **12** and **13** showed parent peaks at $m/z = 248$. The parent peak at $m/z = 280$ of compound **14** indicated addition of methanol; the connectivity and the relative configurations were established by COSY and NOE difference spectroscopy.

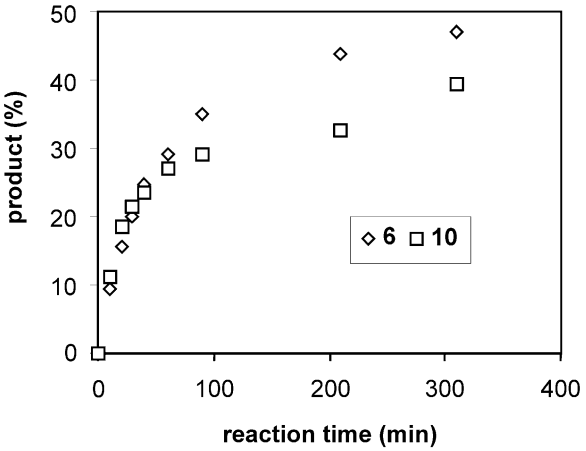

Figure 3. Formation rates of **6** and **10** from acid catalyzed reactions of **1** and **2** (see Experimental).

Pharmacology

The chemotaxis chamber consists of two parts: a lower part, which contains the chemoattractants and is covered with a nitrocellulose filter, and an upper part. In this system, a concentration gradient of the chemoattractant, which is put into wells of the lower part, is built, and cells, which are added into the wells of the upper part of the chamber, migrate toward the chemoattractant gradient. Chemotaxis[12] (migration in the presence of an attractant gradient) can be distinguished from chemokinesis (spontaneous migration in the absence of attractants) when quantifying stained nitrocellulose filters. If substances in the lower compartment of the chamber are chemotactic for cells, these migrate with higher frequency and speed, increasing the distance of migration into the filter micropores with a lead made by a few cells that can be microscopically measured, whereas cells migrating randomly in the absence of chemotactic gradients most often appear as a dense layer of cells just beneath the filter surface. Data are expressed as chemotaxis index, which is the ratio between the distance of migration toward IL-8 and that toward medium. The chemotaxis index of IL-8 has been set as reference (100%).

Results

By reason of our observed diverse reactivity between **1** and **3**, it is necessary to examine the reactions involving the carbonium ions generated from 1(10)-(*E*)- or 1(10)-(*Z*)-parthenolide and to carefully analyze the

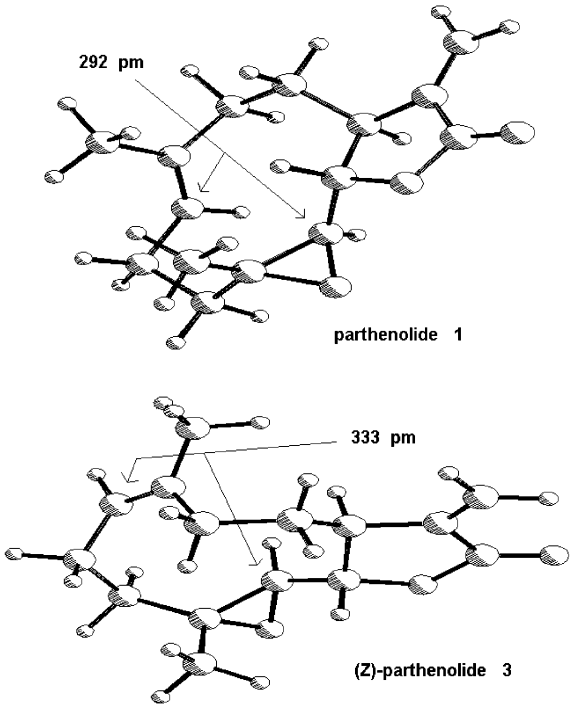

Figure 4. MM-calculated lowest energy conformers of **1** and **3** and their C(1)–C(5) interatomic distances.

stereoelectronic requirements implied in the subsequent rearrangements.

The electrophilic transannular cyclization reaction is strictly dependent on the spatial arrangement of the medium cycle substrate. In general, every one of the four subgroups of germacranolide sesquiterpenes shows a specific conformation which is determined by the (*E*)/(*Z*) combination of the two double bonds; the germacrolide subgroup comprises the molecules having the 1(10)-(*E*) and 4-(*E*) stereochemistry. In the main conformation, the double bonds are approximately perpendicular to the plane of the medium ring and adopt a crossed orientation with the two methyl groups in the position above the plane (Fig. 4). The 4,5-epoxide function doesn't modify the disposition of surrounding atoms, rather it becomes the preferred site for the attack of any acid catalyst. Thus the natural parthenolide can be placed in the germacrolide subgroup. Besides MM calculations allowed to measure 292 pm for the C(1)–C(5) distance.

Mechanistically the transannular cyclization starts with the electrophilic opening of the epoxide ring and the generation of a carbocation susceptible to cyclize: the 3^{ary} C(4) cation would give rise to a [6.2.0]- opposed to a [5.3.0]-decane ring system whereas the 2^{ary} C(5) cation may form a [5.3.0]- opposed to a [4.4.0]-decane skeleton. Compound **1** yielded solely the rearrangement products derived from the final C(10) 3^{ary} carbocation: [6.2.0] and [5.3.0] respectively (Fig. 5). Therefore, the stability order of carbocations may be thought as the main driving force in determining the regiochemistry of transannular cyclizations. To the best of our knowledge, this feature has never been outlined before.

Changing to 1(10)-(*Z*)-parthenolide **3**, MM calculations determined the preferred conformation (Fig. 4) that is typical of the melampolide subgroup: there is an anti arrangement, with C(14) below and C(15) above the medium ring. Here the interatomic distance C(1)–C(5) amounts to 333 pm and this prevents the transannular cyclization from occurring. The acid catalyzed opening of the epoxide ring in **3** led to the exclusive formation of the 3^{ary} carbocation at C(4), then the positive charge is neutralized by losing H^+ or adding methanol (Fig. 2c).

In order to investigate the anti-inflammatory effect in vitro, human neutrophils were exposed to compounds **1**, **2**, **3**, **4**, **6**, **7**, **8**, **9** or medium as control. Compound **4** diminished the chemotactic response slightly, compound **3** was totally ineffective (Fig. 6). The others, as a whole, decreased chemotaxis significantly, in detail the effect of **6** at the lowest concentration (10 pg/mL) was comparable to **1** at the highest concentration (10^7 pg/mL). A linear dose response could be seen for compounds **1, 2**, **6** and **9**; compounds **7** and **8** did not show a concentration-dependent response and might therefore act via non-receptor dependent mechanisms. This however remains speculative and will need further studies. In previous studies homologous deactivation, that means migration toward IL-8 after pre-treatment with IL-8,

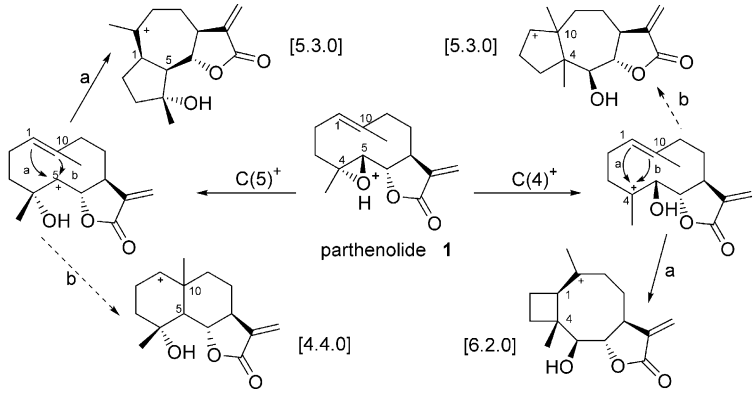

Figure 5. Mechanisms of acid-catalyzed transannular cyclization of **1**. The formation of cation at C(5) is preferred with respect to C(4); the pathways **a** are exclusively observed whilst the pathways **b** do not occur. IUPAC notations for bicyclic decane ring systems are reported in square brackets.

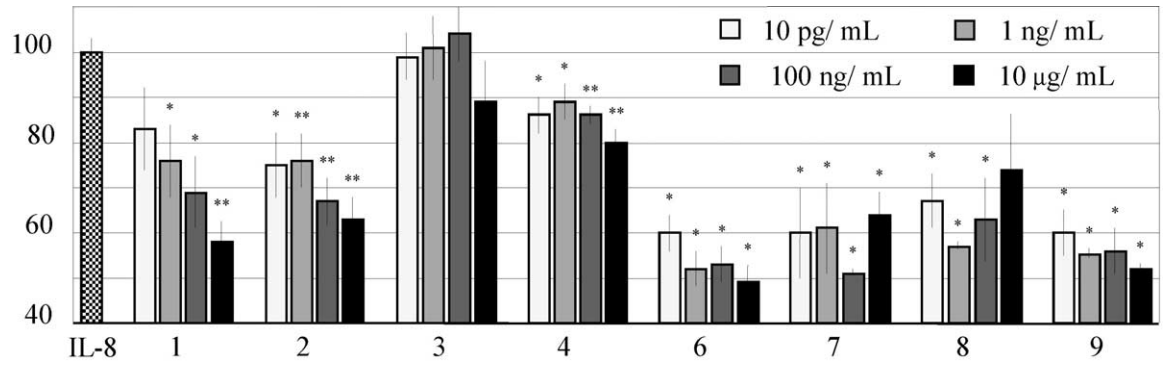

Figure 6. Chemotactic response of **1**, **2**, **3**, **4, 6**, **7**, **8** and **9** at four different concentrations. Data are given as percentual values ± standard error of the mean (SEM). Statistical analysis: Mann–Whitney U (*P <0.05; **P <0.001) after Kruskal–Wallis (P <0.01); n = 6 for **1**, **2**, **3** and **4**, n = 3 for **6**, **7**, **8** and **9**.

was 52.6%.[13] The active compounds were therefore comparable in their ability to reduce chemotactic response.

Discussion

Two fundamental factors emerge from our chemical experimental results: the acid catalyzed rearrangements regioselectively furnish products derived from a 3^{ary} carbocation as final intermediate and the electrophile induced transannular cyclization occurs only when the distance between the bonding carbon atoms is shorter than the rough averaged value of 310 pm.

Following our research protocol, at point (i) we can confidentially state that, within the four tested germacranolides, the exocyclic α-methylene-γ-lactone moiety possesses no or negligible role in chemotaxis indexes. However slight structural changes among **1** or **2** and **3** or **4** exert major influences on their biological properties, because modifications in the molecular spatial arrangement, point (ii), affect largely their chemical reactivity, point (iii). The (*E*)/(*Z*) geometric isomerism drastically modifies the biological activity so it should not originate from a particular set of functional groups (electrophilic, nucleophilic, acidic, basic, etc.). More plausible causes are the tendency to give the electrophilic transannular cyclization or the structure of the reaction products.

In actual fact, the bicyclic compounds **6**, **7** and **9**, derived from acidic treatment of parthenolide, inhibited chemotaxis more than the SL substrate. This lessens the route of alkylation at C(10) by endogenous nucleophiles, as proposed by Fisher et al., and opens the possibility that **1** undergoes metabolic transformations to produce more active binders. We therefore hypothesize that, in the damaged tissue or at the active site of the enzyme, **1** turns into a series of products that are the effective anti-inflammatory agents. The low pH value at the injured tissue coupled to the peculiar tendency of **1** to change into a guaiane skeleton, may constitute a sort of clever trick for delivering the drug to the targeted cells.

In the absence of any knowledge about the receptor, pharmacophores may provide important information in the drug design process. To this end, parthenolide appears to be misleading because it might be a pro-drug which exerts its anti-inflammatory properties by means of a metabolic activation. The chemical features in a proper spatial arrangement, forming the pharmacophore unit, should be searched in the bicyclic core of a sesquiterpene compound. In fact, the modest structural changes among **6**, **7** and **8** have marked influence on the migration of neutrophils. The biological role of the lactone moiety remains to be established.

Conclusions

The present study has corrected a previous literature report and achieved the photochemical conversion of a germacrolide into a melampolide SL. The investigation on the chemical properties of compounds **1**, **2** and **3**, in both basic and acid solutions, explained the regiochemical selectivity and appraised the minimum interatomic distance needed for transannular bonding.

The evaluation of the chemotactic response sheds a new light on the action of **1** as an antiinflammatory natural product. While not exhaustive, this information provides additional hints for drug designers to decide what compounds to make or/and screen for efficacy. The chemical functionalities and the way of biological interaction of a possible major agent, are the subject of on going research in our laboratories.

Experimental

Chemistry. Flash chromatography (FC): Merck RP-18 LiChroprep (40–65 μm). TLC: Merck-Kieselgel 60 PF_{254}. HPLC: Reinin Dynamax 60A CN (8 μm); Machery-Nagel Nucleosil 100-5 C18; 25 × 1 cm column, solvent flux 3 mL/min. Optical rotation: JASCO-DIP-181 polarimeter, $[\alpha]_D$ in deg mL dm^{-1} g. NMR: Varian XL-300 (^1H at 299.94 MHz, ^{13}C at 75.4 MHz), δ in ppm using residual solvent signals as internal standard ($CDCl_3$ = 77.0 ppm, $CHCl_3$ = 7.25 ppm), J values in Hz,

multiplicities and peak assignments from DEPT, ^{1}H, ^{1}H-COSY, $^1J_{CH}$- and $^nJ_{CH}$-COSY. MS: Kratos MS80 with home-built acquisition system. Molecular mechanics (MM) calculations were carried out with the PCMODEL V7.5 computer program, searching for the global energy minima.

1: Parthenolide. *Tanacetum parthenium* was generously given by ISAFA (Villazzano, Trento). The AcOEt extract (3.4 g) of fresh plant material was subjected to FC on silica and eluted with gradient of AcOEt in hexane to afford 10 fractions. Central fractions were evaporated and the residues were submitted to HPLC purification (CN, $\lambda = 225$ nm, hexane/ethanol 80:20), thus furnishing **1** (75 mg).

2: 11,13-Dihydroparthenolide. **2** Was obtained from **1** according to literature procedure.[9]

3: (−)(1a*R*,4*Z*,7a*S*,10a*S*,10b*S*)-2,3,6,7,7a,8,10a,10b-Octahydro-1a,5-dimethyl-8-methyleneoxireno[9,10]cyclodeca[1,2-*b*]furan-9(1a*H*)-one. A solution of **1** (6.5 mg, 0.026 mmol) in 3 mL of benzene was degassed for 30 min by bubbling in N_2, irradiated with UV-light ($\lambda = 254$ nm) for 20 h and the solvent removed. The crude reaction product was purified by means of HPLC (CN, $\lambda = 225$ nm, hexane/ethanol 80:20) to provide unreacted **1** (3.4 mg, 0.014 mmol) and **3** (2.3 mg, 0.009 mmol, 76% yield). $[\alpha]_D^{25}$ −31 ($c = 0.87$, EtOH). ^{1}H NMR ($CDCl_3$): δ 6.23 (d, $J = 3.6$ Hz, 1H, H-13), 5.53 (d, $J = 3.2$ Hz, 1H, H-13), 5.34 (bt, 7 Hz, 1H, H-1), 3.83 (t, $J = 9.4$ Hz, 1H, H-6), 2.90 (d, $J = 9.4$ Hz, 1H, H-5), 2.76 (ddddd, $J = 12.0$, 9.4, 3.6, 3.2, 3.0 Hz, 1H, H-7), 2.46 (td, $J = 13.0$, 5.0 Hz, 1H, H-9β), 2.36 (tdd, $J = 13.0$, 5.0, 3.0 Hz, 1H, H-8α), 2.24 (m, 1H, H-2), 2.15.05 (m, 3H, H-2, H-3β, H-9α), 1.70 (bs, 3H, H-14), 1.63 (m, 1H, H-8β), 1.52 (s, 3H, H-15), 1.07 (m, H-3α). ^{13}C NMR ($CDCl_3$): δ 169.5 (s, C-12), 139.0 (s, C-11), 135.7 (s, C-10), 125.8 (d, C-1), 119.9 (t, C-13), 81.2 (d, C-6), 63.5 (d, C-5), 60.1 (s, C-4), 42.4 (d, C-7), 37.2 (t, C-3), 26.9 (t, C-9), 25.8 (t, C-8), 23.9 (t, C-2), 21.5 (q, C-14), 18.0 (q, C-15). MS: *m*/*z* 248 (M^+, 3%), 43 (100%). HRMS calcd for $C_{15}H_{20}O_3$ (M^+), 248.14124; found, 248.14106.

4: (−)(1a*R*,4*Z*,7a*S*,8*S*,10a*S*,10b*S*)-2,3,6,7,7a,8,10a,10b-Octahydro-1a,5,8-trimethyloxireno[9,10]cyclodeca[1,2-*b*]furan-9(1a*H*)-one. A solution of **2** (9.0 mg, 0.036 mmol) in 3 mL of benzene was degassed for 30 min by bubbling in N_2, irradiated with UV-light ($\lambda = 254$ nm) for 20 h and the solvent removed. The crude reaction product was purified by means of HPLC (CN, $\lambda = 213$ nm, hexane/ethanol 80:20) to provide unreacted **2** (4.8 mg, 0.019 mmol) and **4** (4.1 mg, 0.017 mg, 100%). $[\alpha]_D^{25}$ −74 ($c = 3.4$, EtOH). ^{1}H NMR ($CDCl_3$): δ 5.29 (bt, 7 Hz, 1H, H-1), 3.81 (t, $J = 9.5$ Hz, 1H, H-6), 2.80 (d, $J = 9.5$ Hz, 1H, H-5), 2.45 (td, $J = 13.0$, 5.0 Hz, 1H, H-9β), 2.27 (dq, $J = 12.0$, 7.0 Hz, 1H, H-11), 1.98.20 (m, 5H, 2H-2, H-3β, H-8α, H-9α), 1.85 (tdd, $J = 12.0$, 9.5, 3.0 Hz, 1H, H-7), 1.67 (bs, 3H, H-14), 1.53 (m, 1H, H-8β), 1.52 (s, 3H, H-15), 1.26 (d, 3H, H-13), 1.04 (m, 1H, H-3α). ^{13}C NMR ($CDCl_3$): δ 179.2 (s, C-12), 137.2 (s, C-10), 126.8 (d, C-1), 82.6 (d, C-6), 64.9 (d, C-5), 61.2 (s, C-4), 47.3 (d, C-7), 42.6 (d, C-11), 38.6 (t, C-3), 28.5* (t, C-9), 28.2* (t, C-8), 25.0 (t, C-2), 22.7 (q, C-14), 19.1 (q, C-15) 14.3 (q, C-13). MS: *m*/*z* 250 (M^+, 4.5%), 43 (100%). HRMS calcd for $C_{15}H_{22}O_3$ (M^+), 250.15689; found, 250.15698.

5: (−)(1a*R*,4*Z*,7a*S*,10a*S*,10b*S*)-2,3,6,7,7a,8,10a,10b-Octahydro-8-(methoxymethyl)-1a,5-dimethyloxireno[9,10]cyclodeca[1,2-*b*]furan-9(1a*H*)-one. To a solution of **1** (4.0 mg, 0.016 mmol) in 1 mL of MeOH was added an excess of tetramethylguanidine (100 μL) at room temperature. After 15 min the reaction mixture was purified by TLC (Si60, hexane/ether 3:7) to yield **5** as a white solid (3.6 mg, 0.0128 mmol, 80%). $[\alpha]_D^{25}$ −30 ($c = 1.5$, EtOH). ^{1}H NMR ($CDCl_3$): δ 5.20 (bdd, $J = 12$, 2 Hz, 1H, H-1), 3.82 (t, $J = 9.0$ Hz, 1H, H-6), 3.74 (dd, $J = 10.0$, 3.0 Hz, 1H, H-13), 3.65 (dd, $J = 10.0$, 3.0 Hz, 1H, H-13), 3.38 (s, 3H, MeO), 2.75 (d, $J = 9.0$ Hz, 1H, H-5), 2.34.46 (m, 2H, H-7, H-11), 2.30.40 (m, 1H, H-2β), 2.20.30 (m, 1H, H-9β), 2.05.20 (m, 3H, H-2α, H-3β, H-9α), 1.9.0 (m, 1H, H-8α), 1.69 (bs, 3H, H-14), 1.62 (m, 1H, H-8β), 1.28 (s, 3H, H-15), 1.15.30 (m, 1H, H-3α). ^{13}C NMR ($CDCl_3$): δ 175.0 (s, C-12), 134.5 (s, C-10), 125.2 (d, C-1), 82.2 (d, C-6), 68.2 (t, C-13), 66.3 (d, C-5), 61.5 (s, C-4), 59.4 (q, MeO), 48.6 (d, C-7), 45.5 (d, C-11), 41.0 (t, C-3), 36.6 (t, C-9), 30.0 (t, C-8), 24.1 (t, C-2), 17.2 (q, C-15), 16.9 (q, C-14). MS: *m*/*z* 280 (M^+, 2.0%), 45 (100%). HRMS calcd for $C_{16}H_{24}O_4$ (M^+), 280.16746; found, 280.16710.

Acid treatment of 1. To a solution of **1** (40.0 mg, 0.161 mmol) in 6 mL of MeOH was added pTsOH (55 mg, 0.32 mmol, 2 equiv) at room temperature. After 5 h the reaction mixture was neutralized with Na_2HPO_4 (2 equiv in 1 mL H_2O), added of RP18 stationary phase and dried. The adsorbed material was washed with water and eluted with acetone. This portion (45 mg) was subjected to HPLC (Nucleosil, CH_3CN/H_2O 75: 25, $\lambda = 213$ nm) to give **8** (10.3 mg, 0.042 mmol, 26%), **7** (7.0 mg, 0.028 mmol, 18%) and 22.4 mg of a mixture which by HPLC (CN, hexane/EtOH 80 : 20, $\lambda = 213$ nm) furnished pure **6** (21.0 mg, 0.075 mmol, 47%) and **9** (4.0 mg, 0.014 mmol, 9%).

6: (−)(3a*S*,6*R*,6a*R*,9*R*,9a*S*,9b*S*)-Decahydro-9-hydroxy-6-methoxy-6,9-dimethylazuleno[4,5-*b*]furan-2(3*H*)-one. $[\alpha]_D^{25}$ −31 ($c = 4.2$, EtOH). ^{1}H NMR ($CDCl_3$): δ 6.20 (d, $J = 3.5$ Hz, 1H, H-13), 5.49 (d, $J = 3.1$ Hz, 1H, H-13), 4.18 (dd, $J = 11.7$, 9.7 Hz, 1H, H-6), 3.17 (s, 3H, MeO), 2.80 (dd, $J = 11.5$, 8.0 Hz, 1H, H-1), 2.70.88 (m, 1H, H-7), 2.32 (dd, $J = 11.7$, 11.5 Hz, 1H, H-5), 2.15 (dddd, 13.0, 5.5, 4.7, 4.0 Hz, 1H, H-8α), 1.95 (ddd, 14.0, 5.5, 4.7 Hz, 1H, H-9β), 1.75.90 (m, 3H, H-2, 2H-3), 1.68 (m, 1H, H-9α), 1.44.59 (m, 1H, H-2), 1.22.44 (m, 1H, H-8β), 1.36 (s, 3H, H-15), 1.14 (s, 3H, H-14). ^{13}C NMR ($CDCl_3$): δ 169.6 (s, C-12), 138.9 (s, C-11), 120.1 (t, C-13), 82.7 (d, C-6), 80.4 (s, C-4), 78.1 (s, C-10), 55.4 (d, C-5), 48.3 (q, MeO), 46.8 (d, C-7), 45.9 (d, C-1), 39.0 (t, C-3), 36.0 (t, C-9), 25.5 (t, C-2), 24.4 (t, C-8), 24.4 (q, C-15), 22.5 (q, C-14). MS: *m*/*z* 280 (M^+, 2.4%), 43 (100%). HRMS calcd for $C_{16}H_{24}O_4$ (M^+), 280.16746; found, 280.16734.

9: (−)(3a*S*,6*R*,6a*R*,8a*R*,9*S*,9a*S*)-Decahydro-9-hydroxy-6-methoxy-6,8a-dimethyl-3-methylenecyclobuta[6,7]cycloocta[1,2-*b*]furan-2(3*H*)-one. $[\alpha]_D^{25}$ −45 (c = 1.0, EtOH). ^{1}H NMR ($CDCl_3$): δ 6.25 (d, J = 3.5 Hz, 1H, H-13), 5.58 (d, J = 3.1 Hz, 1H, H-13), 4.18 (dd, J = 9.8, 7.2 Hz, 1H, H-6), 3.58 (d, 9.8 Hz, 1H, H-5), 3.16 (s, 3H, MeO), 3.08.18 (m, 1H, H-7), 2.52 (dd, J = 10.9, 8.0 Hz, 1H, H-1), 2.23 (tdd, 13.5, 5.5, 4.5 Hz, 1H, H-8α), 2.14 (dddd, J = 10.9, 10.7, 10.5, 9.9 Hz, 1H, H-2β), 1.90 (m, 1H, H-2α), 1.60.84 (m, 3H, 2H-3, H-9), 1.40 (ddt, J = 13.5, 11.5, 4.0 Hz, 1H, H-8β), 1.20.28 (m, 1H, H-9), 1.28 (s, 3H, H-15), 1.23 (s, 3H, H-14). ^{13}C NMR ($CDCl_3$): δ 169.2 (s, C-12), 140.6 (s, C-11), 122.2 (t, C-13), 84.5 (d, C-5), 82.1 (d, C-6), 76.6 (s, C-10), 49.3 (q, MeO), 47.6 (d, C-1), 43.7 (d, C-4), 40.3 (t, C-7), 34.2 (t, C-3), 32.5 (t, C-9), 27.0 (t, C-8), 23.6 (q, C-14), 22.3 (t, C-2), 14.0 (s, C-15). MS: m/z 280 (M^+, 0.2%), 85.1 (100%). HRMS calcd for $C_{16}H_{24}O_4$ (M^+), 280.16746; found, 280.16702.

Kinetic investigations of the acid-catalysed reactions of 1 and 2 with MeOH. To each solution of **1** (6.5 mg, 0.026 mmol) and **2** (6.5 mg, 0.026 mmol) in 6.5 mL of MeOH-d_4 was added pTsOH (8.9 mg, 0.052 mmol, 2 equiv) and the reactions at 20 °C were monitored by means of ^{1}H NMR spectroscopy. The relative amounts of the main products **6** and **10** were calculated from the integral ratio of the protons H-13 of **6**/(**1** + **6**) and of the proton H-6 of **10**/(**2** + **10**).

Acid treatment of 3. To a solution of **3** (15.0 mg, 0.0605 mmol) in 3 mL of MeOH was added pTsOH (21 mg, 0.12 mmol, 2 equiv) at room temperature. After 22 days the reaction mixture was neutralized with Na_2HPO_4 (2 equiv in 1 mL H_2O), added of RP18 stationary phase and dried. The adsorbed material was washed with water and eluted with acetone (14 mg). The ^{1}H NMR spectrum of the crude eluate suggested the following composition: **12** (50%), **13** (25%), **14** (12%). The mixture was then purified by HPLC (Nucleosil, CH_3CN/H_2O 38:62, λ = 213 nm) to give **12** (1.7 mg, 0.007 mmol, 11%), **13** (1.2 mg, 0.0043 mmol, 8.0%) and **14** (0.8 mg, 0.0029 mmol, 5%).

12: (−)(3a*S*,6*Z*,9*Z*,11*S*,11a*S*)-3a,4,5,8,11,11a-Hexahydro-11-hydroxy-6,10-dimethyl-3-methylenecyclodeca[*b*]furan-2(3*H*)-one. $[\alpha]_D^{25}$ −53 (c = 0.87, EtOH). ^{1}H NMR ($CDCl_3$): δ 6.26 (d, J = 3.0 Hz, 1H, H-13), 5.56 (d, J = 2.6 Hz, 1H, H-13), 5.51 (bt, J = 8.0 Hz, 1H, H-3), 5.26 (bt, J = 7.5 Hz, 1H, H-1), 4.51 (d, J = 5.1 Hz, 1H, H-5), 4.43 (t, J = 5.1 Hz, 1H, H-6), 2.86–2.96 (m, 1H, H-7), 2.8.9 (overlapped, 1H, H-2), 2.72 (dt, J = 13.0, 7.0 Hz, 1H, H-2), 2.57 (bddd, J = 13.0, 11.0, 2.5 Hz, 1H, H-9β), 2.14 (ddd, J = 13.0, 6.0, 2.5 Hz, 1H, H-9α), 1.90 (dddd, J = 13.0, 11.0, 3.5, 2.5 Hz, 1H, H-8α), 1.78 (d, 1.4 Hz, 3H, H-15), 1.71 (d, J = 1.2 Hz, 3H, H-14), 1.60 (dddd, J = 13.0, 10.5, 6.0, 2.5 Hz, 1H, H-8β). ^{13}C NMR ($CDCl_3$): δ 169.8 (s, C-12), 139.6 (s, C-11), 134.9 (s, C-4), 133.6 (s, C-10), 125.8 (d, C-3), 123.2 (d, C-1), 122.4 (t, C-13), 84.4 (d, C-6), 74.1 (d, C-5), 41.0 (d, C-7), 30.8* (t, C-2), 28.3* (t, C-8), 27.8* (t, C-9), 24.2 (q, C-14), 20.1 (q, C-15). MS: m/z 248 (M^+, 23.8%), 41 (100%). HRMS calcd for $C_{15}H_{20}O_3$ (M^+); 248.14124, found, 248.14097.

13: (−)(3a*S*,6*Z*,11*S*,11a*S*)-3a,4,5,8,9,10,11,11a-Octahydro-11-hydroxy-6-methyl-3,10-bis(methylene)cyclodeca[*b*]furan-2(3*H*)-one. $[\alpha]_D^{25}$ −94 (c = 0.80, EtOH). ^{1}H NMR ($CDCl_3$): δ 6.21 (d, J = 1.7 Hz, 1H, H-13), 5.58 (d, J = 1.5 Hz, 1H, H-13), 5.12 (bdd, J = 12, 4 Hz, 1H, H-1), 4.96 (bs, 1H, H-15), 4.92 (d, J = 1.7 Hz, 1H, H-15), 4.55 (d, J = 9.4 Hz, 1H, H-5), 3.88 (d, J = 9.4 Hz, 1H, H-6), 2.68.82 (m, 2H, H-3α, H-7), 2.60 (bddd, J = 13, 11, 6 Hz, 1H, H-9β), 2.26.42 (m, 2H, H-2β, H-3β), 2.02.20 (m, 2H, H-2α, H-8α), 1.82.92 (m, 1H, H-9α), 1.69 (d, 1.4 Hz, 3H, H-14), 1.65 (m, 1H, H-8β). ^{13}C NMR ($CDCl_3$): δ 169.8 (s, C-12), 144.9 (s, C-4), 139.4 (s, C-11), 132.3 (s, C-10), 126.7 (d, C-1), 122.4 (t, C-13), 120.2 (t, C-15), 82.6* (d, C-5), 81.0* (d, C-6), 38.3 (d, C-7), 31.4** (t, C-3), 30.6** (t, C-2), 27.4 (t, C-9), 26.2 (t, C-8), 22.3 (q, C-14). MS: m/z 248 (M^+, 8.1%), 41 (100%). HRMS calcd for $C_{15}H_{20}O_3$ (M^+), 248.14124; found, 248.14037.

14: (−)(3a*S*,6*Z*,10*R*,11*R*,11a*S*)-3a,4,5,8,9,10,11,11a-Octahydro-11-hydroxy-10-methoxy-6,10-dimethyl-3-methylenecyclodeca[*b*]furan-2(3*H*)-one. $[\alpha]_D^{25}$ −83 (c = 0.53, EtOH). ^{1}H NMR ($CDCl_3$): δ 6.19 (d, J = 2.1 Hz, 1H, H-13), 5.58 (d, J = 1.9 Hz, 1H, H-13), 5.26 (bt, J = 8 Hz, 1H, H-1), 4.82 (dd, J = 5.4, 2.5 Hz, 1H, H-6), 3.58 (bd, J = 5 Hz, 1H, H-5), 3.47 (ddddd, J = 13.0, 5.1, 2.5, 2.1, 1.9 Hz, 1H, H-7), 3.20 (s, 3H, MeO), 2.20.35 (m, 1H, H-2), 1.9.2 (m, 3H, H-2, 2H-9), 1.5.8 (m, 4H, 2H-3, 2H-8), 1.71 (s, 3H, H-14), 1.22 (s, 3H, H-15). ^{13}C NMR ($CDCl_3$): δ # (s, C-12), 140.1 (s, C-11), # (s, C-10), 127.4 (d, C-1), 121.6 (t, C-13), 81.6 (d, C-6), 79.9 (s, C-4), 77.6 (d, C-5), 50.8 (q, MeO), 49.0 (d, C-7), 31.9* (t, C-3), 31.5* (t, C-8), 28.6* (t, C-9), 27.1* (t, C-2), 23.1 (q, C-15), 21.8 (q, C-14). MS: m/z 280 (M^+, 4.8%), 72 (100%). HRMS calcd for $C_{16}H_{24}O_4$ (M^+), 280.16746; found, 280.16750.

*, ** May be interchanged; #missing.

Biology. RPMI 1640 with phenol red was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Bovine serum albumin (BSA) was from Dade Behring (Marburg, Germany), IL-8 from Roche Diagnostics (Mannheim, Germany). Lymphoprep® was from Nycomed Pharma AS (Oslo, Norway), 48-blindwell microchemotaxis chamber from Neuroprobe (Gaithersburg, MD, USA), 5 μm pore-sized nitrocellulose filters from Sartorius (Goettingen, Germany). All experiments were obtained by a single technician who was blinded for test reagents in order to avoid systematic error in answering this sensitive question.

Chemotaxis assay. Neutrophils were obtained from peripheral EDTA anticoagulated blood of healthy volunteers after discontinuous density gradient centrifugation on Lymphoprep® by dextran sedimentation and centrifugation through a layer of Ficoll–Hypaque, followed by hypotonic lysis of contaminating erythrocytes using sodium chloride solution.[14] Cell preparations yielded more than 95% neutrophils (by morphology in GIEMSA stains) and more than 99% viability (by trypan dye exclusion). Experiments were performed in RPMI 1640 with 0.5% BSA.

For deactivation, cells were incubated for 20 min with different substrates at various concentrations or remained untreated and were washed twice in phosphate buffered saline (PBS) before testing for chemotaxis. 50 μL of the cell suspension (1×10^6 cells/mL) was put into the upper compartment of the chemotaxis chamber and cells were allowed to migrate for 30 min at 37 °C in a humidified atmosphere (5% CO_2) toward IL-8 (1 nM), which was put in the lower wells as chemoattractant. After this migration period the nitrocellulose filters were dehydrated, fixed and stained with haematoxylin-eosin. Migration depth was quantified microscopically, measuring the distance (μm) from the surface of the filter to the leading front of three cells.[15]

Data are expressed as mean and SEM. Means were compared by Mann–Whitney *U*-test after Kruskal–Wallis analysis of variance. A *P*-value <0.05 was considered significant. Analyses were performed using the StatView software package (Abacus Concepts, Berkeley, CA).

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