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Anthecularin: A Novel Sesquiterpene Lactone from *Anthemis auriculata* with Antiprotozoal Activity

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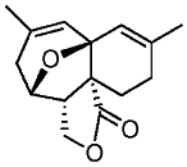

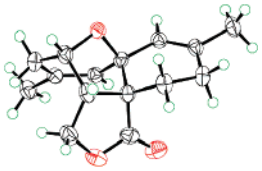

Anthecularin (**1**), a minor sesquiterpene lactone with a novel ring system was isolated from Greek *Anthemis auriculata* (Asteraceae). Its structure was elucidated by means of NMR, HRMS, and X-ray crystallography. Anthecularin showed antitrypanosomal (IC_{50} = 10.1 μg/mL) and antiplasmodial activity (IC_{50} = 23.3 μg/mL) and inhibited two key enzymes of the plasmodial type II fatty acid biosynthesis pathway, *Pf*FabI and *Pf*FabG (IC_{50} values = 14 and 28.3 μg/mL, respectively). A probable biogenesis of **1** is also proposed and discussed.

Infectious parasitic diseases such as trypanosomiasis and malaria still threaten the public health worldwide. Malaria is the most prevalent parasitic disease, claiming the lives of more than a million African children annually. One of the most promising targets that has emerged from the recently available *Plasmodium falciparum* genome sequencing project[1] is the type II fatty acid biosynthesis, which takes place in the newly recognized plastid-like organelle in *Plasmodium*, the apicoplast.[2] Fatty acids are essential to parasite survival because of their role in membrane structure, energy production, and the successful invasion of the host cells.[3] *Plasmodium* fatty acid synthase (*Pf*FAS) is a type II multiple enzyme complex, similar to that found in plants and bacteria, and as such, differs radically from human type I FAS, which is a large multifunctional polypeptide composed of distinct enzyme domains.[4] These structural differences underlie the strategy for the development of new antimalarial agents, which are selectively toxic to the parasite. *Pf*FabG (β-ketoacyl-ACP reductase), *Pf*FabI (enoyl-ACP reductase), and *Pf*FabZ (β-hydroxyacyl-ACP dehydratase) are three key enzymes of the plasmodial FAS-II machinery and represent ideal biological targets for malaria research.[5−7]

In our search for natural products targeting the individual enzymes of the plasmodial FAS-II cascade, we previously reported flavonoid glycosides,[8] flavonoid aglycones,[9] and a monoterpenic iridoidal compound.[10] Herein we describe a novel antimalarial sesquiterpene lactone, anthecularin (**1**), with dual enzyme inhibitory activity against *Pf*FabG and *Pf*FabI enzymes. The compound also exhibits antitrypanosomal activity without any cytotoxicity on primary mammalian cells. Anthecularin (**1**) was isolated from the aerial parts of Greek *Anthemis auriculata* (family Asteraceae), from which we recently reported three linear sesquiterpene lactones, anthecotulide (**2**), 4-hydroxyanthecotulide (**3**), and 4-acetoxyanthecotulide (**4**).[11] The compound was purified from the organic extract of the plant by repeated chromatography on silica gel, and its structure was elucidated by means of NMR (^{1}H, ^{13}C, DEPT-135, HSQC, DQF-COSY, TOCSY, HSQC-TOCSY, HMBC, ROESY), HREIMS, and X-ray crystallography. A literature survey indicates that anthecularin possesses a new skeletal type. This report deals with the isolation, structure elucidation, and the biological activity of the new compound **1**. A plausible biogenesis of anthecularin is also proposed and discussed.

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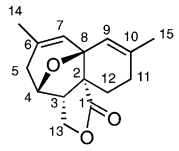

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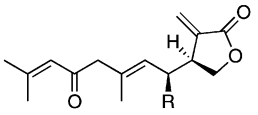

2 R= H
3 R= OH
4 R= OAc

Compound **1** was obtained as colorless crystals (mp 172−173 °C, $[\alpha]^{23}_{D}$ +23.9 (c = 0.15, $CHCl_3$)). Its molecular formula was determined as $C_{15}H_{18}O_3$ by HREIMS (*m*/*z* 246.1261, Δ: + 0.5 mmu). The ^{13}C NMR (150 MHz) spectrum in $CDCl_3$ (Table 1) contained 15 signals, two of which at δ 176.8 (s, C-1) and 66.5 (t, C-13) were readily assigned to a γ-lactone ring. This was consistent with the IR spectrum, which had a typical absorption band at ν_{max} 1775 cm^{-1}. Detailed inspection of the ^{13}C NMR data of **1** in combination with HSQC and DEPT-135 spectra provided evidence for the presence of two olefinic methines (δ 119.8 d, 127.5 d), two olefinic sp^3 carbons (δ 134.0 s, 142.3 s), two olefinic methyls (δ 22.4 q, 24.0 q), three aliphatic methylenes (δ 27.6 t, 30.2 t, 32.5 t), one methine (δ 52.8 d), one oxymethine (δ 76.2 d), one oxygenated quaternary sp^3 carbon (δ 77.7 s), plus a quaternary carbon (δ 60.2 s). The molecular formula indicated seven degrees of unsaturation. Since the lactone carbonyl and two double bonds accounted for three degrees of unsaturation, **1** was determined to be a tetracyclic sesquiterpene.

A combination of DQF-COSY and TOCSY correlations indicated the presence of two spin systems within **1** (Figure 1). The first spin system started with the lactone oxymethylene signals (H-13α δ 4.35 t, J = 10.0 Hz, H-13β δ 3.96 dd, J = 4.9, 10.0 Hz), which coupled scalarly with H-3 (δ 3.15 ddd, J = 4.9, 7.8, 10.0 Hz). The latter in turn showed clear homonuclear cross-peaks with a proton (H-4, δ 4.65, dd, J = 4.9, 7.8 Hz) attached to an oxygenated carbon (C-4, δ 76.2). Finally, H-4 correlated with a pair of methylene protons, which were assigned as H_2-5 (δ 2.68 and 1.93 m), thereby completing the substructure **A**. The second spin system included only two complex methylene signals, H_2-11 (δ 2.68 and 1.92 m), and H_2-12 (δ 2.01 and 1.90 m) (substructure **B**). A further HSQC-TOCSY experiment fully substantiated these substructures.

The interpretation of the HMBC data has played a crucial role for the elucidation of the planar structure of **1**. In particular, the olefinic methyl protons H_3-14 (δ 1.70 s) and H_3-15 (δ 1.81 s) provided convenient starting points to establish long-range connectivities. The H_3-14 signal exhibited couplings with C-6 (^2J), C-5 (^3J), and C-7 (^3J), which led to the fragment **a** (Figure 2). Similarly, the fragment **b** could be generated on the basis of substantial HMBC correlations between H_3-15 and C-10 (^2J), C-9 (^3J), and C-11 (^3J). Both methyl groups showed a four-bond HMBC correlation with C-8 that was significantly deshielded (δ 77.7 s), so an oxygen atom had to be placed here. The olefinic sp^2 protons, H-7 (δ 5.52) and H-9 (δ 5.44) had also long-range couplings with C-8, and with each other (H-7/C-9, H-9/C-7). Since both H-7 and H-9 appeared as broad singlets in the ^1H NMR spectrum, they had to be isolated, i.e., separated from each other by C-8. Thus, fragments **a** and **b** could be connected by the mutual C-8 atom to reveal the fragment **c**. Next, the γ-lactone ring was positioned between C-2 and C-3 on the basis of long-range correlations between C-1/H-3, C-1/H_2-12, C-1/H_2-13; C-2/H-3, C-2/H-4, C-2/H_2-12; C-3/H_2-13, C-3/H_2-12, and C-12/H-3. This formed the fragment **d**. The quaternary carbon atoms C-2 and C-8 had to be between the oxymethine H-4, and the oxygenated sp^3 C-8 pointed to the existence of an epoxy bridge between C-4 and C-8, which forms the fourth ring indicated by the MS data. The absence of an OH band in the IR spectrum and the molecular weight of the compound ruled out the existence of hydroxy groups at C-4 and C-8. Instead, an IR band was observed at ν_{max} 1264 cm^{-1}, further confirming this assumption.

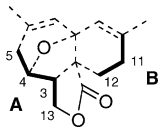

FIGURE 1. Substructures **A** and **B** deduced by DQF-COSY and TOCSY data (shown in bold lines).

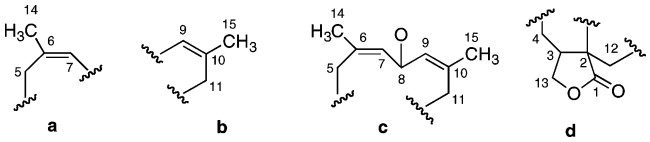

FIGURE 2. Fragments of **1** deduced by HMBC data.

TABLE 1. ^1H and ^{13}C Assignments of 1 (600 MHz, $CDCl_3$)

position	δ_H (mult, J, Hz)	δ_C (mult)
1		176.8 s
2		60.2 s
3	3.15 ddd (4.9, 7.8, 10.0)	52.8 d
4	4.65 dd (4.9, 7.8)	76.2 d
5	2.68 m, 1.93 m	32.5 t
6		134.0 s
7	5.52 br s	127.5 d
8		77.7 s
9	5.44 br s	119.8 d
10		142.3 s
11	2.68 m, 1.92 m	27.6 t
12	2.01 m, 1.90 m	30.2 t
13α	4.35 t (10.0)	66.5 t
13β	3.96 dd (4.9, 10.0)	
14	1.70 s	22.4 q
15	1.81 s	24.0 q

The determination of the relative stereochemistry of **1** presented some challenge and was eventually resolved by X-ray crystallography. The crystal structure confirmed the novel sesquiterpene lactone skeleton, in which both seven- and six-membered rings are present. Due to the minute amounts isolated (1.5 mg) the absolute configuration of anthecularin could not be determined.

The biosynthetic origin of anthecularin (**1**) is of great interest, given its unique structure. The long-held view that the isoprenoid compounds, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), are universally derived from mevalonic acid (MVA) has been overturned with the discovery of methylerythritol phosphate (MEP) pathway more than a decade ago. Current knowledge indicates that these two pathways are separated by compartmentalization, i.e., the MEP pathway is operative in plastids and is responsible for the biosynthesis of hemiterpenoids, monoterpenoids, diterpenoids, and carotenoids, whereas the MVA pathway is located in the cytoplasm and synthesizes sesquiterpenoids, triterpenoids, and steroids.[12] On

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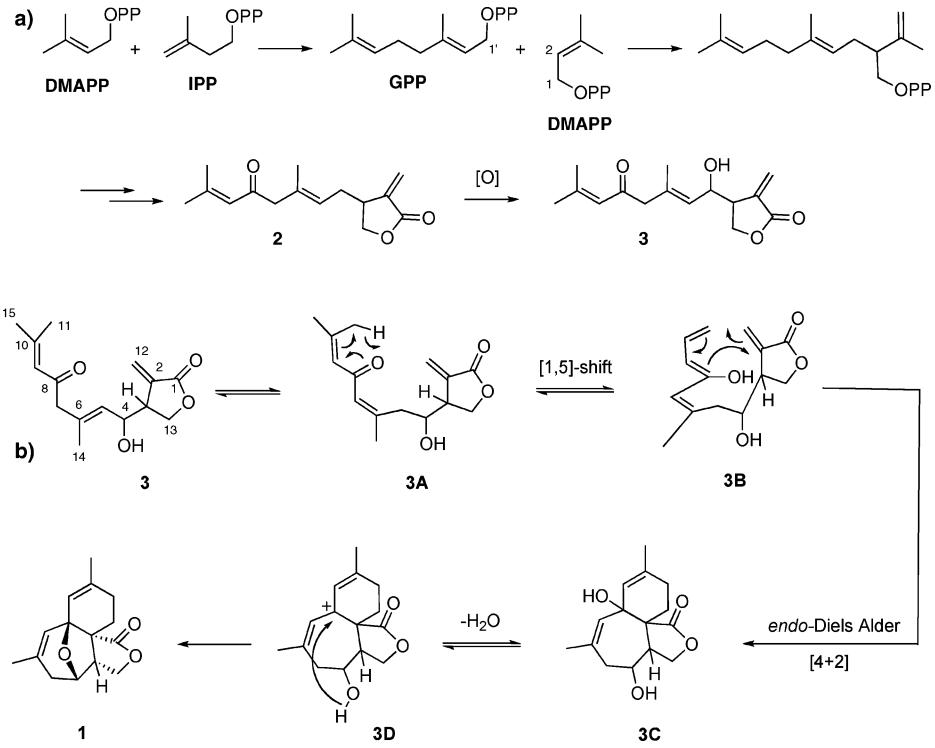
SCHEME 1. (a) Proposed Biosynthetic Route to 4-Hydroxyanthecotulide (3) from Anthecotulide (2) via the Head-to-Middle (1′-2) Linkage of GPP to DMAPP (adapted from Ref 16); (b) Proposed Biogenesis of Anthecularin (1) from 3



the other hand, it is generally believed that all sesquiterpenoids originate from farnesyl diphosphate (FPP), which derives from the head-to-tail (i.e., the regular 1′-4) coupling of geranyl diphosphate (GPP) and isopentenyl diphosphate (IPP). However, there are a few reports on sesquiterpenes originating from a non-FPP precursor.[13] The biosynthetic origin of anthecotulide (**2**), an allergenic sesquiterpene lactone found in *Anthemis cotula* and other Asteraceae plants,[14,15] has been the subject of a recent investigation by van Klink et al.[16] Feeding studies with various isotopically labeled glucose precursors into *A. cotula* plantlets indicated that the isoprene building blocks of **2** are formed exclusively via the MEP biosynthetic pathway, contrasting with the commonly held view that sesquiterpenes are synthesized exclusively in the cytoplasm from MVA. Furthermore, a deuterium-labeling experiment showed that anthecotulide (**2**) is an irregular sesquiterpene lactone that derived from the head-to-middle (i.e., 1′-2) coupling of GPP and DMAPP, and not from FPP.[16] The coexistence of anthecularin (**1**) with anthecotulide (**2**) and its oxygenated derivatives 4-hydroxyanthecotulide (**3**) and 4-acetoxyanthecotulide (**4**) in *Anthemis auriculata*[11] indicates a strong biosynthetic relationship. It has been suggested that FPP may not be the universal sesquiterpene precursor in the Asteraceae family.[16] Here we propose that anthecularin is also an irregular, non-farnesyl-derived sesquiterpene and compound **3** is its biosynthetic precursor. A plausible biogenetic pathway is illustrated in Scheme 1a, in which compound **3** is derived from anthecotulide (**2**) via the aforementioned alternative 1′-2 coupling. As outlined in Scheme 1b, **3** can interconvert to **3A** via double bond migration. Intermediate **3A** could then tautomerize to **3B**, which yields the complex polycyclic core framework of **3C** through a key *endo*-Diels–Alder cycloaddition. Compound **1** could be considered as arising by formation of the cyclic ether bridge by S_N1 substitution of the stabilized *bis*-allylic carbocation intermediate **3D**, itself formed through loss of water from intermediate **3C**.

Compound **1** showed dual inhibitory potential against recombinant *Pf*FabI and *Pf*FabG enzymes from the FAS-II pathway of *P. falciparum* with IC_{50} values of 14.0 and 28.3 μg/mL, respectively. No inhibition was observed against *Pf*FabZ. The dual enzyme inhibition activity was well correlated with its in vitro antiplasmodial potential on the drug-resistant (K1) *P. falciparum* strain (IC_{50} 23.3 μg/mL). Compound **1** also showed trypanocidal activity against *Trypanosoma brucei* rhodesiense (IC_{50} 10.1 μg/mL) and was devoid of any cytotoxicity on mammalian L6 cells (IC_{50} > 90 μg/mL).

Sesquiterpene lactones comprise a large class of natural products with diverse biological activities. Artemisinin, a blockbuster antimalarial drug from *Artemisia annua* is a cadinane-type sesquiterpene lactone with an unusual endoperoxide function. Both artemisinin and anthecularin (**1**) originate from the plants of the tribe Anthemidae of the family of Asteraceae. The antimalarial activity of artemisinin has been postulated to result from the inhibition of SERCA, a *P. falciparum* Ca^{2+}-ATPase.[17] In comparison to that of artemisinin (IC_{50} 0.002 μg/mL), the antimalarial activity of anthecularin is low. However, it is still important to note that anthecularin is

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the first sesquiterpene lactone interacting with the *Plasmodium* FAS-II cascade, thus giving first evidence for *Pf*FAS-II as its possible intracellular target. Considering the structural novelty, biological activity, and safety, anthecularin (**1**) could be regarded as a new drug lead for the design of novel antimalarial agents. Trypanocidal activity of anthecularin is also promising, and synthetic studies toward compound **1** are underway. These studies will not only allow the assessment of the activities of **1** and derivatives against a larger panel of parasitic protozoa but might also allow deeper insight into the proposed biosynthesis of **1** from anthecotulide-type unusual linear sesquiterpene lactones, which do not appear to derive from FPP, the universal precursor of all sesquiterpenes.

Experimental Section

Plant Material, Extraction, and Isolation. Plant material and the detailed isolation procedure has been described previously.[11] Briefly, the fresh aerial parts of *A. auriculata* (0.48 kg) were finely ground and extracted at room temperature with cyclohexane/Et_2O/MeOH (1:1:1; extract A) and MeOH/H_2O (1:1; extract B), successively. Extract A was washed with brine, and the aqueous layer was re-extracted with EtOAc. The organic layer was dried with Na_2SO_4 and concentrated under reduced pressure. The residue (5.75 g) was fractionated by VLC on SiO_2, using cyclohexane/EtOAc/Me_2CO mixtures of increasing polarity to give 11 fractions (A−K). Fraction D (402.8 mg) was subjected to column chromatography on silica gel (CH_2Cl_2/EtOAc 100:0 to 100% MeOH) and yielded 16 fractions (D_1−D_{16}). Fraction D_5 (8.7 mg) was further purified by prep. TLC to afford compound **1** (1.5 mg).

Anthecularin (**1**): colorless crystals; $[\alpha]_D^{23}$ +23.9 (c = 0.15, $CHCl_3$); IR (film, $CHCl_3$) ν_{max} 2915, 1775, 1457, 1264 cm^{-1}; ^1H and ^{13}CNMR data, see Table 1; HREIMS m/z $[M]^+$ 246.1261 (Δ: + 0.5 mmu), calcd for $C_{15}H_{18}O_3$ 246.1256.

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Supporting Information Available: All NMR and MS data, general experimental procedures, biological assay descriptions, X-ray crystal structure, crystallographic data and crystallographic information file (CIF) for anthecularin (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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