Antiplasmodial Activities of Sesquiterpene Lactones from *Eupatorium semialatum*

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Eupatorium semialatum is a member of the Asteraceae, which occurs in Guatemala. Previously, we reported the occurrence of sesquiterpene lactones of the eudesmanolide type as main constituents in the leaves. This paper deals with the isolation and identification of the first guaianolide found in *E. semialatum*. Since this plant is used against malaria and other diseases in the Guatemalan folk medicine, the main sesquiterpene lactones were tested for their activities against *Plasmodium falciparum in vitro*.

Introduction

Eupatorium semialatum Benth. is a member of the Asteraceae, which occurs in the highland regions of Guatemala (Nash and Williams, 1976). This species was the subject of a controversial discussion between different botanists from the United States and Guatemala (Nash and Williams, 1976; King and Robinson, 1987). Whereas King and Robinson (1987) after review of several voucher specimens concluded that *E. semialatum* is identical with *E. ligustrinum*, a south-Mexican species, which was recently redirected into the genus *Ageratina* (*A. ligustrina*), *E. semialatum* was described as completely different from *E. ligustrinum* by Nash and Williams (1976) in the Flora of Guatemala. Since phytochemical investigations often help to clarify botanical problems, we investigated the leaves of *E. semialatum* from Guatemala and found sesquiterpene lactones of the eudesmanolide type (Lang *et al.*, 2000, Lang *et al.*, 2001), which have not been isolated from *A. ligustrina* so far (Tamayo-Castillo *et al.*, 1988). In contrast to the eudesmanolides **1–7**, we now report a new compound (**8**), which was found to be a sesquiterpene lactone of the guaianolide type. The

isolation and structure elucidation of the hitherto unknown compound is described here for the first time and the implications of this finding with respect to the classification of *E. semialatum* are discussed.

E. semialatum is interesting not only on account of its botany, but also because of its medicinal use in Central America. The leaves of this shrub or small tree are used in the district Alta Verapaz against malaria and dysentery, but also against other diseases (Cáceres, 1996). Since sesquiterpene lactones have been found to be active against *Plasmodium* parasites (François *et al.*, 1996; Woerdenbag *et al.*, 1994), we continued our pharmacological studies on the biological activities of sesquiterpene lactones and tested the main compounds (**1–8**) found in *E. semialatum* against a chloroquine resistant strain (K1) of *Plasmodium falciparum*.

Results and Discussion

The sesquiterpene lactones **1–7** were isolated from *E. semialatum* as described previously (Lang *et al.*, 2000; Lang *et al.*, 2001). The new compound **8** was obtained after separation of the methanol

soluble part of the dichloromethanic extract using CC, preparative TLC and HPLC. The ^{13}C NMR spectrum of **8** displayed signals for 26 carbons, ten of them detected at shift values characteristic for the complex 4-hydroxy-5-(5-hydroxytigloyloxy)-tigloyloxy ester side chain, previously found in **3**, **5** and **7**. The remaining 16 carbon signals could be attributed to a substituted sesquiterpene which includes a methoxy group as indicated by the signal at δ 49.9 ppm (Budešínský and Šaman, 1995). The ^{1}H NMR displayed a total number of 23 signals representing 31 protons. As expected, seven signals belonged to the 4-hydroxy-5-(5-hydroxy-tigloyloxy)tiglic acid moiety (Lang *et al.*, 2001; Budešínský and Šaman, 1987). The remaining signals were assigned using the additionally recorded 2D-COSY, HMQC and HMBC spectra. The typical proton signals (H-6, H-7, H-13) and the corresponding carbon signals for a five membered 7(12),6α-methylene-γ-lactone ring were found at shift values characteristic for sesquiterpene lactones (see M & M) (Budešínský and Šaman, 1995; Yoshioka *et al.*, 1973). By analogy with the previously reported eudesmanolides (Lang *et al.*, 2000; Lang *et al.*, 2001) we also found three signals for the protons at C-8 (δ 5.65 dd) and C-9 (2.79 dd and 2.29 dd), which clearly showed that C-9 is not substituted, while C-8 bears the ester group (see M & M).

The signal at δ 4.84 ppm (H-6) displayed one additional coupling to a triplet (1H) at δ 2.44 (H-5), which showed a vicinal coupling to the proton at C-1 (δ 3.08 ppm). Therefore **8** must be a guaianolide sesquiterpene lactone. The relative stereochemistry at C-5 and C-1 followed from the coupling constants $J_{5,6}$ and $J_{1,5}$ = 11.4 Hz, which indicated that both protons possess an α-orientation (Bohlmann *et al.*, 1977). The cyclopentane ring was therefore found to be cis-annelated. H-1 was further coupled with two protons attached to C-2 (δ 2.33 m and 1.80 m, respectively), which are connected with a one proton singlet at δ 4.26 ppm (H-3). The shift value of the latter one showed the presence of an additional hydroxylation at this carbon. Four further singlets showed the presence of one methyl- (δ 1.50 ppm), one methoxy- (δ 3.26 ppm) and one exocyclic methylene- (δ 5.09 and 4.89 ppm) group, which were all attached to quartenary carbons. The positions of these three groups could be assigned by interpretation of the HMBC spectrum, which showed, that the exocyclic methylene group was build by an double bond between C-10 and C-14, while the methyl group (C-15) and the methoxy group are attached to C-4. From their shift values and the signals found in the NOESY spectrum C-15 was found to be β-oriented, which subsequently leads to an α-oriented methoxy group. The sesquiterpene lactone alcohol was therefore identified as 3β,8β-dihydroxy-4α-methoxy-10(14),11(13)-guaiadien-12,6-olide, which was found for the first time in nature. The ester derivative isolated from *E. semialatum* is a new natural compound. The relative stereochemistry of **8** was additionally confirmed by the interpretation of its NOESY spectrum, which showed all required connectivities. For this compound we propose the trivial name semialatolide.

The occurrence of semialatolide also supports Nash's opinion, that *E. semialatum* is quite different from *E. ligustrinum* and subsequently *A. ligustrina*, because the two guaianolides found there possess different 1,5-annelations of the bicyclo[5.3.0]decane ring system (Tamayo-Castillo *et al.*, 1988; Romo *et al.*, 1968).

Since sesquiterpene lactones are biologically active compounds (Picman, 1986) and some of them have been shown to be active against *P. falciparum* (François *et al.*, 1996; Woerdenbag *et al.*, 1994; Quinghaosu Antimalarial Coordinating Research Group, 1979), we continued our studies on the antiplasmodial activity of sesquiterpene lactones by testing compounds **1**–**8** using the pLDH-assay (Makler *et al.*, 1993). All compounds were solved in culture medium and diluted to twelve different concentrations. All samples were tested six times. The results are shown in Table I. All the

Table I. IC$_{50}$ values ± SE of **1**–**8** and chloroquine diphosphate against *P. falciparum* (strain K1) *in vitro* (*n* = 6).

Group	Compound	IC$_{50}$ [μM]
A	1	31.7 ± 4.0
	2	28.5 ± 3.2
	3	27.0 ± 3.7
	4	26.2 ± 1.5
B	5	16.3 ± 1.9
	6	8.9 ± 1.8
	7	13.3 ± 4.1
	8	11.4 ± 2.4
	chloroquine	0.18 ± 0.03

compounds were found to have some activity against *P. falciparum* but they could be divided into two groups of compounds, one containing **5**–**8** having lower IC_{50} values (group B) and one containing **1**–**4**, which possess higher IC_{50} values (group A). Within both groups no significant differences were found. The presence of a 4,15-exomethylene group in the eudesmanolides **1**–**4** obviously led to a decrease in activity, since all compounds possessing this structure element are listed in group A. The fact that the isomeric pairs of compounds **3/5** and **3/7**, respectively, and **4/6**

show up in different groups, led to the assumption that the acid side chain does not influence the activity of the respective compound, but the configuration in the ring system is important. This can be explained by the conformation of the cyclohexane ring system, which adopts a full chair conformation, despite the sp2-hybridization at C-4 in compounds **1**–**4**, while the other compounds (**5**–**7**) do not, as seen from our molecular studies.

Although the compounds only possess moderate activities, they may explain the use of the plant extracts in traditional medicine. The content of
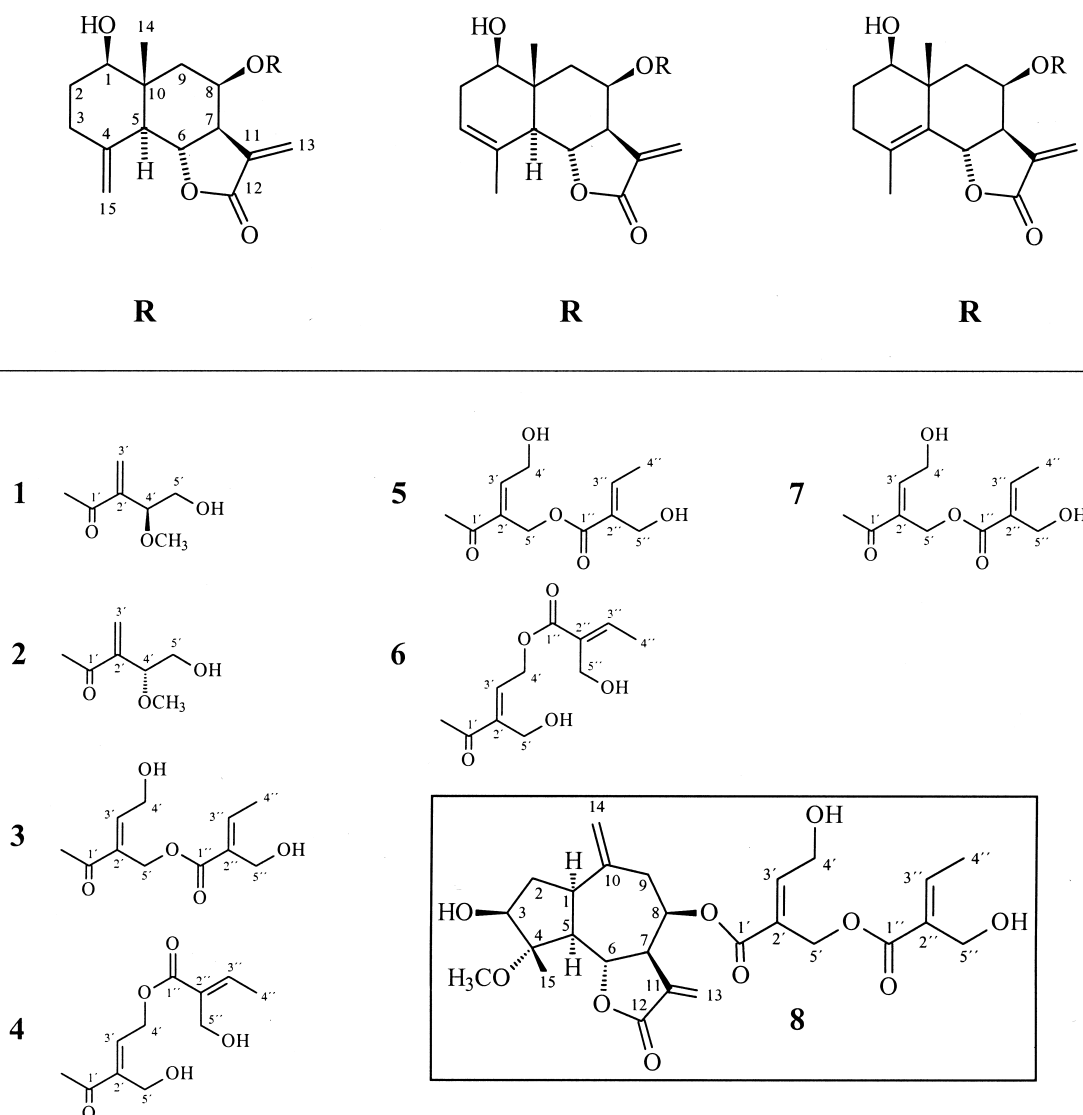

Fig. 1. Sesquiterpene lactones isolated from *E. semialatum*.

sesquiterpene lactones in *E. semialatum* leaves is not yet known, but from the bitter taste we can estimate it to be relatively high. It is possible that other more active compounds are present in the aqueous extracts of *E. semialatum* and further investigations on the more hydrophilic compounds are planned.

Material and Methods

Plant material

The dried leaves of *Eupatorium semialatum* Benth. (725 g, voucher no. JC2541), collected near Purulhá, Baja Verapaz, Guatemala, were ground and extracted exhaustively with CH_2Cl_2 (1 l) in a Soxhlet apparatus. The dried extract (81 g) was then dissolved in MeOH, which yielded 36.86 g methanol-soluble material.

Purification by repeated CC on Sephadex LH-20 with MeOH, silica gel 60 with CH_2Cl_2/MeOH (9:1 v/v), silica gel 60 with CH_2Cl_2/acetone (2:1 v/v), followed by prep. TLC and prep. HPLC gave 6 mg of a glassy solid, **8**.

NMR: Bruker ARX 500.

GC-MS: EI (70 eV) HP MSD 5972 with GC 5890 plus (HP); Optima-1 (MN), 25 m × 0.25 mm; 150 °C (3 min) to 280 °C at 10° min^{-1}; R_t (min.): 14.32.

D-MS: CI (NH_3), Finnigan MAT INCOS 50.

HPLC: HP 1050, DAD. 215 and 260 nm, Hibar RP 18 LiChrosorb (7 μm, 25.0 × 7 mm), flow 3.0 ml min^{-1}. CH_3CN-H_2O (25:75). R_t (min.): **8**: 15.6.

TLC: Silica gel 60 F_{254}; CH_2Cl_2/acetone (2:1); anisaldehyde/H_2SO_4; R_f: 0.2.

3β-Hydroxy-8β-[4-hydroxy-5-(5-hydroxytigloyl-oxy)tigloyloxy]-4α-methoxy-10(14),11(13)-guaiadien-12,6-olid (**8**): λ_{max} MeOH/H_2O (1:1): 218 nm, ε 5729. EI-MS *m/z* (rel. int.): 294 [M+H-Acyl]$^{+\bullet}$ (1), 262 [294-MeOH]$^{+\bullet}$ (2), 249 (8), 244 [262-H_2O]$^{+\bullet}$ (4), 235 (13), 166 (13), 109 (18), 101 (83), 91 (42), 88 (48), 79 (31), 77 (39), 69 (52), 67 (30), 59 (53), 55 (29), 43 (100), 41 (98). DCI-MS m/z (rel. int.): 524 [M+NH_4]$^+$ (100), 426 (7), 409 (16), 366 (54), 312 (10), 298 (9), 151 (28), 134 (86).

^1H NMR (500 MHz, $CDCl_3$, TMS): δ 7.05 (1H, t, *J* = 6.3 Hz, H-3′), 6.90 (1H, dd, *J* = 6.9 Hz, H-3″), 6.22 (1H, d, *J* = 3.2 Hz, H-13a), 5.65 (1H, dd, *J* = 3.8, 5.7 Hz, H-8), 5.55 (1H, d, *J* = 3.2 Hz, H-13b), 5.09 (1H, s, H-14a), 5.06 (1H, d, *J* = 12.0 Hz, H-5′a), 4.89 (1H, s, H-14b), 4.88 (1H, d, *J* = 12.0 Hz, H-5′b), 4.84 (1H, dd, *J* = 9.5, 11.4 Hz, H-6), 4.49 (2H, dd, *J* = 6.3, 13.9 Hz, H-4′), 4.31 (2H, s, H-5″), 4.26 (1H, d, *J* = 3.8 Hz, H-3), 3.26 (3H, s, OCH_3), 3.08 (2H, m, H-3, H-7), 2.79 (1H, dd, *J* = 3.8, 13.9 Hz, H-9β), 2.44 (1H, t, *J* = 11.4 Hz, H-5), 2.33 (1H, m, H-2β), 2.29 (1H, dd, *J* = 3.8, 13.9 Hz, H-9α), 1.92 (3H, d, *J* = 6.9 Hz, H-4″), 1.80 (1H, dd, *J* = 8.2, 13.9 Hz, H-2α), 1.50 (3H, s, H-15).

^{13}C NMR (125 MHz, $CDCl_3$, TMS): δ 169.61 (C-12), 167.40 (C-1″), 165.36 (C-1′), 147.13 (C-3′), 142.11 (C-3″), 141.43 (C-10), 135.06 (C-11), 131.52 (C-2″), 127.12 (C-2′), 121.57 (C-13), 116.41 (C-14), 86.16 (C-4), 76.52 (C-6, assignment interchangeable), 76.11 (C-3, assignment interchangeable), 67.60 (C-8), 59.62 (C-4′), 58.28 (C-5′), 56.68 (C-5″), 53.72 (C-5), 50.38 (C-7), 49.92 (OCH_3), 43.84 (C-1, C-9), 36.47 (C-2), 16.91 (C-15), 14.32 (C-4″).

IR (KBr), ν_{max} cm^{-1}: 3476 br (OH), 2941 (CH), 1763 (γ-lactone), 1714 (unsaturated ester), 1648.

Antiplasmodial assay

A culture of the K1 strain of *P. falciparum* was kindly supplied by Professor D. C. Warhurst of the London School of Hygiene and Tropical Medicine. Human A$^+$ erythrocytes and plasma were supplied by the Yorkshire Blood Transfusion Centre in Leeds.

Malaria parasites (*P. falciparum*, strain K1) were maintained in human A$^+$ erythrocytes suspended in RPMI 1640 medium supplemented with A$^+$ serum (prepared from recalcified plasma) and D-glucose according to the methods of Trager and Jensen (1976) and Fairlamb *et al.* (1985). Cultures containing predominantly early ring stages of the malaria parasite were used for testing. Extracts and compounds were dissolved or micronised in DMSO and further diluted with RPMI 1640 medium (the final DMSO concentration did not exceed 1% which has been shown not to affect parasite growth). Two-fold serial dilutions were made in 96-well microtitre plates in duplicate and infected erythrocytes were added to give a final volume of 100 μl with a 1% haematocrit and 2% parasitaemia. Chloroquine diphosphate was used as a positive control and uninfected and infected erythrocytes without compounds were included in each test. Plates were placed into a modular incubator

chamber, gassed with a mixture made up of nitrogen 93%, oxygen 3%, carbon dioxide 4% and incubated at 37 °C for 48 hours. Parasite growth was assessed by measuring lactate dehydrogenase (LDH) activity as described by Makler *et al.* (1993). The reagent used contained the following in each ml: 3-acetylpyridine adenine dinucleotide (APAD), 0.74 mg, lithium lactate, 19.2 mg, diaphorase, 0.1 mg, Triton X-100, 2 µl, nitroblue tetrazolium, 1 mg, phenazine ethosulfate, 0.5 mg. 50 µl of this reagent was added to each well and mixed, and plates were incubated for 15 min. at 37 °C. Optical densities were read at 550 nm using a microplate reader and% inhibition of growth was calculated by comparison with control values. IC_{50}

values were determined using linear regression analysis.

Test compounds. Compounds **1–7** were previously isolated from *E. semialatum*. For details see Lang *et al.* (2000) and Lang *et al.* (2001).

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