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TRITERPENOIDS FROM *MELILOTUS MESSANENSIS*; SOYASAPOGENOL G, THE FIRST NATURAL CARBONATE DERIVATIVE*

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Key Word Index—*Melilotus messanensis*; Leguminoseae; triterpenoids; gammacerane-3-ene-2-one; messagenolide; soyasapogenol G; natural carbonate derivative; paleobiomarkers; allelopathy.

Abstract—Three new rare triterpenes, the paleo-biomarkers gammacer-16-ene-3-one and messagenolide, and the first natural carbonate derivative soyasapogenol G, were isolated from allelopathic bioactive fractions of *Melilotus messanensis* L. Their structures were established by chemical correlations and spectroscopic (^{1}H NMR, ^{1}H–^{1}H COSY, ^{13}C NMR, ^{13}C–^{1}H HETCOR and mass spectrometry) methods.

INTRODUCTION

Melilotus species are native to the temperate and subtropical regions in Eurasia and north Africa, some of them being cultivated as forage [1]. *M. messanensis*, a small shrub endemic to the Mediterranean basin [2], is a member of only 23 species that comprises the genus *Melilotus*. Due to their ability to grow in saline soils, the potential of *M. messanensis* and *M. segetalis* as a forage resource, a green manure and a source of biocide compounds is being evaluated [2–6].

Chemical studies of *M. messanensis* have shown the presence of low levels of flavonols, simple coumarins [7] and the steroid diosgenin [8]. In a continuation of our research focused on the search for natural product models as allelochemicals, we have evaluated the allelopathic bioactive medium polar fractions of *M. messanensis*, for which we previously reported lupane triterpenes as major constituents [6]. In this paper, we report on the isolation and structural elucidation of the minor rare bioactive triterpenes, the two paleo-biomarkers gammacer-16-ene-3-one (**1**) and 18-$\alpha(H)$-oleanane messagenolide (**2**) and the first natural carbonate soyasapogenol G (**3**) (Fig. 1).

Gammaceranes and 18-$\alpha(H)$-oleananes are rare families of triterpenes. They are usual constituents of petroleum and are used as paleo-biomarkers. Gammacerane triterpenes have been isolated from the more primitive protozoans [9] and ferns [10, 11]. From higher plants, gammaceranes have only been isolated from two species, *Picris hieracioides* [12] (Compositae) and *Swertia chirata* [13, 14] (Gencianaceae). The isolation of gammacerane triterpenes is extremely significant, not only because of their rareness, but also from the biogenetic point of view as they are considered to be the key intermediates in the biosynthesis of swertane skeletons through non-classical carbocations [15].

18-$\alpha(H)$-Oleananes have been previously isolated from Nigerian and Egyptian petroleum and coal [16–18] and their significance as paleo-biomarkers has been discussed in detail by Whitehead [19, 20]. This is one of the few examples where they are reported from a plant source. They are generally considered to be key intermediates in the biosynthetic pathway between squalene and the lupane, ursane and oleanane triterpenoids [21].

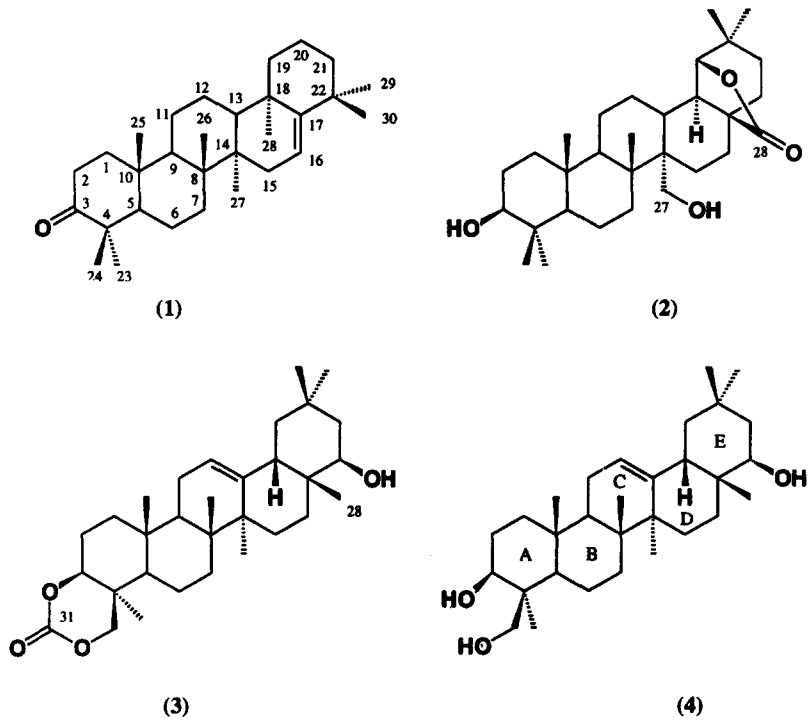
Soyasapogenol G (**3**) (soyasapogenol B carbonate derivative) represents to our knowledge a new type of functionalization in the chemistry of the natural products.

RESULTS AND DISCUSSION

Fraction **B** from the extraction of *M. messanensis* L. afforded, in addition to long chain fatty acids, 5 mg of **1** as major component. The EI mass spectrum showed a $[M]^+$ at *m/z* 424, corresponding to the molecular formula $C_{30}H_{48}O$. Peaks at *m/z* 150 and 135 are in good agreement with a retro-Diels–Alder fragmentation and the subsequent loss of a methyl radical from the gammacerane skeleton, respectively [22].

The ^{1}H NMR spectrum showed eight tertiary methyl groups, corresponding to the gammacerane structure. A signal at δ5.44 (1H, *dd*, $J_{16,5\alpha} = 5$ Hz and $J_{16,15\beta} = 3$ Hz, H-16) was assigned to an olefinic proton in a trisubstituted double bond $\Delta^{16,17}$. Two more signals, δ2.49 (1H, *ddd*, $J_{2\alpha,2\beta} = 16$ Hz, $J_{2\alpha,1\beta} = 10$ Hz and $J_{2\alpha,1\alpha} = 8$ Hz, H-2α) and 2.41 (1H, *ddd*, $J_{2\alpha,2\beta} = 16$ Hz, $J_{2\beta,1\alpha} = 8$ Hz and $J_{2\beta,1\beta} = 4$ Hz, H-2β), were assigned to a methylene group attached to a carbonyl group at C-3. Signals at δ117.7 (C-16) and 147.6 (C-17), olefinic carbons, and 218.0 (C-3, carbonyl group) in the ^{13}C NMR

*Part 4 in the series 'Natural Product Models as Allelochemicals'. For part 3 see ref. [6].



(1) (2) (3) (4)

Fig. 1. Compounds isolated from *M. messanensis*.

spectrum (Table 1) confirmed the structure as gammacer-16-ene-3-one, which is reported for the first time as a natural product. A comprehensive series of NOE experiments showed that the initial assignments [13] for the methyl signals need to be corrected. The new assignments, based on the selected NOE effects shown in Fig. 2 are δ1.11 for H-29 and 1.05 for H-30, respectively (previous assignments for the same protons were δ0.84 and 1.13, respectively). The proposed structure was further substantiated by a comparison of the ^{13}C NMR spectral data with those for gammacer-16-ene-3β-ol for the C, D and E ring moieties [14], whilst those of the chiral-16-en-3-one support the assignments for the A, B and C rings [15].

From fraction **M**, 3 mg of **2** was isolated. The IR spectrum had absorption bands at 3351 cm^{-1} (OH) and 1767 cm^{-1} (γ-lactone). From the EI mass spectrum, the major diagnostic peaks were those at *m/z* 207 and 189, which arise from the cleavage of the C-9(11) and C-8(14) bonds, and contain the A and B rings with a hydroxyl group of a pentacyclic triterpene [23]. The remaining modifications have to be placed in the C, D and E rings.

The ^{1}H NMR spectrum was characteristic of a pentacyclic triterpene with six tertiary methyl groups and two modified methyls as hydroxymethylene and γ-lactone groups. The signal at δ3.18 (1H, *dd*, $J_{3\alpha,2\beta}$ = 11.5 Hz and $J_{3\alpha,2\alpha}$ = 5 Hz, H-3α) was assigned to a typical H-3α proton attached to a carbon which supports a β-oriented hydroxyl group. The hydroxymethylene group (signals at δ3.32 [*d*, $J_{27,27'}$ = 11 Hz, H-27] and δ3.55 [*d*, $J_{27,27'}$ = 11 Hz, H-27']) and the lactonic proton (δ4.28, *s*, H-19) had to be located at C-27 and C-28; since any modification of methyls C-29 or C-30 would shift to lower field values the remaining methyl [24]. The only disposition that allows a singlet lactonic proton is the one with a 28β,19β-γ-lactone in an 18α-oleanane skeleton. The γ-lactone moiety forces a 'quasi' 90° angle between H-19α and H-18α and nulls the coupling constant. MMX calculations [25] (Fig. 3) give 81° and 0.1 Hz as theoretical values for the H18α–H19α dihedral angle and coupling constant, respectively. Thus, the hydroxymethylene group must be located at the C-27 position in accord with the structure 3β,27-dihydroxy-18Hα-oleane-28β,19β-olide (messagenolide, **2**) first reported in the literature.

Compound **3** was isolated as a solid powder from fraction **M**. Direct comparison of its spectroscopic data with those of soyasapogenol B [26, 27] (**4**) showed a great similarity, except for the following items: in the EI mass spectrum a [M]$^+$ at *m/z* 484, according with the molecular formula $C_{31}H_{48}O_4$, implied a 26 amu increment (CO); the base peak, corresponding to the retro-Diels–Alder cleavage of C ring remained at *m/z* 234 (D and E rings), which implied that the modifications have to be placed at the A–B ring system. From the ^{1}H NMR data, the only differences were those of the H-3α, H-24 and H-24' chemical shifts. Thus, H-24 and H-24' appeared at δ4.67 (*d*, $J_{24,24'}$ = 11 Hz) and 3.85 (*dd*, $J_{24,24'}$ = 11 Hz and $J_{24',3\alpha}$ = 2 Hz), respectively, while H-3α appeared at δ4.03 (*ddd*, $J_{3\alpha,2\beta}$ = 14 Hz, $J_{3\alpha,2\alpha}$ = 6 Hz and $J_{3\alpha,24'}$ = 2 Hz) and were clearly deshielded with respect to those of **4**. The absorption at 1738 cm^{-1}

Table 1. ^{13}C NMR data for triterpenes **1–4** (100 MHz, $CDCl_3$)*

C	1	2	3	4
1	39.5	38.9‡	36.7	38.4
2	34.1	27.4	25.9	25.9
3	218.0	78.9	86.1	80.9
4	47.3	39.9	34.9	42.0‖
5	54.8	55.5	54.4	55.8
6	19.5	18.1	18.2	18.4
7	33.4†	36.0	33.0	33.1
8	41.1	40.6	39.8	39.7
9	49.7	51.2	47.6	47.7
10	36.7	37.3	37.3	37.4‖
11	21.8	20.8	23.6	23.7
12	22.6	25.6	121.8	122.3
13	46.6	38.1‡	144.1	143.9
14	39.4	46.5	42.1	42.8
15	33.3†	31.1	28.2	28.2
16	117.7	31.9	28.1	27.6
17	147.6	55.5	36.4	36.6
18	37.6	38.8	44.6	44.7
19	41.5	81.3	46.2	46.1
20	18.6	26.5	30.5	30.5
21	41.7	30.0	41.5	41.5
22	36.1	33.7	76.5	76.6
23	26.8	27.9	25.5	22.4
24	21.1	15.3	70.6	64.5
25	16.1	16.5	16.3	16.1¶
26	16.7	15.5	17.0	16.8¶
27	17.4	69.7	25.5	25.4
28	20.6	184.1	28.2	28.2
29	29.8	27.4§	32.8	32.8
30	32.8†	27.9§	20.0	20.0
31			148.5	

*The degree of protonation was obtained by APT multi-phase programs. Assignments based on COSY, APT and HECTOR experiments.

†‡§‖¶Values may be interchanged within the same column.

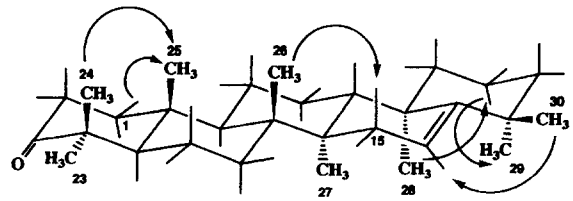


Fig. 2. Observed NOE effects for gammacerane (**1**).

(carbonate carbonyl group) in the IR spectrum, along with the previous assertions, allowed us to deduce the presence of a cyclic carbonate between the C-3 and C-24 hydroxyl groups. The additional coupling constant between the H-24′ and H-3α signals (2 Hz) can be explained by the long distance interaction through the conjugated system O–CO–O observed in the ^1H NMR COSY 2D experiment.

The ^{13}C NMR spectrum showed an additional signal at δ148.5 assigned to the carbonyl group of the carbonate moiety (C-31) [28]. A comparison of the C-3, C-22 and C-24 values with those of soyasapogenol B [26, 27] confirmed the structure.

In order to confirm the proposed structure, a chemical correlation from **4**, also present in this plant [7], was carried out. Treatment of **4** with *N,N′*-carbonyldiimidazole as previously described [29] afforded the cyclic carbonate **3**, whose physical and spectroscopic data were in full agreement with those for the natural product. The 22-carbonylimidazolide derivative of the carbonate (**5**) was also obtained as a minor product (Fig. 4).

Compound **4** is a widespread triterpene in the plant kingdom and has been isolated from many sources [30–32]. The fact that its carbonate derivative (**3**) has never been isolated is rather strange. It was thought possible that, due to the hard Soxhlet extraction methods traditionally used, the carbonate could decompose to **4**. However, treatment of the carbonate with ethanol under

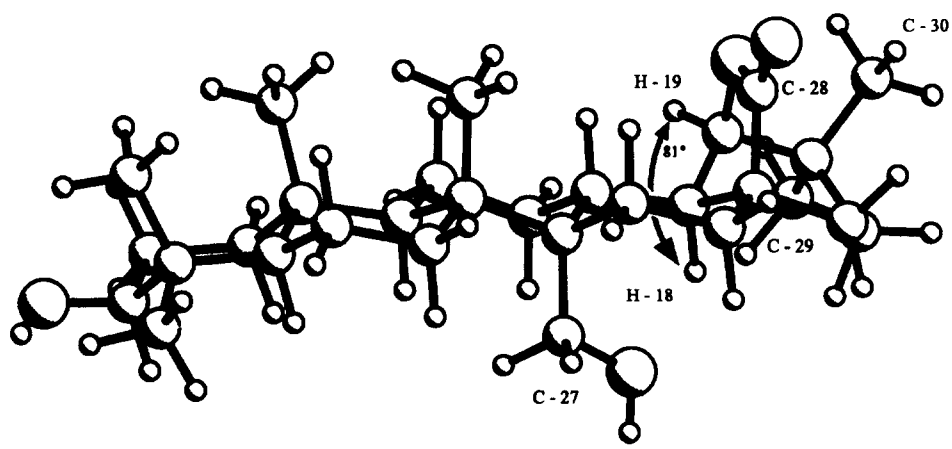


Fig. 3. Theoretical conformation of messagenolide (**2**) as determined by MMX calculations.

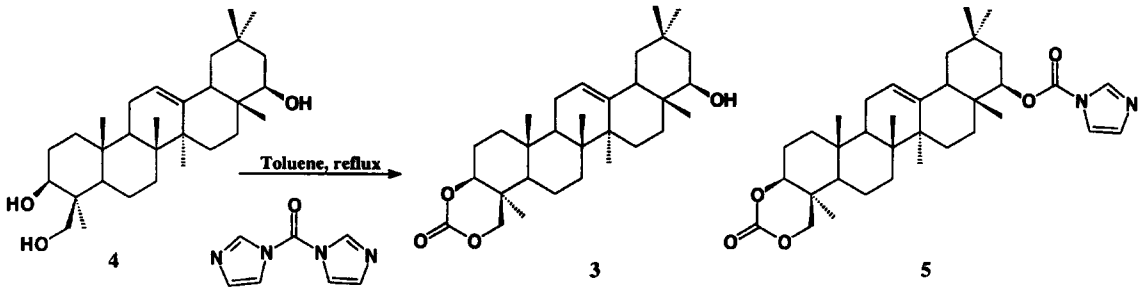


Fig. 4. Chemical correlation between soyasaponegols B (4) and F (3).

reflux for 24 hr demonstrated that it was quite stable and does not decompose to 4 or other compounds.

Treatment of 4 with CO_2 in methanolic or chloroform solution over 24 hr leads to no product formation. The same results were obtained after stirring 4 in chloroform and methanol solutions in a CO_2 atmosphere for three days. So, we can conclude that 3 is a secondary metabolite from *M. messanensis* and is not an artefact. This compound is of particular interest since it is the first bioactive natural product, to our knowledge, containing a carbonate function.

Based on the bioassay results of compounds 1–3 [33, 34], it is likely they could be involved in the allelopathic action of *M. messanensis*.

EXPERIMENTAL

^1H and ^{13}C NMR: 400 and 100 MHz, respectively, using $CHCl_3$ as int. reference; MS: VG 12-250 spectrometer, 70 eV; HRMS: KRATOS model MS-80-RFA using EI technique (70 eV); Mps uncorr.; CC: silica gel (Merck) employing hexane–EtOAc mixts; HPLC: L-6200A Merck-Hitachi apparatus, using an RI detector and Merck analyt. (Si 60-Lichrospher, 5 μm) and semi-prep. (Si 60-Lichrosorb, 7 μm) columns. All solvents were distilled from glass prior to use.

Extraction and isolation. Melilotus messanensis was collected in July 1991 in Trebujena, Cádiz, Spain (the voucher is deposited at the University of Seville Herbarium, Spain, SEV, 7992). Fresh plant material (328 g) was soaked with H_2O (wt plant : solvent, 1:3) for 24 hr at 25° in the dark. The aq. extract was extracted (10 ×) (0.5:1, CH_2Cl_2–H_2O) and the combined extracts dried over Na_2SO_4 and then evapd *in vacuo* to yield 1.2 g crude extract. The CH_2Cl_2 extract was sepd by CC on silica gel using *n*-hexane–EtOAc mixts of increasing polarity, yielding 214 × 50 ml frs, which were reduced to 13 frs (A–M) after comparison by CCF.

Fraction **B** provided, after CC and HPLC purification (*n*-hexane–EtOAc, 99:1), 5 mg **1**; from fraction **M**, 3 mg **2** and 4 mg **3** were isolated [HPLC: *n*-hexane–EtOAc (3:2) and CH_2Cl_2, respectively].

Gammacer-16-ene-3-one (1). $C_{30}H_{48}O$, amorphous solid, mp 159°–165°; $[\alpha]_D^{25}$ + 23° ($CHCl_3$; *c* 0.1); IR $\nu_{max}^{KBr,\,neat}$ cm^{-1}: 2951, 1696 (CO); HRMS EI (70 eV): calc. for $C_{30}H_{48}O$ 424.3705, found 424.3709; EIMS (70 eV) *m/z* (rel. int.): 424 $[M]^+$ (44), 409 $[M - CH_3]^+$ (31), 391 (14), 205 (48), 191 (60), 190 (49), 189 (100), 187 (73), 177 (75), 150 (45), 135 (63); ^1H NMR: 5.44 (*dd*, 1H, $J_{16,15\alpha}$ = 5 Hz, $J_{16,15\beta}$ = 3 Hz, H-16), 2.49 (*ddd*, 1H, $J_{2\beta,2\alpha}$ = 16 Hz, $J_{2\beta,1\alpha}$ = 10 Hz, $J_{2\beta,1\beta}$ = 8 Hz, H-2β), 2.41 (*ddd*, 1H, $J_{2\alpha,2\beta}$ = 16 Hz, $J_{2\alpha,1\alpha}$ = 8 Hz, $J_{2\alpha,1\beta}$ = 4 Hz, H-2α), 2.20 (*ddd*, 1H, $J_{15\beta,15\alpha}$ = 17 Hz, $J_{15\beta,16}$ = 2 Hz, H-15β), 1.94 (*dd*, 1H, $J_{1\alpha,1\beta}$ = 13 Hz, $J_{1\beta,2\beta}$ = 7 Hz, $J_{1\beta,2\alpha}$ = 4 Hz, H-1β), 1.75 (*m*, 1H, H-20)*, 1.11 (*s*, 3H, H-29), 1.07 (*s*, 6H, H-23, H-28), 1.05 (*s*, 3H, H-30), 1.02 (*s*, 3H, H-24), 0.97 (*s*, 6H, H-26, H-27), 0.93 (*s*, 3H, H-25); ^{13}C NMR: Table 1. *Assignments based on trace ^1H NMR COSY 2D analyses.

Messagenolide (2). $C_{30}H_{48}O_4$, gum; $[\alpha]_D^{25}$ + 39° ($CHCl_3$; *c* 0.1); IR $\nu_{max}^{KBr,\,neat}$ cm^{-1}. 3351 (OH), 1767 (γ-lactone); HRMS EI (70 eV): calc. for $C_{30}H_{48}O_4$ 472.3552, found 472.3544; $[M - H_2O]^+$ calc. 454.3447, found 454.3445; EIMS (70 eV) *m/z* (rel. int.): 454 $[M - H_2O]^+$ (30), 439 (11), 411 (16), 207 (24), 206 (24), 189 (54), 43 (100); ^1H NMR: 4.28 (*s*, 1H, H-19), 3.55 (*d*, 1H, $J_{27,27'}$ = 11 Hz, H-27'), 3.32 (*d*, 1H, $J_{27,27'}$ = 11 Hz, H-27), 3.18 (*dd*, 1H, $J_{3\alpha,2\beta}$ = 11 Hz, $J_{3\alpha,2\alpha}$ = 5 Hz, H-3α), 1.76 (*d*, 1H, $J_{13,18}$ = 11 Hz, H-18), 1.61 (*m*, 1H, H-2)*, 1.54 (*m*, 1H, H-2)*, 1.50 (*m*, 1H, H-6)*, 1.35 (*m*, 1H, H-13)*, 1.34 (*m*, 1H, H-6)*, 1.23 (*m*, 1H, H-7),* 0.99 (*s*, 3H, H-30), 0.95 (*s*, 3H, H.23), 0.90 (*s*, 3H, H-26), 0.87 (*s*, 3H, H-29), 0.82 (*s*, 3H, H-25), 0.75 (*s*, 3H, H-24), 0.67 (*br d*, 1H, $J_{5,6\beta}$ = 9 Hz, H-5); ^{13}C NMR: Table 1. *Assignments based on trace ^1H NMR COSY 2D analyses.

Soyasapogenol G (3). $C_{31}H_{48}O_4$, amorphous solid, mp 148°–152°; $[\alpha]_D^{25}$ + 29° ($CHCl_3$; *c* 0.1); IR $\nu_{max}^{KBr,\,neat}$ cm^{-1}: 3481 (OH), 1738 (–O–CO–O–); HRMS EI (70 eV): calc. for $C_{31}H_{48}O_4$ 484.3553, found 484.3566; EIMS (70 eV) *m/z* (rel. int.): 484 $[M]^+$ (3), 469 $[M - CH_3]^+$ (1), 451 (1) $[M - H_2O - CH_3]^+$ (1), 422 $[M - H_2CO_3]^+$ (0.3), 251 (9), 234 (100), 219 (48), 206 (2), 201 (15), 176 (31); ^1H NMR: 5.24 (*dd*, 1H., $J_{12,11\alpha} = J_{12,11\beta}$ = 5 Hz, H-12), 4.67 (*d*, 1H, $J_{24,24'}$ = 11 Hz, H-24), 4.04 (*ddd*, 1H, $J_{3\alpha,2\beta}$ = 12 Hz, $J_{3\alpha,2\alpha}$ = 5 Hz, $J_{3\alpha,24'}$ = 2 Hz, H-3α), 3.85 (*dd*, 1H, $J_{24,24'}$ = 11 Hz, $J_{24',3\alpha}$ = 2 Hz, H-24'), 3.43 (*dd*, 1H, $J_{22\alpha,21\alpha} = J_{22\alpha,21\beta}$ = 5 Hz, H-22α), 1.28 (*s*, 3H, H-23), 1.11 (*s*, 3H, H-27), 1.03 (*s*, 3H, H-28), 1.00 (*s*, 3H, H-26), 0.96 (*s*, 3H, H-25), 0.90 (*s*, 3H, H-29), 0.86 (*s*, 3H, H-30); ^{13}C NMR: Table 1.

Preparation of soyasapogenol G (**3**). A toluene soln of 8 mg **4** was treated with of *N,N'*-carbonyldiimidazole (1:4 molar excess) under a N_2 atmosphere and reflux for 4 hr. The reaction mixt. was extracted with H_2O to remove the unreacted imidazole and the organic layer dried over Na_2SO_4. The solvent was then evapd *in vacuo* and the crude extract subjected HPLC (*n*-hexane–EtOAc, 3:2) to yield **3** (4 mg) and **5** (1 mg), and 3 mg **4**.

Soyasapogenol G imidazole derivative (**5**). $C_{35}H_{50}N_2O_5$, gum; **IR** $\nu_{max}^{KBR,neat}$ cm^{-1}: 3481 (OH), 1738 (–O–CO–O–); ^1H NMR: 7.42 (*dd*, 1H, $J_{2',4'} = J_{2',5'} = 1.5$ Hz, H-2'), 7.07 (*dd*, 2H, $J_{5',2'} = 1.5$ Hz, $J_{5',4'} = 0.9$ Hz, H-4', H-5'), 5.31 (*dd*, 1H, $J_{12,11\alpha} = J_{12,11\beta} = 4$ Hz, H-12), 4.87 (*dd*, 1H, $J_{22\alpha,21\alpha} = J_{22\alpha,21\beta} = 4$ Hz, H-22α), 4.68 (*d*, 1H, $J_{24,24'} = 11$ Hz, H-24), 4.04 (*ddd*, 1H, $J_{3\alpha,2\beta} = 12$ Hz, $J_{3\alpha,2\alpha} = 4$ Hz, $J_{3\alpha,24'} = 2$ Hz, H-3α), 3.86 (*dd*, 1H, $J_{24,24'} = 11$ Hz, $J_{24',3\alpha} = 2$ Hz, H-24'), 2.27 (*br d*, 1H, $J = 14$ Hz, H-21), 1.29 (*s*, 3H, H-23), 1.17 (*s*, 3H, H-27), 1.02 (*s*, 3H, H-28), 1.00 (*s*, 3H, H-26), 0.96 (*s*, 3H, H-25), 0.94 (*s*, 3H, H-29), 0.90 (*s*, 3H, H-30).

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