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^{1}H and ^{13}C NMR study of copteroside E derivatives†

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ABSTRACT: The triterpenoid saponin copteroside E (**1**) was isolated from the flowers and leaves of *Verbesina suncho*. Detailed ^{1}H and ^{13}C NMR assignment of its acetyl derivatives **2** and **3** was achieved by 2D NMR techniques including COSY, TOCSY, NOESY, HMQC and HMBC. The presence of a glucuronolactone moiety in **2** is supported by the calculated vs observed $^{1}H-^{1}H$ coupling constants. Molecular mechanics calculations on **1** were useful for understanding the formation of glucuronolactone **2** and decaacetate **3.** Copyright © 2000 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{1}H NMR; ^{13}C NMR; 2D NMR; copteroside E; oleanolic acid saponins; glucuronic acid derivative; glucuronolactone derivative; molecular mechanics calculations

INTRODUCTION

Copteroside E or 3β-{([*O*-β-D-xylopyranosyl-(1 → 2)-*O*-β-D-xylopyranosyl-(1 → 4)]-*O*-β-D-glucopyranuronosyl)-oxy}olean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester (**1**) is a triterpenoid saponin which was originally isolated from the epigeal parts of *Climacoptera transoxana*.[1] This compound belongs to a relevant group of substances which are classified as oleanolic acid derivatives containing a glucuronic acid unit at C-3. In general, these compounds have shown an important cytoprotective activity against CCl_4-induced hepatotoxicity.[2] In previous work,[1] the structure elucidation of copteroside E (**1**) was carried out by chemical transformations and mass spectral analyses of some derivatives. However, neither ^{1}H nor ^{13}C NMR data were reported. These facts prompted us to perform detailed NMR studies of two acetyl derivatives of **1**, glucuronolactone **2** and decaacetate **3**. In addition, molecular mechanics calculations on **1** allowed us to understand its particular reactivity towards acetylation.

† This paper is dedicated to Professor Dr Harald Günther on the occasion of his 65th birthday.

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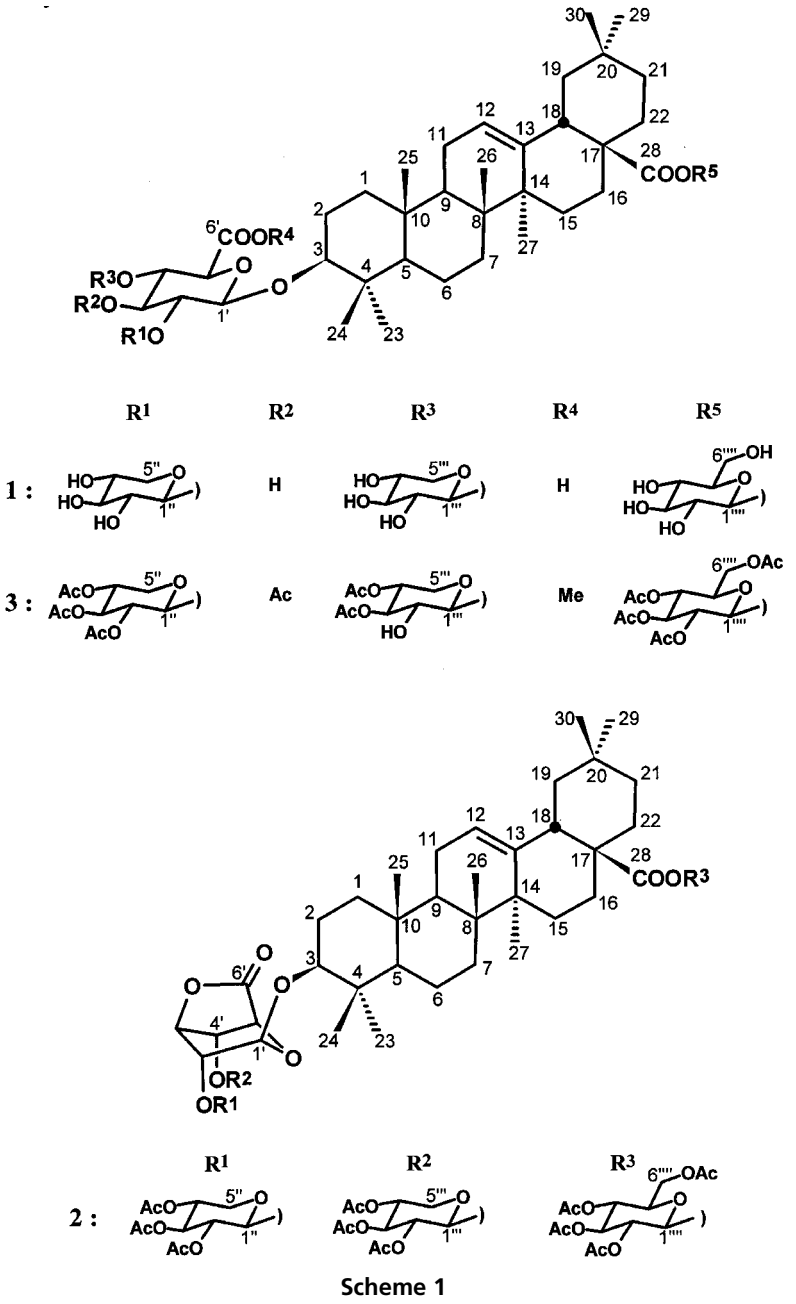
RESULTS AND DISCUSSION

Copteroside E (**1**) was isolated, after several chromatographic procedures, from the complex mixture of saponins present in the methanolic extract of the flowers and leaves of *Verbesina suncho* Blake, a shrub which grows in northern Argentina. Its elemental composition as $C_{52}H_{82}O_{22}$ was confirmed by positive fast atom bombardment mass spectrometry using Na or K atoms, which gave quasi-molecular ion peaks at *m*/*z* 1104 corresponding to $[M - H + 2Na]^+$ (calculated mass 1103.5) or *m*/*z* 1136 corresponding to $[M - H + 2K]^+$ (calculated mass 1135.5). Similar quasi-molecular ion peaks have been observed with triterpenoid saponins containing glucuronic acid moieties.[2,3] In addition, acid hydrolysis of **1** yielded oleanolic acid, D-glucuronic acid, D-glucose and D-xylose, which were identified by direct comparison with authentic samples.

The ^{1}H NMR spectrum of **1** in pyridine-$d_5 + D_2O$ showed the four anomeric proton signals at δ 6.23, 5.21, 5.05 and 4.81 as doublets ($J = 7-8$ Hz), indicating the presence of the four β-linked sugar units. This was in agreement with the ^{13}C NMR spectrum, which showed four anomeric carbon signals at δ 106.1, 105.8, 104.7 and 95.8 ppm. Careful comparison of the ^{13}C NMR spectrum of **1** (Table 1) with that of 3β-([*O*-β-D-xylopyranosyl-(1 → 3)-*O*-β-D-glucopyranuronosyl]oxy)olean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester, isolated from *Beta vulgaris*,[4] and with that of momordin IIc, isolated from *Momordica cochinchinensis*,[5] was in agreement with the presence of an olean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester unit further substituted at C-3.

Acetylation of **1** under standard reaction conditions, that is, using Ac_2O in pyridine followed by neutralization with



Scheme 1

aqueous HCl, extraction with EtOAc and washing with aqueous HCl and $NaHCO_3$, yielded decaacetate lactone **2.** Its mass spectrum showed a molecular ion peak $[M - H + Na]^+$ at m/z 1484 in agreement with the molecular formula $C_{72}H_{100}O_{31}$. The 500 MHz ^{1}H NMR spectrum of **2** (Table 2), in combination with COSY and TOCSY contour plots indicated the presence of the 24 protons of the four sugar residues. The multiplicities and coupling constants of the two β-xylose and a β-glucose units were clearly recognized. However, the coupling constant values of the glucuronic acid moiety were not those expected when a typical glucuronic acid moiety is taken into consideration. Instead, these values, shown in Table 3, were in full agreement with the presence of a glucuronolactone unit in which the carboxylic acid of **1** has reacted with the hydroxy group at C-3, with the consequent inversion of the pyranoside ring conformation. A molecular model of **2** (E_{MMX}63.0 kcal mol^{-1}) (1 kcal = 4.184 kJ) is depicted in Fig. 1, showing the axial orientation for the two xylose moieties. In this work, the molecular models were generated using the MMX force field calculations as implemented in the PCMODEL program. In order to simplify the calculations, only the sugar residues at C-3 and rings A and B of the triterpene moiety were taken into consideration.

A coupled ^{13}C spectrum showed that the anomeric carbon of the glucuronolactone moiety had a one-bond coupling constant of J(C,H) = 172 Hz, in agreement with the axial orientation of the oxygen atom at C-1′.[6] The calculated H—C—C—H dihedral angles of this model allowed us to obtain a set of calculated ^{1}H–^{1}H vicinal coupling constants for the glucuronolactone unit by means of a generalized Karplus-type relationship.[7,8] The couplings were in close correspondence with those observed in the experimental spectrum, as can be seen in Table 3.

Table 1. ^{13}C NMR chemical shifts for compounds **1–3**[a]

C	**1**[b]	**2**[c]	**3**[d]	C	**1**[b]	**2**[c]	**3**[d]
1	38.7	38.5	38.6	27	26.1	25.6	25.7
2	26.5	25.3	25.8	28	176.5	175.6	175.6
3	89.3	90.0	91.1	29	33.2	33.0	33.0
4	39.5	39.0	38.7	30	23.7	23.4	23.5
5	55.9	55.4	55.6	GlucA 1′	105.8	103.6	103.7
6	18.5	18.2	18.1	2′	81.6	77.5	76.9
7	33.2	33.0	33.0	3′	78.0	73.6	74.1
8	39.9	39.3	39.3	4′	83.4	71.7	78.8
9	48.0	47.6	47.7	5′	76.9	67.2	73.4
10	36.9	36.7	36.7	6′	174.9	170.6	170.5
11	23.8	22.8	22.7	Xyl(2′) 1″	104.7	102.1	101.0
12	122.7[e]	123.0	123.0	2″	76.4	70.7	71.2
13	144.2	142.8	142.9	3″	78.0	71.3	72.5
14	42.2	41.7	41.7	4″	71.1	68.8	68.2
15	28.3	27.7	27.8	5″	67.4	62.1	62.5
16	23.5	23.4	23.5	Xyl(4′) 1‴	106.1	100.0	104.5
17	47.0	46.8	46.8	2‴	75.1	70.7	71.7
18	41.8	41.0	41.2	3‴	76.7	71.6	73.4
19	46.3	45.7	45.8	4‴	70.6	69.1	69.0
20	30.8	30.6	30.6	5‴	67.4	62.8	62.8
21	34.1	33.8	33.9	Glu 1⁗	95.8	91.6	91.6
22	32.6	31.8	31.9	2⁗	74.1	70.0	69.9
23	27.9	27.8	27.5	3⁗	79.3	72.9	72.9
24	16.5	16.4	16.0	4⁗	71.1	68.0	68.0
25	15.6	15.3	15.4	5⁗	78.9	72.5	72.5
26	17.5	16.9	17.0	6⁗	62.3	61.5	61.5

[a] TMS as internal standard.
[b] Measured at 75.4 MHz in C_5D_5N.
[c] Measured at 125.7 MHz in $CDCl_3$. Acetates: δ 170.6, 170.3, 170.2, 170.1, 169.9 (×2), 169.4, 169.0, 168.9 (×2), 20.7 and 20.5 (×10).
[d] Measured at 75.4 MHz in $CDCl_3$. Acetates: δ 170.6, 170.2, 170.1, 170.0, 169.8, 169.7 (×2), 169.4, 169.0, 168.9, 21.2, 21.0, 20.8, 20.7 (×5), 20.6, 20.5. MeO: 53.2.
[e] Overlapped with the solvent signal.

The ^{13}C spectrum of **2** (Table 1) was assigned with the aid of HMQC and HMBC diagrams. In particular, the HMBC spectrum was also very helpful to corroborate the sugar sequence since it revealed that the anomeric proton H-1′ showed correlation with C-3, C-2′, C-3′ and C-5′; H-2′ showed correlations with C-1′, C-3′, C-4′ and with the anomeric carbon C-1″; H-3′ correlated with C-4′; H-5′ correlated with C-3′, C-4′ and C-6′; and H-1‴ showed correlation with C-4′.

In order to increase the acetylation yields we decided to avoid the extraction procedure, considering that the aqueous $NaHCO_3$ washings could contribute to an important loss of those compounds containing a carboxylic acid moiety. Therefore, we again treated **1** with Ac_2O in pyridine, but the product was isolated by evaporation of the volatile reagents under a stream of N_2 and immediate dissolution in MeOH. Surprisingly, this procedure yielded the decaacetate methyl ester **3**, a non-lactonized compound which contains a free hydroxy group at C-2‴, as could be deduced from the chemical shift of H-2‴ (δ 3.71 ppm). The 500 MHz ^{1}H NMR spectrum of this compound **3** (Table 2) clearly exhibited the 24 protons of the sugar residues whose multiplicities and coupling constants corresponded to the characteristic values for a β-glucuronic acid, two β-xylose and a β-glucose units. Assignment of the signals

Table 2. ^{1}H NMR data for compounds **2** and **3**[a]

^{1}H	**2**[c]	**3**[b]
3	3.07 (dd, $J = 5, 12$)	3.09 (dd, $J = 5, 12$)
12	5.32 (br t, $J = 3$)	5.32 (br t, $J = 3$)
18	2.82 (dd, $J = 4, 13$)	2.82 (dd, $J = 4, 13$)
23	0.89 (s)	1.00 (s)
24	0.74 (s)	0.81 (s)
25	0.89 (s)	0.90 (s)
26	0.72 (s)	0.73 (s)
27	1.12 (s)	1.12 (s)
29	0.90 (s)	0.90 (s)
30	0.91 (s)	0.90 (s)
GlucA 1′	5.05 (br s)	4.50 (d, $J = 7$)
2′	3.99 (br d, $J = 4$)	3.71 (dd, $J = 7, 9$)
3′	4.61 (br dd, $J = 4, 5$)	5.16 (dd, $J = 9, 10$)
4′	4.19 (dd, $J = 3, 5$)	3.86 (t, $J = 10$)
5′	4.04 (br d, $J = 3$)	3.96 (d, $J = 10$)
Xyl(2′) 1″	4.59 (d, $J = 7$)	4.61 (d, $J = 7$)
2″	4.99 (dd, $J = 7, 9$)	4.86 (dd, $J = 7, 9$)
3″	5.16 (dd, $J = 9, 10$)	5.12 (dd, $J = 9, 10$)
4″	4.94 (dt, $J = 6, 10$)	4.94 (dt, $J = 6, 10$)
5ax″	3.31 (dd, $J = 10, 12$)	3.27 (dd, $J = 10, 12$)
5ec″	4.07 (dd, $J = 6, 12$)	4.08 (m)
Xyl(4′) 1‴	4.60 (d, $J = 7$)	4.27 (d, $J = 7$)
2‴	5.02 (dd, $J = 7, 9$)	3.42 (dd, $J = 7, 9$)
3‴	5.21 (dd, $J = 9, 10$)	5.04 (dd, $J = 9, 10$)
4‴	5.03 (dt, $J = 6, 10$)	4.87 (dt, $J = 6, 10$)
5ax‴	3.32 (dd, $J = 10, 12$)	3.28 (dd, $J = 10, 12$)
5ec‴	4.14 (dd, $J = 6, 12$)	4.00 (dd, $J = 6, 12$)
Glu 1⁗	5.58 (d, $J = 8$)	5.59 (d, $J = 8$)
2⁗	5.18 (dd, $J = 8, 10$)	5.20 (dd, $J = 8, 10$)
3⁗	5.25 (dd, $J = 9, 10$)	5.26 (dd, $J = 9, 10$)
4⁗	5.13 (dd, $J = 9, 10$)	5.14 (dd, $J = 9, 10$)
5⁗	3.79 (ddd, $J = 3, 5, 10$)	3.80 (m)
6a⁗	4.28 (dd, $J = 5, 13$)	4.28 (dd, $J = 5, 13$)
6b⁗	4.05 (dd, $J = 3, 13$)	4.06 (m)

[a] Measured at 500 MHz from $CDCl_3$ solutions containing TMS as internal reference. Coupling constants in Hz.
[b] Acetates: δ 2.08, 2.06, 2.05, 2.05, 2.05, 2.04, 2.04, 2.02, 2.02, 2.01 (10 s).
[c] MeO: δ 3.79 (s). Acetates: δ 2.10, 2.07, 2.07, 2.04, 2.03, 2.02, 2.02, 2.02, 2.02, 2.01 (10 s).

Table 3. Observed vs calculated vicinal coupling constants and the corresponding MMX dihedral angles of glucuronolactone **2**

Fragment	Observed J (Hz)	Calculated J (Hz)	Dihedral angle (°)
H1′—C1′—C2′—H2′	~1	1.0	−92
H2′—C2′—C3′—H3′	4	4.0	+59
H3′—C3′—C4′—H4′	5	5.8	−47
H4′—C4′—C5′—H5′	3	3.7	+50

was further confirmed by the correlations observed in the COSY contour plot, where the four independent proton systems were recognized. The sugar sequence was verified from the correlations found in the HMBC diagram of **3**. A correlation between C-28 and H-1⁗ confirmed the position of the glucose unit at C-28, while a correlation between C-3 and H-1′ confirmed the presence of the glucuronic acid residue at C-3. The complete ^{13}C NMR

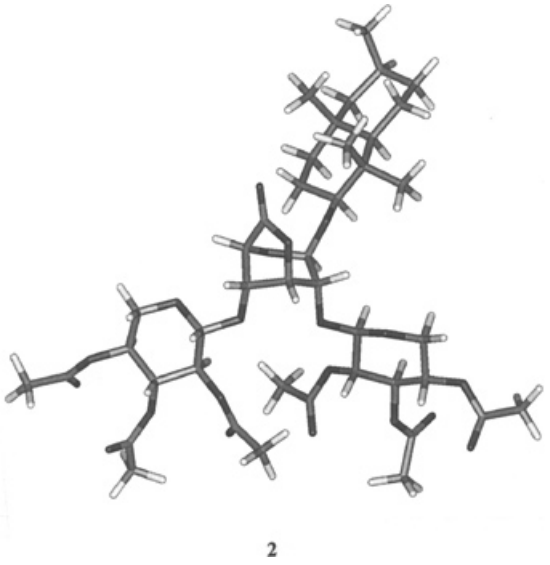


Figure 1. MMX molecular model of glucuronolactone **2** in the minimum energy conformation.

assignment of the glucuronic acid moiety was achieved from the HMBC diagram, where H-1′ showed correlation with C-3, H-2′ showed correlations with C-1′, H-3′ with C-2′ and C-4′, H-4′ with C-3′ and C-5′, and H-5′ with C-4′ and C-6′. Individual assignment of the two xylose moieties attached to C-2′ and C-4′ of the glucuronic acid unit was supported by the HMBC correlations of C-2′ with H-1″ and of C-4′ with H-1‴, respectively.

It seemed noteworthy that the hydroxy group at C-2‴ in **3** remained unchanged after the acetylation procedure. Inspection of an MMX molecular model of **1** (E_{MMX} 46.6 kcal mol^{-1}) in the minimum energy conformation[9] shown in Fig. 2 revealed that in fact the hydroxy group at C-2‴ remains close to the carboxylic acid group at C-5′ (O-2‴—C-6′ distance = 3.8 Å). Therefore, these groups easily could form a lactone intermediate **4** (E_{MMX} 67.8 kcal mol^{-1}) during the acetylation procedure (Fig. 2), the subsequent methanolysis of which could yield **3.**

At this point, the seven-membered ring lactone **4** can also be postulated as a reaction intermediate in the formation of the five-membered ring lactone **2.** Conversion of **4** into **2** may occur in the acid media during the extraction procedure through a series of transesterification reactions. Hydrolysis of the seven-membered ring lactone **4** and rotation of the C4′—O4′ and O4′—C1‴ bonds gives intermediate **5** (E_{MMX} 55.4 kcal mol^{-1}), as shown in Fig. 3, in the proper conformation to allow a transesterification reaction of the acetyl group from O-3′ to O-2‴. Once the hydroxy group at C-3′ is free, the pyranoside ring can invert its conformation to yield intermediate **6** (E_{MMX} 52.5 kcal mol^{-1}) as represented in Fig. 3. Intermediate **6** meets the stereochemical requirements to undergo lactonization yielding glucuronolactone **2**.

The data described here contribute to the knowledge of the spectral properties of oleanolic acid derivatives containing a glucuronic acid unit at C-3 and open new avenues for the understanding of their chemical behavior, which can be useful in future pharmacological studies of this class of substances.

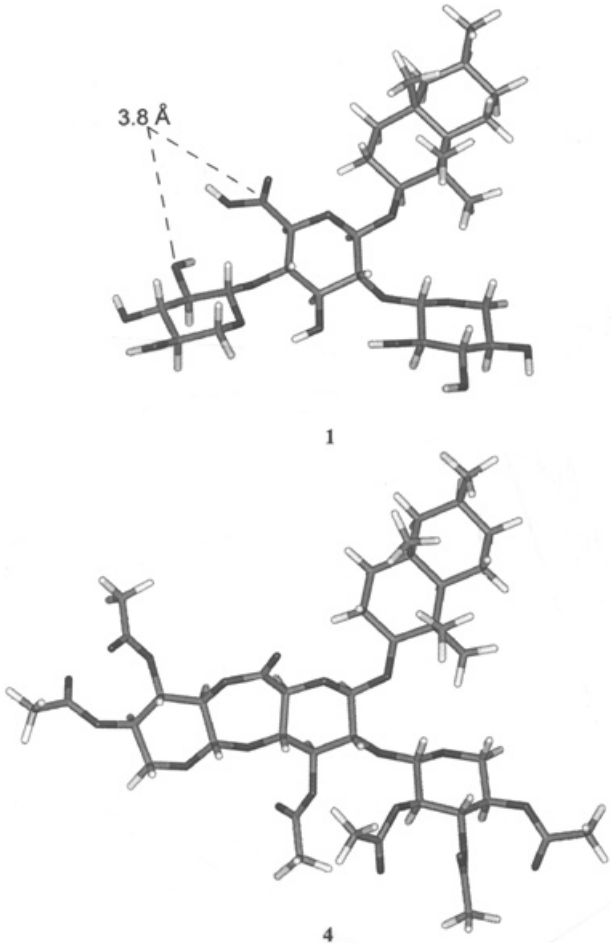


Figure 2. MMX molecular models of copteroside E (**1**) showing the distance between O-2‴ and C-6′ and the putative seven-membered-ring lactone **4**, reaction intermediate during the acetylation of **1**.

EXPERIMENTAL

NMR spectra

NMR measurements were performed on a Varian Unity Plus 500 or a Varian XL300GS spectrometer using TMS as the internal reference. NMR spectra of **1** were measured in pyridine-d_5 and those of **2** and **3** in $CDCl_3$. All 500 MHz spectra were recorded using an indirect detection probe. COSY, phase-sensitive NOESY and TOCSY spectra were obtained with a spectral window of 4850 Hz in both dimensions, using 1024 data points, 128 time increments with 16 transients for each time increment and linear prediction to 1024 points (zero-filled to 2048). A recycle delay of 1 s was used for each experiment. The NOESY mixing time was 1.5 s and the TOCSY mixing time was 70 ms. HMQC spectra were measured with a ^{13}C spectral width of 20 400 Hz and a ^{1}H spectral window of 4850 Hz; 32 transients were acquired for each 256 time increments with a recycle delay of 1 s. Application of linear prediction to 2048 points was performed in the indirect detected time. HMBC spectra were recorded with f_2 and f_1 spectral windows of 4850 and 25 921 Hz, respectively, using 1024 data points in f_2, with f_1 linear prediction to 1024 points. Both dimensions were zero-filled to 2048. The recycle

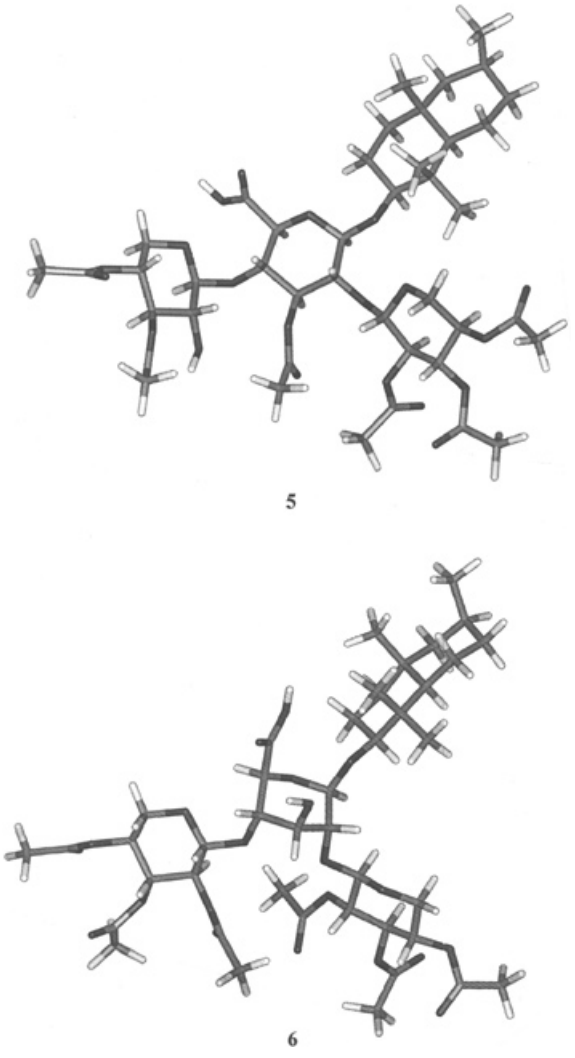


Figure 3. MMX molecular models of intermediates **5** and **6**.

delay was 1 s and a value of 9 Hz was used for the average long-range C–H coupling constant.

Structure calculations

Molecular modeling calculations were performed using the MMX force field, which is a derived version of the MM2 program[9] as implemented in the PCMODEL program V 6.00 (Serena Software, Bloomington, IN, USA). A conformational search for the sugar units, the methoxy groups, and the acetyl groups was carried out by the analysis of the rotational energy barrier plots in combination with the E_{MMX} convergence parameter using the dihedral driver option.

Isolation of copteroside E (1)

The flowers and leaves of *Verbesina suncho* Blake were collected near El Cadillal dike, Tucumán province, Argentina. A voucher specimen (CC 540) is deposited in the Instituto Miguel Lillo, Tucumán, Argentina. Air-dried flowers and leaves (948 g) were extracted at room temperature with MeOH for 3 days. After evaporation under vacuum, the extract was partitioned between *n*-BuOH–H_2O (1 : 1). The BuOH layer was evaporated to dryness, yielding a brown residue (70 g) which was dissolved in a small amount of MeOH and precipitated with acetone (700 ml). A portion of the precipitate (13 g) was chromatographed on silica gel (70–230 mesh) using $CHCl_3$–MeOH–H_2O (35 : 15 : 2) as the eluent. A total of 148 fractions were collected and monitored by TLC. Fractions 72–86 (1.240 g) were combined and rechromatographed over silica gel with *n*-BuOH–AcOH–H_2O (100 : 3 : 10). Successive rechromatography under the same conditions yielded 89 mg of pure copteroside E (**1**) as a white powder which decomposes above 250 °C (lit.[1] 236–240 °C); ^{1}H NMR ($C_5D_5N + D_2O$, 300 MHz), δ 6.23 (1H, d, J = 8 Hz), 5.44 (br t, J = 3 Hz, H-12), 5.21 (1H, d, J = 7 Hz), 5.05 (1H, d, J = 7 Hz), 4.81 (1H, d, J = 7 Hz), 3.20 (2H, m, H-3 and H-18), 1.28, 1.19, 1.07, 1.01, 0.93, 0.89, 0.80 (3H each, 7 s, 7 Me); ^{13}C NMR, see Table 1; FAB-MS, *m/z* (relative intensity (%)), 1136 $[M - H + 2K]^+$ (100), 705 (24), 647 (35), 613 (87); FAB-MS, *m/z* (relative intensity, %), 1104 $[M - H + 2Na]^+$ (100), 1103 (72), 972 (51), 745 (38), 529 (42), 501 (56), 463 (85), 393 (54), 369 (76).

Acid hydrolysis of copteroside E (1)

Compound **1** (10 mg) was refluxed with 5 ml of HCl (2%) in 60% aqueous MeOH (5 ml) for 3 h. The reaction mixture was extracted with $CHCl_3$ (×2). The sapogenin was identified as oleanolic acid by direct comparison with an authentic sample. The aqueous layer was percolated through an Amberlite Monobed Resin MB-3 column and concentrated under vacuum. The residue was compared with standard sugars on silica gel (EtOAc–MeOH–H_2O–HOAc, 13 : 3 : 3 : 5) and cellulose plates (*n*-BuOH–pyridine–H_2O, 6 : 4 : 3). The spots were revealed with H_2SO_4 for silica gel plates and $AgNO_3$–sugar reagent for cellulose plates.

Preparation of glucuronolactone 2

A solution of **1** (20 mg) in C_5H_5N (5 ml) was treated with Ac_2O (3 ml). The mixture was stored at room temperature for 5 days. The reaction mixture was poured over 10% HCl and extracted with EtOAc. The organic layer was washed with HCl (10%), H_2O, aqueous $NaHCO_3$ and H_2O, dried with anhydrous Na_2SO_4, filtered and evaporated under vacuum. The residue was chromatographed over silica gel using hexane–AcOEt (1 : 1) to yield **2** as a viscous oil (7 mg, 25%); IR, $\nu_{max}(CHCl_3)(cm^{-1})$ 1750, 1366, 1220; ^{1}H NMR, see Table 2; ^{13}C NMR, see Table 1.

Preparation of decaacetate 3

A solution of **1** (20 mg) in C_5H_5N (5 ml) was treated with Ac_2O (3 ml). The mixture was stored at room temperature for 5 days. The volatile components were removed using a stream of N_2. Treatment with MeOH for 30 min, followed by evaporation with the stream of N_2, yielded an oily residue which was chromatographed over silica gel using hexane–AcOEt 2 : 1. Fractions 6–8 afforded pure **3** as a viscous oil (12 mg, 42%); IR, $\nu_{max}(CHCl_3)(cm^{-1})$ 2930, 1756, 1368, 1228; ^{1}H NMR, see Table 2; ^{13}C NMR, see Table 1.

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