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Antioxidant phenolic and quinonemethide triterpenes from *Cheiloclinium cognatum*

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Abstract

The triterpenes, 22β-hydroxypristimerin and cognatine, were isolated together with the known compounds pristimerin, maytenin, 20α-hydroxymaytenin, 22β-hydroxymaytenin, netzahualcoyol, netzahualcoyondiol and netzahualcoyone from root bark of *Cheiloclinium cognatum*. The structures of the isolated compounds were elucidated by interpretation of their spectral data, including gHMQC and gHMBC experiments.The isolates were investigated for their radical scavenging abilities through a spectrophotometric assay involving reduction of 2,2-diphenyl-picryl hydrazyl (DPPH).

Keywords: *Cheiloclinium cognatum*; Hippocrateaceae; Quinonemethide triterpenes; Phenolic triterpene; Antioxidant activity

1. Introduction

The Celastraceae and Hippocrateaceae families are rich sources of quinonemethide triterpenes (Brüning and Wagner, 1978; Gunatilaka, 1996), and these compounds have a variety of biological activities such as antitumor (Bhatnagar et al., 1979; Gonçalves de Lima et al., 1971), antimicrobial (Ferreira de Santana et al., 1971); antibiotic (Gonçalves de Lima et al., 1969; Gonzalez et al., 1977); cytotoxic (Setzer et al., 1998) and antimalarial (Pavanand et al., 1989; Figueiredo et al., 1998) properties. Systematic studies on Celastraceae and Hippocrateaceae plant species (Corsino et al., 1998a,b,c, 2000, 2003; Buffa Filho et al., 2002) have been concerned with biosynthesis of quinonemethide triterpenes, the main secondary metabolites in this taxon. The biosynthetic study dealing on *Maytenus aquifolium* (Celastraceae) and *Salacia campestris* (Hippocrateaceae) indicated that friedelane triterpenes are putative precursors of quinonemethide triterpenes, which are biosynthesized in the leaves and translocated to the root bark, where these compounds would be transformed into quinonemethide triterpenes (Corsino et al., 2000). These findings have led us to investigate other species of Celastraceae and Hippocrateaceae. *Cheiloclinium cognatum* (Miers) A.C. Sm. (Hippocrateaceae) a tree widely distributed in the Cerrado region of Brazil, was selected to investigate the oxidative transformation on the E ring of the quinonemethide triterpenes. With this objective, we carried out a chemical study on this plant species, which led to the isolation of two new triterpenes, 22β-hydroxypristimerin (**1**) and cognatine (**2**), besides seven known quinonemethide triterpenes; pristimerin (**3**), maytenin (**4**), 22β-hydroxymaytenin (**5**), 20α-hydroxymaytenin (**6**), netzahualcoyonol (**7**), netzahualcoyondiol (**8**) and netzahualcoyone (**9**). The free radical scavenging activities of the isolates have been evaluated through a spectrophotometric assay using the stable free radical 2,2-diphenyl-picryl hydrazyl(DPPH).

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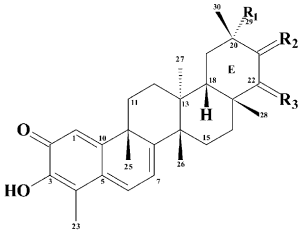
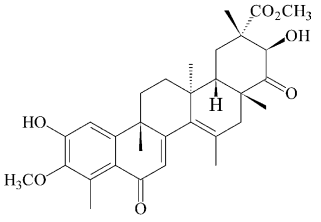
2. Results and discussion

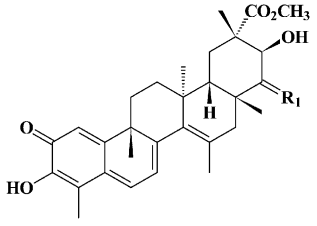
A CH_2Cl_2 soluble fraction obtained from the ethanolic extract of the root bark *C. cognatum* was subjected to silica gel column chromatography. The fractions obtained were further purified by preparative TLC and/or HPLC, affording compounds **1–9**.





2

	R_1	R_2	R_3
1	CO_2CH_3	H_2	βOH, αH
3	CO_2CH_3	H_2	H_2
4	H	O	H_2
5	H	O	βOH, αH
6	OH	O	H_2



	R_1
7	H_2
8	βOH,αH
9	O

22β-Hydroxypristimerin (**1**), obtained as a red amorphous solid, was shown to have the molecular formula $C_{30}H_{40}O_5$ by analysis of its ESI/MS spectrum, elemental analysis and ^{13}C NMR spectral data. The IR spectrum exhibited absorption bands at 3450 cm^{-1} (hydroxyl group) and 1724 and 1645 cm^{-1} (carbonyl groups). The ^1H NMR spectrum of **1** revealed the presence of six methyl singlets at δ 2.13, 1.38, 1.22, 1.15, 0.99 and 0.41, attributed to H_3-23, H_3-25, H_3-26, H_3-30, H_3-28 and H_3-27, respectively, (Table 1). The quinonemethide moiety was characterized by a doublet at δ 6.26 (1H, $J = 6.5$ Hz), a singlet at δ 6.41 (1H) and a doublet at δ 6.94 (1H, $J = 6.5$ Hz) attributed to H-7, H-1 and H-6, respectively. The singlet at δ 2.13, characteristic of a methyl group attached to a sp^2 carbon (H_3-23), corroborated the presence of a quinonemethide system. Further oxygenated functional groups were assumed to be hydroxyl and ester on the basis of NMR spectral analysis. The ^{13}C NMR, gHMBC and gHMQC spectra (Table 2) showed the presence of 30 carbons in **1**. In the ^{13}C NMR spectrum two carbonyl carbon signals

Table 1
^1H NMR spectral data of compounds **1** and **2** [500 MHz, δ (ppm), multiplicity, J (Hz), $CDC1_3$]

Position	**1**	**2**
1	6.41 (*s*)	6.85 (*s*)
6	6.94 (*d*, $J_{6,7} = 6,5$ Hz)	–
7	6.26 (*d*, $J_{7,6} = 6,5$ Hz)	6.00 (*s*)
21	–	4.95 (*s*)
22	4.02 (*dd*, $J_{22ax,21ax} = 11.0$ Hz and $J_{22ax,21eq} = 4.0$ Hz)	–
23	2.13 (*s*)	2.67 (*s*)
25	1.38 (*s*)	1.33 (*s*)
26	1.22 (*s*)	1.72 (*s*)
27	0.41 (*s*)	0.90 (*s*)
28	0.99 (*s*)	1.38 (*s*)
30	1.15 (*s*)	0.95 (*s*)
OCH_3 at C-29	3.50 (*s*)	3.74 (*s*)
OCH_3 at C-3	–	3.73 (*s*)

(δ 178.2 and δ 178.3) attributed to C-29 and C-2, respectively, six methyl carbons (δ 38.1, 32.2, 24.0, 21.6, 19.0 and 10.1), six methylene carbons (δ 38.1, 36.1, 33.5, 30.8, 29.5 and 28.2), four methine carbons (δ 134.0, 119.5, 118.0 and 46.1), 10 quaternary carbons (δ 169.7, 164.6, 146.0, 127.3, 117.2, 44.5, 42.6, 42.4, 39.2 and 38.2), one methoxyl carbon (δ 51.6) and one hydroxymethine carbon (δ 67.9) were observed. The assignments were based on gHMQC and gHMBC spectra and by comparison with published data on pristimerin (Gunatilaka, 1989, 1996). The correlations observed in the gHMBC spectrum from the singlet at δ 0.99 (H_3-28) to the hydroxymethine carbon at δ 67.9 (Fig. 1) and the signals at δ 36.1 (C-16), 38.1 (C-17) and 46.1 (C-18), confirmed the attachment of the hydroxyl group at C-22. The relative stereochemistry of H-22 was deduced by the coupling constant values (δ 4.02, $J_{22ax/21ax} = 11.0$ Hz and $J_{22ax/21eq} = 4.0$ Hz), which corroborated the *equatorial* orientation of the hydroxyl group. The ester group was confirmed by a singlet at δ 3.50 (3H) attributed to a methoxyl portion of the ester group at C-29, which was confirmed by means of correlation of the methyl group at δ 1.15 (C-30) with the carbonyl carbon of the ester group at δ 178.2 (C-29) observed in the gHMBC spectrum (Fig. 1). The structure of **1** was determined as 22β-hydroxypristimerin.

Cognatine (**2**), isolated as a pale yellow amorphous solid, was shown to have the molecular formula $C_{31}H_{38}O_7$ by means of analysis of ESI/MS data, elemental analysis and ^{13}C NMR spectral data. The IR spectrum showed absorption bands for hydroxyl group at 3438 cm^{-1} and carbonyl groups at 1718 and 1643 cm^{-1}. Its ^1H NMR spectrum (Table 1) showed the presence of two singlets at δ 6.85 (1H), attributed to H-1 and at δ 6.00 (1H) assigned to H-7. Also, two methoxyl groups signals at δ 3.73 and 3.74 (3H, each) were observed, which were characterized as methoxyl groups attached to C-3 and belonging to the ester group at C-

Table 2
^{13}C NMR spectral data for compounds **1**, **2**, **7**–**9** (125 MHz, δ (ppm), multiplicity, $CDCl_3$)[a]

Position	1	2	7	8	9
1	119.5 *d*	110.1 *d*	119.4 *d*	120.0 *d*	120.1 *d*
2	178.3 *s*	151.7 *s*	178.8 *s*	178.1 *s*	178.0 *s*
3	146.0 *s*	144.7 *s*	146.1 *s*	146.2 *s*	146.3 *s*
4	117.2 *s*	134.0 *s*	118.6 *s*	116.7 *s*	116.8 *s*
5	127.3 *s*	122.4 *s*	127.6 *s*	127.7 *s*	128.2 *s*
6	134.0 *d*	185.8 *s*	137.8 *d*	134.5 *d*	134.1 *d*
7	118.0 *d*	129.3 *d*	122.2 *d*	121.5 *d*	122.2 *d*
8	169.7 *s*	160.1 *s*	160.2 *s*	159.0 *s*	158.4 *s*
9	42.4 *s*	42.2 *s*	45.5 *s*	44.5 *s*	44.1 *s*
10	164.6 *s*	151.2 *s*	163.5 *s*	159.9 *s*	157.1 *s*
11	33.5 *t*	37.1 *t*	37.3 *t*	37.4 *t*	37.1 *t*
12	29.5 *t*	35.8 *t*	35.2 *t*	34.8 *t*	35.9 *t*
13	39.2 *s*	40.7 *s*	43.3 *s*	42.4 *s*	42.1 *s*
14	44.5 *s*	134.7 *s*	135.5 *s*	136.0 *s*	135.9 *s*
15	28.2 *t*	125.9 *s*	129.8 *s*	126.6 *s*	126.3 *s*
16	36.1 *t*	38.0 *t*	39.0 *t*	37.9 *t*	38.2 *t*
17	38.1 *s*	48.8 *s*	35.6 *s*	38.9 *s*	48.9 *s*
18	46.1 *d*	46.2 *d*	43.6 *d*	38.7 *d*	46.3 *d*
19	30.8 *t*	34.2 *t*	34.2 *t*	33.9 *t*	34.2 *t*
20	42.6 *s*	50.3 *s*	48.0 *s*	47.4 *s*	50.3 *s*
21	38.1 *t*	74.3 *d*	68.0 *d*	69.2 *d*	74.4 *d*
22	67.9 *d*	214.0 *s*	39.0 *t*	79.4 *d*	213.7 *s*
23	10.1 *q*	15.1 *q*	10.5 *s*	10.3 *s*	10.3 *s*
25	38.1 *q*	21.6 *q*	29.4 *s*5	29.5 *s*	28.9 *s*
26	21.6 *q*	21.7 *q*	21.9 *s*	21.8 *s*	21.9 *s*
27	19.0 *q*	23.9 *q*	24.3 *s*	24.5 *s*	24.2 *s*
28	24.0 *q*	22.5 *q*	31.1 *s*	27.0 *s*	22.4 *s*
29	178.2 *s*	175.2 *s*	178.8 *s*	179.0*s*	175.3 *s*
30	32.2 *q*	13.7 *q*	17.5 *s*	13.7 *s*	13.6 *s*
OCH_3-29	51.6 *q*	52.7 *q*	52.3*s*	52.7 *s*	52.6 *s*
OCH_3-3	–	61.7 *q*	–	–	–

[a] Multiplicities of carbons were determined by a DEPT 134° experiment.

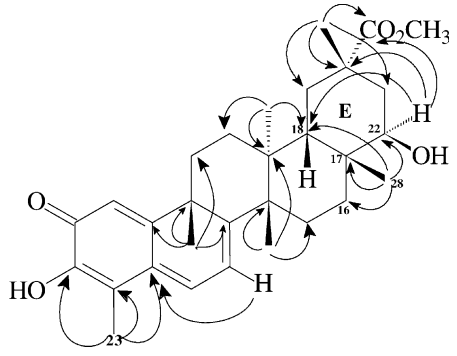


Fig. 1. Selected gHMBC correlations observed for compound **1**.

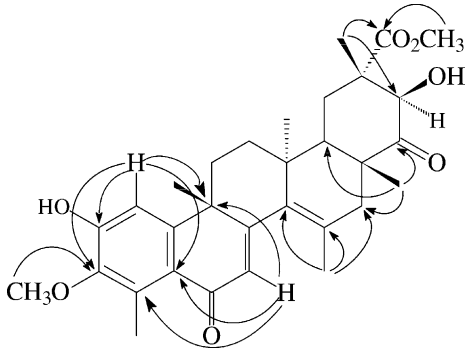


Fig. 2. Selected gHMBC correlations observed for compound **2**.

29, respectively, as evidenced by gHMBC experiments (Fig. 2). Other singlets integrating for 3H each were observed at δ 2.67, assigned to a methyl group on aromatic ring (H_3-23), at δ 1.72 assigned to one methyl group on double bond (C-26) and at δ 1.38, 1.33, 0.95 and 0.90, corresponding to tertiary methyl groups (H_3-28, H_3-25, H_3-30 and H_3-27, respectively).

The analysis of the ^{13}C NMR (Table 2), gHMQC and gHMBC spectral data indicated the presence of three carbonyl carbons at δ 214.0, 185.8 and 175.2 attributed to C-22, C-6 and C-29, respectively, six methyl groups at δ 27.6, 23.9, 22.5, 21.7, 15.1 and 13.7, attributed to C-25, C-27, C-28, C-26, C-23 and C-30, respectively, two vinylic carbons at δ 134.7 and 125.9, two vinylic carbons conjugated to carbonyl groups at δ 160.1 and 129.3, four methylene carbons at δ 38.0, 37.1, 35.8 and 34.2, one methine carbon at δ 46.2, five quaternary aromatic carbons at δ 151.7, 151.2, 144.7, 134.0 and 122.4. The remaining signal was an unsubstituted aromatic carbon (δ 110.1), which was assigned to C-l. Additionally, sig-

nals for four quaternary carbons (δ 42.2, 40.7, 48.8 and 50.3), two sp^3 carbons belonging to methoxyl groups (δ 52.7 and 61.7) and one hydroxymethine carbon at δ 74.3 were also observed.

The gHMBC data (Fig. 2) showed the correlation of the singlet at δ 6.85 (H-l) to the carbons at δ 151.7 (C-2), δ 144.7 (C-3), δ 122.4 (C-5) and δ 42.2 (C-9) and the singlet at δ 6.00 (H-7) to the carbons at δ 134.7 (C-4), δ 122.4 (C-5) and δ 42.2 (C-9) corroborating unambiguously the assignments of H-l and H-7. Besides, the signal at δ 6.85 (1H, H-l) was correlated to δ 110.1 (C-l) and the signal at δ 6.00 (1H, H-7) showed correlation to δ 129.3 (C-7) in the HMQC spectrum. The correlation between the methoxy signal at δ 3.73 and the carbon signal at δ 144.7 confirmed the position of the methoxyl group at C-3. Also, the gHMBC spectrum showed correlations from the signal at δ 1.22 (H_3-26) to signals at δ 125.9 and δ 134.7, assigned to carbons C-14 and C-15, respectively. The signal at δ 1.38 (H_3-28) showed correlations with the carbon signal at δ 38.0 (C-16) and also with the carbonyl signal at δ 214.0, which confirmed the position of one carbonyl group at C-22. An additional correlation from the methyl signal at δ 1.72 (H_3-26) to the carbon at δ 38.0 (C-16) corroborated the attribution of the other carbonyl at C-6, with the chemical shift at δ 185.6 being characteristic of an α,β-unsaturated carbonyl and C-16, a methylenic carbon. The remaining gHMBC correlations from H_3-30 (δ 0.95) to C-19 (δ 34.2) and the hydroxymethine carbon at C-21 (δ 74.3), besides correlation of methoxyl hydrogens at δ 3.74 with the carbonyl signal at C-29 (δ 175.2), established the proposed structure **2** for cognatine.

The known compounds pristimerin (**3**), maytenin (**4**), 22β-hydroxymaytenin (**5**) and 20α-hydroxymaytenin (**6**) were identified by comparison with published data (Gunatilaka, 1989; Gunatilaka, 1996; Dhanabalasingham et al., 1996; Furlan et al., 1990). Compounds **7**–**9** had been previously isolated from *Orthosphenia mexicana* (González et al., 1983), but a number of inconsistencies were found regarding the assignments of published ^{13}C NMR data. The analysis of 2D NMR spectra allowed accurate assignment of ^{13}C NMR signals mostly based on gHMQC and gHMBC experiments.

The spectrophotometric assay using DPPH indicated a prominent free radical scavenging activity for some of the isolated quinonemethide triterpenes (Fig. 3). The stable free radical DPPH has an absorption maximum at 517 nm, which decreases upon reduction through reaction with an antioxidant compound. The free radical scavenging activity of quinonemethide triterpenes is mostly due to the α,β-unsaturated carbonyl moiety with extended conjugation through ring B, e.g. in compounds **1** and **3**–**6** or B–D, e.g. in compounds **7**–**9**. These features stabilize the quinonemethide free radical promptly formed after loss of one radical hydrogen, donated to the DPPH. The more extended conjugations of triterpenes **7**–**9** account for their higher free radical scavenging abilities (ca. 45% inhibition at 60 μM) when compared to compounds **1**–**6** (less than 30% inhibition at 60 μM). The phenolic triterpene **2** exhibited the lowest activity (18% at 60 μM) probably due to the lack of conjugation through rings B–D. The free radical scavenging ability of the quinonemethide triterpenes and a standard compound rutin (66% inhibition at 60 μM) are due to catechol moiety present in the B ring of rutin the C2–C3 double bond (van Acker et al., 1996). The synthetic antioxidant BHT (11% inhibition at 60 μM) exhibited the weakest antiradical activity as expected due to its slow reaction rate with DPPH (Bondet et al., 1997).

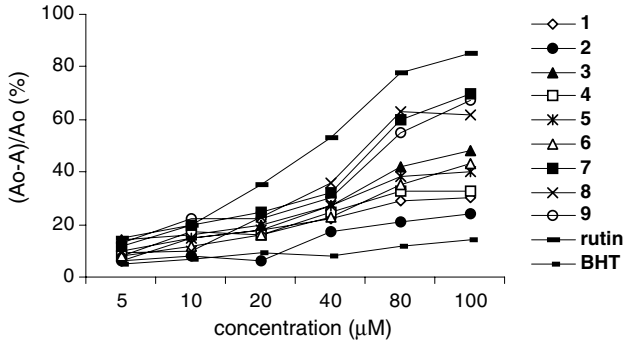


Fig. 3. Radical scavenging activity of compounds **1**–**9** and standards rutin and BHT.

3. Experimental

3.1. Plant material

Root barks of *C. cognatum* (Hippocrateaceae) were collected at Universidade Federal de Goiás, Goiânia, GO and identified by Dr Júlio Antônio Lombardi (Departamento de Botânica do Institute de Ciências Biolôgicas – Universidade Federal de Minas Gerais, UFMG). A voucher specimen (No. 19797) is deposited at Herbarium of Institute de Ciências Biolôgicas – UFG, Goiás.

3.2. Instrumentation and chromatography

Silica gel (Merck, 230–400 and 70–230 mesh) were used for CC unless otherwise stated and solvents were distilled prior to use. ^1H and ^{13}C NMR spectra were recorded on a Varian INOVA spectrometer at 500 and 125 MHz, respectively, using $CDCl_3$ as solvent and TMS as reference. HPLC separations were performed using a Shimadzu LC-10AS. Preparative HPLC separation were performed on a Varian PROSTAR, using a reversed phase column (Phenomenex-Luna C_{18}, 21.2 × 250 mm,

10 μm), eluted with MeOH:H_2O (7:3, with 0.05% H_3PO_4) detection at 420 nm. IR spectra were obtained on a Nicolet spectrometer. ESI-MS were recorded on a VG Platform II spectrometer. Elemental analyses were performed on an Elemental Analyser 2400 CHN Perkin–Elmer.

3.3. Extraction and isolation of constituents

Dried and powdered root barks of *C. cognatum* (690.0 g) were extracted with EtOH. The resulting EtOH extract was filtered and concentrated in vacuo to afford a brown gum (210.6 g), which was submitted to liquid–liquid partitioning. The CH_2C1_2 soluble part of the EtOH extract was concentrated in vacuo (25.5 g), submitted to a silica gel column (600 g, 70–230 mesh) eluted with a hexane/EtOAc gradient, yielding 41 fractions (A_1–A_{41}). Fraction A_9–A_{11} (1.48 g) was submitted to a flash silica gel CC (230–400 mesh), eluted with a hexane/EtOAc gradient resulting in 32 fractions (B_1–B_{41}). Fraction B_{10}–B_{14} (0.154 g) was submitted to prep. TLC [hexane:EtOAc (4:1)] yielding compound **3** (55 mg). Fraction B_{18}–B_{21} (0.085 g) was submitted to prep. TLC [hexane:EtOAc (4:1)] affording compound **4** (34 mg). Fraction A_{16}–A_{17} (3.55 g) was submitted to flash silica gel CC (70–230 mesh), eluted with hexane/EtOAc gradient, resulting in 46 fractions (C_1–C_{46}). Fraction C_{19}–C_{23} (0.221 g) was submitted to a reversed phase prep. HPLC [MeOH:H_2O (7:3), $\lambda = 420$ nm, 10 ml min^{-1}] to afford compounds **1** (63 mg) and **8** (65 mg). Fraction A_{22}–A_{26} (2.46 g) was subjected to a flash silica gel CC eluted with hexane/EtOAc gradient, resulting in 77 fractions (D_1–D_{77}). Fraction D_{23}–D_{27} was submitted to prep. TLC [hexane:EtOAc (4:1), AcOH 0.05%] affording compounds **5** (13 mg) and **6** (23 mg). Fraction D_{17}–D_{22} was submitted to prep. TLC [hexane:EtOAc (4:1), AcOH 0,05%, three elutions] and further purified by reversed phase prep. HPLC [MeOH:H_2O (7:3), $\lambda = 420$ nm, 10 mL min^{-1}] affording compounds **7** (18 mg) and **2** (4 mg).

3.4. Radical scavenging activity on DPPH radical

Radical scavenging activity was assayed according to the Blois method (Blois, 1958). The free radical scavenging capacities of quinonemethide triterpenes and standard compounds rutin and BHT were evaluated based on their ability to reduce the free radical already assigned (DPPH). Each compound (1 mg) was solubilized in methanol (10 ml) to yield a stock solution. Several dilutions of each compound from 5 to 100 μM were then prepared in MeOH and to each sample a solution of DPPH (0.004%) was added. Absorbance at 517 nm was determined after 30 min on a Milton Roy 20D spectrophotometer and the percentage of activity was calculated (see Fig. 3).

3.5. 22β-Hydroxypristimerin (*1*)

Amorphous solid. UV λ^{MeOH}: nm 420 (2670); IR ν_{max} (KBr): 3450; 2929; 1724; 1645; 1598, 1222; 1089, 1081 cm^{-1}; ESI/MS m/z (rel. int.) 481 [M + 1] (100%); for ^1H and ^{13}C NMR spectral data see Tables 1 and 2; Elemental analysis: Found: C, 74.97; H, 8.39; O, 16.65. $C_{30}H_{40}O_5$ requires C, 74.97; H, 8.39; O, 16.64.

3.6. Cognatine (*2*)

Amorphous solid. UV λ^{MeOH} nm: 320 (2880); IR ν_{max} (KBr): 3438; 2927; 1718; 1643; 1579, 1448, 1218; 1120, 1018 cm^{-1}; ESI/MS m/z (rel. int.) 523 [M + 1] (100%); for ^1H and ^{13}C NMR spectral data see Tables 1 and 2; Elemental analysis: Found: C, 71.28; H, 7.27, O, 21.46. $C_{31}H_{38}O_7$ requires C, 71.27; H, 7.28; O, 21.46.

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