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Note

Glycosylated nervogenic acid derivatives from *Liparis condylobulbon* (Reichb.f.) leaves

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ABSTRACT

Three new nervogenic acid glycosides, 1-*O*-α-L-rhamnopyranosyl 3,5-bis(3-methyl-but-2-enyl)-4-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]-benzoate, 3,5-bis(3-methyl-but-2-enyl)-4-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]-benzoic acid, and bis{3,5-bis(3-methyl-but-2-enyl)-4-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]-benzoyl} 1,2-*O*-β-D-glucopyranose, which we named condobulbosides A–C, were isolated from a methanol extract of the leaves of *Liparis condylobulbon* together with an apigenin C-glycoside, schaftoside. Their structures were established on the basis of spectral techniques, namely, UV, IR, HR-MS spectroscopy, both 1D and 2D NMR experiments, and chemical reactions.

Liparis is an orchid genus consisting of over 400 terrestrial epiphytic species widely distributed in tropical and temperate regions of the world.[1] It is notable that despite the wide distribution of *Liparis* spp., there is only limited knowledge about natural products obtained from this genus. Previously reported phytoconstituents of *Liparis* were glycosylated nervogenic acids, and bitter choline and pyrrolizidine alkaloids.[2–5] These compounds were considered as chemotaxonomical markers for *Liparis* species.[6] Some of the compounds that have been isolated from this genus were also considered for their bioactivity. In particular nervogenic acid exhibited moderate anti-inflammatory activity,[7] and prenylated benzoic acid derivatives displayed significant antibacterial and molluscicidal activities.[8] In our ongoing search for new bioactive natural products, we considered *Liparis condylobulbon* (Reichb.f) as a possible source of new chemical entities due to the scarce knowledge about the phytochemistry of this plant. Herein, we report the isolation and structural elucidation of three novel nervogenic acid derivatives and apigenin C-glycoside schaftoside from the MeOH extract of *L. condylobulbon* leaves.

The lipophilic compounds were removed from the dried MeOH extract of the *L. condylobulbon* leaves by dissolving in $CHCl_3$. The MeOH soluble part of the total extract was repeatedly separated by semi-preparative RP-HPLC. This separation process resulted in the isolation of three new glycosides of nervogenic acid and the apigenin C-glycoside, in the literature it was found as schaftoside.[9]

The basic characteristics of the structures of the compounds **1–4** were obtained by analyzing the UV and IR spectra. Similar UV absorptions were observed for **1–3**, with maximum values at $\lambda \sim 230$ nm, ~250 nm, and ~290 (sh) nm. A search of the UV spectra in library showed some conformity with benzoic acid. For compound **4**, a spectrum with maxima at $\lambda \sim 270$ and 335 nm, typical for the presence of apigenine skeleton, was observed. A parallel series of absorption bands was evident from the analysis of the IR spectra of **1–3**: ν_{max} 3300 cm^{-1}, a broad, intensive band corresponding to OH vibrations; 2970–2870 cm^{-1}, corresponding to CH vibrations (the absence of a rocking band at 720 cm^{-1} excluded the presence of a long aliphatic chain); 1715–1691 cm^{-1}, assigned to the C=O stretch of an ester group conjugated with aryl; bands in the region 1600–1400 cm^{-1}, assigned to the aromatic carbon stretch; strong absorption bands 1200–1000 cm^{-1}, showing the presence of a sugar moiety (C–O stretch and bend). Similar absorption bands were observed for **4**, with exception of bands typical for prenyl (2950–2850 cm^{-1}) and carbonyl band was shifted to 1645 cm^{-1}.

Compound **1** was isolated as a white amorphous solid. Its molecular formula was established as $C_{35}H_{52}O_{16}$ on the basis of the molecular ion peak $[M+Na]^+$ at *m/z* 751.3145, observed in the HR-MS spectrum (calcd 751.3153). The ESI-MS analysis of compound **1** under negative ESI conditions showed three major ionic species, namely, the ions at *m/z* 727, *m/z* 581, and *m/z* 273, corresponding to $[M-H]^-$, the loss of the rhamnose moiety, and nervogenic acid, respectively.

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The ^{1}H NMR spectrum displayed a singlet at δ 7.68 integrating for two protons, supporting the presence of an aromatic ring. In addition, signals due to two prenyl groups were present, namely, one doublet of triplet at δ 5.28 (dt, 2H, J 1.3, 6.9 Hz), multiplets at δ 3.67–3.40 (4H), and one singlet at δ 1.78 integrating for 12 protons. Three signals were assigned to anomeric protons, in particular the doublets at δ 6.12 (d, 1H, J 1.7 Hz) and at δ 4.85 (d, 1H, J 8.0 Hz), and the broad singlet at δ 5.38 (1H). Further information was obtained from 2D NMR experiments. Non-quaternary carbon resonances were revealed in the HSQC spectrum, while long range C–H correlations were observed in the HMBC experiment. Long-range correlations were observed from H-2 and H-6 (δ 7.68) with the carbon resonance at δ 165.9 (C-7), supporting the presence of a carboxylic acid group. More HMBC correlations were observed from the same proton signal and the resonances at δ 130.1 (C-2, C-6), 157.8 (C-4), and 137.2 (C-3, C-5), supporting the presence of a benzoic acid derivative. The HMBC correlations observed from H-1′ (δ 3.67–3.40) with C-4, C-3, and C-5; C-3′, C-2, and C-6 revealed the linkage of prenyl units to C-3 and C-5. These data let us to establish the aglycone moiety as 4-hydroxy-3,5-bis-(3-methyl-but-2-enyl)-benzoic acid, previously described as nervogenic acid.[8] Next, the proton resonances of the sugar units were observed. The analysis of HSQC, COSY, and ROESY spectra indicated the presence of two rhamnose units and one glucose unit (see Table 1) which were confirmed by the hydrolysis experiments. The absolute configuration of monosaccharides was established by GC–MS analysis of 2-*R*-butanol derivatives using modified method of Gerwig et al.[10,11] Sugar units were identified as β-D-glucopyranoside and α-L-rhamnopyranosides. The chemical shift of H-1$^{\text{Rha I}}$ (δ 6.12) indicated that this rhamnose unit was linked with an ester linkage. This was also supported by the HMBC correlation observed from the same proton signal H-1$^{\text{Rha I}}$ with C-7. The positions of the remaining sugar units were determined on the basis of the HMBC and ROESY correlations. Long-range correlations were observed from H-1$^{\text{Glc I}}$ (δ 4.85) with C-4 (δ 157.8). Furthermore, ROESY correlations were observed from the same proton signal (H-1$^{\text{Glc I}}$) and H-2′ (δ 5.28). The interglycosidic linkage of the second rhamnose unit and the glucose moiety was deduced from the HMBC correlation from H-1$^{\text{Rha II}}$ (δ 5.38) and C-2$^{\text{Glc I}}$ (δ 78.3).

Thus the structure of compound **1** was determined as 1-*O*-α-L-rhamnopyranosyl 3,5-bis(3-methyl-but-2-enyl)-4-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]-benzoate, and we named it condylobulboside A.

Compound **2** was isolated as a white amorphous solid. Its molecular formula was established as $C_{29}H_{42}O_{12}$ on the basis of the molecular ion peak $[M+Na]^+$ at m/z 605.2753, observed in the HR-MS spectrum (calcd 605.2574). The ESI-MS analysis of compound **2** under negative ESI conditions showed two major ionic species, namely, the ions at m/z 581 and m/z 273, corresponding to [M−H] and the nervogenic acid moiety, respectively.

The ^{1}H NMR spectrum of compound **2** was almost superimposable on the previous one except for the absence of the signals ascribable to the esterified rhamnose unit. Complete structure assignments were obtained from exhaustive analysis of the HMQC, HMBC, COSY, and NOESY data (see Table 1).

The structure and absolute configuration of sugars of **2** were confirmed by the same method as described above. Thus the structure of compound **2** was determined as 3,5-bis(3-methyl-but-2-enyl)-4-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]-benzoic acid, and we named it condylobulboside B.

Compound **3** was isolated as a yellowish amorphous solid. Its molecular formula was established as $C_{64}H_{92}O_{28}$ on the basis of the molecular ion peak $[M+Na]^+$ at m/z 1331.5587, observed in the HR-MS spectrum (calcd 1331.5673). The ESI-MS analysis of compound **3** under negative ESI conditions showed three major ionic species, namely, the ions at m/z 1307, m/z 581, and m/z 273, the last corresponding to nervogenic acid.

The ^{1}H NMR spectrum of compound **3** revealed the presence of the same aglycone as observed with the MS data. A difference was observed because of the presence of two different signals ascribable to the H-2 and H-6 protons of the aromatic ring (at δ 7.66 and 7.61, respectively). Furthermore signals due to the presence of the prenyl double bond (δ 5.23) looked broader compared with the previous compounds, suggesting the presence of two similar nervogenic acid units. Further signals were assigned to anomeric protons, namely, the doublet at δ 5.96 (d, 1H, J 7.9 Hz, H-1$^{\text{Glc III}}$) and the broad singlet δ 5.33 (H-1$^{\text{Rha I}}$, H-1$^{\text{Rha II}}$), the latter integrating for two proton signals. From the analysis of HMQC, COSY, and TOCSY data, two more anomeric protons, partially overlapped to other signals, were revealed, namely, at δ 4.82 and δ 93.2 (H-1$^{\text{Glc I}}$, H-1$^{\text{Glc II}}$). Relative integration of the ^{1}H NMR spectrum revealed the presence of two nervogenic acids and five sugar moieties, consistent with the previous data. Sugar units were determined as glucose and rhamnose moieties, ascribable to the anomeric signals at δ 4.82 (H-1$^{\text{Glc I}}$, H-1$^{\text{Glc II}}$) and δ 5.33 (H-1$^{\text{Rha I}}$, H-1$^{\text{Rha II}}$), as in compounds **1** and **2**, and a further glucose unit with an anomeric signal at δ 5.96 (H-1$^{\text{Glc III}}$). The chemical shift of H-1$^{\text{Glc III}}$ (δ 5.96) and H-2$^{\text{Glc III}}$ (δ 5.23) suggested the presence of an ester linkage at position H-1$^{\text{Glc III}}$ and H-2$^{\text{Glc III}}$. Thus Glc III is esterified at C-1$^{\text{Glc III}}$ and C-2$^{\text{Glc III}}$ by two different nervogenic acid units.

The glycosylation positions of both the nervogenic acids were analogous to those in compound **1** for sugar units Glc I and Rha I, as supported by NMR data which are superimposable for those two sugar units, and the presence of D-glucose and L-rhamnose was confirmed by acid hydrolysis and methods described above. All these data are consistent with those of the ESI-MS analysis and with the major ionic species, which could be assigned to the $[M-H]^-$ molecular ion (m/z 1307), a fragment less the rhamnoglucoside moiety (m/z 999), a fragment composed of a nervogenic acid and a rhamnoglucoside moiety (m/z 581), and nervogenic acid (m/z 273), respectively. Thus compound **3** is a new nervogenic acid derivative, bis{3,5-bis(3-methyl-but-2-enyl)-4-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]-benzoyl} 1,2-*O*-β-D-glucopyranose, and we named it condobulboside C.

Compound **4** has been identified as schaftoside by comparison of spectral data with those found in the literature.[9] The presence of schaftoside (**4**) in *L. condylobulbon* is reported for the first time.

Other nervogenic acid glycosides have been previously reported in several *Liparis* species but the presence of rhamnose-linked nervogenic acid derivatives, as well as the glycoside esters **1–3**, is here described for the first time. Moreover, to our knowledge, the presence of rhamnose-substituted nervogenic acid derivatives has not been reported either in *Liparis* spp. or in other botanical families. Considering the previously published structures, compound **3** is the first example of a branched nervogenic acid derivative incorporating two rhamnose and three glucose units.

1. Experimental

1.1. General methods

Optical rotations were measured on a JASCO P-2000 polarimeter in a 1-dm tube at the D line of sodium for MeOH solutions at 25 °C. IR spectra were recorded on a Nicolet Impact 400D FT-IR spectrophotometer. 1D and 2D NMR spectra in CD_3OD were obtained at room temperature at 600 MHz and 150 MHz, respectively, on a Bruker AVANCE DRX 600 spectrometer for **1** and at 300 MHz and 75 MHz, respectively, on a Bruker AMX 300 spectrometer for **2–4**, with TMS as an internal standard. Chemical shifts are expressed in δ units (parts per million). All assignments were confirmed with the aid of 2D experiments (COSY, HMBC and HSQC, ROESY for **1** and HMQC,

Table 1
NMR spectral data for compounds **1–3**[a]

Position	**1**[b]		**2**[c]		**3**[c]	
	δ_H (multiplicity, *J* in Hz)	δ_C	δ_H (multiplicity, *J* in Hz)	δ_C	δ_H (multiplicity, *J* in Hz)	δ_C
Aglycon I						
1		130.5		130.5		128.5
2	7.68 (s)	130.1	7.64 (s)	130.3	7.61 (s)	129.7
3		137.2		136.6		136.1
4		157.8		156.8		156.3
5		137.2		136.6		136.1
6	7.68 (s)	130.1	7.64 (s)	130.3	7.61 (s)	131.9
7		165.9		170.4		165.1
1′	3.67–3.40 (m)	28.7	3.65–3.36 (m)	29.7	3.65–3.40 (m)	28.4
2′	5.28 (dt, 1.3, 6.9)	123.9	5.27 (t, 6.9)	124.4	5.23 (m)	123.7
3′		134.6		133.4		133.4
4′	1.78 (s)	18.2	1.75 (s)	18.4	1.72 (s)	17.1
5′	1.78 (s)	26.0	1.75 (s)	25.9	1.72 (s)	24.7
Aglycon II						
1						128.5
2					7.66 (s)	129.7
3						136.1
4						156.3
5						136.1
6					7.66 (s)	131.9
7						165.1
1′					3.65–3.40 (m)	28.4
2′					5.23 (m)	123.7
3′						133.4
4′					1.72 (s)	17.1
5′					1.72 (s)	24.7
Glc-I						
1	4.85 (d, 8.0)	103.9	4.78 (d, 8.0)	104.0	4.82 (d, 8.2)	93.2
2	3.77 (dd, 8.0, 9.0)	78.5	3.75 (dd, 9.2, 8.0)	78.4	3.76 (dd, 9.0, 8.2)	78.2
3	3.54 (t, 9.0)	79.1	3.51 (t, 9.2)	79.2	3.51 (dd, 9.0, 8.5)	79.1
4	3.36 (t, 9.4)	71.9	3.33 (dd, 9.0, 9.4)	72.9	3.32 (dd, 8.5, 9.0)	72.2
5	3.09 (m)	78.3	3.06 (m)	78.4	3.05 (m)	78.0
6	3.67 (dd, 7.9, 12.0)	62.8	3.73 (dd, 12.0, 6.6)	62.7	3.70 (dd, 12.0, 8.0)	62.7
	3.62 (dd, 5.6, 12.0)		3.61 (dd, 12.0, 4.2)		3.55 (dd, 12.0, 5.0)	
Glc-II						
1					4.82 (d, 8.2)	93.2
2					3.76 (dd, 9.0, 8.2)	78.2
3					3.51 (dd, 9.0, 8.5)	79.1
4					3.32 (dd, 8.5, 9.0)	72.2
5					3.05 (m)	78.0
6					3.70 (dd, 12.0, 8.0)	62.7
					3.55 (dd, 12.0, 5.0)	
Glc-III						
1					5.96 (d, 7.9)	94.5
2					5.23 (m)	74.6
3					3.50 (m)	79.2
4					3.33 (m)	72.9
5					3.72 (m)	77.8
6					3.94 (dd, 12.0, 6.0)	62.1
					3.75 (dd, 12.0, 8.0)	62.1
Rha-I						
1	6.12 (d, 1.7)	95.8	5.37 (s)	102.3	5.33 (br s)	102.3
2	3.92 (dd, 1.7, 3.5)	71.2	3.94 (dd, 1.7, 3.6)	72.2	3.98 (dd, 1.8, 3.3)	72.0
3	3.76 (m)	72.6	3.74 (m)	72.8	3.75 (m)	72.2
4	3.39 (m)	73.8	3.37 (m)	72.9	3.40 (m)	73.6
5	4.02 (dd, 6.0, 12.0)	70.2	4.01 (dd, 6.3, 11.3)	71.1	4.07 (dd; 6.2, 12.0)	70.1
6	1.16 (d, 6.4)	17.8	1.16 (d, 6.3)	18.0	1.15 (d, 6.2)	17.7
Rha-II						
1	5.38 (s)	102.1			5.33 (br s)	102.3
2	3.99 (m)	70.1			3.98 (dd, 1.8, 3.3)	72.0
3	3.76 (m)	73.1			3.75 (m)	72.2
4	3.49 (t, 9.4)	73.0			3.40 (m)	73.6
5	3.71 (m)	72.2			4.07 (dd; 6.2, 12.0)	70.1
6	1.28 (d, 6.4)	18.0			1.15 (d, 6.2)	17.7

[a] NMR data were measured in CD_3OD.
[b] NMR data were obtained at 600/150 MHz for $^1H/^{13}C$.
[c] NMR data were obtained at 300/75 MHz for $^1H/^{13}C$.

TOCSY, NOESY for **2–4**). NMR experiments were performed using the standard Bruker software. HRESIMS and HREIMS data were measured with a Mariner Biosystem (API-TOF) mass spectrometer. Solutions of the isolated compounds in a mixture of methanol and water (50/50) with 0.2% of ammonia were prepared and injected directly into the API source at a rate of 10 μl/min.

GC–MS analysis was performed on a Varian Saturn 2000, using a HP-5 capillary column (30 m × 25 mm; flow rate 1 ml/min). Injection-port temperature 250 °C. The elution program was isothermal 2 min at 110 °C; followed by a temperature gradient of 5 °C/min up to 180 °C and then succeeded with a temperature gradient of 10 °C/min up to final temperature 300 °C. Instrument was equipped with ion-trap MS detector (detector temperature 200 °C).

1.2. Plant material

The fresh leaves of *L. condylobulbon* were collected in the greenhouse of the University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic, October 2007; the sample was identified by Dr. Petr Babula, Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic. A voucher specimen (LC-07O) has been deposited at the herbarium of the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic.

1.3. Extraction and isolation

The fresh plant material (50 g) was extracted with MeOH in an ultrasonic bath (3 × 200 ml, 60 min each). The solvent was removed under vacuum and residual water was evaporated by lyophilization. The dry extract (20 g) was suspended in $CHCl_3$ and filtered. The residue on the filter was dissolved in MeOH and used for analytical and semi-preparative RP-HPLC to gain **1–3**. The hydrophilic water soluble part of filter residue afforded material for isolation of **4**. RP-HPLC: gradient elution with MeCN (A) and 0.2% HCOOH (B) was used. Analytical HPLC: 0 min 10% of A, 36 min 100% A, flow rate 1 ml/min, column temperature 40 °C; semi-preparative HPLC: 0 min 20% of A, 16 min 32.8% of A, flow rate 5 ml/min, column temperature 40 °C. Fractions were collected based on the DAD response (λ 280 nm), t_R 19–19.5 min for **1**, t_R 22–23 min for **2**, and 24–24.3 min for **3**, respectively. A similar HPLC procedure was used for isolation of **4**, semi-preparative HPLC: 0 min 5% of A, 22 min 22.3% of A, flow rate 5 ml/min, column temperature 40 °C, t_R 19.3–20 min. The solvent was evaporated from each fraction by lyophilization and the pure compounds precipitated.

1.4. Monosaccharide identification

Compounds **1–3** (0.5 mg of each) were hydrolyzed (1.0 ml 3 M TFA, 100 °C, 2 h), the solution was evaporated to dryness, the residue dissolved in MeOH (0.5 ml), reduced with $NaBH_4$ (~5 mg, 30 min), treated with AcOH (0.25 ml), dried under a stream of N_2, and then MeOH (1 ml) was added. The mixture was dried, and the residue was acetylated with Ac_2O (2 ml, 100 °C, 1 h, 0.2 ml of AcOH). The mixture was dried and the residue was diluted with H_2O and extracted with Et_2O. The Et_2O phase was concentrated and subjected to GC analysis. Compounds **1–3** displayed peaks for D-glucose and L-rhamnose at t_R 28.175 min and 18.109 min, respectively. It was identical to the acetyl derivates of D-glucose and acetyl monosaccharide derivatives of hydrolyzed rutin treated in the same manner.

1.5. Absolute configuration of the monosaccharides

Alkylating reagent (2% HCl in 2-*R*-butanol) was prepared by the addition of 0.008 ml acetyl chloride to 0.3 ml of 2-*R*-butanol cooled to 5 °C. This reaction mixture was stirred at room temperature for 1 h. To 0.3 ml of alkylating reagent, 0.5 mg of sample (compounds **1–3**) was added and reaction mixture was stirred at room temperature for 72 h. The reaction mixture was then neutralized with saturated aqueous solution of Na_2CO_3 and evaporated to dryness under a steam of nitrogen. The residue was acetylated with Ac_2O as described above and subjected to GC analysis. Retention times were compared with those of standard samples (prepared from D-glucose and rutin) in the same manner (t_R β-D-glucose 27.702 min and t_R α-L-rhamnose 16.050 min).

1.6. 1-*O*-α-L-Rhamnopyranosyl 3,5-bis(3-methyl-but-2-enyl)-4-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]-benzoate; condobulboside A (1)

White amorphous powder (9 mg): $[\alpha]_D^{25}$ − 28 (*c* 0.25, MeOH); IR (ATR); *ν* 3314, 2974, 2918, 1706, 1625, 1417, 1126, 1053 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD, 600 and 150 MHz), see Table 1; HR-ESI-MS (positive) $[M+Na]^+$ *m/z*: 751.3145 (Anal. Calcd for $C_{35}H_{52}NaO_{16}$: 751.3153).

1.7. 3,5-Bis(3-methyl-but-2-enyl)-4-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]-benzoic acid; condobulboside B (2)

White amorphous powder (12 mg): $[\alpha]_D^{25}$ − 40 (*c* 0.8, MeOH); IR (ATR); *ν* 3305, 2962, 2920, 1689, 1601, 1542, 1382, 1039 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD, 300 and 75 MHz), see Table 1; HR-ESI-MS (positive) $[M+Na]^+$ *m/z*: 605.2753 (Anal. Calcd for $C_{29}H_{42}NaO_{12}$: 605.2574).

1.8. Bis{3,5-bis(3-methyl-but-2-enyl)-4-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]-benzoyl} 1,2-*O*-β-D-glucopyranose; condobulboside C (3)

Yellowish amorphous powder (6 mg): $[\alpha]_D^{25}$ − 148 (*c* 0.25, MeOH); IR (ATR); *ν* 3312, 2970, 2919, 1693, 1599, 1542, 1382, 1052 cm^{-1}; ^1H and ^{13}C NMR (CD_3OD, 300 and 75 MHz), see Table 1; HR-ESI-MS (positive) $[M+Na]^+$ *m/z*: 1331.5587 (Anal. Calcd for $C_{64}H_{92}NaO_{28}$: 1331.5673).

1.9. Apigenin 6-*C*-β-D-glucopyranosyl-8-*C*-α-L-arabinopyranoside; Schaftoside (4)

Yellowish amorphous powder (4 mg): Spectral data identical to the literature.[9]

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Supplementary data

Supplementary data (^1H and ^{13}C NMR spectra) associated with this article can be found, in the online version, at doi:10.1016/j.carres.2009.06.012.

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