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Anthocyanins from flowers of the orchids *Dracula chimaera* and *D. cordobae*

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Abstract

The main anthocyanins from flowers of the orchids *Dracula chimaera* and *D. cordobae* were isolated from a purified methanolic extract by preparative HPLC. Their structures were determined to be cyanidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside), cyanidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside), cyanidin 3-*O*-β-glucopyranoside, peonidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) and peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside). The structure determinations were mainly based on extensive use of 2D and 1D NMR spectroscopy, UV–vis spectroscopy and MS. The anthocyanin contents of species belonging to the subtribe Pleurothallidinae including genus *Dracula* Luer (Orchidaceae) have previously not been determined. The high content of anthocyanin rutinosides found in *D. chimaera* and *D. cordobae* (78 and 28% of the total anthocyanin content, respectively) differs from previously analysed orchid species, in which glucose is found as the only anthocyanin sugar moiety.

Keywords: Orchidaceae; Pleurothallidinae; *Dracula chimaera*; *Dracula cordobae*; Flowers; Anthocyanins; Chemotaxonomy; NMR; MS

1. Introduction

The genus *Dracula* Luer belongs to the subtribe Pleurothallidinae, of the family Orchidaceae. The genus was created by Dr. Carlyle Luer (1978) by splitting off certain species with hairy flowers and different type of lip from the large genus *Masdevallia*. The first *Dracula* spp. were found by plant hunters in the 1870s, and they are still being discovered mainly in the cloud forests of Colombia, Ecuador and Peru (Luer, 1993). There are now over 100 recognised species. Colours range from white through shades of yellow, pink, blood red to dark maroon—almost black. Usually combinations of several of these colours are present as shading, fine or large spots, or lines. Although the anthocyanin content of some orchid genera have been thoroughly investigated, the anthocyanin content of species belonging to the subtribe Pleurothallidinae including genus *Dracula* has not previously been determined. In this paper we report the isolation and complete structure determination of anthocyanins from flowers of *Dracula chimaera* and *D. cordobae*.

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2. Results and discussion

The HPLC profile of the acidified methanolic extract of flowers of *D. chimaera* detected in the visible region showed three major (**2**, **3** and **4**) and several minor anthocyanins (Fig. 1). The UV/vis spectra of all pigments **1–5** had λ_{max} at 520–524 nm and $A_{440}/A_{Vis\text{-}max}$ ratios of 27–31% (Table 1) in accordance with 3-substituted cyanidin or peonidin derivatives (Harborne, 1958). The lack of extra absorptions in the UV region of the spectra indicated the absence of aromatic acylation. Pigment **1** co-chromatographed (HPLC) with authentic cyanidin 3-*O*-β-glucopyranoside. A fragment ion *m*/*z* 287 corresponding to cyanidin aglycone and the molecular ion *m*/*z* 449 in the electrospray MS spectrum of **1** confirmed this identity.

Pigment **5** co-chromatographed (HPLC) with authentic peonidin 3-(6″-malonylglucoside). A fragment ion *m*/*z* 301 corresponding to peonidin aglycone and a molecular ion *m*/*z* 549 corresponding to peonidin malonylglucoside in the electrospray MS spectrum of **5** confirmed this identity.

The downfield region of the ^{1}H NMR spectrum of **2** showed a 3H ABX system at δ 9.01 (*d*, 0.8 Hz; H-4), δ 6.97 (*dd*, 1.9 Hz, 0.8 Hz; H-8) and δ 6.76 (*d*, 1.9 Hz; H-6)

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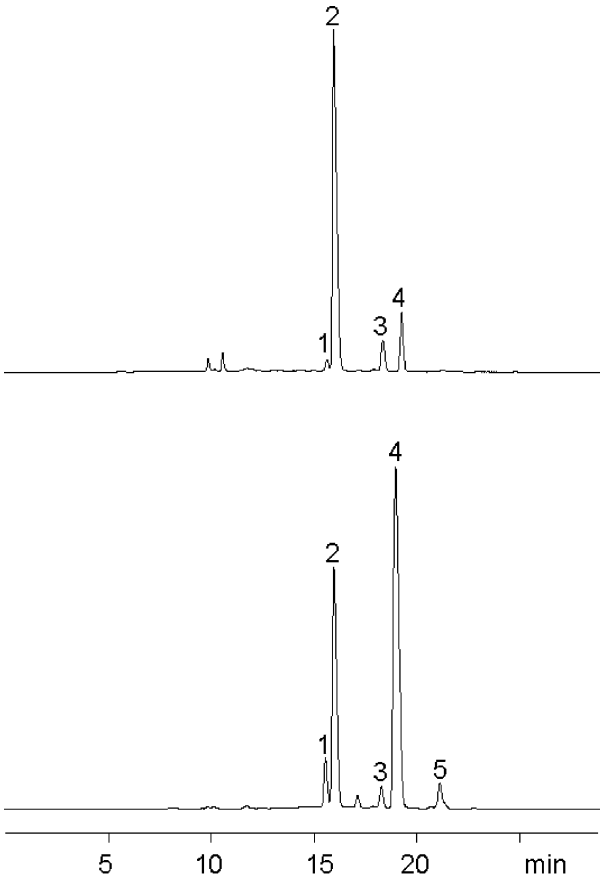


Fig. 1. HPLC chromatogram of anthocyanins from flowers of *D. chimaera* (top) and *D. cordobae* (bottom) detected at 520 nm.

Table 1
Chromatographic and spectral data of cyanidin 3-*O*-β-glucopyranoside (**1**), cyanidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (**2**), peonidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (**3**), cyanidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) (**4**) and peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) (**5**) from flowers of *D. chimaera* and *D. cordobae*

Compd	Content[a] (%)		ES-MS[c]		On-line		HPLC-DAD	
	D. chimaera[b]	*D. cordobae*	*m/z* A^+	*m/z* M^+	λ_{max} (nm)	$A_{440}/A_{vis\text{-}max}$ (%)	t_R (min)	
1	2	12	287	449	520	31	15.51	
2	72	25	287	595	520	30	15.93	
3	6	3	301	609	522	29	18.23	
4	10	56	287	535	523	28	18.92	
5	1	4	301	549	524	27	21.12	

[a] Relative anthocyanin amounts.
[b] Also contain seven uncharacterized trace anthocyanins (together 9%).
[c] A^+ = aglycone fragment ion; M^+ = molecular ion.

and a 3H AMX system at δ 8.34 (*dd,* 8.7 Hz, 2.2 Hz; H-6′), δ 8.10 (*d,* 2.2 Hz; H-2′) and δ 7.10 (*d*, 8.7 Hz; H-5′) (Table 2) which is in accordance to cyanidin aglycone. The sugar regions of the 1D ^{1}H and 1D ^{13}C CAPT NMR spectra, respectively, of **2** were in accordance

Table 2
^{1}H NMR spectral data for cyanidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (**2**), peonidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (**3**) and cyanidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) (**4**) in CF_3COOD-CD_3OD (5:95; v/v) at 25 °C

	2	**3**	**4**
Aglycone			
4	9.01 *d*, 0.8 Hz	9.08 *d*, 0.8 Hz	9.04 *d*, 1.0 Hz
6	6.76 *d*, 1.9 Hz	6.78 *d*, 1.9 Hz	6.76 *d*, 1.9 Hz
8	6.97 *dd*, 0.8 Hz, 1.9 Hz	7.03 *dd*, 0.8 Hz, 1.9 Hz	6.99 *dd*, 1.9 Hz, 1.0 Hz
2′	8.10 *d*, 2.2 Hz	8.32 *d*, 2.3 Hz	8.11 *d*, 2.2 Hz
5′	7.10 *d*, 8.7 Hz	7.16 *d*, 8.8 Hz	7.11 *d*, 8.7 Hz
6′	8.34 *dd*, 8.7 Hz, 2.2 Hz	8.37 *dd*, 8.8 Hz, 2.3 Hz	8.37 *dd*, 8.7 Hz, 2.2 Hz
OMe		4.11 *s*	
3-O-β-Glucopyranoside			
1″	5.37 *d*, 7.7 Hz	5.39 *d*, 7.7 Hz	5.36 *d*, 7.9 Hz
2″	3.75 *dd*, 7.7 Hz, 9.1 Hz	3.74 *dd*, 7.7 Hz, 9.2 Hz	3.76 *dd*, 7.9 Hz, 9.1 Hz
3″	3.64 *t*, 9.1 Hz	3.63 *m*	3.63 *t*, 9.1 Hz
4″	3.51 *dd*, 9.1 Hz, 9.4 Hz	3.49 *dd*, 9.2 Hz, 9.4 Hz	3.50 *dd*, 9.1 Hz, 9.5 Hz
5″	3.81 *m*	3.81 *m*	3.90 *ddd*, 9.5 Hz, 7.3 Hz, 1.9 Hz
6A″	4.16 *dd*, 11.3 Hz, 1.6 Hz	4.14 *dd*, 11.3 Hz, 1.5 Hz	4.64 *dd*, 12.0 Hz, 1.9 Hz
6B″	3.69 *dd*, 11.3 Hz, 5.0 Hz	3.69 *m*	4.37 *dd*, 12.0 Hz, 7.3 Hz
6″-O-α-Rhamnopyranosyl			
1‴	4.75 *d*, 1.6 Hz	4.73 *d*, 1.6 Hz	
2‴	3.89 *dd*, 1.6 Hz, 3.1 Hz	3.88 *dd*, 1.6 Hz, 3.3 Hz	
3‴	3.71 *m*	3.69 *m*	
4‴	3.41 *m*	3.40 *m*	
5‴	3.65 *m*	3.63 *m*	
6	1.25 *d*, 6.3 Hz	1.24 *d*, 6.3 Hz	
6″-O-Malonyl			
2			3.44 *s*

with one rhamnose and one glucose unit. All the ^{1}H sugar resonances were assigned by the 2D ^{1}H-^{1}H DQF-COSY experiment and the corresponding ^{13}C resonances were then assigned by the 2D HSQC spectrum and the 1D ^{13}C CAPT spectrum (Table 2 and Table 3). The crosspeak at δ 5.35/145.6 (H-1″/C-3) in the HMBC spectrum confirmed that the glucose unit was attached to the aglycone at the 3-hydroxyl. The downfield shift of C-6″ (67.79 ppm) in the CAPT spectrum of **2** indicated the linkage between the 3-glucose and the rhamnose unit to be at the 6″-hydroxyl. The crosspeaks at δ 4.73/67.7 (H-1‴/C-6″), δ 4.14/102.1 (H-6A″/C-1‴) and δ 3.67/102.1 (H-6B″/C-1‴) in the HMBC spectrum confirmed the linkage between the rhamnose and the inner glucose unit to be at the 6″-hydroxyl. Thus, **2** was identified as cyanidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside). A fragment ion *m/z* 287 corresponding to cyanidin aglycone and the molecular

ion m/z 595 corresponding to cyanidin rutinoside in the electrospray MS spectrum of **2** confirmed this identity.

The NMR spectra of pigment **3** showed many similarities with the corresponding spectra of **2** (Tables 2 and 3, respectively). After assignments of the proton resonances, pigment **3** revealed an asymmetric anthocyanidin B-ring with one methoxy group and one hydroxyl group in accordance with peonidin. Thus, **3** was identified as peonidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside). A fragment ion m/z 301 corresponding to peonidin aglycone and the molecular ion m/z 609 corresponding to peonidin rutinoside in the electrospray MS spectrum of **3** confirmed this identity. ^{13}C NMR data for this pigment have previously not been reported.

Table 3
^{13}C NMR spectral data for cyanidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (**2**), peonidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (**3**) and cyanidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) (**4**) in CF_3COOD–CD_3OD (5:95; v/v) at 25 °C

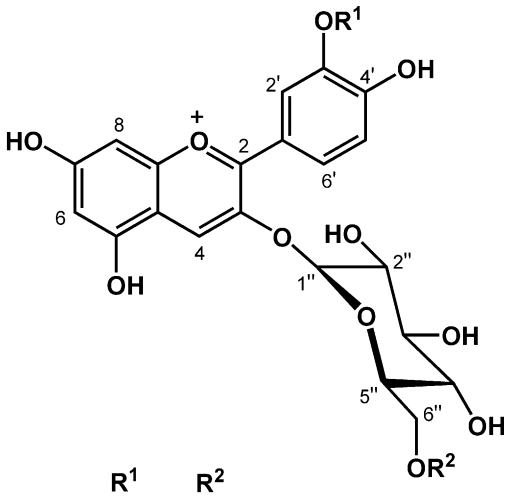
	2	**3**[a]	**4**
Aglycone			
2	164.17	164.3	164.58
3	145.62	145.6	145.59
4	136.21	136.8	136.90
5	159.06	159.2	159.2
6	103.48	103.5	103.64
7	170.45	nd[b]	170.41
8	95.23	95.4	95.25
9	157.63	157.8	157.76
10	113.24	113.3	113.28
1′	121.20	121.2	121.24
2′	118.40	115.4	118.43
3′	147.44	149.7	147.50
4′	155.88	156.8	155.89
5′	117.43	117.8	117.39
6′	128.42	129.1	128.48
OMe		57.0	
3-O-β-Glucopyranoside			
1″	103.53	103.8	103.62
2″	74.69	74.9	74.65
3″	78.02	78.1	77.92
4″	71.22	71.3	71.33
5″	77.44	77.5	75.94
6″	67.79	67.9	65.45
6″-O-α-Rhamnopyranosyl			
1‴	102.19	102.2	
2‴	71.87	71.9	
3‴	72.44	72.5	
4‴	73.92	74.0	
5‴	69.77	69.9	
6‴	17.87	17.9	
6″-O-Malonyl			
1			168.59
2			[c]
3			170.08

[a] Chemical shifts from the 2D HSQC and HMBC spectra.
[b] nd = not detected.
[c] Exchange with deuterium.

The downfield region of the ^{1}H NMR spectrum of **4** showed a 3H ABX system at δ 9.04 (*d,* 1.0 Hz; H-4), δ 6.99 (*dd,* 1.9 Hz, 1.0 Hz; H-8) and δ 6.76 (*d,* 1.9 Hz; H-6) and a 3H AMX system at δ 8.37 (dd, 8.7 Hz, 2.2 Hz; H-6′), δ 8.11 (*d,* 2.2 Hz; H-2′) and δ 7.11 (*d*, 8.7 Hz; H-5′) (Table 2) which is in accordance to cyanidin aglycone. The sugar regions of the ^{1}H and ^{13}C CAPT NMR spectra of **4** were in accordance with one glucose unit. The anomeric coupling constant (7.9 Hz) and the 6 ^{13}C resonances belonging to the sugar in the CAPT spectrum of **4** were in accordance with a β-glucopyranose unit. All the ^{1}H sugar resonances were assigned by the 2D ^{1}H-^{1}H DQF-COSY experiment and the corresponding ^{13}C resonances were then assigned by the 2D HSQC spectrum and the 1D ^{13}C CAPT spectrum (Table 3). The crosspeak at δ 5.36/145.5 (H-1″/C-3) in the HMBC spectrum confirmed that the glucose unit was attached to the aglycone at the 3-hydroxyl. The acyl moiety was identified as malonic acid by the 2H singlet at δ 3.44 (H-2) and the ^{13}C resonances at δ 168.59 (C-1) and δ 170.08 (C-3). The downfield shift of H-6A'' (δ 4.64), H-6B'' (δ 4.37) and C-6″ (δ 65.45) indicated the linkage between the glucose and the acyl group to be at the 6″-hydroxyl. The crosspeaks at δ 4.65/168.5 (H-6A''/C-1) and δ 4.37/168.5 (H-6B''/C-1) in the HMBC spectrum confirmed the linkage between the acyl and the sugar unit to be at the 6″-hydroxyl. Thus, **4** was identified as cyanidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside). A fragment ion m/z 287 corresponding to cyanidin aglycone and the molecular ion m/z 535 corresponding to cyanidin malonylglucoside in the electrospray MS spectrum of **4** confirmed this identity.

The same anthocyanins (pigments **1**–**5**) were also identified by co-chromatography (HPLC) and LC-MS in flowers of *D. cordobae*, however in different quantitative proportions (Table 1). Structures of pigments **1**–**5** are shown in Fig. 2.

Earlier studies on the anthocyanin content in Orchidaceae species revealed a complex anthocyanin pattern. Uphoff (1979) described the widespread occurrence of cyanidin 3-monoglucoside, 3-diglucoside and 3,5-diglucoside together with complex acylated anthocyanins. Lowry and Keong (1973) reported the presence of cyanidin 3-glucoside in the flowers of *Dendrobium crocatum* and also cyanidin glycosides in the flowers of *Dendrobium cornutum*. By analysing 28 *Dendrobium* species Kuehnle et al. (1997) found anthocyanins based on the aglycones cyanidin (major), pelargonidin and peonidin (minor). The anthocyanins recently identified in orchids (*Dendrobium* spp. *Laelia pumila* Rchb. F. × *Cattleya walkeriana* Gardn.; *Bletilla striata*; *Phalaenopsis* spp.



	R^1	R^2
1	H	H
2	H	rhamnosyl
3	CH_3	rhamnosyl
4	H	malonyl
5	CH_3	malonyl

Fig. 2. Structures of anthocyanins isolated from *D. chimaera* and *D. cordobae*.

Sophronitis coccinea) are mainly polyacylated derivatives of cyanidin 3,7,3′-triglucoside including the acyl moieties malonic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid (Saito et al., 1994, 1995; Tatsuzawa et al., 1994, 1995, 1997, 1998; Williams et al., 2002). Strack et al. (1986, 1989) reported, however, the widespread occurrence of cyanidin 3-glucoside (chrysanthemin), cyanidin 3,5-diglucoside (cyanin), cyanidin 3,7-diglucoside (seranin), cyanidin 3-oxalylglucoside (ophrysanin) and cyanidin oxalyl 3,5-diglucoside (orchicyanin II) in European orchids. They also indicated the presence of a covalent anthocyanin-flavonol complex cyanidin oxalyl-3,5-diglucoside-kaempferol 7-glucoside (orchicyanin I).

Anthocyanins from flowers of *D. chimaera* and *D. cordobae* are based on cyanidin with the corresponding peonidin derivatives as minor pigments. They are substituted with glucose at the aglycone 3-hydroxyl and with the exception of pigment **1** the glucose unit is further substituted at the 6″-hydroxyl with either a malonyl or a rhamnosyl unit. The anthocyanins previously identified in orchids are almost exclusively based on cyanidin as aglycone and glucose as the only glycosylating sugar, in addition to various acyl moieties. Thus, the relatively high proportions of anthocyanins containing the disaccharide rutinose identified in flowers of *D. chimaera* and *D. cordobae* (72 and 25%, respectively, of the total anthocyanin content) may have a chemotaxonomic importance on the subtribe level.

3. Experimental

3.1. Plant material

Specimens of *D. chimaera* and *D. cordobae* were purchased commercially and grown in the tropical greenhouse of Botanical garden, Bergen (BG), and flowers were collected and immediately frozen as soon as they emerged. Voucher specimens have been deposited in Bergen (BG) as H-401 and H-402. *D. chimaera* (Rchb.f.) Luer has petals with a yellowish basic colour, blotched with dark red-purple spots and strongly pubescent with dark purple–red hairs. The petals end in long dark purple–red tails. The flowers are somewhat smaller than the measurements given by Luer (1993) but we believe that this is due to sub-optimal conditions in the greenhouse were the specimen has been grown. *D. cordobae* Luer has petals creamy white with a dark purple–red colouring on the margins and tails.

3.2. Isolation of pigments

Anthocyanins in colourful flowers of *D. chimaera* (75 g) were extracted for 24 h at 4 °C with MeOH–TFA (99.5:0.5, v/v). The concentrated crude extract was purified by partition (three times) against ethyl acetate, and further purified by XAD-7 Amberlite absorption chromatography. Pure anthocyanins were then isolated by preparative HPLC (Gilson 305/306 pump equipped with an HP-1040A detector), which was performed with an ODS-Hypersil column (25×2.2 cm, 5 μm) using the solvents HCOOH–H_2O (1:19, v/v) (A) and HCOOH–H_2O–MeOH (1:9:10, v/v) (B). The elution profile consisted of a linear gradient from 10 to 100% B for 45 min, isocratic elution (100% B) for the next 13 min, followed by linear gradient from 100 to 10% B for 1 min. The flow rate was 14 ml min^{-1}, and aliquots of 300 μl were injected. Prior to injection the sample was filtered through a 0.45 μm Millipore membrane filter.

3.3. Analytical chromatography

Analytical HPLC (HP 1050) was performed with a ODS-Hypersil column (25×0.4 cm, 5 μm) using the solvents HCOOH-H_2O (1:19, v/v) (A) and HCOOH–H_2O–MeOH (1:9:10, v/v) (B). The elution profile consisted of a linear gradient from 10 to 100% B for 23 min, isocratic elution, 100% B, for the next 5 min, followed by linear gradient from 100 to 10% B for 1 min. The flow rate was 0.75 ml min^{-1}, and aliquots of 15 μl were injected. Prior to injection all samples were filtered through a 0.45 μm Millipore membrane filter.

3.4. Spectroscopy

UV–Vis absorption spectra were recorded on-line during HPLC analysis. Spectral measurements were

made over the wavelength range 240–600 nm in steps of 2 nm. Relative amounts of each anthocyanin are reported as percentages of total peak area in each chromatogram without taking into account the different molar absorption coefficients.

Mass spectral data on pigments **1**–**5** were achieved by a LCMS system (Waters 2690 HPLC-system connected to Micromass LCZ mass spectrometer) with electrospray ionization in positive mode (ESP+). The following ion optics were used: Capillary 3 kV, cone 30 V and 60 V, and extractor 7 V. The source block temperature was 120 °C and the desolvation temperature was 150 °C. The electrospray probe-flow was adjusted to 100 μl/min. Continuous mass spectra were recorded over the range m/z 150–800 with scan time 1s and interscan delay 0.1 s.

The NMR experiments were obtained at 600.13 MHz and 150.92 MHz for ^{1}H and ^{13}C respectively, on a Bruker DRX-600 instrument equipped with a multinuclear inverse probe for all but the ^{13}C 1D CAPT experiment which was performed on a $^{1}H/^{13}C$ BBO probe. Sample temperatures were stabilized at 25 °C. The deuteriomethyl ^{13}C signal and the residual ^{1}H signal of the solvent (CF_3CO_2D–CD_3OD; 5:95, v/v) were used as secondary references (δ 49.0 and 3.40 from TMS, respectively). The one-bond proton–carbon shift correlations were established using phase-sensitive gradient-selected Heteronuclear Single Quantum Coherence, HSQC (Braun et al., 1996). The experiment was optimised for a one-bond proton–carbon coupling constant of 145 Hz. 256 FIDs were recorded in t_1 and 2 K data points in t_2, and 140 transients were collected for each t_1 increment. The spectral widths were 21,128 Hz in f_1 and 5165 Hz in f_2. The proton–carbon shift correlations by long range coupling were established using the heteronuclear multiple bond correlations (HMBC) experiment (Braun et al., 1996). 256 FIDs were recorded in t_1 and 2K data points in t_2, and 200 transients were collected for each t_1 increment. The spectral widths were 25,656 Hz in f_1 and 5165 Hz in f_2. The one-bond proton–proton shift correlations were established using phase-sensitive gradient-selected Double Quantum Filtered Correlation Spectroscopy (DQF-COSY) with solvent suppression (Braun et al., 1996). The experiment was optimized for a one-bond proton-proton coupling constant of 7.5 Hz. 256 FIDs were recorded in t_1 and 4 K data points in t_2, and 64 transients were collected for each t_1 increment. The spectral width was 1860 Hz.

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