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An unambiguous structural elucidation of a 1,3-β-D-glucan obtained from liquid-cultured *Grifola frondosa* by solution NMR experiments

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ABSTRACT

Grifolan LE (GRN-LE), a purified β-D-glucan, which is obtained from liquid-cultured *Grifola frondosa*, exhibits various biological activities, including antitumor effects. Significant progress has been made in the study of these effects. However, an unambiguous structural characterization of GRN-LE using NMR spectroscopy has not been carried out as yet. It is well accepted that the biological effects of a β-glucan depend on its primary structure, conformation, and molecular weight. In the present study, we unambiguously elucidate the primary structure of GRN-LE using NMR spectroscopy. The data presented here reveal that GRN-LE comprises a 1,3-β-D-glucan backbone with a single 1,6-β-D-glucosyl side branching unit on every third residue.

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β-Glucan is a well-known biological response modifier (BRM) widely distributed in nature. A variety of β-glucans have been isolated from various sources, for example, fungi, plants, and seaweeds. The physicochemical properties of β-glucans differ according to their primary structure, including linkage type, degree of branching (DB), degree of polymerization (DP), and conformation, (e.g., triple helix, single helix, or random coil), and molecular weight.[1–3] Recent reports have highlighted a significant role of β-glucans in the treatment of cancer and infectious diseases in both modern medicine and traditional oriental therapies. They also play an important role as dietary substances because in addition to possessing antitumor and immunomodulating properties, they lower the plasma cholesterol level and enhance the hematopoietic response.[4–6] For instance, lentinan from *Lentinus edodes*[7] and sonifilan (SPG) from *Schizophyllum commune*[8] have been clinically used in cancer therapy in Japan.

We have previously obtained and reported a 1,3-β-D-glucan isolated from the edible/medicinal mushroom *Grifola frondosa* (*G. frondosa*), which is termed grifolan (GRN), mainly comprising six-branched 1,3-β-D-glucan and exhibiting immunopotentiating action, such as antitumor activity.[9–13] So far, we have obtained various β-glucans from the fruiting bodies[9] and matted mycelia[10] of *G. frondosa*; isolation methods, such as hot water extract,[11] cold alkaline extract, hot alkaline extract,[12] and obtained from the culture supernatant.[13] Despite these isolation methods, the basic structures of the β-glucans are fundamentally closed. Thus, owing to ease of preparation, we used the β-D-glucan that was enzymatically synthesized using liquid-cultured mycelium of *G. frondosa* (GRN-LE) in this study. However, there is limited information about the mechanism(s) of action of GRN. Host molecules that serve as β-glucan receptors have recently been reported, for example, complement receptor type 3 (CR3)[14] and lactosylceramide (LacCer).[15] More recently, dectin-1 has also been identified as an essential 1,3-β-glucan.[16,17] It is important to know the relationship between the structure and the receptor-binding ability of β-glucans.[18,19] We have previously reported that GRN-LE is capable of binding to dectin-1.[18] Although it is well established that the biological effects of β-glucan depend on its primary structure, conformation, and molecular weight,[1,2] the precise determination of the structure of a β-glucan (GRN-LE) by nuclear magnetic resonance (NMR) spectroscopy has not been performed till date. A previous structural analysis was conducted using 1D-^{13}C NMR spectroscopy and methylation.[13] 1D-^{13}C NMR spectroscopy is often used for the structural analysis of β-glucans because it offers better signal dispersion than 1D-^{1}H NMR spectroscopy; however, only limited structural information is obtained from the former because of low sensitivity and loss of coupling information. There have been remarkable advancements in NMR techniques in the last three decades, and thus, sophisticated techniques such as multidimensional NMR are currently available.[20] Thus, the use of 1D-^{1}H and both homo- and heteronuclear 2D-NMR is vital for the accurate structural elucidation of GRN-LE. Furthermore, characterization of the GRN-LE structure by NMR is important not only for promoting its clinical usage in immunotherapy, but also for understanding the mechanisms underlying its biological effects. In the present study, we report the primary structure of GRN-LE and assign all its protons and carbons by 1D- and 2D-NMR spectroscopies, as well as correlation

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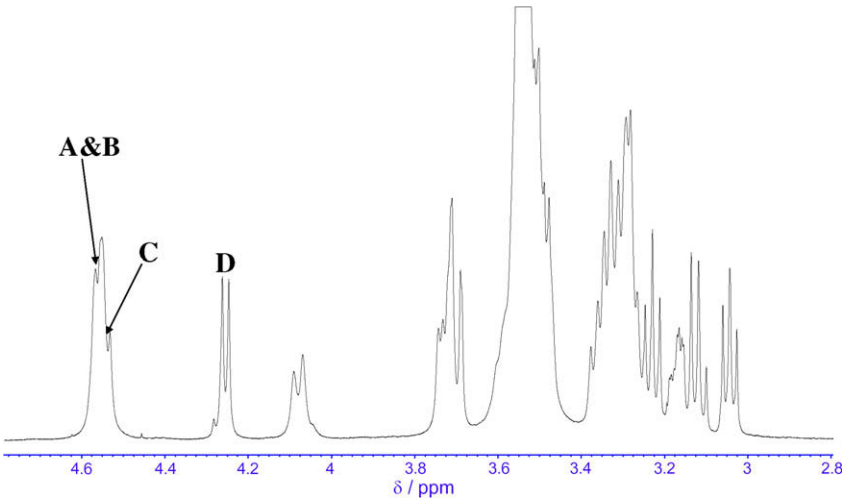

Figure 1. 1D-^{1}H NMR spectrum of GRN-LE at 70 °C.

Table 1
Coupling constants ($^{1}J_{H1,C1}$ and $^{3}J_{H1,H2}$) for GRN-LE

		δ_H (ppm)	$^{3}J_{H1,H2}$ (Hz)	$^{1}J_{H1,C1}$ (Hz)
Residue	A	4.559	7.7	155
	B	4.559	7.7	155
	C	4.542	n.d.	155
	D	4.254	7.7	155

n.d.: not detected due to heavy overlapping.

spectroscopy (COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC) spectroscopy, HSQC-TOCSY, heteronuclear multiple bond coherence (HMBC) spectroscopy, and heteronuclear two-bond correlation (H2BC) spectroscopy.

To elucidate the precise structure of a β-glucan isolated from *G. frondosa* used in this study (Griforan LE; GRN-LE), we tried to unambiguously assign all proton and carbon signals observed in the NMR spectrum of GRN-LE. We first confirmed that the differences between the ^{13}C NMR spectra of GRN-LE in a solution of Me_2SO-d_6/D_2O (6:1) and that of GRN-LE in a solution of Me_2SO-d_6 because we had previously observed that some β-glucans underwent sol to gel transition when water was gradually added to their Me_2SO solution, resulting in the disappearance of the signals in the ^{13}C NMR spectrum.[21,22] However, we did not observe any difference (data not shown) between the two above-mentioned spectra; in other words, all the signals due to GRN-LE in Me_2SO-d_6/D_2O (6:1) could be clearly observed in the spectrum. The 1D-^{1}H NMR spectrum of GRN-LE in Me_2SO-d_6/D_2O (6:1) at 70 °C is shown in Figure 1. The anomeric region (δ_H 4.2–4.7 ppm) contained four signals, three of which heavily overlapped with one another (δ_H

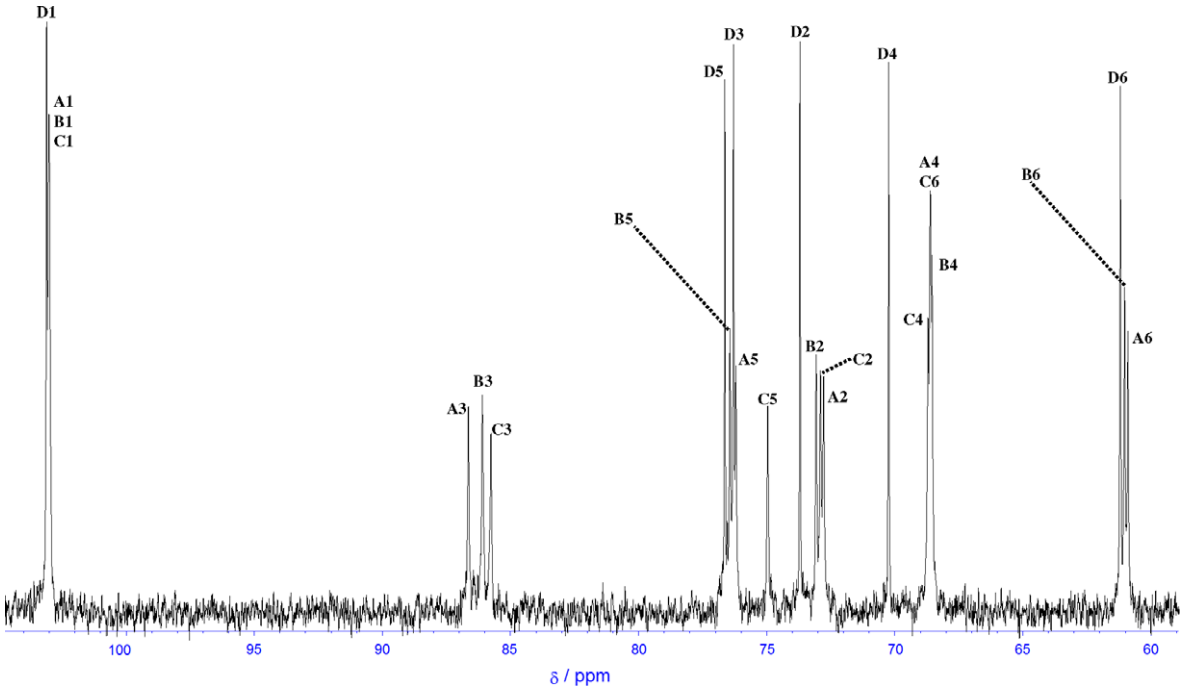

Figure 2. 1D-^{13}C NMR spectrum of GRN-LE at 70 °C.

4.55 ppm). The other peak was of a well-resolved doublet (δ_H 4.245 ppm). The overlap of the anomeric doublets was confirmed by additional NMR experiments, including a COSY experiment. The four sugar residues in GRN-LE were arbitrarily labeled as A, B, C, and D, as described in Figure 1. On the basis of their observed chemical shifts, $^3J_{H1,H2}$ and $^1J_{H1,\ C1}$ (Table 1), all the residues were assigned as β-hexapyranosyl residues. The 1D-^{13}C NMR spectra in Figure 2 show two signals in the anomeric region (δ_C 95–110 ppm); one signal was were assigned to residues A, B, and C (δ_C 103.02 ppm) and the other to residue D (δ_C 103.11 ppm), which were confirmed by cross-peaks in the ^{1}H, ^{13}C-HSQC spectrum (data not shown).

Next, we attempted to assign all ^{1}H resonances in the ^{1}H NMR spectrum on the basis of COSY and TOCSY experiments. In the 2D-TOCSY spectrum (Fig. 3), a complete series of cross-peaks was observed between A H-1 and A H-2, 3, 4, 5, 6a, and 6b, as well as between B, C, and D H-1 and between B, C, D H-2, 3, 4, 5, 6a, and 6b. However, the assignments of some of the protons were ambiguous owing to extensive overlap with the neighboring protons. Thus, we further studied these ambiguously assigned protons from the overlaid spectra of ^{13}C-edited HSQC and H2BC, in order to separate the neighboring protons in the ^{13}C dimension. In other words, the overlaid HSQC and H2BC spectra showed the entire intra-ring assignment, as reported previously.[23,24] Thus, all the protons in GRN-LE were assigned unambiguously.

In addition, the combination of the ^{13}C-edited HSQC and HSQC-TOCSY spectra at various TOCSY spin-lock periods (30–120 ms), and the H2BC experiment allowed the complete assignment of the ^{13}C spectrum. Table 2 summarizes the ^{1}H and ^{13}C NMR spectral assignments of GRN-LE. These assignments are based on the COSY, TOCSY, HSQC, HSQC-TOCSY, and H2BC spectra.

In the spectrum of GRN-LE, the four cross-peaks (i.e. between A H-1 and B H-3, between B H-1 and C H-3, between C H-1 and A H-3, and between D H-1 and C H-6), which are shown in the ^{1}H, ^{13}C-HMBC spectrum (Fig. 4), were assigned to glycosidic linkages, as indicated by A(1→3)B, B(1→3)C, C(1→3)A, and D(1→6)C, respectively. These results were also confirmed by the NOESY experiment (data not shown).

Table 2
Chemical shifts (ppm) of ^{1}H and ^{13}C NMR signals for GRN-LE

Sugar residue	^{1}H/^{13}C						
	1	2	3	4	5	6a	6b
→3)-β-D-Glc*p*-(1→ A	4.559 103.02	3.330 72.78	3.526 86.64	3.284 68.61	3.297 76.20	3.722 60.90	3.488
→3)-β-D-Glc*p*-(1→ B	4.559 103.02	3.331 73.07	3.520 86.10	3.268 68.55	3.283 76.43	3.732 61.03	3.488
→3,6)-β-D-Glc*p*-(1→ C	4.542 103.02	3.366 72.90	3.547 85.76	3.326 68.70	3.522 74.96	4.080 68.61	3.586
β-D-Glc*p*-(1→ D	4.245 103.11	3.043 73.70	3.228 76.30	3.118 70.24	3.169 76.63	3.700 61.20	3.495

The ratio of the main chain (residues A, B, and C) to the side chain (residue D) was approximately 3:1 when calculated on the basis of the accurate integrities of the anomeric protons. Thus, these results strongly suggested that the primary structure of GRN-LE contains 1,3-β-glucan and 1,6-β-glucan units, as described in Figure 5. As shown in this figure, GRN-LE analyzed in this study has a structure comprising a 1,3-β-D-glucan backbone with every third residue bearing a single 1,6-β-D-glucopyranosyl side-branching unit.

In conclusion, in the present study, we clearly elucidated the primary structure of the purified β-D-glucan GRN-LE obtained from *G. frondosa*, which is a 1,3-β-D-glucan backbone with a single 1,6-β-D-glucopyranosyl side-branching unit on every third residue. This structural elucidation is, at least in part, useful for revealing the precise mechanisms underlying the biological effects of GRN-LE

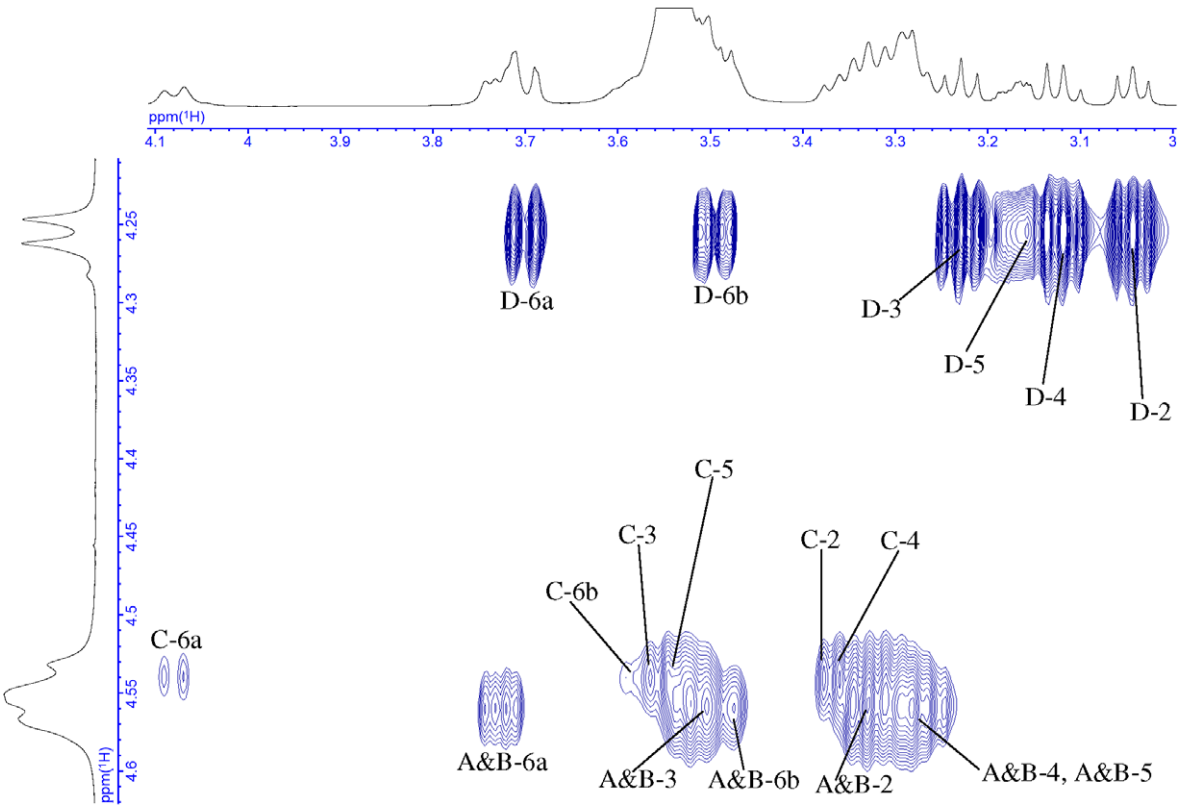

Figure 3. Anomeric region of the 2D-TOCSY spectrum of GRN-LE at 70 °C. The 2D-TOCSY experiment was performed with a spin-lock mixing time of 150 ms.

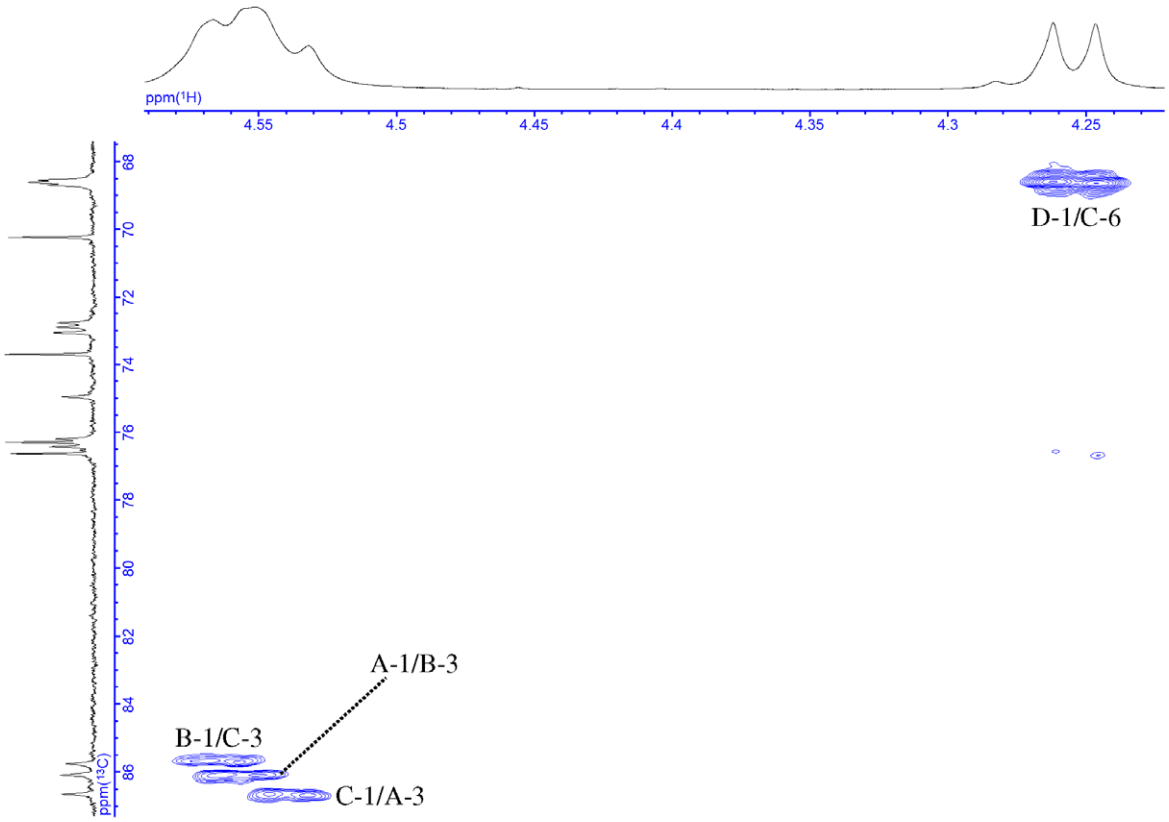

Figure 4. ^{1}H, ^{13}C-HMBC spectrum of GRN-LE at 70 °C.

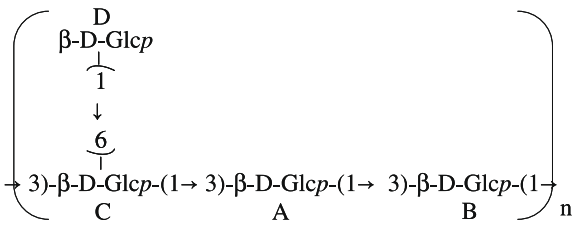

Figure 5. Structure of GRN-LE obtained from *G. frondosa*. Glc*p*: glucopyranose.

and in the promotion of food-based therapies. Furthermore, this structural information can be used to gain an insight into the molecular mechanisms involved in β-D-glucan-induced immune responses such as β-D-glucan-receptor interactions (by NMR spectroscopy), as well as to study the ligand specificity of the β-D-glucan receptors.

1. Experimental

1.1. Materials

GRN-LE was prepared as described previously.[13] D_2O (deuteration degree min 99.96%) and Me_2SO-d_6 (99.96%) were purchased from Merck.

1.2. NMR spectroscopy

Exchangeable protons were removed by suspending GRN-LE in D_2O, and subsequent lyophilization. This exchange process was repeated thrice. All the spectra were recorded in a mixed solvent, Me_2SO-d_6/D_2O (6:1) (15 mg/mL), at 70 °C using the method proposed by Kim et al.[3] on a Bruker Avance 500 spectrometer equipped with a TXI-*xyz* three gradient probe for ^{1}H detection or a BBO-*z* gradient probe for ^{13}C detection. The chemical shifts are provided in ppm, using the internal Me_2SO signal (δ_H = 2.53 ppm) for ^{1}H and the internal Me_2SO signal (δ_C = 39.5 ppm) for ^{13}C as references. The 1D-^{1}H experiment was performed using the Bruker standard pulse sequence with 3157 Hz in 64 K complex data points. The relaxation delay used was $5T_1$ in order to obtain accurate signal integrations. Prior to Fourier transformation, zero filling was used four times, and noise was reduced using the TRAF function. The 1D-^{13}C experiment was performed using the Bruker standard pulse sequence with 30,581 Hz in 64 K complex data points. Prior to Fourier Transformation, zero filling was used four times, and noise was reduced using exponential multiplication. Two-dimensional COSY was performed with 256 increments of 2048 data points with four scans per t_1 increment using the Bruker standard pulse sequence. The spectral width was 1383 Hz in each dimension. TOCSY was carried out at a TOCSY spin-lock period of 30–150 ms using the pulse sequence suggested by Griesinger et al.[25] in order to suppress the rotating-frame Overhauser effect (ROE) signals. The spectral width was 3004 Hz in each dimension, and 512 increments of 4096 data points with 16 scans per t_1 increment were recorded. 2D NOESY was performed with a mixing time of 200 ms using the Bruker standard pulse sequence. The spectral width was 2140 Hz in each dimension, and 256 increments of 2048 data points with 32 scans per t_1 increment were recorded. 2D ^{13}C-edited HSQC was performed with 512 increments of 2048 data points with 32 scans per t_1 increment using the Bruker standard pulse sequence. The spectral width was 2741 Hz for t_2 and 9432 Hz for t_1. 2D ^{1}H, ^{13}C-HMBC spectroscopy was performed with 256 increments of 2048 data points with 128 scans per t_1 increment using the Bruker standard pulse sequence. The delay time for the evolution of long-range couplings was set to 62.5 ms (opti-

mized for 8 Hz). The spectral width was 2741 Hz for t_2 and 9432 Hz for t_1. 2D ^{1}H, ^{13}C-HSQC-TOCSY was conducted with 512 increments of 2048 data points with 48 scans per t_1 increment using the Bruker standard pulse sequence. The spectral width was 2741 Hz for t_2 and 9432 Hz for t_1. 2D H2BC was carried out with 512 increments of 2048 data points with 96 scans per t_1 increment using the pulse sequence provided by Nyberg et al.[23,26,27] The spectral width was 5000 Hz for t_2 and 26,000 Hz for t_1. All the 2D experiments were zero-filled to 2k, and 2k in both dimensions prior to Fourier transformation. In all the experiments, a square cosine-bell window function was applied in both dimensions, except in the COSY and H2BC experiments. A sine-bell window function was applied in both dimensions for the COSY experiment. The H2BC experiment was treated with a cosine window function in t_1 and a $\pi/4$ shifted sine in t_2.

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