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Note

A novel water-soluble β-(1→6)-D-glucan isolated from the fruit bodies of *Bulgaria inquinans* (Fries)

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ABSTRACT

A low molecular-weight polysaccharide named BIWP2 was purified from the fruit bodies of *Bulgaria Inquinans* (Fries) via hot-water extraction, followed by freeze-thawing and gel filtration chromatography on Sephadex G-75. Monosaccharide composition analysis revealed that BIWP2 contained exclusively glucose. High performance size exclusion chromatography (HPSEC) showed that it was a homogeneous polysaccharide fraction. Its molecular weight was estimated to be 2.6 KD and the polydispersity index (M_w/M_n) was calculated to be 1.4. Periodate oxidation, methylation, and NMR analyses indicated that BIWP2 was a linear β-(1→6)-D-glucan without side chains. This is the first time to report a linear β-(1→6)-D-glucan with low molecular weight in non-lichenized ascomycete.

Fungi polysaccharides are mainly glucans with various structures.[1,2] β-(1→3)- and (1→3,1→6)-linked glucans are common in the Basidiomycota[3–7] and Ascomycota[8–10] fungi. β-(1→6)-glucans have been isolated from basidiomycete mushrooms,[11–13] lichenized ascomycetes,[14,15] and yeast.[16,17] Iorio reported that β-D-glucans of the opportunistic fungal pathogen *Candida albicans* were mainly composed of β-(1→6)-glucans with O-3 branched side chains, a structure very close to that of β-glucans from *Saccharomyces cerevisiae* yeast.[18] It is well known that the glucans with β-(1→3)-glucosidic linkages are biological response modifiers (BRMs).[19,20] Recent experimental results have shown that some β-(1→6)-glucans also have remarkable biological activities.[11,12,21–25]

Non-lichenized ascomycete *Bulgaria inquinans* (Fries) is an edible wood-inhabiting ascomycete growing on freshly felled oak commonly in the Changbai Mountain area of China.[26] It has been used as food and folk antitumor medicine for many years. Eating too much will make one's lips swelling valgus as pig's mouth because of photosensitivity dermatitis. Therefore, it is usually called 'pig-mouth mushroom'. Several benzofluoranthrene derivatives, dihydroxyperylenequinones, and azaphiones have been isolated from the fruit bodies of *B. inquinans* (Fries).[27–30] Bioassay tests showed that these compounds had antitumor,[31] antipruritic, and antierythema activities.[32] Although some small molecules from *B. inquinans* (Fries) have been studied, as far as our knowledge, there are no reports about the studies on polysaccharides from it. The present paper describes the isolation and characterization of a novel β-(1→6)-D-glucan from non-lichenized ascomycete *B. inquinans* (Fries).

The crude polysaccharide fraction BIW (8% yield of dried fruit bodies) was extracted by hot water from the fruit bodies of *B. inquinans* (Fries), purified by re-dissolving them in water to remove the insoluble impurities and de-proteined by the Sevag method. BIW exhibited a wide molecular weight distribution (from 3 KD to 50 KD) on Sepharose CL-6B gel filtration chromatography. The monosaccharide composition analysis indicated that BIW contained glucose, mannose, and galactose in a molar ratio of 1:1.3:1. BIW was fractionated by freeze-thawing process to give two fractions, BIWS (supernatant, 63%) and BIWP (precipitate, 19%).

BIWS was a heteropolysaccharide fraction consisting of glucose, mannose, and galactose in a molar ratio of 1:1:1. Its molecular weight showed a heterogeneous distribution from 15 KD to 50 KD. These results indicated that BIWS might contain several polysaccharides such as glucans, glucomannans, and galactomannans.

BIWP contained 95.2% of glucose, 2.1% of mannose, and 2.7% of galactose. It appeared at total volume position on Sepharose CL-6B gel filtration chromatography, which meant that its molecular weight was quite small. BIWP gave two peaks on Sephadex G-75 gel filtration chromatography and was further separated into two fractions: BIWP1 and BIWP2 in a ratio of 1:42. Probably, a small amount of the polysaccharides from BIWS was precipitated by the process of freeze-thawing, which resulted in generation of BIWP1. BIWP1 consisted of glucose, mannose, and galactose in a molar ratio of 1:2.8:0.9. Its molecular weight distribution was heterogeneous.

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BIWS and BIWP1 were composed of more than one monosaccharide. In contrast, BIWP2 contained 99.02% of glucose and a trace amount of mannose (0.88%). HPSEC indicated that the molecular weight distribution of BIWP2 was homogeneous and M_w was estimated to be 2.6×10^3 Da ($M_w/M_n = 1.4$), which indicated that BIWP2 was a homogenous glucan. The trace amount of mannose might be impurities.

BIWP2 was water-soluble white powder. The specific rotation value was $[\alpha]_D^{20}$ −77.5 (*c* 0.2, H_2O) which corresponded to a β-glucosidic configuration.[11,13,33] The FT-IR spectrum showed that the absorption bands appeared at 3332.43 cm^{-1} (hydroxyl stretching vibration), 2914.82 (C–H stretching vibration), 1645.09 cm^{-1} (bound water), 1041.39 cm^{-1} (pyranose ring), and 902.54 cm^{-1} (β-glycosidic linkages).

Methylation analysis of BIWP2 produced two methylated alditol acetates detected by GC–MS. The main product was 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucitol (94%), and the other was 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol (6%). This revealed that BIWP2 was a (1→6)-linked glucan and contained (1→6)-linked and non-reducing terminal glucosyl residues in a molar ratio of 15:1. The results of periodate oxidation indicated that BIWP2 consumed 1.78 mol periodate and produced 0.92 mol formic acid per mol of glucosyl residual on average. GC analysis indicated that the hydrolyzed products contained a large amount of glycerol, but no erythritol and monosaccharide. These results support that BIWP2 was a (1→6)-linked glucan.

^1H NMR and ^{13}C NMR spectra were shown in Figure 1 and the data were listed in Table 1. All signals were identified with respect to those of β-(1→6)-D-glucans reported in the literatures.[11–15,34,35]

Table 1
^1H and ^{13}C NMR chemical shifts of BIWP2 in D_2O

Residue	δ $^{13}C/^1H$ (ppm)						
	1	2	3	4	5	6 (6a)	6b
→6)-β-Glc*p*-(1→	104.66 4.42	74.71 3.22	76.57 3.40	71.14 3.35	77.25 3.53	70.48 4.12	 3.75

A C-1 signal for typical β-glycosidic configuration appeared at 104.66 ppm in the ^{13}C NMR spectrum and a H-1 signals for typical β-glycosidic configuration appeared at 4.42 ppm in the ^1H NMR spectrum. The coupling constant values were $J_{H\text{-}1,\ H\text{-}2}$ ~8.5 Hz and $J_{H\text{-}1,\ C\text{-}1}$ ~160 Hz. These NMR data suggested that BIWP2 was a β-glycosidic-linked glucan.

The HMBC spectrum of BIWP2 is shown in Figure 2, which provided the signals corresponding to long-range connections among protons and the carbons placed at two and three bonds. For explanation of the HMBC results, two units of glucose (A and A′) were considered. The interresidual $^1H/^{13}C$ cross-peaks were identified between H-1 (4.42 ppm) of residue A and C-6 (70.48 ppm) of residue A′ (A H-1, A′ C-6), C-1 (104.66 ppm) of residue A and H-6a (4.12 ppm) and H-6b (3.75 ppm) of residue A′ (A C-1, A′ H-6a; A C-1, A′ H-6b), and vice versa.

The NMR results summarized above showed that BIWP2 was a linear (1→6)-D-glucan. Based on the percentage of non-reducing terminal residues, BIWP2 was composed of about 16 sugar residues, which was consistent with the molecular weight (2.6×10^3 Da) estimated by HPSEC.

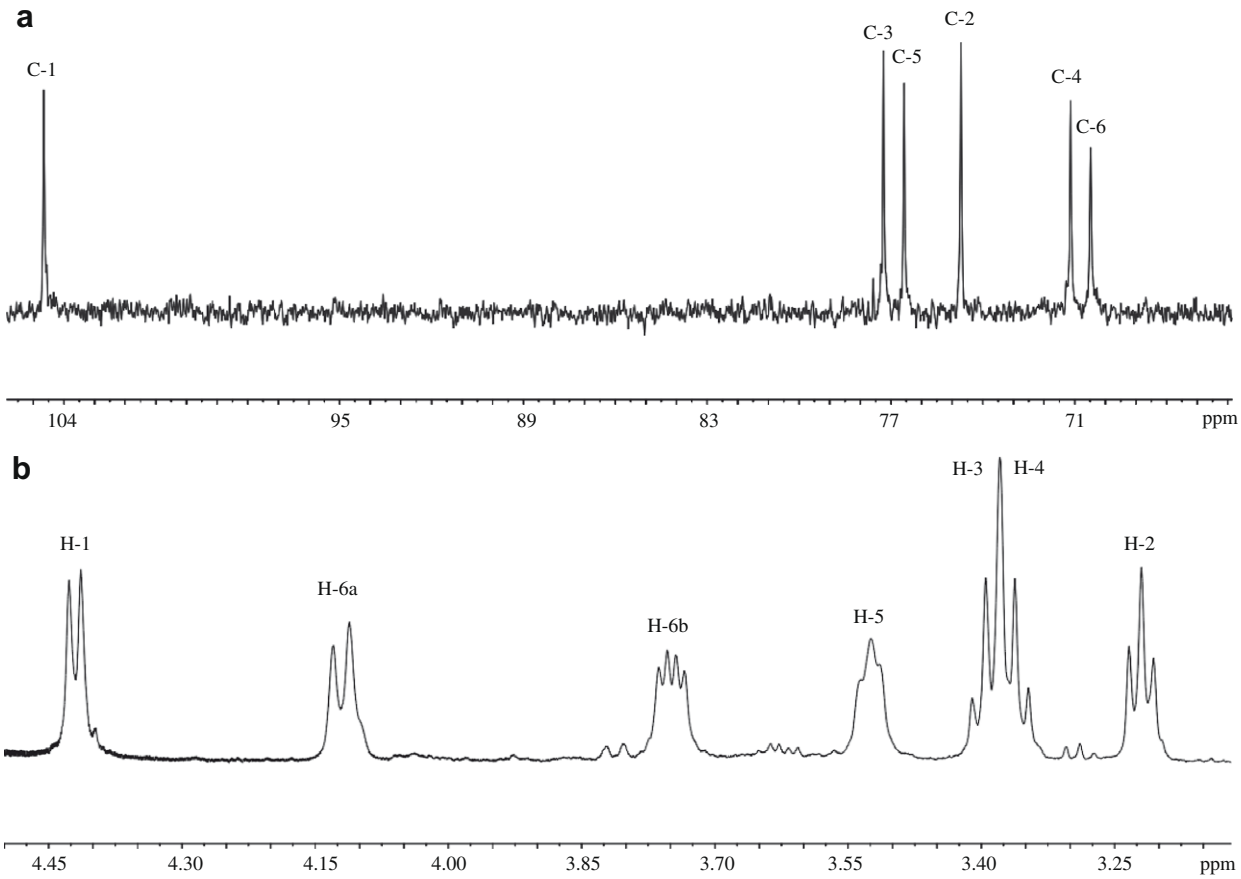


Figure 1. NMR spectra of the fraction BIWP2: (a) ^{13}C NMR spectrum (150 MHz); (b) ^1H NMR spectrum (600 MHz). Solvent: D_2O, temperature: 20 °C.

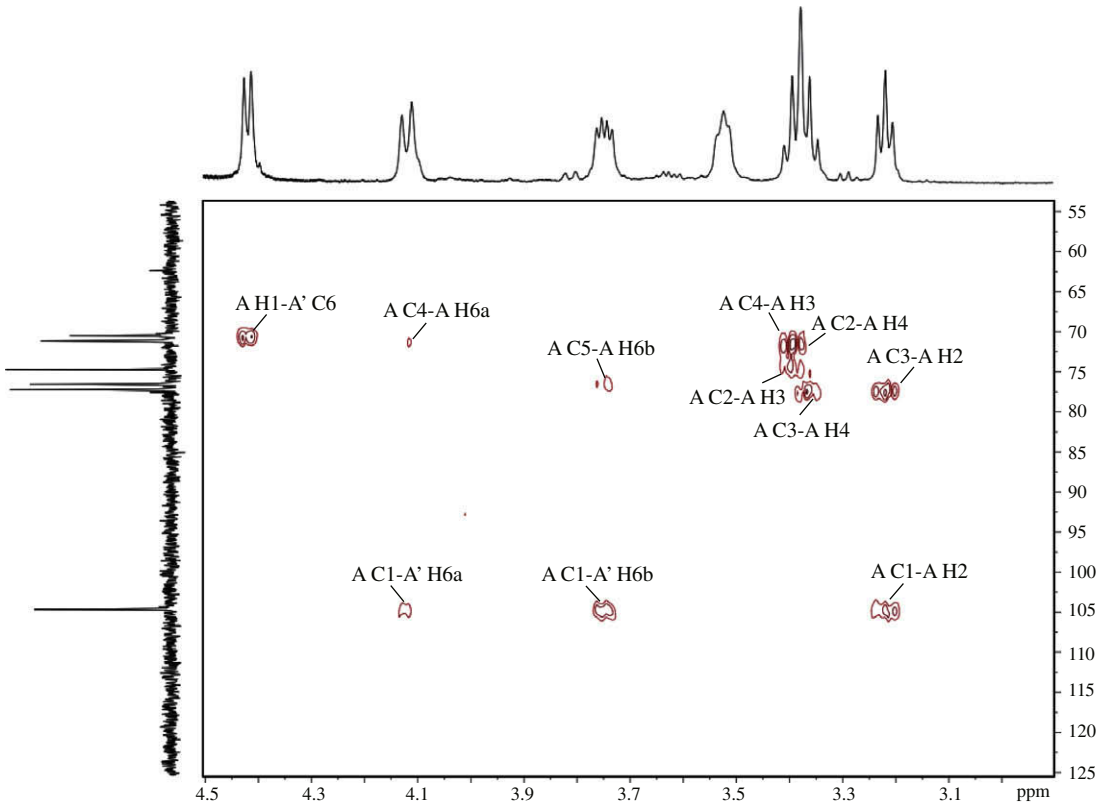
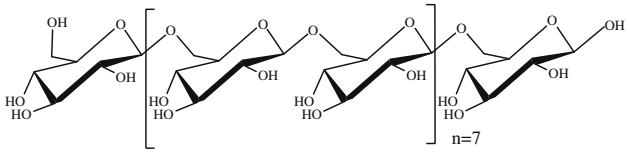


Figure 2. The HMBC spectrum for the fraction BIWP2. Relevant cross-peaks are labeled.

The specific rotation, IR and NMR all identified BIWP2 to be β configuration. The structure of BIWP2 is shown below:



To date, to our knowledge, linear β-(1→6)-D-glucans have been found in the fungi *Agaricus blazei*,[11] *Agaricus bitorquis*,[13] *Botryosphaeria rhodiana*,[14,15] *Grifola frondosa*[19], *Umbilicaria mammulata*,[34] and prokaryotic organism *Actinobacillus*,[35] and branched β-(1→6)-D-glucans have been massively found in yeast.

It has been known that β-(1→6)-D-glucan is an essential fungal-specific component of yeast cell wall, approximately 10% of the cell wall dry weight, and plays a critical role in the cell wall architecture by anchoring mannoproteins to other cell wall components.[16,17] The molecular weights of the β-(1→6)-D-glucans isolated from fungi mentioned above are among the range from 1×10^4 Da to 1×10^6 Da. But the molecular weight of BIWP2 is about 2.6×10^3 Da. BIWP2 has a novel character of relative low molecular weight. As *B. Inquinans* (Fries) contains, besides BIWP2, quite a lot of mannoproteins and large amount of high molecular weight β-(1→6)-D-glucans (Yifa Zhou, unpublished data), we deduce that BIWP2 plays a similar role in the cell wall architecture to β-glucans in *S. cerevisiae* and *C. albicans*, anchoring mannoproteins to other cell wall components. In addition, it might be intermediate in the biogenesis of more complex β-D-glucans.

It is well known that β-(1→3)-D-glucans or β-(1→3)-D-glucans with O-6 branched side chains have remarkable biological activities because of their interaction with the receptors on the surface of macrophages and other white blood cells.[36] Recently, it has been found that β-(1→6)-glucans showed marked biological activities, too. β-(1→6)-D-glucans from *Gyrophora esculenta* (partially acetylated at O-3),[21] from *G. frondosa* (branched at O-3, M_w 1×10^6 Da),[22–24] from *A. blazei* (M_w 1–5 $\times$ 10^6 Da),[11] and from *Lyophyllum decastes* Sing. (branched at O-3 for every 6–5 backbone residues, M_w 1.4×10^4 Da)[12] have remarkable antitumor activity. In addition, pustulan, a β-(1→6)-glucan partially acetylated at O-3, has the potential to induce anti-inflammatory and pro-inflammatory effects.[25] Based on the knowledge of structure–activity relationship of polysaccharides, the molecular weight and branches influence the activities. However, it is unclear what the rules are exactly. From the activities of the β-(1→6)-glucans listed above, it has been seen that all linear or branched, low or high molecular weight β-(1→6)-glucans might have biological activities. Therefore, BIWP2, as the β-(1→6)-glucans found in fungi, may have potential biological and pharmaceutical activities. Studies on the immunological and anticancer activities of BIWP2 are in progress in our research group.

In conclusion, a homogenous water-soluble polysaccharide BIWP2 was isolated and purified from the fruit bodies of *B. Inquinans* (Fries). It was characterized to be a linear β-(1→6)-D-glucan with a low molecular weight of 2.6×10^3 Da. BIWP2 massively existed in the non-lichenized ascomycete *B. Inquinans* (Fries), which probably has biological and pharmaceutical activities. Therefore, *B. inquinans* (Fries) is a resource of low molecular weight β-(1→6)-D-glucan and BIWP2 has potential application in medicine.

1. Experimental

1.1. Materials

Fruit bodies of *B. inquinans* (Fries) were purchased from Fusong in Jilin Province, China and identified by Professor Hongxing Xiao,

School of Life Sciences, Northeast Normal University in Changchun, China. Sepharose CL-6B and Sephadex G-75 were purchased from Amersham Pharmacia Biotech. Dextrans and the monosaccharides (D-Gal, D-Ara, D-Fuc, D-Rha, D-Man, D-Xyl, D-Glc, Glucuronic acid, and Galacturonic acid) were purchased from Sigma. All other reagents were of analytical grade made in China.

1.2. General methods

The total carbohydrate content was determined by phenol–H_2SO_4 method using glucose as standard.[37] All gel filtration chromatographies were monitored by assaying carbohydrate content. Protein content was determined according to the method of Sedmak and Grossberg, with Coomassie brilliant blue reagent and bovine serum albumin as the standard.[38] Dialysis was carried out using tubing with M_w cut-off 3500 Da (for globular protein).

Gas chromatography was performed with a Shimadzu GC-14C instrument equipped with a hydrogen flame ionization detector on Rtx-2330 column (0.32 mm × 15 m i.d., 0.2 μm) at a temperature program of 175 °C (hold 2 min) followed by 8 °C/min to 240 °C (hold 1 min) and 8 °C/min to 265 °C (hold 17 min). The hydrogen flow rate was 10 mL/min and the ion-source temperature was 275 °C.

HPLC was carried out on a Shimadzu 10Avp HPLC system equipped with 10Avp HPLC Pump, SPD-10Avp UV-VIS Detector, and RID-10A Refractive Index Detector.

The specific rotation was determined at 20 ± 1 °C with an automatic polarimeter (Model WZZ-2B, China). UV–vis absorbance spectra were recorded with a UV–vis spectrophotometer (Model SP-752, China).

1.3. Extraction and purification

The fruit bodies of *B. inquinans* (Fries) were first exhaustively extracted with 95% ethanol under reflux for 12 h to remove hydrophobic compounds such as lipids. This step was repeated three times. After filtration, the residue was dried at room temperature, and then extracted with hot distilled water (90–95 °C, 1:20 w/w) three times (6 h for each). The aqueous filtrates were combined and concentrated to small volume (one-tenth of the original volume), then 95% ethanol was added to the aqueous filtrates up to 80% to precipitate the polysaccharides which were collected by centrifugation and dried in vacuum. The precipitate was dissolved in water (15% w/w) to remove the insoluble substances by centrifugation. The supernatant was submitted to the Sevag method to remove free proteins.[39] After removing the remaining Sevag reagent by vacuum evaporation, the water phase was freeze-dried, which gave rise to BIW. BIW was dissolved in water (10% w/w) and submitted to freeze-thawing process, centrifuged to separate the polysaccharides into two fractions, supernatant (BIWS) and precipitate (BIWP). BIWP was further fractionated by Sephadex G-75 gel filtration chromatography to give two fractions: BIWP1 collected at void volume position and BIWP2 collected as main portion.

1.4. Homogeneity and molecular weight

Determination of homogeneity and molecular weight was carried out by HPSEC-linked gel filtration column of TSK-G3000 PWXL, eluting with 0.2 M NaCl at a flow rate of 0.67 mL/min at 35.0 ± 0.1 °C. The gel filtration column was calibrated by standard dextrans (50 KD, 25 KD, 12 KD, 5 KD, 1 KD) using linear regression. Sample concentration was 5 mg/mL and injection amount was 20 μL.

1.5. Monosaccharide component analysis

The monosaccharide analysis was performed by HPLC method as described by Honda[40] and Yang.[41] Briefly, the sample (2 mg) was first methanolyzed using 0.5 mL anhydrous methanol containing 2 M HCl at 80 °C for 16 h. Then the methanolyzed products were hydrolyzed with 0.5 mL of 2 M CF_3COOH at 120 °C for 1 h.[42] The hydrolysis product monosaccharides were derivatized to be 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives and subsequently analyzed by HPLC on a Shim-pak VP-ODS column (150 × 4.6 mm i.d.) with a guard column on a Shimadzu HPLC system (LC-10ATvp pump and UV–vis detector) and monitored by UV absorbance at 245 nm.

1.6. Methylation analysis

The methylation analysis was carried out according to the method of Needs and Selvendran.[43] In brief, BIWP2 (10 mg) was dissolved in DMSO (1.5 mL) and methylated by treatment with NaOH/DMSO (1 mL) suspension and iodomethane (1.0 mL). The reaction mixture was extracted with $CHCl_3$, and then the solvent was removed by vacuum evaporation. Complete methylation was confirmed by the disappearance of the –OH band (3200–3400 cm^{-1}) in the FT-IR spectrum. The per-O-methylated polysaccharide was hydrolyzed subsequently by HCOOH (85%, 0.5 mL) for 4 h at 100 °C, and then by CF_3COOH acid (2 M, 1 mL) for 6 h at 100 °C. The partially methylated sugars in the hydrolysate were reduced by $NaBH_4$ and acetylated.[44] The resulting mixture of alditol acetates was analyzed by GC–MS.

1.7. Periodate oxidation

The periodate oxidation followed the procedure described by Chaplin and Kennedy.[45] BIWP2 (25 mg) was dissolved in 0.015 M $NaIO_4$ (25 mL), and maintained at 4 °C in dark. The reaction was monitored every 6 h with spectrophotometric method at 223 nm. After the oxidation was completed (64 h), the excessive $NaIO_4$ was decomposed with ethylene glycol (0.1 mL). The $NaIO_4$ consumption was calculated according to the decrease in absorbance (A223). The formic acid production was determined by titration with 0.1 M NaOH. The reaction mixture was dialyzed against tap water and distilled water in turn, then the retentate was reduced with $NaBH_4$ overnight. After neutralization and dialysis, the retentate was freeze-dried, completely hydrolyzed, reduced by $NaBH_4$, acetylated and then analyzed for sugar composition by GC.

1.8. FT-IR analysis

FT-IR spectra were obtained on a Nicolet 560 FT-IR spectrometer with DTGS detector in a range of 400–4000 cm^{-1}. The samples were measured as a film on KBr discs.

1.9. NMR analysis

The ^1H, ^{13}C and HMBC NMR spectra were recorded using a Bruker 5 mm broadband observe probe at 20 °C with a Bruker Avance 600 MHz spectrometer (Germany), operating at 600 MHz for ^1H and 150 MHz for ^{13}C. β-Glucan sample (20 mg) was dissolved in D_2O (99.8% D, 0.5 mL), freeze-dried, redissolved in D_2O (0.5 mL), and centrifuged to remove the excessive sample. All the experiments were recorded using standard Bruker software. The heteronuclear multiple-bond correlation (HMBC) experiment was performed with 128 increments of 2048 real points over a spectral width of 3623.19 Hz in the acquisition domain F2 and 33333.33 Hz in the time domain F1. A total of 64 scans were used per increment with a delay of 65 ms for the evolution of long-range couplings.

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