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Note

Substrate Specificity of GH29 α-L-Glucosidases from *Cecembia lonarensis*

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Abstract: We recently found two α-L-glucosidases, which can hydrolyze *p*-nitrophenyl α-L-glucopyranoside (PNP L-Glc) rather than *p*-nitrophenyl α-L-fucopyranoside, in glycoside hydrolase family 29. This study evaluated their substrate specificity for *p*-nitrophenyl α-L-rhamnopyranoside (PNP L-Rha), α-L-quinovopyranoside (PNP L-Qui), and α-L-xylopyranoside (PNP L-Xyl), of which structure is similar to PNP L-Glc. The two α-L-glucosidases had little activity toward PNP L-Rha. They exhibited higher k_{cat}/K_m values for PNP L-Qui but smaller for PNP L-Xyl than for PNP L-Glc. The molecular docking studies indicated that these specificities were correlated well with the active-site structure of the α-L-glucosidases. The finding that α-L-quinovoside, which has been suggested to occur in nature, is also a substrate for α-L-glucosidases indicates that this enzyme are not solely dedicated to α-L-glucoside hydrolysis.

Key words: α-L-glucosidase, glycoside hydrolase family 29, substrate specificity, molecular docking

Only D-glucose exists in nature, whereas the L-glucose is believed to be non-existent. However, some bacteria can metabolize L-glucose, and its metabolic pathway has even been clarified in *Paracoccus laeviglucosivorans* [1], implying the existence of L-glucose in nature. In addition, our recent finding of α-L-glucosidases in the *Cecembia lonarensis* [2] raises the possibility of α-L-glucosides in nature. Organisms have various glycosidases that break down the different types of glycosidic linkages in oligosaccharides, polysaccharides, and glycosides found in nature. The presence of a particular glycosidase implies that its substrate carbohydrate exists.

C. lonarensis has two α-L-glucosidases, ClAgl29A and ClAgl29B, belonging to the glycoside hydrolase family 29 (GH29), a family of α-L-fucosidases [2]. Pairwise alignment of these proteins shows 56.1 % identity and 70.3 % similarity and contains a 19.8 % gap. X-ray crystallography showed that the structures of the active sites of both enzymes are well conserved, with no differences in the spatial arrangement of amino acid residues [2]. In the homology search using ClAgl29B as the query sequence and PDB as the search database, it shows the highest similarity to GH29 α-L-fucosidase from *Thermotoga maritima* (TmaFuc), with a sequence identity of 29 % (87 % of query coverage). In addition, α-L-galactosidase from *Phocaeicola plebeius* DSM 17135 (PDB id, 7snk) and tissue α-L-fucosidase from *Homo sapiens* (PDB id, 7pls) hit as homologous proteins were at the top of the list. In the Foldseek Search using ClAgl29B (PDB id, 7xsg) as the query structure, the most similar structure is TmaFuc (PDB id, 2zxd). While showing apparent sequence similarity to α-L-fucosidases, ClAgl29A and ClAgl29B display substrate selectivity towards *p*-nitrophenyl α-L-glucopyranoside (PNP L-Glc) rather than *p*-nitrophenyl α-L-fucopyranoside (PNP L-Fuc). α-L-Glucopyranoside differs from α-L-fucopyranoside in the orientation of the hydroxyl group at C4 (OH-4) and in bearing a hydroxyl group at C6 (OH-6) (Fig. 1). α-L-Glucosidases have distinctly different recognition machinery for the equatorial OH-4 of α-L-glucoside compared to GH29 α-L-fucosidases. GH29 α-L-fucosidases recognize the axial OH-4 of α-L-fucoside through hydrogen bonds with the side chains of two His residues corresponding to His34 and His128 of TmaFuc. ClAgl29A and ClAgl29B lack the His residue corresponding to His34, and a carboxy group of Asp residue, Asp127 (ClAgl29A) and Asp139 (ClAgl29B), recognizes the equatorial OH-4 of α-L-glucoside [2]. On the other hand, recognition around C6 of α-L-glucoside by the α-L-glucosidases is relatively loose, with the thiol group of Cys406 (ClAgl29A) and Cys418 (ClAgl29B) within hydrogen bonding distance to the OH-6. TmaFuc also have relaxed recognition for C6 of α-L-fucoside and can hydrolyze *p*-nitrophenyl α-L-galactopyranoside as substrates [2].

This study investigated which α-L-glycosides other than α-L-glucoside are substrates for ClAgl29A and ClAgl29B. We evaluated the specificity for *p*-nitrophenyl derivatives of α-L-quinovopyranoside (PNP L-Qui), α-L-xylopyranoside (PNP L-Xyl), and α-L-rhamnopyranoside (PNP L-Rha) chemically synthesized (Fig. 1). L-Quinovose has no hydroxy group at C6 compared to L-glucose. The pentose L-xylose forms the pyranose structure, which has no hydroxymethyl group at the C-6 position of L-glucopyranose. By evaluating the enzyme reaction to PNP L-Qui and PNP L-Xyl, the relaxed substrate recognition of ClAgl29A and ClAgl29B for C6 can be evaluated kinetically. α-L-Rhamnoside, the 2-epimer of α-L-quinovoside, is widely found in nature and is included in

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Abbreviations: PNP L-Glc, *p*-nitrophenyl α-L-glucopyranoside; PNP L-Qui, *p*-nitrophenyl α-L-quinovopyranoside; PNP L-Rha, *p*-nitrophenyl α-L-rhamnopyranoside; PNP L-Xyl, *p*-nitrophenyl α-L-xylopyranoside

the main chain of rhamnogalacturonan I and the side chain of rhamnogalacturonan II. It also exists as glycosides such as quercetin and kaempferol. Given the possibility that ClAgl29A and ClAgl29B might be more reasonable as enzymes that hydrolyze α-L-rhamnopyranoside, which is abundant in nature, as a substrate than uncertain α-L-glucoside, we decided to investigate their reactivity with PNP L-Rha.

L-Xylose (Combi-Blocks, Inc.) and L-rhamnose (Nacalai Tesque, Inc., Kyoto, Japan) were *O*-acetylated by acetic anhydride in pyridine. L-Quinovose was obtained by microwave-enhanced Mo(VI)-catalyzed conversion of L-rhamnose [3]. The L-quinovose and L-rhamnose mixture obtained was *O*-acetylated and separated by flash column chromatography (hexane : ethyl acetate = 2:1). In the presence of stannic tetrachloride, *p*-nitrophenol was acted on per *O*-acetylated L-sugars in dichloromethane to give *p*-nitrophenyl per *O*-acetyl α-L-glycosides [4]. Flash column chromatography (hexane : ethyl acetate) of the crude products provided pure per *O*-acetyl α-L-glycosides. The acetylated glycosides were deacetylated with sodium methoxide in methanol. The chemical shifts of the NMR for each PNP α-L-glycoside are shown in the later section. Trace amounts of PNP L-Rha were found on the NMR analysis for PNP L-Qui but were ignored because ClAgl29A and ClAgl29B had little activity toward PNP L-Rha, as described below.

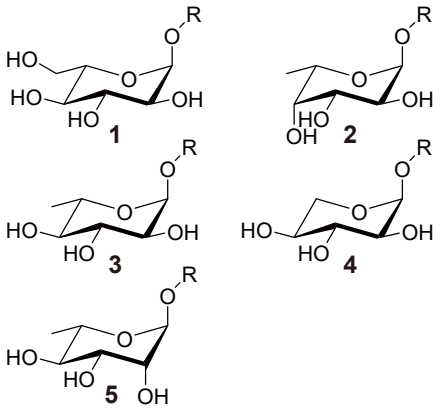

Fig. 1. Structures of α-L-glucopyranoside (1), α-L-fucopyranoside (2), α-L-quinovopyranoside (3), α-L-xylopyranoside (4), and α-L-rhamnopyranoside (5).

ClAgl29A and ClAgl29B were prepared as previously reported [2], and their hydrolysis activities toward 2 mM PNP L-Qui, PNP L-Xyl, and PNP L-Rha were measured. The reactions were performed in 40 mM sodium acetate buffer (pH 5.5) at 35 ˚C, and the reaction rate was determined from the increased amount of liberated *p*-nitrophenol per unit of time. The enzymes hydrolyzed PNP L-Qui and PNP L-Xyl but were barely able to hydrolyze PNP L-Rha. The hydrolysis rate of PNP L-Rha was 1/860 of PNP L-Qui in ClAgl29A and 1/1,200 in ClAgl29B. We performed molecular docking of methyl α-L-rhamnoside and ClAgl29B using GNINA [5], and compared it to the β-L-glucose binding to ClAgl29B determined by the X-ray crystal structure analysis (Fig. 2). In the structure of ClAgl29B with L-glucose bound to the active site, the equatorial OH2 can form hydrogen bonds with Nε1 of Trp140 and Nε2 of His199 (Fig. 2A). These non-covalent bonds could neutralize the electron-withdrawing property of the O2 atom of the substrate and

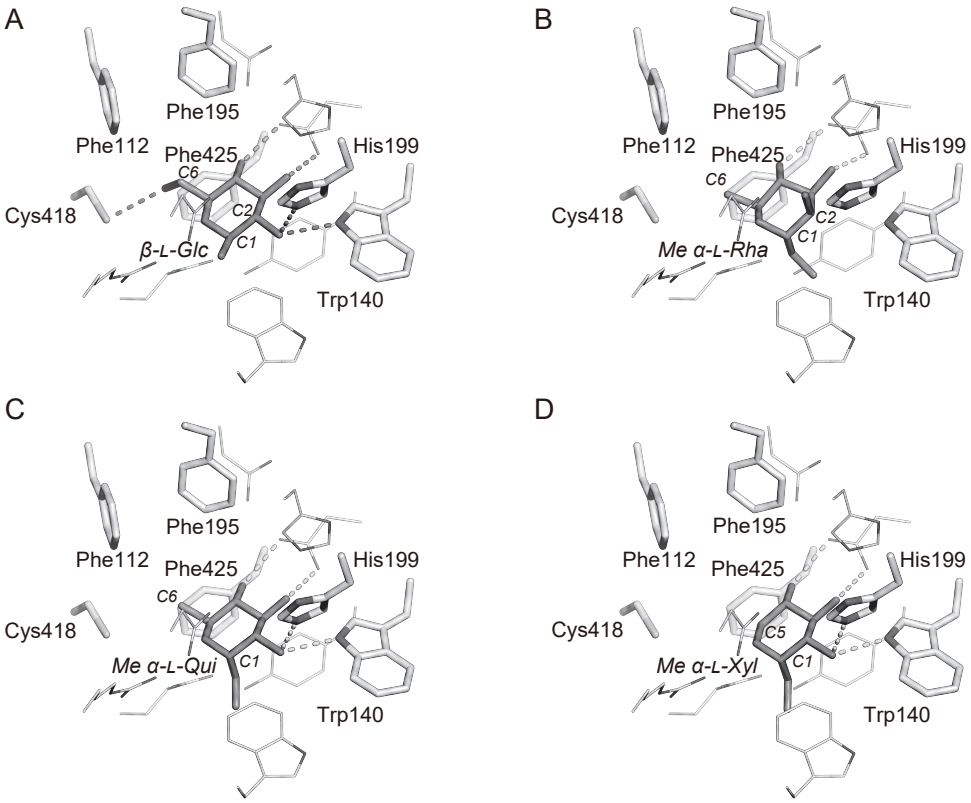

Fig. 2. Docking simulation of methyl α-L-glycosides.

The binding of β-L-glucose to the active site pocket of ClAgl29B (PDB ID, 7xsh) with neighboring residues within 4 Å (A). The predictions of binding of methyl α-L-rhamnoside (B), methyl α-L-quinovoside (C), and methyl α-L-xyloside (D). Molecular docking was performed by GNINA docking software using ClAgl29B (chain A in 7xsh) as a receptor protein. Amino acid residues critical for the recognition of α-L-glucoside are highlighted in the stick model. Dashed lines represent the expected hydrogen bonds.

contribute to the stabilization of the oxocarbenium ion-like transition state. α-L-Rhamnoside with the axial OH2 would not benefit from this hydrogen bonding acceleration effect and would be less likely to be a substrate (Fig. 2B). Meanwhile, the hydrolysis activity to PNP L-Rha, albeit feeble, suggests that no absolute steric hindrance exists between the axial hydroxyl group and the enzyme, as shown in the docking simulations. The active-site pocket of ClAgl29A bears corresponding Trp128 and His187 at respective sites and would show a similar substrate recognition mechanism.

The initial reaction rates for PNP L-Qui and PNP L-Xyl at each substrate concentration (0.025−2.0 mM for PNP L-Qui and 0.25−5 mM for PNP L-Xyl) of ClAgl29A (used 50.4 nM for PNP L-Qui and 75.7 nM for PNP L-Xyl) and ClAgl29B (used 37.2 nM for PNP L-Qui and 62.0 nM for PNP L-Xyl) were determined, and the kinetic parameters were calculated. The reaction rate for hydrolysis of PNP L-Qui followed the Michaelis-Menten equation with the substrate concentration tested. The experimentally obtained reaction rates were non-linearly regressed on the Michaelis-Menten equation to obtain Michaelis-Menten kinetic parameters. On the other hand, for PNP L-Xyl, the reaction rate did not follow the Michaelis-Menten equation, and a decrease in reaction rate was observed at high substrate concentrations for both enzymes. A reduction in reaction rate at high substrate concentrations would be attributed to transglycosylation or substrate inhibition in the double displacement mechanism, which ClAgl29A and ClAgl29B employed. The reaction products with 5 mM PNP L-Xyl were analyzed by reversed-phase HPLC equipped with UV detection at a wavelength of 312 nm, and no transglycosylation products were detected. We thus infer that the decrease in reaction rate was due to substrate inhibition and calculated the kinetic parameters by fitting the experimental results to the following reaction rate equation: $v = k_{cat}[E]_0[S]/([S]+K_m+[S]^2/K_i)$. In all cases, GraFit 7.02 (Erithacus Software Limited) was used to fit the reaction data to the reaction rate equations and to calculate the rate parameters. The parameters obtained are summarized in Table 1, with those previously reported for PNP L-Glc and PNP L-Fuc [2]. The kinetic study revealed that ClAgl29A and ClAgl29B exhibited higher k_{cat}/K_m values for PNP L-Qui than PNP L-Glc. While there was no marked difference in the k_{cat} values of both substrates, the K_m value of PNP L-Qui was smaller than that of PNP L-Glc. K_m is the apparent dissociation constant, which in the double displacement mechanism is expressed as $K_m = [E][S]/([ES]+[ES'])$, where ES is the Michaelis complex and ES′ is the enzyme-substrate covalent intermediate. Since the intermediate is unlikely to be stable, the low K_m for PNP L-Qui should be a measure that PNP L-Qui is more likely to bind than PNP L-Glc. The structure of ClAgl29B bound with β-L-glucose indicates that the C6 of L-glucose is in a hydrophobic environment surrounded by Phe112, Phe195, and Phe425 (Fig. 2A). ClAgl29A also has Phe101, Phe183, and Phe413, corresponding to the three Phe residues in ClAgl29B. Docking simulation of methyl α-L-quinovoside with ClAgl29B using GNINA showed that it binds to the enzyme in much the same way as L-glucose (Fig. 2C), and PNP L-Qui, which has no hydroxyl group on C6, seems to achieve a more suitable hydrophobic interaction. On the other hand, in binding with PNP L-Glc, it may adapt to the hydrophobic environment by neutralizing the polarity of OH6 by forming a hydrogen bond with Cys418 (Fig. 2A). The k_{cat}/K_m values for PNP L-Xyl of both α-L-glucosidases were as small as that for PNP L-Fuc. Thus, even with PNP L-Xyl with an equatorial OH4, the selectivity of these enzymes was found to be low. The high K_m and low k_{cat}/K_m of PNP L-Xyl can be attributable to the low affinity of α-L-xylopyranoside for the enzyme. GNINA docking simulations showed that methyl α-L-xylopyranoside could bind to the enzyme like L-glucose and methyl α-L-quinovoside (Fig. 2D). Still, the calculated affinity of methyl α-L-xylopyranoside was −5.55 kcal/mol, which was less binding than the affinity of methyl α-L-quinovopyranoside of −6.29 kcal/mol. As noted earlier, α-L-glucosidases hardly hydrolyzed PNP L-Rha. The calculated affinity for methyl α-L-rhamnoside was lower than the affinities for methyl α-L-quinovopyranoside and methyl α-L-xylopyranoside at −4.90 kcal/mol, suggesting reliable calculation. The lower affinity of α-L-xylopyranoside may be ascribed to the lack of the hydroxymethyl or methyl group found in L-glucopyranoside or L-quinovoside; that is, it may be because it cannot take advantage of the hydrophobic interaction provided by the three Phe residues mentioned earlier. The substrate inhibition in hydrolysis of PNP L-Xyl might be caused by the binding of another PNP L-Xyl to the enzyme-substrate covalent intermediate ES′. Enzymes with a double displacement mechanism catalyze transglycosylation by binding a second substrate to the intermediate ES′. The second substrate attacks the intermediate instead of water, forming a new glycosidic linkage. However, if the reactivity of the second substrate is poor and cannot form a

Table 1. Kinetic parameters of ClAgl29A and ClAgl29B for *p*-nitrophenyl α-L-glycosides.

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_i (mM)
ClAgl29A				
PNP L-Qui	0.268 ± 0.015	1.97 ± 0.09	7.35 ± 0.47	
PNP L-Xyl	4.13 ± 1.20	0.940 ± 0.230	0.232 ± 0.024	4.07 ± 1.91
PNP L-Glc[a]	2.01 ± 0.26	2.00 ± 0.11	1.00 ± 0.08	
PNP L-Fuc[a]	0.279 ± 0.005	0.0658 ± 0.0008	0.236 ± 0.005	
ClAgl29B				
PNP L-Qui	0.417 ± 0.029	4.59 ± 0.37	12.2 ± 0.7	
PNP L-Xyl	7.90 ± 2.24	3.98 ± 0.92	0.509 ± 0.044	0.287 ± 0.140
PNP L-Glc[a]	1.79 ± 0.18	7.76 ± 0.41	4.34 ± 0.22	
PNP L-Fuc[a]	0.240 ± 0.011	0.0844 ± 0.0019	0.352 ± 0.007	

[a] Data from Shishiuchi *et al.* (2022) (Ref. [2]).

glycosidic bond even if it binds, neither the water nor the substrate can attack the intermediate, resulting in an inactive ES′S complex that causes substrate inhibition. In the transglycosylation, the second substrate is believed to bind to the +1 subsite and attack the intermediate. Substrate inhibition with PNP L-Xyl may indicate that α-L-xylosyl moiety has a high affinity for the +1 subsite.

ClAgl29A and ClAgl29B can hydrolyze both α-L-glucoside and α-L-quinovoside. This suggests that these α-L-glucosidases are not enzymes specialized for α-L-glucoside hydrolysis. Similar observations have been described in reports of the L-glucose metabolic pathway, suggesting that this pathway is shared by the inositol metabolic pathway [1]. In other words, no metabolic enzymes specific for L-glucose or α-L-glucoside have yet been identified, which would not be sufficient to endorse the natural occurrence of these compounds. Unlike α-L-glucoside, α-L-quinovoside, or more precisely α-L-quinovosyl, has been found in the *O*-antigens of *Providencia stuartii* O44, which is associated with urinary tract infections [6] and *Yersinia pseudotuberculosis*, which is a cause of pseudotuberculosis [7]. *P. stuartii* is also found in soil, freshwater, and sewage. It is, therefore, possible that lake-dwelling *C. lonarensis* may encounter the *O*-antigen of this bacterium. *P. stuartii* and *Y. pseudotuberculosis* have been studied for their *O*-antigen structures due to their virulence, and the LPS of *C. lonarensis* and other Gram-negative bacteria would also contain α-L-quinovosyl moieties, and α-L-glucosidases may be involved in their metabolism.

EXPERIMENTAL

^{1}H NMR (500 MHz) and ^{13}C (126 MHz), COSY, HSQC, HSQC-TOCSY, H2BC, and HMBC NMR spectra were recorded with a Bruker AVANCE I system. Since each compound was insoluble in deuterium oxide or methanol-d4, they were washed several times with methanol-d4 and dissolved in dimethyl sulfoxide-d6 for NMR measurement. However, the H/D substitutions were insufficient, and the ^{1}H signals derived from the hydroxyl groups were detected.

p-nitrophenyl α-L-quinovopyranoside (PNP L-Qui). ^{1}H NMR (500 MHz, DMSO-d6) δ (ppm): 8.19 (d, J = 9.3 Hz, 2H, PNP), 7.22 (d, J = 9.3 Hz, 2H, PNP), 5.58 (d, J = 3.6 Hz, 1H, H-1), 5.24 (d, J = 6.2 Hz, 1H, OH-2), 5.14 (d, J = 5.7 Hz, 1H, OH-4), 5.01 (d, J = 4.9 Hz, 1H, OH-3), 3.58 (m, 1H, H-3), 3.45 (m, 1H, H-5), 3.44 (m, 1H, H-2), 2.92 (td, J = 5.6 and 9.2 Hz, 1H, H-4), 1.06 (d, J = 6.3 Hz, 3H, H-6); ^{13}C NMR (126 MHz, DMSO-d6) δ (ppm): 162.3 (PNP), 141.8 (PNP), 126.0 (PNP), 116.9 (PNP), 97.6 (C-1), 75.9 (C-3), 75.5 (C-4), 71.6 (C-2), 69.2 (C-5), 18.0 (C-6); $^{1}J_{CH}$ = 173.1 Hz.

p-nitrophenyl α-L-rhamnopyranoside (PNP L-Rha). ^{1}H NMR (500 MHz, DMSO-d6) δ (ppm): 8.18 (d, J = 9.3 Hz, 2H, PNP), 7.21 (d, J = 9.3 Hz, 2H, PNP), 5.54 (d, J = 1.7 Hz, 1H, H-1), 5.18 (d, J = 5.2 Hz, 1H, OH-2), 4.95 (d, J = 5.0 Hz, 1H, OH-4), 4.78 (d, J = 4.8 Hz, 1H, OH-3), 3.86 (t, J = 4.6 Hz, 1H, H-2), 3.64 (ddd, J = 3.5, 5.7 and 9.1 Hz, 1H, H-3), 3.38 (qd, J = 6.1 and 9.6 Hz, 1H, H-2), 3.30 (td, J = 5.6 and 9.3 Hz, 1H, H-4), 1.08 (d, J = 6.1 Hz, 3H, H-6); ^{13}C NMR (126 MHz, DMSO-d6) δ (ppm): 161.4 (PNP), 141.8 (PNP), 125.9 (PNP), 116.8 (PNP), 98.6 (C-1), 71.8 (C-4), 70.4 (C-3), 70.2 (C-5), 70.0 (C-2), 18.0 (C-6); $^{1}J_{CH}$ = 172.8 Hz.

p-nitrophenyl α-L-xylopyranoside (PNP L-Xyl). Two sugar ring conformations, $^{1}C_4$ and $^{4}C_1$, were detected in an H-1 integral ratio of 1.18:0.84. ^{1}H NMR (500 MHz, DMSO-d6) δ (ppm): 8.19 (d, J = 9.3 Hz, PNP), 7.22 (d, J = 9.3 Hz, PNP), 5.61 (d, J = 3.5 Hz, H-1, $^{1}C_4$), 5.46 (d, J = 4.1 Hz, H-1, $^{4}C_1$), 5.26 (d, J = 6.3 Hz, 1H, OH-2, $^{1}C_4$), 5.21 (d, J = 5.3 Hz, OH-2, $^{4}C_1$), 5.13 (d, J = 5.3 Hz, OH-4, $^{1}C_4$), 5.06 (d, J = 4.9 Hz, OH-3, $^{1}C_4$), 5.04 (d, J = 4.4 Hz, OH-4, $^{4}C_1$), 4.97 (d, J = 4.8 Hz, OH-3, $^{4}C_1$), 3.82 (m, H-2, $^{4}C_1$), 3.70 (m, H-3, $^{4}C_1$), 3.69 (m, H-5a, $^{4}C_1$), 3.68 (m, H-4, $^{4}C_1$), 3.57 (m, H-3, $^{1}C_4$), 3.53 (m, H-5a, $^{1}C_4$), 3.45 (m, H-2, $^{1}C_4$), 3.40 (m, H4, $^{1}C_4$), 3.32 (dd, J = 6.8 and 10.9 Hz, H-5b, $^{4}C_1$), 3.26 (t, J = 10.7 Hz, H-5b, $^{1}C_4$); ^{13}C NMR (126 MHz, DMSO-d6) δ (ppm): 162.1 (PNP), 141.9 (PNP), 126.0 (PNP), 116.9 (PNP), 98.7 (C-1, $^{4}C_1$), 97.7 (C-1, $^{1}C_4$), 73.3 (C-3, $^{1}C_4$), 71.4 (C-2, $^{1}C_4$), 71.0 (C-3, $^{4}C_1$), 69.5 (C-4, $^{1}C_4$), 69.0 (C-2, $^{4}C_1$), 67.1 (C-4, $^{4}C_1$), 64.6 (C-5, $^{4}C_1$), 63.2 (C-5, $^{1}C_4$); $^{1}J_{CH}$ ($^{1}C_4$) = 177.0 Hz, $^{1}J_{CH}$ ($^{4}C_1$) = 170.9 Hz.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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