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Synthesis of an aryl 2-deoxy-β-glycosyl tetrasaccharide to probe retaining *endo*-glycosidase mechanism

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Dedicated to Professor George Fleet on occasion of his 65th birthday

ABSTRACT

The synthesis of the 1,3–1,4-β-glucanase substrate analogue 4-nitrophenyl *O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-2-desoxi-β-D-glucopyranoside **2** is reported. Starting from the main tetrasaccharide obtained by enzymatic depolymerization of barley β-glucan, the synthetic scheme involves preparation of the corresponding 3-*O*-substituted glycal which was converted into a 2-deoxy-α-glycosyl iodide as a glycosyl donor. The key glycosylation step was successfully achieved by nucleophilic substitution of the iodide donor with 4-nitrophenolate with high β-selectivity.

1. Introduction

Deoxy glycosides have been traditionally used in mechanistic and kinetic studies of glycosidases as analogues of their natural substrates to probe hydrogen bonding interactions in the active site of the enzyme and their role in specificity and catalytic efficiency. For retaining β-glycosidases the 2-OH of the sugar unit in subsite −1 has a major contribution to transition state stabilization. Kinetic studies for a number of glycosidases[1–6] have shown that the substrate 2-OH group contributes 5–10 kcal·mol^{-1} to transition state stabilization whereas other hydroxyl groups may only contribute 1–2 kcal·mol^{-1}. Even though 2-deoxy glycosides are more reactive than the parent 2-hydroxy compounds in terms of spontaneous hydrolysis,[7,8] they are often poor substrates or inhibitors of retaining β-glycosidases. The hydrogen bond interactions between the 2-OH group of the substrate and active site residues of the enzyme are optimized upon ring distortion leading to the transition state, which transiently increases the acidity of the hydroxyl group resulting in stabilization of the oxocarbenium-like transition state in the enzymatic mechanism of the enzyme.[9,10] A number of X-ray structures of enzyme–ligand complexes and covalent glycosyl–enzyme intermediates show strong H-bond interactions of the 2-OH group with the catalytic nucleophile, the short distance between oxygen atoms (ca. 2.5 Å) close to a low barrier hydrogen bond (LBHB).[4,5,10–13]

We have been extensively working with *Bacillus* 1,3–1,4-β-glucanases that belong to family 16 of glycoside hydrolases.[14] They are endo-glycosidases that hydrolaze mixed-linked β1→3 and β1→4 glucans such as barley β-glucan and lichenan, with a strict cleavage specificity for β1→4 linkages in 3-*O*-substituted glucopyranosyl units. Due to a number of mechanistic differences as compared to the canonical mechanism of retaining glycosidases, in particular the high efficiency shown by the enzyme for hydration of glycals in which the 1,2-double bond of the sugar unit in subsite −1 is hydrated to the 2-deoxy glycoside, prompted us to evaluate the importance of the 2-OH group of the substrates in the hydrolytic mechanism of the enzyme.

Herein we report the synthesis of the 2-deoxy analogue **2** of the aryl tetrasaccharide substrate **1** (Fig. 1). Compound **1** is a good substrate of *B. licheniformis* 1,3–1,4-β-glucanase, which catalyzes the hydrolysis of the glycosidic bond between the 3-*O*-substituted glucosyl unit and the aglycon with release of 4-nitrophenol.[15,16] The 2-deoxy analogue **2** may follow the same enzyme-catalyzed hydrolysis and comparative kinetic analysis will allow evaluation of the role of the 2-OH in the enzymatic mechanism.

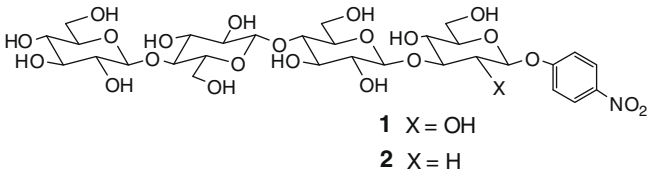

Figure 1. Aryl β-glycoside substrates for 1,3–1,4-β-glucanases.

2. Results and discussion

The main challenge in the synthesis of **2** is the construction of the aryl 2-deoxy-β-glucopyranosidic linkage. The stereoselective preparation of 2-deoxy-β-glycosides is difficult due to the lack of substituents at C-2 that provide anquimeric assistance to direct β-glycosylation.[17–19] The most common methods involve the use of a heteroatom substituent at C2 of the glycosyl donor followed by its reductive removal after glycosylation.[20] Other methods

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include the use of α-glycosyl phosphates,[21] displacement of α-glycosyl halides,[22] palladium-catalyzed glycosylation reactions,[23] and utilization of alkoxy-substituted anomeric radicals.[24] However most of these methods fail or provide low stereoselectivity for the glycosylation of phenols to obtain aryl 2-deoxy-β-glycosides. Only few methods[25–28] show β-selectivity. They involve the preparation of rather complex glycosyl donors, usually 2-deoxy-2-phenylthio- or 2-deoxy-2-phenylseleno-glycosyl donors, and need further reactions for removal of the temporary group at C-2. In selecting a direct β-glycosylation method, α-glycosyl halides have shown to readily undergo substitution with aryl alkoxy anions to provide aryl 2-deoxy-β-glycosides.[29]

The synthesis (Fig. 2) started from readily available per-O-acetylated tetrasaccharide **3** (obtained by enzymatic hydrolysis of barley β-glucan)[15] which was transformed to the α-glycosyl bromide **4** with HBr in acetic acid and then reduced to the glycal **5**. Reduction of the bromide **4** was studied by different methods because the presence of a good leaving group (the 3-*O*-cellotriosyl group) in the allylic position of the formed glycal double bond leads to rearrangements and bond cleavages that lower the yield of glycal formation when compared with the reduction of other glycosyl bromides. Reduction with Zn/acetic acid in the presence of Cu^{2+} rendered the glycal **5**, but best yields after optimization through an experimental design approach were 36%. Other methods attempted were reduction with Cr^{2+} in basic media,[30] reduction with Zn/silver/graphite[31], and electrochemical reduction in aprotic solvents,[32] but the yields did not surpass 40%. The best results were obtained by using Mn and bis(cyclopentadienyl)titanium(III) chloride (Cp_2TiCl_2) in THF,[33] with an overall yield of 50% from **4**. The glycal **5** was converted in the 2-deoxy-tetrasaccharide **6** through the mercuration–demercuration procedure of Bettelli et al.[34] First, addition of mercurium acetate to the double bond of **5** gave an organomercuric intermediate at C-2 which was directly reduced by addition of $NaBH_4$. Again, allylic reactions seemed to be responsible for the low yield obtained in this step (ca. 50%). Acetylation of the reducing end of **6** quantitatively yielded the per-O-acetylated 2-deoxy-tetrasaccharide **7**, which was ready for β-glycosylation.

The α-glycosyl iodide **8** used as glycosyl donor was obtained by treatment of **7** with iodotrimethyl silane in anhydrous CH_2Cl_2 at 0 °C,[29] and subsequently used without purification in the glycosylation step with sodium 4-nitrophenoxide. The desired aryl 2-deoxy glycoside **9** was obtained with a β:α selectivity higher than 20:1 (the α-anomer was detected by ^1H NMR in enriched fractions during the purification), along with the expected E2-elimination product (glycal **5**) and the tetrasaccharide **7**, generated by the reaction of **8** with remains of TMSAcO which were not efficiently eliminated after the glycosyl iodide synthesis. After careful chromatographic purification, the 4-nitrophenyl 2-deoxy-β-glycoside **9** was isolated with an overall 30% yield starting from **7**. Deprotection of the *O*-acetyl groups using sodium methoxide in methanol (88% yield) gave the desired analogue **2**.

Since the ^1H NMR data of compound **6** showed that the anomeric hydroxyl group at C-1 was mainly in the α-configuration, a one-step glycosylation of **6** by the method reported by Roush and Li[35] based on a Mitsunobu reaction was also attempted. Treatment of the 2-deoxy tetrasaccharide **6** with 4-nitrophenol in the presence of triphenylphosphine and diethylazodicarboxylate (DEAD) in CH_2Cl_2/toluene afforded the per-O-acetylated 4-nitrophenyl-2-deoxy saccharide **9** (α + β) in 48% overall yield. Despite of the good yield, stereoselectivity was poor, with a β:α ratio of 2.2 to 1.

3. Conclusion

In conclusion, the target 4-nitrophenyl 2-deoxy-β-glycoside **2** was obtained in seven steps from per-O-acetylated 3-*O*-β-cellotriosyl-D-glucopyranose with an overall yield of 6.1%. The synthetic sequence involves preparation of the glycal **5** under mild conditions since it its prone to rearrangements (3-O-substituted glycal). Mercuration–demercuration yielded the 2-deoxy saccharide which was converted into the 2-deoxy-α-glycosyl iodide **8**. The key glycosylation step was successfully achieved by nucleophilic substitution of the α-glycosyl iodide **8** with 4-nitrophenolate with high β-selectivity. Comparative kinetics of the 2-deoxy analogue **2** and substrate **1** to evaluate the contribution of the 2OH group of the substrate in the enzymatic hydrolysis by 1,3–1,4-β-glucanases will be reported elsewhere.

4. Experimental

4.1. General

All materials were purchased from commercial sources, except tetrasaccharide **3**, which was obtained following the procedure de-

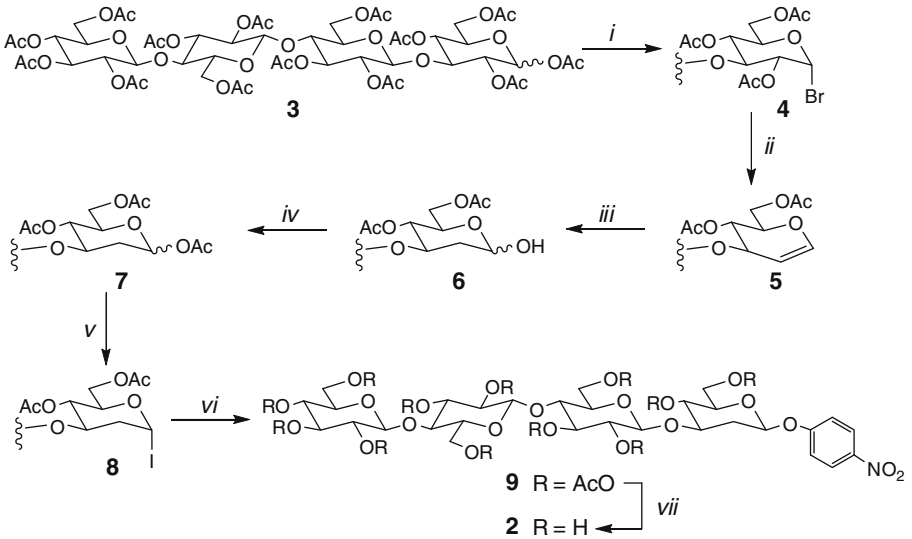

Figure 2. (i) HBr/HAcO 33%, rt, 30 min (99%); (ii) Cp_2TiCl_2, Mn powder, THF, rt, N_2 atm, 13 h (50% from **3**); (iii) (a) $Hg(AcO)_2$, THF/H_2O (4:1), 0 °C, 45 min, (b) $NaBH_4$, H_2O/THF (4:1), 0 °C, 1 min (47%); (iv) Ac_2O/Py (1:1), rt, 7 h (99%); (v) TMSI, CH_2Cl_2, 0 °C, N_2 atm, 45 min; (vi) 4-nitrophenol, NaHMDS, 15-crown-5, THF, rt, 45 min (30% from **7**); (vii) 20 mM NaOMe in MeOH, rt, 13.5 h (88%).

scribed in the literature.[15] Mn powder was dried under vacuum at 60 °C for 6 h before being used. All anhydrous solvents were distilled prior to use: THF was distilled from $LiAlH_4$; CH_2Cl_2 was distilled from CaH_2; toluene was distilled from P_2O_5; and MeOH was distilled from magnesium/iodine. TLC was performed on Macherey-Nagel Polygram SIL G/UV_{254} (0.20 mm) plates, and detection was achieved by observation under UV light at 254 and 360 nm, and by spraying with 50% H_2SO_4 and heating. Flash chromatography was carried out on silica gel (SDS, 35–70 μm). NMR spectra were recorded on a Varian-Gemini 300 instrument using tetramethylsilane as internal standard. Yields are not optimized.

4.1.1. *O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-(1→4)-*O*-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→3)-4,6-di-*O*-acetyl-1,5-anhydro-2-deoxy-D-arabino-hex-1-enitol 5

At first, 5.48 g (4.37 mmol) of tetrasaccharide **3** was suspended in 45 mL of 33% HBr in HAcO. The system was protected with a $CaCl_2$ tube and was stirred vigorously for 30 min. The resulting solution was poured onto 300 mL of ice/water and the precipitated solid was filtered and redissolved in 150 mL of $CHCl_3$. The filtrate was extracted with 3 × 150 mL of $CHCl_3$. The organic layers were combined, washed with 4 × 150 mL of saturated aqueous $NaHCO_3$, and dried over $MgSO_4$. The solvent was distilled under reduced pressure and the glycosyl bromide was dried under vacuum. Compound **4** was obtained as a white solid and was used in the next synthetic step without further purification.

Next, 2.36 g (9.48 mmol) of Cp_2TiCl_2 and 1.00 g (18.18 mmol) of Mn powder were added to the glycosyl bromide **4**. The reaction flask was sealed under an N_2 atmosphere. Then, 35 mL of anhydrous THF was added with a syringe and the solution was stirred for 13 h. The reaction mixture was filtered over Celite and collected over 30 g of silica gel. The solvent was distilled off and the silica was dried under vacuum and charged in a chromatographic column, which was eluted with cyclohexane–ethyl acetate 2:1→3:4 to yield 2.49 g (2.19 mmol, 50%) of glycal **5**.

^{1}H NMR ($CDCl_3$): δ_H = 6.45 (dd, $J_{1,2}$ = 6.5, $J_{1,3}$ = 1.0, 1H; H-1^I), [5.23–4.80, 4.59–3.54] (m, 24H; H-2$^{I–IV}$, H-3$^{I–IV}$, H-4$^{I–IV}$, H-5$^{I–IV}$, H-6$^{I–IV}$), 4.65 (d, $J_{1,2}$ = 8.0, 1H; H-1^{II}), 4.47-4.48 (2d, $J_{1,2}$ = 8.0, 2H; H-1$^{III,\ IV}$), 2.15–1.97 (12s, 36H; C*H*$_3$CO); ^{13}C NMR ($CDCl_3$): δ_C = 170.3–169.0 (CH_3CO), 144.7 (C-1^I), [99.4, 99.2, 98.1, 97.4] (C-2^I, C-1$^{II–IV}$), [76.3, 76.0, 73.2, 72.3, 72.2, 71.9, 71.8, 71.4, 71.3, 71.1, 71.0, 70.4, 69.8, 67.7, 67.1] (C-2 $^{II–IV}$, C-3 $^{I–IV}$, C-4 $^{I–IV}$, C-5 $^{I–IV}$), [62.2, 62.0, 61.4, 61.0] (C-6 $^{I–IV}$), 20.5–20.2 (*C*H$_3$CO).

4.1.2. *O*-(2,3,4,6-Tetra-*O*-acetyl)-β-D-glucopyranosyl-(1→4)-*O*-(2,3,6-tri-O-acetyl)-β-D-glucopyranosyl-(1→4)-*O*-(2,3,6-tri-*O*-acetyl)-β-D-glucopyranosyl-(1→3)-4,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranose 6

A solution of 1.83 g (5.74 mmol) of $Hg(AcO)_2$ in 15 mL of water at 0 °C was added to a solution of 2.16 g (1.90 mmol) of the glycal **5** in 60 mL of THF at 0 °C. The resulting yellow suspension was stirred at 0 °C for 45 min, then it was diluted with 225 mL of water at 0 °C and 430 mL (11.4 mmol) of $NaBH_4$ was added to the mixture. It was stirred for 1 min observing the appearance of a gray solid, and it was neutralized adding crushed CO_2. The reaction mixture was extracted with 4 × 100 mL of AcOEt and the combined organic layers were washed with 4 × 100 mL of saturated aqueous NaCl, dried over $MgSO_4$, and the solvent was distilled under vacuum. The crude reaction mixture was purified by flash chromatography (cyclohexane–ethyl acetate 3:2→1:3) and 1.03 g (891 μmol, 47%) of **6** was obtained as a white solid. ^{1}H NMR ($CDCl_3$): δ_H = 5.41 (s, 1H; H-1^I), 5.15–3.52 (m, 26H; H-1$^{II–IV}$, H-2$^{II–IV}$, H-3$^{I–IV}$, H-4$^{I–IV}$, H-5$^{I–IV}$, H-6$^{I–IV}$), 2.15–1.97 (m, 38H; C*H*$_3$CO, H-2^{I}a, H-2^{I}b); ^{13}C NMR ($CDCl_3$): δ_C = 170.9–169.1 (CH_3CO), [100.8, 100.5, 99.3] (C1$^{II–IV}$), 91.6 (C1^I), [76.2, 76.1, 74.8, 72.8, 72.7, 72.6, 72.6, 72.5, 72.0, 71.7, 71.7, 71.5, 69.4, 68.3, 67.7] (C2$^{II–IV}$, C3$^{I–IV}$, C4$^{I–IV}$, C5$^{I–IV}$), [62.6, 62.1, 61.8, 61.4] (C6$^{I–IV}$), 35.3 (C2^I), 20.8–20.4 (*C*H$_3$CO).

4.1.3. Acetyl *O*-(2,3,4,6-tetra-*O*-acetyl)-β-D-glucopyranosyl–(1→4)-*O*-(2,3,6-tri-*O*-acetyl)-β-D-glucopyranosyl-(1→4)-*O*-(2,3,6-tri-*O*-acetyl)-β-D-glucopyranosyl-(1→3)-4,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside 7

At first, 2.07 g (1.79 mmol) of **6** was dissolved in 48 mL of Ac_2O/pyridine (1:1) and the mixture was stirred at rt, protected from light and moisture, for 7 h. The mixture was poured into 300 mL of ice water and the solid was filtered and redissolved in 200 mL of $CHCl_3$. The filtrate was extracted with 3 × 100 mL of $CHCl_3$. These organic layers were combined, and were washed with 3 × 100 mL of saturated aqueous $NaHCO_3$, dried over $MgSO_4$, and distilled off. The obtained white solid was dried under vacuum to yield 2.13 g (1.78 mmol, 99%) of **7** as a 4:1 mixture of β:α anomers. ^{1}H NMR ($CDCl_3$): δ_H = 6.23 (dd, $J_{1eq,2ax}$ = 3.0 Hz, $J_{(1eq,2eq)}$ = 1.0 Hz, 0.2H; H-1eq^I), 5.73 (dd, $J_{1ax,2ax}$ = 9.5 Hz, $J_{1ax,2eq}$ = 2.0 Hz, 0.8H; H-1ax^I), 5.16–3.56 (m, 26H; H-1$^{II–IV}$, H-2$^{II–IV}$, H-3$^{I–IV}$, H-4$^{I–IV}$, H-5$^{I–IV}$, H-6$^{I–IV}$), 2.15–1.97 (m, 40H; C*H*$_3$CO, H-2eq^I), 1.90-1.70 (m, 1H; H-2ax^I); ^{13}C NMR ($CDCl_3$): δ_C = 170.7-169.0 (CH_3CO), [100.8, 100.5, 98.9, 91.2] (C-1$^{I–IV}$), [77.4, 76.2, 76.1, 75.8, 72.9, 72.8, 72.7, 72.6, 72.4, 72.0, 71.7, 71.6, 71.5, 68.4, 67.7] (C-2$^{II–IV}$, C-3$^{I–IV}$, C-4$^{I–IV}$, C-5$^{I–IV}$), [62.3, 62.1, 61.7, 61.5] (C-6$^{I–IV}$), 34.5 (C-2^I), 21.0-20.5 (*C*H$_3$CO).

4.1.4. 4-Nitrophenyl *O*-(2,3,4,6-tetra-*O*-acetyl)-β-D-glucopyranosyl–(1→4)-O-(2,3,6-tri-*O*-acetyl)-β-D-glucopyranosyl-(1→4)-*O*-(2,3,6-tri-*O*-acetyl)-β-D-glucopyranosyl-(1→3)-4,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside 9

At first, 2.13 mg (1.78 mmol) of tetrasaccharide **7** was added to 15 mL of freshly distilled CH_2Cl_2 and the resulting solution was cooled to 0 °C. Two hundred and seventy microliters (1.98 mmol) of iodotrimethylsilane were added and the mixture was stirred at 0 °C for 45 min. After evaporation of the solvent under reduced pressure, the residue was dissolved in 10 mL of anhydrous toluene and the solvent was evaporated again. The orange oil thus obtained (the α-glycosyl iodide **8**) was redissolved in 15 mL of anhydrous THF and added, under a nitrogen atmosphere, to a 15 mL anhydrous THF solution of 401 mg (2.88 mmol) 4-nitrophenol, 430 μL (2.17 mmol) of 15-crown-5, and 2.20 mL sodium hexamethyldisilazide (1 M in THF). After stirring for 45 min, the mixture was diluted with 100 mL of $CHCl_3$ and washed with 4 × 100 mL of aqueous 1 N NaOH and 100 mL of saturated aqueous NaCl. The organic layer was dried over $MgSO_4$, the solvent was evaporated, and the residue was purified by flash chromatography ($CHCl_3$–ethyl acetate 3:2), to yield 390 mg (0.31 mmol) of **9**. Other fractions containing **9** were purified again by flash chromatography, to yield an additional 291 mg (0.23 mmol) of **9**, resulting in a total yield of 30% from **7**.

^{1}H NMR ($CDCl_3$): δ_H = 8.20–7.08 (2d, J = 9.5 Hz, 4H; $C_6H_4NO_2$), 5.30 (dd, $J_{1,2ax}$ = 8.5 Hz, $J_{1,2eq}$ = 2.5 Hz, 1H; H-1^I), 5.19–3.54 (m, 26H; H-1$^{II–IV,}$ H-2$^{II–IV}$, H-3$^{I–IV}$, H-4$^{I–IV}$, H-5$^{I–IV}$, H-6a$^{I–IV}$, H-6b$^{I–IV}$), 2.45 (ddd, J_{gem} = 13.0 Hz, $J_{2eq,3}$ = 4.5 Hz, $J_{1,2eq}$ = 2.0, 1H; H-2eq^I), 2.15-1.98 (m, 37H; C*H*$_3$CO, H-2ax^I); ^{13}C NMR ($CDCl_3$): δ_C = 170.5-169.1 (CH_3CO), 161.3 (C-1), 142.7 (C-4), 125.7 (C-3, C-5), 116.2 (C-2, C-6), [100.8, 100.5, 99.0, 96.0] (C-1$^{I–IV}$), 76.2-67.6 (C-2$^{II–IV}$, C-3$^{I–IV}$, C-4$^{I–IV}$, C-5$^{I–IV}$), 62.7-61.4 (C-6$^{I–IV}$), 34.7 (C-2^I), 20.8-20.4 (*C*H$_3$CO).

4.1.5. 4-Nitrophenyl *O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-2-deoxy-β-D-glucopyranoside 2

At first, 403 mg (0.32 mmol) of **9** was suspended in 12 mL of freshly distilled MeOH, and then 2.2 mL of a 0.165 M solution of

NaOMe in MeOH was added. After stirring with exclusion of light and moisture for 13.5 h, the precipitated solid was filtered and dried under vacuum to yield 215 mg (0.28 mmol, 88%) of **2**. ^{1}H NMR (DMSO-d_6): δ_H = 8.22–7.23 (2d, J = 9.5 Hz, 4H; $C_6H_4NO_2$), 5.49 (dd, $J_{1,2ax}$ = 9.0 Hz, $J_{1,2eq}$ = 1.0 Hz, 1H; H-1^I), [4.39, 4.33, 4.24] (3d, $J_{1,2}$ = 8.0 Hz, 3H; H-1$^{II–IV}$), 3.85–2.96 (m, 23H; H-2$^{II–IV}$, H-3$^{I–IV}$, H-4$^{I–IV}$, H-5$^{I–IV}$, H-6a$^{I–IV}$, H-6b$^{I–IV}$), 2.45 (ddd, J_{gem} = 11.0 Hz, $J_{2eq,3}$ = 4.5 Hz, $J_{1,2eq}$ = 1.0 Hz, 1H; H-2eq^I), 1.67 (ddd, J_{gem} = 11.0 Hz, $J_{1,2ax}$ = $J_{2ax,3}$ = 9.5 Hz, 1H; H-2ax^I); ^{13}C NMR (DMSO-d_6): δ_C = 161.7 (C_{Ar}-1′), 141.7 (C_{Ar}-4′), 125.7 (C_{Ar}-3′, C_{Ar}-5′), 116.5 (C_{Ar}-2′, C_{Ar}-6′), [103.2, 102.8, 100.9, 96.2] (C-1$^{I–IV}$), [80.6, 80.4, 78.4, 77.1, 76.8, 76.5, 74.9, 74.8, 74.8, 74.8, 73.2, 73.0, 72.7, 70.0, 68.6] (C-2 $^{II–IV}$, C-3 $^{I–IV}$, C-4 $^{I–IV}$, C-5 $^{I–IV}$), [61.0, 60.5, 60.4, 60.4] (C-6 $^{I–IV}$), 36.1 (C-2^I).

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