Casuarine-6-*O*-α-D-glucoside and its analogues are tight binding inhibitors of insect and bacterial trehalases

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SYNTHETIC PROCEDURES

Commercial reagents were used as received. All reactions were carried out under magnetic stirring and were monitored by TLC on 0.25 mm silica gel plates (Merck F_{254}) and column chromatography was carried out on Silica Gel 60 (32-63 μm). Yields refer to spectroscopically and analytically pure compounds unless otherwise stated. ^{1}H NMR spectra were recorded on a Varian Mercury-400. ^{13}C NMR spectra were recorded on a Varian Gemini-200. Infrared spectra were recorded with a Perkin-Elmer Spectrum BX FT-IR System spectrophotometer. Mass spectra were recorded on a QMD 1000 Carlo Erba instrument by direct inlet; relative percentages are shown in brackets. ESI full MS were recorded on a Thermo LTQ instrument by direct inlet; relative percentages are shown in brackets. Elemental analyses were performed with a Perkin-Elmer 2400 analyzer. Optical rotation measurements were performed on a JASCO DIP-370 polarimeter.

(2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→6)-*O*-tris(benzyloxy)-7-deoxy-5-oxo casuarine (11). A solution of 2,3,4,6-tetra-*O*-benzyl glucopyranosyl trichloroacetimidate **10**[1] (190 mg, 0.29 mmol) and pyrrolizidine **8** (79 mg, 0.17 mmol) in dry Et_2O (3 mL) was stirred for 10 minutes at room temperature (rt) under nitrogen atmosphere in the presence of 3 Å molecular sieves (150 mg). After cooling to –20 °C and addition of TMSOTf (20 μL, 0.09 mmol), stirring was continued for 40 min.; during this period the temperature was raised to rt. The mixture was washed with a saturated aqueous solution of Na_2CO_3 (2 mL), the combined organic layers were dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified by flash column chromatography on silica gel (pentane/EtOAc 5:1, increasing polarity) to afford pure **11** as a

[1] H. Rathore, T. Hashimoto, K. Igarashi, H. Nukaya, D. S. Fullerton, *Tetrahedron*, 1985, **41**, 5427-5438.

colorless oil (140 mg, 88 % yield). $[\alpha]_D^{22}$ = +44.0 (c 1.23, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.48-7.46 (m, 2 H, H-Ar), 7.38-7.25 (m, 31 H, H-Ar), 7.17-7.14 (m, 2 H, H-Ar), 5.68 (d, *J* = 3.5 Hz, 1 H, H-1), 5.05 (d, *J* = 11.3 Hz, 1 H, H-Bn), 5.02 (d, *J* = 12.1 Hz, 1 H, H-Bn), 4.86 (d, *J* = 10.5 Hz, 1 H, H-Bn), 4.79 (d, *J* = 10.9 Hz, 1 H, H-Bn), 4.75 (d, *J* = 11.7 Hz, 1 H, H-Bn), 4.65 (dd, *J* = 10.1, 8.6 Hz, 1 H, H-6'), 4.60-4.46 (m, 9 H, H-Bn), 4.37 (dd, *J* = 5.5, 5.1 Hz, 1 H, H-2'), 4.05 (m, 1 H, H-3'), 4.01 (t, *J* = 9.4 Hz, 1 H, H-4), 3.83 (dd, *J* = 7.4, 5.5 Hz, 1 H, H-1'), 3.76-3.58 (m, 7 H, H-2, H-3, H-5, Ha-6, Hb-6, H-7a', Ha-8'), 3.54 (dd, *J* = 9.8, 3.5 Hz, 1 H, Hb-8'), 2.60 (m, 1 H, Ha-7'), 2.01 (m, 1 H, Hb-7'). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 171.3 (s, C-5'), 138.8-137.4 (s, 7 C, C-Ar), 128.5-127.4 (d, 35 C, C-Ar), 95.6 (d, C-1), 89.0 (d, C-1'), 86.1 (d, C-2'), 81.5 (d, C-4), 78.8, 77.4 (d, 2 C), 75.7, 75.2 (t, C-Bn), 73.6 (d, C-6'), 73.5, 73.3, 72.5, 72.3, 71.6 (t, C-Bn), 70.8 (d, 1 C), 69.0 (t, C-8'), 68.6 (t, C-6), 59.2 (d, 1 C), 58.3 (d, C-3'), 34.9 (t, C-7'). IR ($CDCl_3$): 3090, 3067, 3033, 2868, 1702, 1454, 1363, 1099, 1071 cm^{-1}. MS *m/z*: 1018 (100, $[M+Na]^+$), 996 (35, M^+). Anal. Calcd for $C_{63}H_{65}NO_{10}$ (996.19): C, 75.96; H, 6.58; N, 1.41. Found: C, 75.75; H, 6.82; N, 1.44.

(2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→6)-*O*-tris(benzyloxy)-7-deoxy casuarine (13). To a cooled (0°C) solution of **11** (73 mg, 0.053 mmol) in dry THF (0.75 mL) a 1 M solution of $LiAlH_4$ in THF (0.250 mL) was added under nitrogen atmosphere. The mixture was stirred at RT for 1 h, then, after cooling at 0°C, a saturated aqueous solution of Na_2SO_4 (0.3 mL) was added dropwise. The suspension was then filtered through Celite®, washed with EtOAc and concentrated under reduced pressure to afford the crude product, which was purified by flash column chromatography on silica gel (EtOAc /petroleum ether 7:3, R_f=0.35) to give pure **13** as a colorless oil (30 mg, 58 %). $[\alpha]_D^{29}$ = +36.7 (c 1.20, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.35-7.17 (m, 35 H, H-Ar), 4.95 (d, *J* = 10.9 Hz, 1 H, H-Bn), 4.87 (d, *J* = 10.7 Hz, 1 H, H-Bn), 4.84 (d, *J* = 3.5 Hz, 1 H, H-1), 4.78 (d, *J* = 10.7 Hz, 1 H, H-Bn), 4.73 (d, *J* = 11.7 Hz, 1 H, H-Bn), 4.72 (d, *J* = 12.1 Hz, 1 H, H-Bn), 4.65 (d, *J* = 12.1 Hz, 1 H, H-Bn), 4.63 (d, *J* = 12.5 Hz, 1 H, H-Bn), 4.59-4.48 (m, 7 H, H-

Bn), 4.33 (m, 1 H, H-6'), 4.13 (dd, *J* = 6.6 Hz, 1 H, H-1'), 4.07 (dd, *J* = 7.4, 7.0 Hz, 1 H, H-2'), 3.95 (t, *J* = 9.4 Hz, 1 H, H-3), 3.79 (m, 1 H, H-5), 3.75 (dd, *J* = 10.5, 3.5 Hz, 1 H, Ha-6), 3.67 (dd, *J* = 9.4 Hz, 1 H, H-4), 3.65 (dd, *J* = 10.5, 1.7 Hz, 1 H, Hb-6), 3.58-3.46 (m, 5 H, H-2, H-3', H-7a', Ha-8', Hb-8'), 3.19 (dd, *J* = 11.3, 4.3 Hz, 1 H, Ha-5'), 3.05 (m, 1 H, Hb-5'), 2.21 (m, 1 H, Ha-7'), 1.93 (m, 1 H, Hb-7'). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 138.7, 138.6, 138.5, 138.3, 138.2, 138.1, 137.9 (s, C-Ar.), 128.6-127.4 (d, 35 C, C-Ar), 96.3 (d, C-1), 88.1 (d, C-1'), 84.6 (d, C-2'), 81.8 (d, C-3), 79.9 (d, 1 C), 78.8 (d, C-6'), 77.6 (d, C-4), 75.6-72.2 (t, 7 C, C-Bn), 72.1 (t, C-8'), 70.7 (d, C-5), 68.5 (t, C-6), 67.6 (d, 1 C), 66.0 (d, 1 C), 58.7 (t, C-5'), 37.8 (t, C-7'). IR ($CDCl_3$): 3088, 3066, 3031, 2926, 2866, 1496, 1453, 1363, 1207, 1070 cm^{-1}. MS *m/z*: 1004 (32, $[M+Na]^+$), 982 (100, M^+). Anal. Calcd for $C_{63}H_{67}NO_9$ (982.21): C, 77.04; H, 6.88; N, 1.43. Found: C, 77.38; H, 6.74; N, 1.73.

6-α-D-Glucopyranosyl-(1→6)-*O*-7-deoxy-casuarine (5). To a stirred solution of **13** (46 mg, 0.05 mmol) in MeOH (5 mL), 20 mg of Pd (10% on C) and 2 drops of conc. HCl were added. The suspension was stirred at rt under hydrogen atmosphere for 12 h then filtered through Celite® and washed with MeOH. Evaporation under reduced pressure afforded a vitreous solid that was transferred to a column of DOWEX 50WX8 and then washed with MeOH (15 mL), H_2O (10 mL) to remove non amine containing products and then with 7% NH_4OH (25 mL) to elute product **5**. Evaporation of the solvent afforded **5** as a colorless highly hygroscopic solid (13 mg, 77% yield). $[\alpha]_D^{29}$ = +97.8 (c 0.88, H_2O). ^{1}H-NMR (400 MHz, D_2O) δ 4.89 (d, *J* = 3.9 Hz, 1 H, H-1), 4.38 (m, 1 H, H-6'), 4.03 (t, *J* = 8.2 Hz, 1 H, H-1'), 3.77-3.55 (m, 7 H, H-2', Ha,b-8', H-3, H-4, H-5, Ha-6), 3.46 (dd, *J* = 9.9, 3.9 Hz, 1 H, H-2), 3.36-3.28 (m, 2 H, H7a', Hb-6), 3.22 (ddd, *J* = 9.6, 6.0, 3.5 Hz, 1 H, H-3'), 3.19-3.16 (m, 1 H, Ha-5'), 3.01 (dd, *J* = 13.1, 3.7 Hz, 1 H, Hb-5'), 2.20-2.13 (m, 1 H, Ha-7'), 2.09-2.06 (m, 1 H, Hb-7'). ^{13}C-NMR (50 MHz, D_2O) δ 99.6 (d, C-1), 81.8 (d, C-1'), 81.0 (d, C-6'), 77.9 (d, C-2'), 74.8, 74.4, 73.2, 71.8, 71.4, 68.3 (d, C-2, C-3, C-4, C-5, C-3', C-7a'), 63.5, 62.6 (t, C-6, C-8'), 60.2 (t, C-5'), 37.7 (t, C-7'). ^{1}H-NMR (400 MHz, CD_3OD) δ 4.92 (d, *J* = 3.7 Hz,

1 H, H-1), 4.51 (m, 1 H, H-6'), 4.13 (t, *J* = 8.0 Hz, 1 H, H-1'), 3.89-3.63 (m, 7 H, H-2', Ha,b-6, Ha,b-8'), 3.47 (dd, *J* = 9.8, 3.8 Hz, 2 H), 3.45-3.43 (m, 1 H, H7a'), 3.39-3.31 (m, 3 H, H-3', Ha-5'), 3.12 (dd, *J* = 12.7, 3.6 Hz, 1 H, Hb-5'), 2.30-2.19 (m, 2 H, Ha,b-7'). ^{13}C-NMR (50 MHz, CD_3OD) δ 96.9 (d, C-1), 79.3 (d, C-1'), 77.5 (d, C-6'), 75.4 (d, C-2'), 72.3, 72.0, 70.9, 69.6, 69.2 (d, C-2, C-3, C-4, C-5, C-3'), 66.4 (d, C-7a') 60.4, 60.3 (t, C-6, C-8'), 57.1 (t, C-5'), 35.1 (t, C-7'). MS: *m/z* 350 (0.4 M^{+}-1), 320 (100), 172 (9), 158 (42), 68 (21) cm^{-1}. Anal. Calcd for $C_{14}H_{25}NO_9$ (351.35): C, 47.86; H, 7.17; N, 3.99. Found: C, 47.51; H, 7.11; N, 3.52.

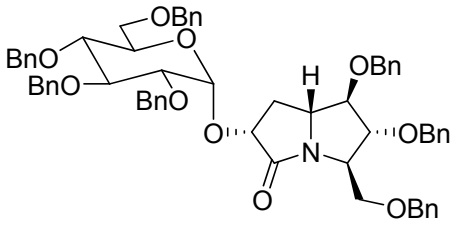
(2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→6)-*O*-tris(benzyloxy)-7-deoxy-7-methoxycarbonyl-5-oxo-casuarine (12). A solution of glucopyranosyl trichloroacetimidate **10**[1] (890 mg, 1.30 mmol) and pyrrolizidine **9** (429 mg, 0.81 mmol) in Et_2O/DCM 3:1 was stirred for 10 min. at rt under nitrogen atmosphere in the presence of 3 Å molecular sieves (200 mg). After cooling to -20 °C and addition of TMSOTf (73 μL, 0.40 mmol), stirring was continued for 1 h, during which the temperature was raised to rt. The mixture was washed with 8 mL of a saturated aqueous solution of Na_2CO_3 and the combined organic layer was dried over Na_2SO_4, filtered and concentrated to dryness. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc 7:1) to afford pure **12** (R_f=0.26 petroleum ether/EtOAc 3:1) as a colorless oil (598 mg, 75%). $[\alpha]_D^{21}$ = +32.7 (c 0.2, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.45-7.11 (m, 35 H, H-Ar), 5.80 (d, *J* = 3.5 Hz, 1 H, H-1), 5.01 (d, *J* = 10.6 Hz, 2 H, H-Bn), 5.00 (d, *J* = 9.6 Hz, 1 H, H-6'), 4.82 (d, *J* = 10.5 Hz, 1 H, H-Bn), 4.75 (d, *J* = 10.8 Hz, 1 H, H-Bn), 4.71 (d, *J* = 12 Hz, 1 H, H-Bn), 4.64 (d, *J* = 12 Hz, 1 H, H-Bn), 4.57-4.46 (m, 8 H, H-Bn), 4.27 (t, *J* = 4 Hz, 1 H, H-2'), 4.17-4.14 (m, 1 H, H-3'), 3.96 (dd, *J* = 6.4, 4.4 Hz, 1 H, H-1'), 3.94 (dd, *J* = 10.4 Hz, 1 H, H-3), 3.86 (dd, *J* = 6.4, 1 H, H-7a'), 3.77 (dd, *J* = 10 Hz, 1 H, H-5), 3.71-3.62 (m, 7 H, OCH_3, H-6a, H-6b, H-4, H-2), 3.57 (dd, *J* = 9.6 Hz, 1 H, H-8a'), 3.51 (dd, *J* = 9.6, 4.4 Hz, 1 H, H-8b'), 3.26 (dd, *J* = 10, 2 Hz, 1 H, H-7'). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 170.7, 170.0 (s, C=O). 138.6-137.1 (s, 7 C, C-Ar.), 128.3-

127.2 (d, 35 C, C-Ar), 95.2 (d, C-1), 87.8 (d, C-1'), 85.2 (d, C-2'), 81.1 (d, C-3), 78.5 (d, C-2), 76.7 (d, C-6'), 75.5 (t, C-Bn), 75.2 (d, C-5), 74.9-71.8 (t, 6 C, C-Bn), 70.5 (d, C-4), 68.3 (t, C-8'), 67.8 (t, C-6), 61.8 (d, C-7a'), 58.5 (d, C-3'), 52.3 (q, Me), 51.84 (d, C-7'). IR ($CDCl_3$): 2925, 2867, 1737 (C=O), 1711 (C=O), 1454, 1071 cm^{-1}. HRMS (ESI) for $C_{65}H_{67}NO_{12}Na$ $[M+Na]^+$ calculated: 1076.4556; found: 1076.4553. Anal. Calcd for $C_{65}H_{67}NO_{12}$ (1054.23): C, 74.05; H, 6.41; N, 1.33. Found: C, 74.05; H, 6.47; N, 1.00.

(2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→6)-*O*-tris(benzyloxy)-7-deoxy-7-[(hydroxy)methyl]-casuarine (14). To a cooled (0°C) solution of **12** (100 mg, 0.09 mmol) in dry THF (1.5 mL) a 2 M solution of $LiBH_4$ in THF (0.9 mL) and a 1 M solution of BH_3 in THF (1.8 mL) were added dropwise. The reaction mixture was stirred at rt for 5 days and then, after cooling to -20°C, H_2O was added dropwise. The mixture was filtered through Celite® and washed with $CHCl_3$, then concentrated under reduced pressure to afford a viscous oil. Purification by flash column chromatography on silica gel (EtOAc/hexane 1:3) afforded pure **14** (R_f=0.28) as a colorless oil (89 mg, 98%). $[\alpha]_D^{20}$ = +25.3 (c 0.6, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.35-7.14 (m, 35 H, H-Ar), 4.88-4.82 (m, 2H, H-Bn), 4.79 (d, *J* = 3.6 Hz, 1 H, H-1), 4.74-4.45 (m, 10 H, H-Bn), 4.37-4.28 (m, 3H, H-1', H-Bn), 4.17-4.02 (m, 3 H), 3.89-3.84 (m, 2 H), 3.77-3.73 (m, 1 H), 3.66-3.42 (m, 9 H), 3.34 (dd, *J* = 13.5, 5.3 Hz, 1 H), 2.36 (m, 1 H, H-7'). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 138.2-137.8 (s, 7 C, C-Ar.), 128.6-127.5 (d, 35 C, C-Ar), 95.5 (d, C-1), 86.9, 83.2, 81.8, 79.7, 79.6, 78.9, 77.8 (d, 1C), 75.7, 75.3, 73.7, 73.3, 72.9, 72.7, 72.2 (t, 1C), 71.2, 70.3 (d, 1 C), 69.0, 68.1, 66.9, 61.7 (t, 1 C), 53.9 (d, C-7'). IR ($CDCl_3$): 3480 (OH), 3032, 2925, 2870, 2386, 1454, 1078 cm^{-1}. HRMS (ESI) for $C_{64}H_{69}NO_{10}$ $[M^+]$ calculated: 1012.4994; found: 1012.4993. Anal. Calcd for $C_{64}H_{69}NO_{10}$ (1012.23): C, 75.94; H,6.87; N,1.38; Found: C, 75.65; H, 7.02; N, 1.19.

6-α-D-Glucopyranosyl-(1→6)-*O*-7-deoxy-7-hydroxymethyl-casuarine (6). To a stirred solution of **14** (89 mg, 0.09 mmol) in MeOH (8 mL), 20 mg of Pd (10% on C) and 2 drops of conc. HCl

were added. The suspension was stirred at rt under hydrogen atmosphere for 2 days and then filtered through Celite® and washed with MeOH. Evaporation under reduced pressure afforded a vitreous solid that was transferred to a column of DOWEX 50WX8 and then washed with MeOH (15 mL), H_2O (10 mL) to remove non amine containing products and then with 7% NH_4OH (25 mL) to elute the product **6**. Evaporation of the solvent afforded the product as a colorless highly hygroscopic solid (27 mg, 79%). $[\alpha]_D^{26}$ = + 102.5 (c 0.105, MeOH). ^{1}H-NMR (400 MHz, D_2O) δ 4.89 (d, *J* = 3.7 Hz, 1 H, H-1), 4.26-4.25 (m, 1 H, H-6'), 4.08 (t, *J* = 7.7 Hz, 1 H, H-1'), 3.77-3.42 (m, 10 H), 3.32 (dd, *J* = 9.8, 9.0 Hz, 1 H, H-3'), 3.24-3.15 (br m ,3 H, Ha-5'), 3.04 (br m, 1 H, H-7a'), 2.50-2.48 (m, 1 H, H-7'). ^{13}C-NMR (50 MHz, D_2O) δ 96.9 (d,C-1), 79.8 (d, C-1'), 79.1 (d, C-2'), 76.0 (d, C-6'), 72.3, 71.9, 70.6, 69.2, 68.6 (d, 6 C, C-2, C-3, C-4, C-5, C-3', C-7a'), 61.2, 60.9, 60.0 (t, C-6, C-8', C-9'), 51.3 (t, C-5'). IR (KBr):3399, 1419, 1023 cm^{-1}. MS (EI) *m/z*: 351 (26); 350 (85); 207 (22), 188 (37); 110 (19), 91 (22), 73 (44), 60 (100), 55 (64). Anal. Calcd for $C_{15}H_{27}NO_{10}$ (381.38): C, 47.24; H, 7.14; N, 3.67; Found: C, 46.97; H, 7.25; N, 3.56.



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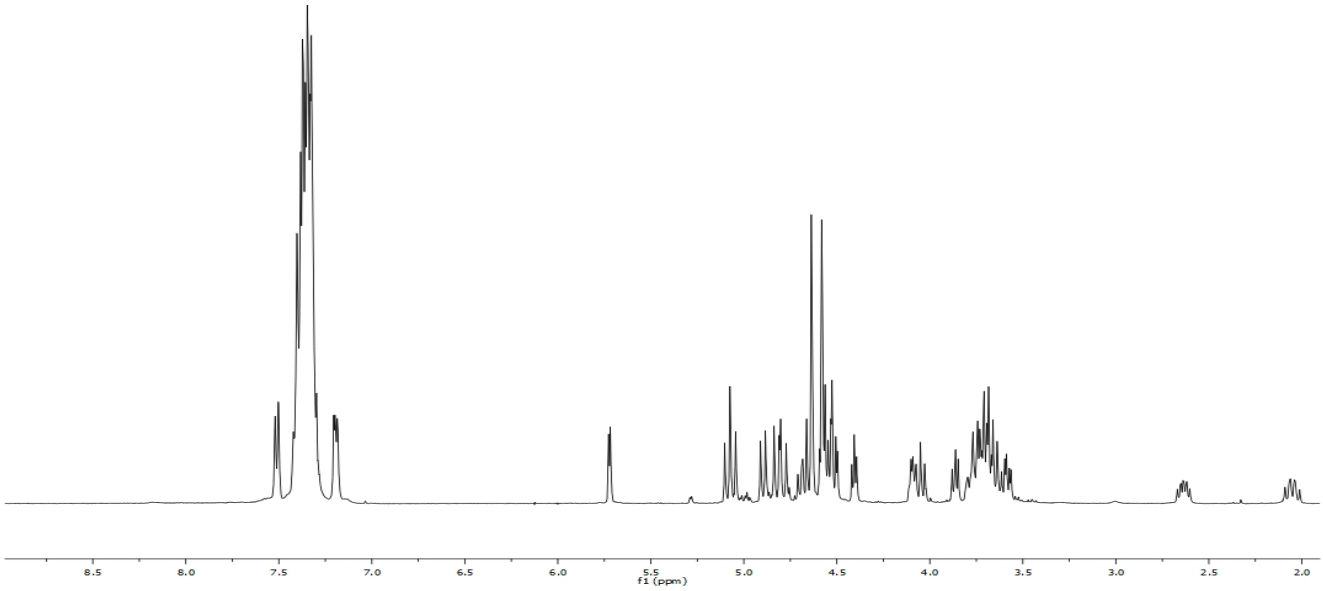


Figure S1 ^{1}H-NMR spectrum of compound 11 (400 MHz, $CDCl_3$).

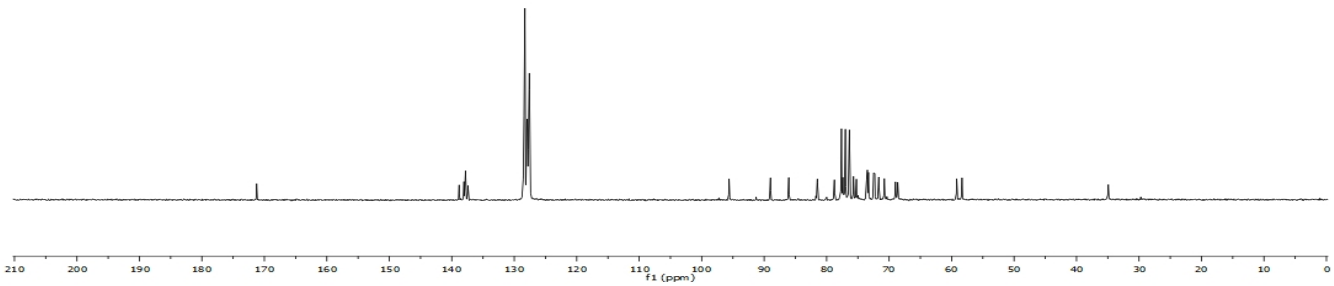
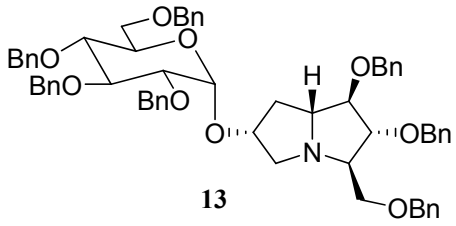


Figure S2. ^{13}C-NMR spectrum of compound 11 (50 MHz, $CDCl_3$).



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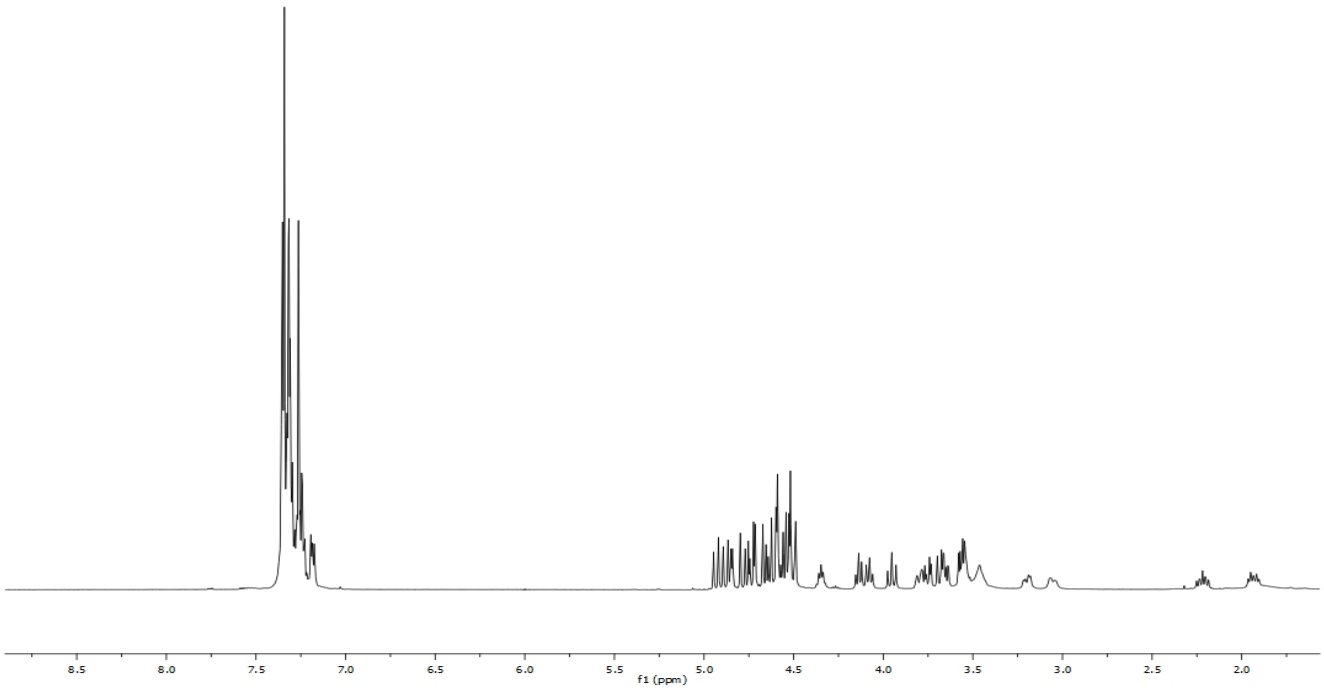


Figure S3. ^{1}H-NMR spectrum of compound 13 (400 MHz, $CDCl_3$).

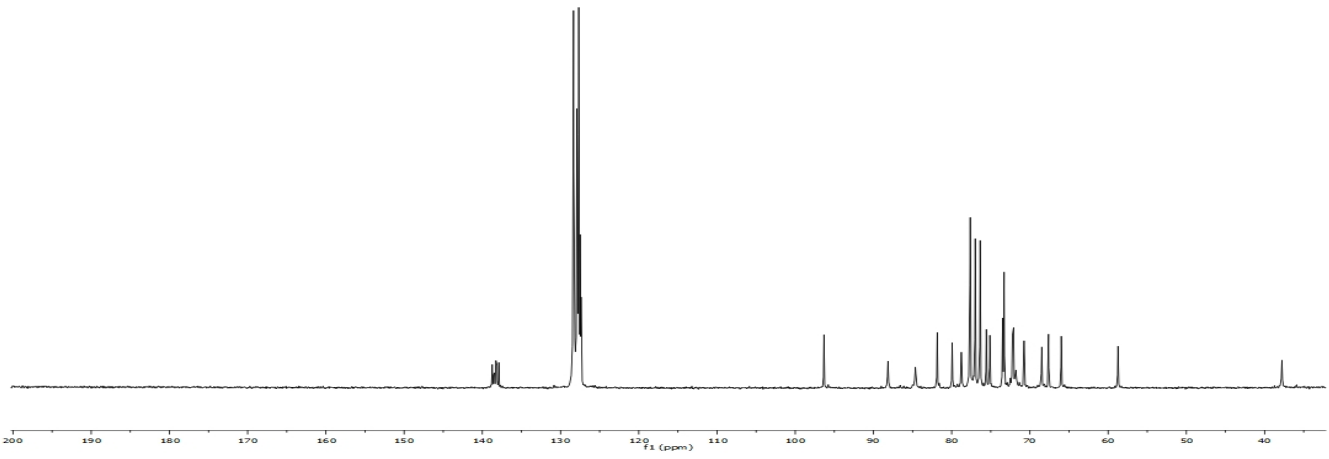
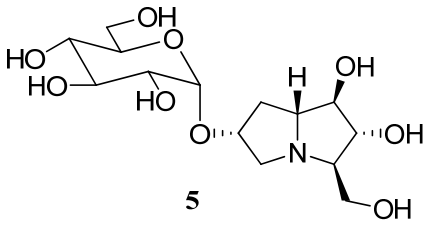


Figure S4. ^{13}C-NMR spectrum of compound 13 (50 MHz, $CDCl_3$).



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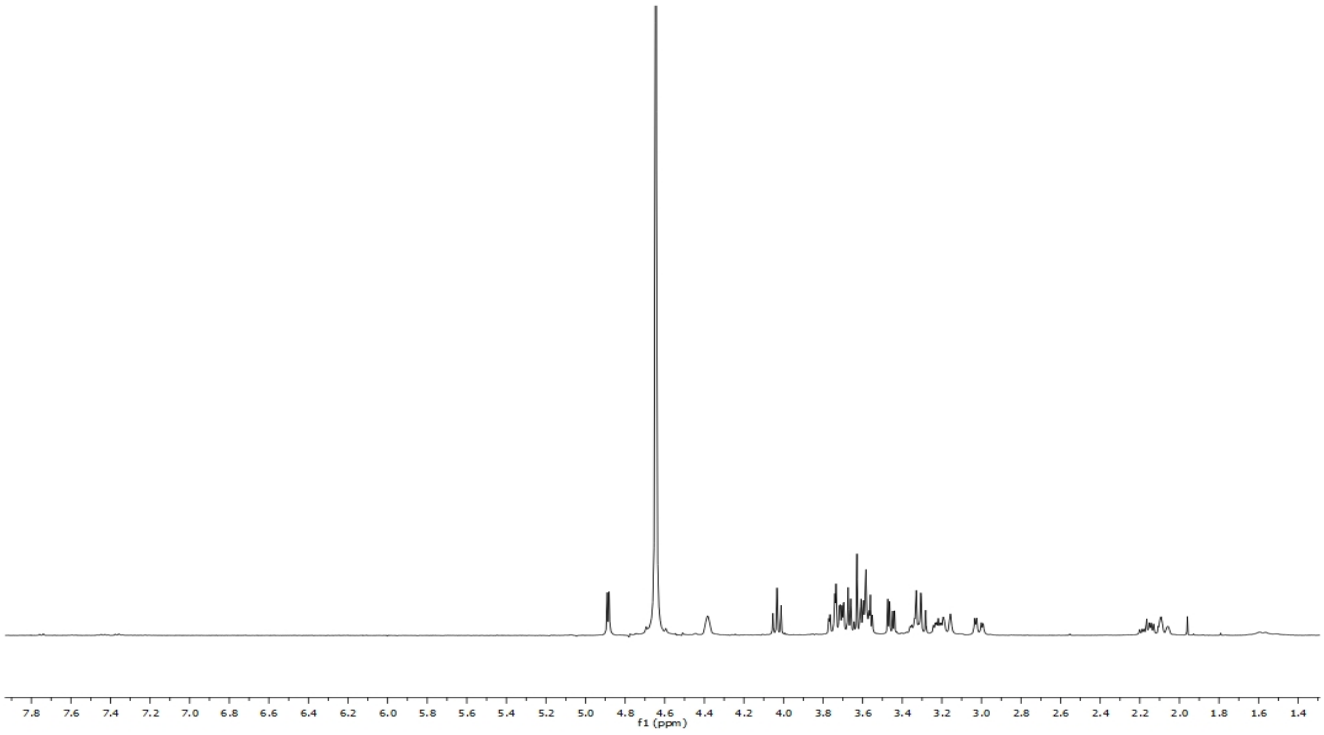


Figure S5. ^{1}H-NMR spectrum of compound 5 (400 MHz, D_2O).

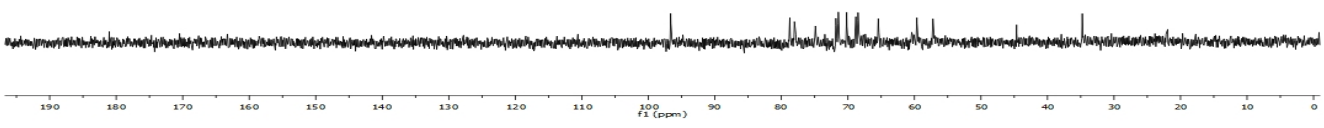
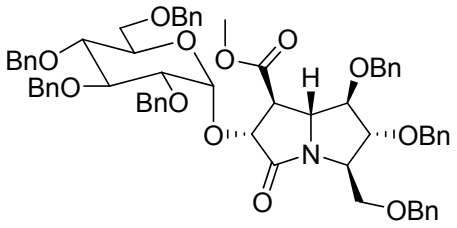


Figure S6. ^{13}C-NMR spectrum of compound 5 (50 MHz, D_3O).



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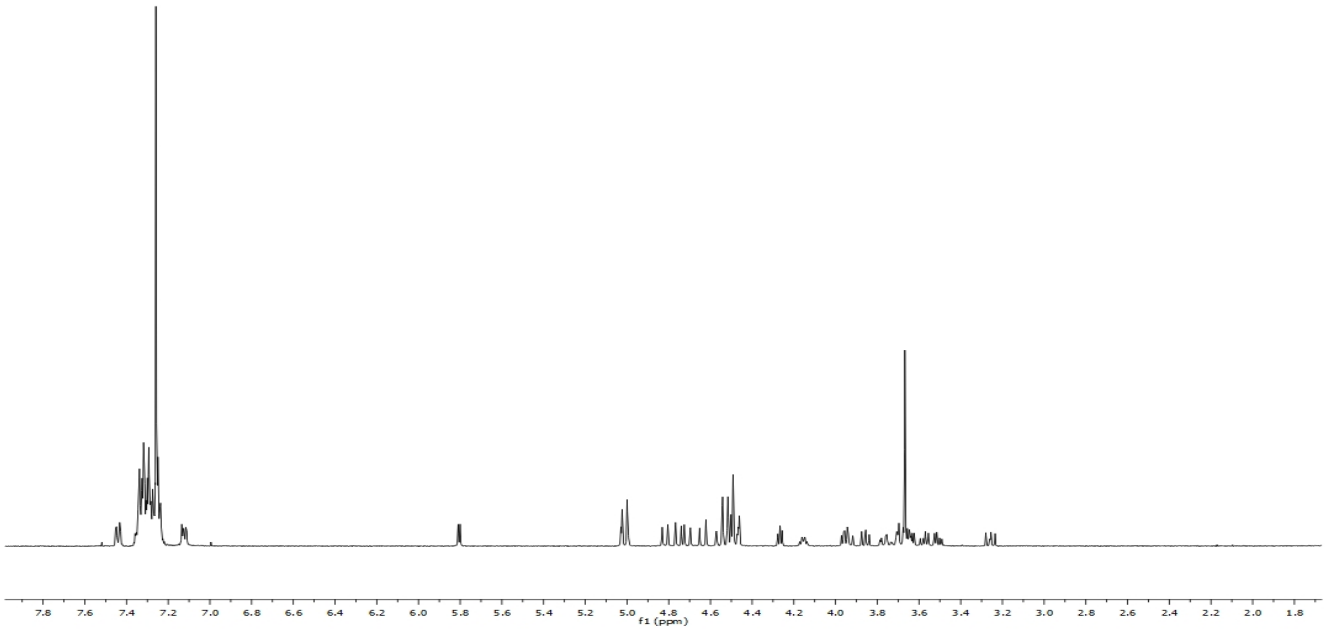


Figure S7. ^{1}H-NMR spectrum of compound 12 (400 MHz, $CDCl_3$).

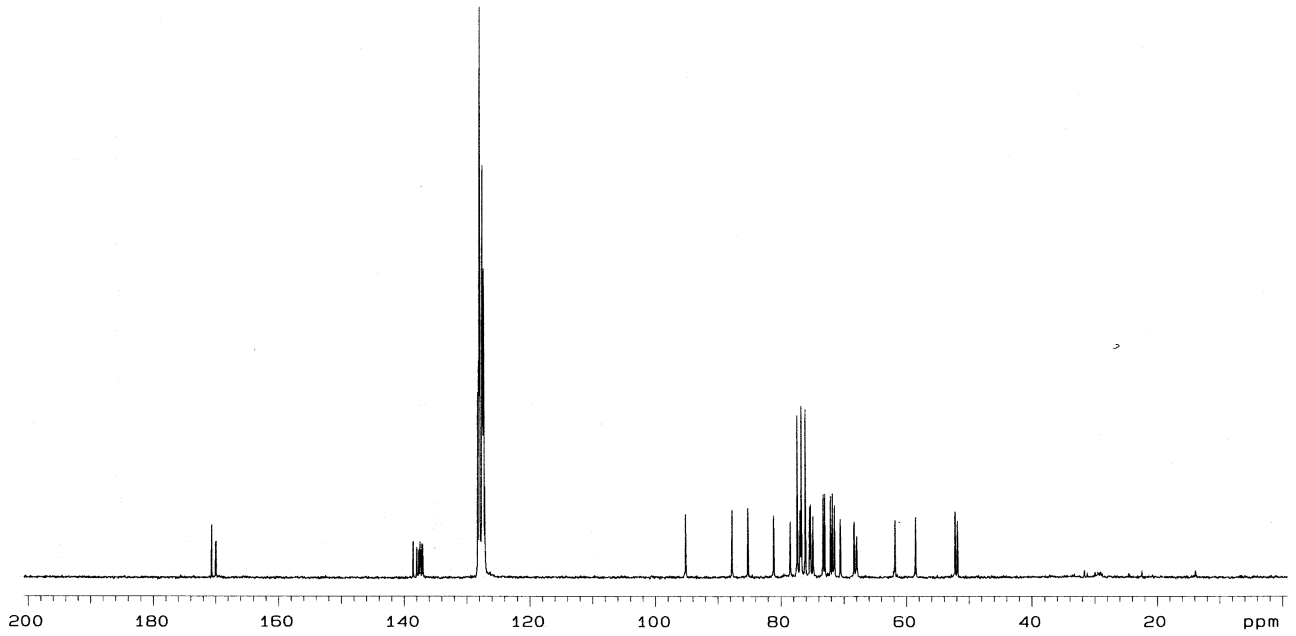
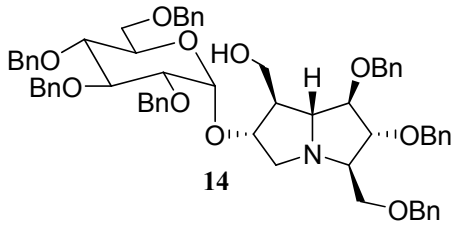


Figure S8. ^{13}C-NMR spectrum of compound 12 (50 MHz, $CDCl_3$).



14

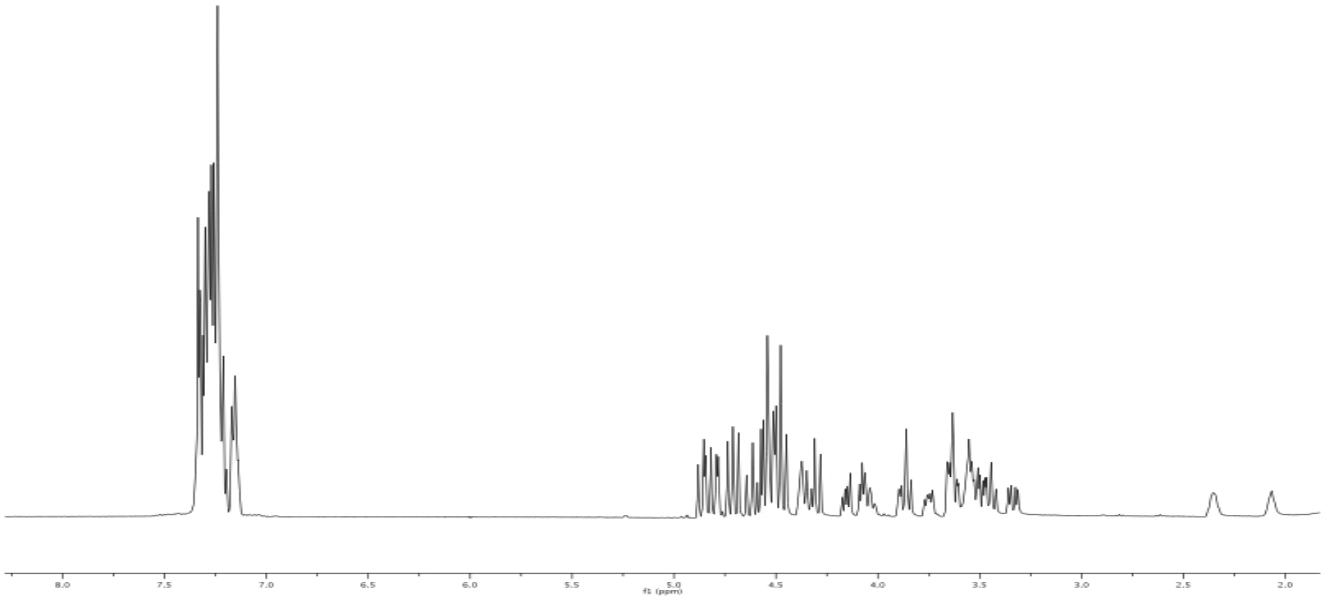


Figure S9. ^{1}H-NMR spectrum of compound 14 (400 MHz, $CDCl_3$).

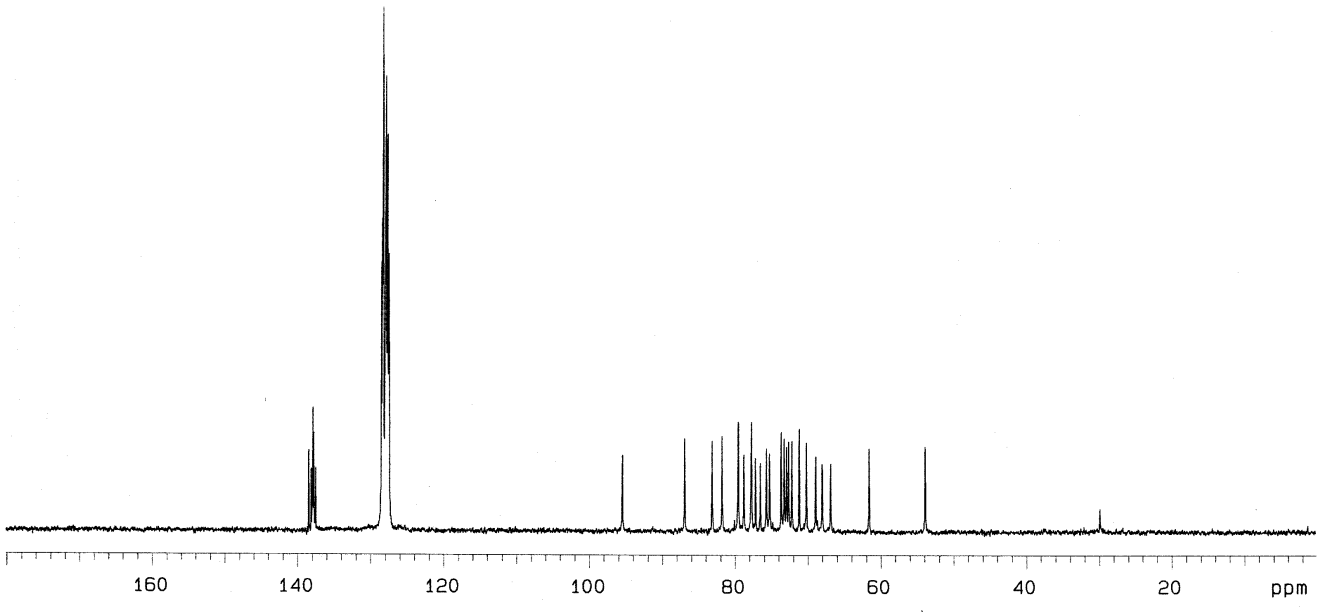
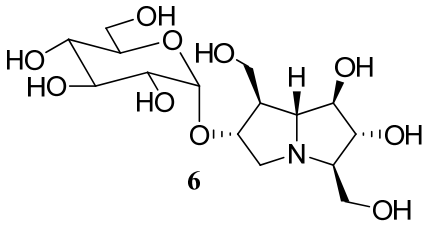


Figure S10. ^{13}C-NMR spectrum of compound 14 (50 MHz, $CDCl_3$).



6

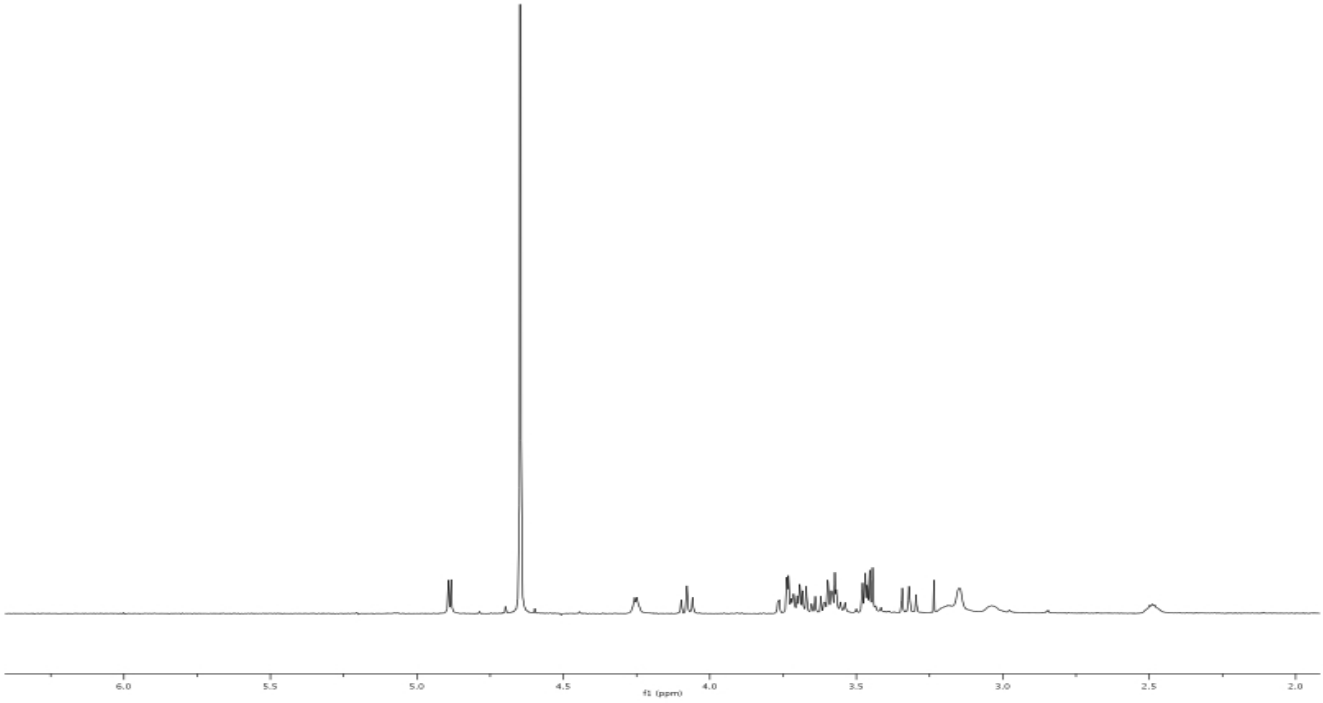


Figure S11. ^{1}H-NMR spectrum of compound 6 (400 MHz, D_2O).

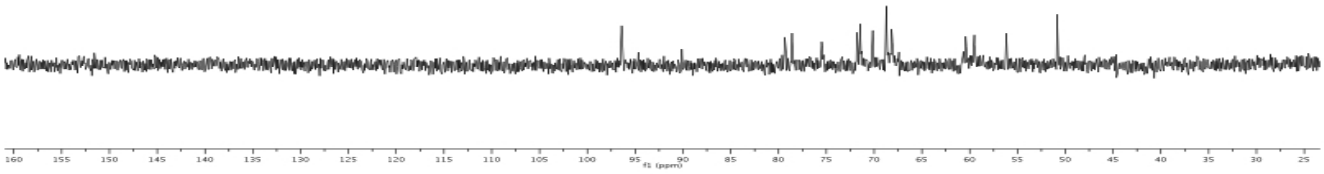


Figure S12. ^{13}C-NMR spectrum of compound 6 (50 MHz, D_2O).

ENZYME PRODUCTION AND PURIFICATION: Trehalase from *Chironomus riparius* was prepared from fresh larvae as described elsewhere.[2] Briefly, larvae were homogenized in a warring blender in 50 mM MES, pH 6.5, containing 0.1 mM phenylmethylsulfonyl fluoride and 0.01% sodium azide. The crude extract was centrifuged at 100,000 × *g* for 1 h at 4 °C and the sediment was resuspended in the homogenization buffer with 30 mM CHAPS and stirred gently at room temperature for 30 min. The preparation was centrifuged at 100,000 × *g* as above and the supernatant containing the solubilized membrane-bound trehalase was used for further processing. The suspension was applied to DEAE Sephacel (Pharmacia Biotech) column equilibrated with 20 mM Tris-HCl, pH 6.8 and trehalase was eluted with 1 M NaCl in the same buffer. Fractions containing trehalase activity were combined, concentrated by ultrafiltration under N_2 and further purified on a Mono Q column (using a FPLC system); fractions were eluted with increasing concentrations of NaCl. Active fractions were combined and subjected to affinity chromatography on a concanavalin A column. The enzyme was eluted with 0.2 M α-methyl-D-mannoside in 20 mM Tris-HCl, pH 6.8 containing 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$. The protein concentration was determined by the method of Bradford[3] using bovine serum albumin as a standard.

Tre37A was recombinantly over-expressed and purified as described previously.[4]

Trehalase from porcine kidney was a commercial preparation purchased from Sigma-Aldrich.

ENZYME KINETIC ASSAYS: Activity for the trehalases from *C. riparius* and porcine kidney was measured using a coupled assay with glucose-6-phosphate dehydrogenase and hexokinase according to the methods used by Wegener et al.[5] Enzyme assays were performed in triplicate at 30 °C using a Cary3 UV-Vis spectrophotometer. Enzyme activity was analyzed by Cary Win UV application software for Windows XP. The effect of compounds **4**-**6** on enzyme activity was evaluated at a fixed substrate concentration in the presence of increasing inhibitor concentrations. Substrate concentrations were 0.5 mM and 2.5 mM for *C. riparius* trehalase and porcine kidney

[2] M. Forcella, F. Cardona, A. Goti, C. Parmeggiani, L. Cipolla, M. Gregori, R. Schirone, P. Fusi, P. Parenti, *Glycobiology*, submitted.

[3] M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248-252.

[4] R. P. Gibson, T. M. Gloster, S. Roberts, R. A. Warren, I. Storch de Gracia, A. Garcia, J. L. Chiara and G. J. Davies, *Angew. Chem. Int. Ed.*, 2007, **46**, 4115-4119.

[5] G. Wegener, V. Tschiedel, P. Schlöder, O. Ando, *J. Experim. Biol.*, 2003, **206**, 1233-1240.

trehalase, respectively: these values are the corresponding K_m values as determined by kinetic studies. The assay was performed in sodium acetate buffer at pH 6.5 as described by Wegener et al.[5] Previous studies[2] revealed that the optimum pH is 6.5, and the assays were performed at an enzyme concentration of 4 nM and 37 nM for *C. riparius* and pig kidney trehalase, respectively. Initial rates as a function of inhibitor concentration were fitted in Sigma Plot (Jandel, CA) to the following equation:

$$\frac{v_i}{v_0} = \frac{1}{1 + \dfrac{[I]}{IC_{50}}}$$

where v_i and v_0 are the initial rates in the presence and in the absence of inhibitor, respectively, [I] the inhibitor concentration, and IC_{50} the inhibitor concentration producing half-maximal inhibition. Enzyme activities were determined in the presence of 1-1000 nM **4,** 0.01-100 μM **5** and 0.1-1000 μM **6**. K_i values were calculated using the Cheng-Prusoff relationship $IC_{50} = K_i\left(1 + \dfrac{[S]}{K_M}\right)$ assuming competitive mechanism of inhibition. Competition was assessed by measuring enzyme activity at fixed inhibitor concentration and increasing trehalose concentrations. As the estimated IC_{50} values of **4** and **5** approached the enzyme concentration (E_T), to calculate the corresponding K_i values data were fitted to the Morrison equation for tight binding inhibition:[6]

$$\frac{v_i}{v_0} = 1 - \frac{\left([E]_T + [I]_T + K_i^{app}\right) - \sqrt{\left([E]_T + [I]_T + K_i^{app}\right)^2 - 4[E]_T[I]_T}}{2[E]_T}$$

where K_i^{app} is equivalent to the IC_{50}.

Kinetics for Tre37A were performed using a stopped assay essentially as described previously.[4] Glucose release was detected using glucose oxidase/peroxidase linking enzymes (Megazyme, Bray, Eire). Assays were performed at 37 °C, in 75 mM sodium maleate buffer, pH 5.5, containing 0.38 mg mL^{-1} bovine serum albumin. Measurements were made at trehalose concentrations between 0.1 and 4 mM; Tre37A was present at a final concentration of 6.8 nM. 200 μL aliquots of the reaction

[6] R.A. Copeland, *Evaluation of Enzyme Inhibitors in Drug Discovery,* 2005, Wiley & Sons, NJ.

were taken at appropriate time intervals, boiled for 2 min to denature the enzyme, and 1 mL of the glucose oxidase/peroxidase solution was added. Assays were otherwise performed as described in the manufacturer's instructions. Rates were determined and the data fitted to the Michaelis-Menten equation in GRAFIT (Erithacus Software Ltd., Horley, UK). K_i values were determined in the same way, in the presence of 100-200 nM **5** or 3-6 μM **6**. Tre37A was pre-incubated with each of the inhibitors for 20 min prior to the start of the assay to prevent any complications with slow onset inhibition. Data were similarly fitted in GRAFIT to obtain an apparent K_M (K_M^{app}), and K_i values were determined using the equation $K_M^{app} = K_M (1+[I]/K_i)$.

CRYSTALLISATION AND STRUCTURE DETERMINATION

Tre37A crystals were grown from protein (11 mg mL^{-1}) with 5 mM compound **6**, in 1.8 M ammonium sulphate and 0.1 M citrate buffer, pH 3.5 and 10 nM sarcosine. Crystals were flash frozen using liquid nitrogen in the above conditions with the addition of 5 mM **6** and 20% ethylene glycol as cryo-protectant. Data were collected, to 2.10 Å, at the European Synchrotron Radiation Facility, Grenoble, on station ID14-4 at 100K and were processed with the HKL SUITE.[7] The data reduced to space group $P2_12_12_1$ with cell dimensions a = 94.3 Å, b = 117.0 Å and c = 203.6 Å, and there were four molecules in the asymmetric unit. The structure of the complex was solved using rigid body refinement in REFMAC[8] with PDB entry 2JJB. The model was refined with manual building in COOT[9] interspersed with refinement of geometrical restraints in REFMAC.[7] Data quality and refinement statistics are shown in Table S1. The coordinates and structure factors have been deposited in the Protein Data Bank with accession code 2WYN.

[7] Z. Otwinowski and W. Minor, *Methods Enzymol.*, 1997, **276**, 307-326.

[8] G. N. Murshudov, A. A. Vagin and E. J. Dodson, *Acta Crystallogr. D Biol. Crystallogr.*, 1997, **53**, 240-255.

[9] P. Emsley and K. Cowtan, *Acta Crystallogr. D Biol. Crystallogr.*, 2004, **60**, 2126-2132.

Table S1. Data processing and refinement statistics for Tre37A in complex with **6**.

Resolution (outer shell), Å	20-2.10 (2.17-2.10)
R_{merge} (outer shell)	0.133 (0.509)
Mean I/σI (outer shell)	13.9 (3.9)
Completeness (outer shell), %	100 (100)
Multiplicity (outer shell)	6.7 (6.3)
No. unique reflections	131010
R_{cryst}	0.16
R_{free}	0.21
RMSD bonds (Å)	0.014
RMSD angles (°)	1.48
PDB code	2WYN