Organic & Biomolecular Chemistry

PAPER

View Article Online
View Journal | View Issue

Cite this: *Org. Biomol. Chem.*, 2014, **12**, 7786

Exploring functional cyclophellitol analogues as human retaining beta-glucosidase inhibitors

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Received 29th July 2014,
Accepted 19th August 2014

DOI: 10.1039/c4ob01611d

www.rsc.org/obc

The natural product, cyclophellitol and its aziridine analogue are potent mechanism-based retaining β-glucosidase inhibitors. In this paper we explore the inhibitory potency of a number of cyclophellitol analogues against the three human retaining β-glucosidases, GBA, GBA2 and GBA3. We demonstrate that *N*-alkyl cyclophellitol aziridine is at least equally potent in inhibiting the enzymes evaluated as its *N*-acyl congener, whereas the *N*-sulfonyl analogue is a considerably weaker inhibitor. Our results complement the literature on the inhibitory potency of cyclophellitol analogues and hold promise for the future design of more effective activity-based retaining glycosidase probes with respect to probe stability in physiological media.

Introduction

Cyclophellitol **1**, first isolated in 1990 from *Phellinus* sp., is a potent and selective mechanism-based inhibitor of retaining β-glucosidases.[1] Cyclophellitol **1** is a close structural mimic of β-glucopyranose that upon binding reacts covalently and irreversibly with the active site nucleophile of retaining β-glucosidases.

Retaining β-glucosidases, and retaining β-glycosidases in general, employ a catalytic mechanism that is commonly referred to as the Koshland double displacement mechanism.[2] This process (see Fig. 1A), involves two acidic amino acid residues (Glu, Asp) residing in the enzyme active site. In retaining β-glucosidases, these acidic residues are positioned about 5–6 Å apart such that one can act as the nucleophile and the other as a general acid/base catalyst. When a retaining β-exoglucosidase binds to cyclophellitol **1**, the inhibitor is positioned in such a way that the epoxide can undergo transdiaxial opening by consecutive protonation and nucleophilic attack of the two catalytic carboxylic acid residues (Fig. 1B).[3] An ester linkage is formed that is considerably more stable than the corresponding acylal linkage that would result from the initial step in the enzymatic cleavage of beta-glucosidic linkages. As a result, hydrolysis of the covalently bound enzyme-cyclophellitol adduct is slowed down to a point that the enzyme is irreversibly inhibited.

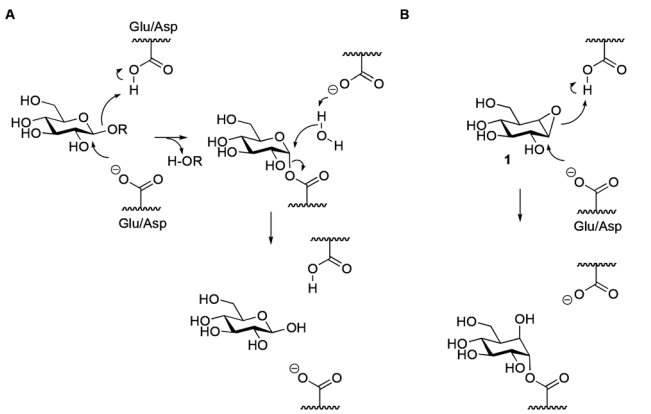

Fig. 1 Mechanism of retaining β-exoglucosidase-mediated hydrolysis of β-glucosidic linkages (A) and mechanism-based, covalent and irreversible inhibition of β-glucosidases by the natural product, cyclophellitol **1** (B).

Following the discovery of cyclophellitol **1**, Tatsuta and coworkers reported the synthesis of cyclophellitol thiirane **2** and cyclophellitol aziridine **3** (Fig. 2) as well as a number of *N*-alkyl- and *N*-acyl cyclophellitol aziridine derivatives.[4] Inhibition studies that were performed on almond β-glucosidase revealed that the nature of the *N*-substituent on cyclophellitol aziridine influences inhibitory potency. Following these initial studies, research on cyclophellitol derivatives as glycosidase inhibitors has been relatively limited[5] until recently, especially

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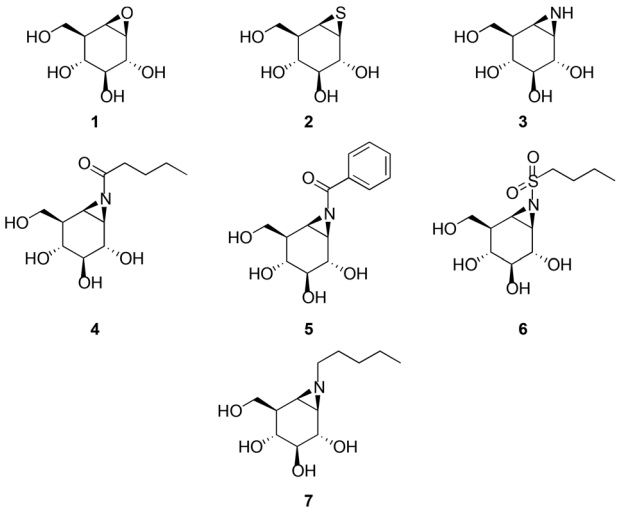


Fig. 2 Target structures subject of the here-presented studies.

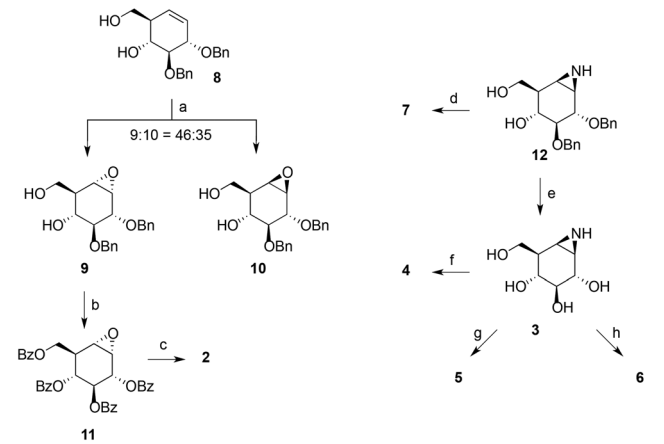
when compared to the multitude of reports describing the synthesis and evaluation of deoxynojirimycin-type competitive glycosidase inhibitors.[6] Arguably, this lack of interest stems from the covalent irreversible mode of action exerted by this compounds class – a feature not normally considered to be ideal for drug development. Indeed, research on deoxynojirimycin derivatives is often conducted with drug discovery aims in mind, with a number of *N*-alkyl deoxynojirimycin derivatives currently in clinical use or in development.[7] Cyclophellitol derivatives and more in general mechanism-based glycosidase inactivators are however receiving increasing attention as discovery tools in chemical glycobiology research.[8] 2-Deoxy-2-fluoroglycosides have proven indispensable tool to unravel the mode of action of retaining glycosidases and to confirm the double displacement mechanism originally proposed by Koshland.[9] Cyclophellitol derivatives in turn proved good starting points for the development of reagents for activity-based profiling of retaining glycosidases.[10]

We have previously shown that substitution of cyclophellitol **1** at the primary alcohol with a BODIPY reporter group yielded a highly potent and specific activity-based probe to monitor the human lysosomal retaining β-glucosidase, GBA, in cell extracts, *in situ* and *in vivo*.[11] *N*-acyl-cyclophellitol aziridine with (BODIPY or biotin) reporter groups occupying the space normally reserved for the aglycon within an exoglucosidase active site in turn proved broad-spectrum retaining β-glucosidase probes.[12] These activity-based retaining β-glucosidase probes efficiently modified all four known human retaining β-glucosidases, GBA, GBA2, GBA3 and LPH. These latter results invite a more in-depth study of modified cyclophellitol derivatives as mechanism-based inhibitors, and possible starting points for activity-based probe development for retaining β-glucosidases. We here report on the synthesis and inhibitory potential of a number of *N*-substituted cyclophellitol aziridine derivatives on the human retaining β-glucosidases, GBA, GBA2 and GBA3, in comparison with cyclophellitol (**1**), cyclophellitol thiirane (**2**) and cyclophellitol aziridine (**3**).

Results and discussion

Cyclohexene derivative **8** was reported in 2005 by Madsen and co-workers in their work[13] on the total synthesis of the natural product, cyclophellitol **1**. This intermediate, accessible in gram quantities following essentially the literature route,[13,14] proved suitable for the synthesis of all target compounds. We previously reported that mCPBA-mediated epoxidation of the homo-allylic alcohol in **8** furnished the required β-epoxide in the natural product, cyclophellitol **1** with good stereoselectivity.[14] The synthesis of β-thiirane **2** however required access to 1,6-*epi*-cyclophellitol **9**, which we could obtain in good quantities through epoxidation of **8** with *in situ* formed methyl(trifluoromethyl)-dioxirane. In this way, the desired protected 1,6-*epi*-cyclophellitol **9** was obtained as the major isomer (46%), with partially protected cyclophellitol **10** as the minor isomer. $Pd(OH)_2$-catalysed hydrogenolysis followed by perbenzoylation gave 1,6-*epi*-cyclophellitol **11**. Transformation of epoxide **11** into thiirane analogue **2** with complete inversion of configuration was performed by refluxing **11** with *N*,*N*-dimethylthioformamide (DMTF) and trifluoroacetic acid. Ensuing deacetylation under Zemplen conditions furnished cyclophellitol thiirane **2** in poor overall yield, but in sufficient quantities to conduct the planned inhibition studies. It should be noted that in their original synthesis of thiirane **2**, Tatsuta and co-workers employed a per-(methoxy)benzylated 1,6-*epi*-cyclophellitol derivative.[4] We attempted to adopt this scheme but could not reproduce this intermediate following the literature procedure (Scheme 1).

The synthesis of substituted cyclophellitol aziridines **4**–**7** started from partially protected cyclophellitol aziridine **12**, which we prepared from the Madsen intermediate as we reported recently.[14] Selective N-alkylation of **12** with iodopentane



Scheme 1 Reagents and conditions: (a) oxone, CF_3OCH_3, $NaHCO_3$, acetonitrile, 4 mM Na_2EDTA (46% **9**, 35% **10**); (b) (i) H_2, $Pd(OH)_2$/C, MeOH (97%); (ii) Bz_2O, DMAP, pyridine (26%); (c) (i); *N*,*N*-dimethylthioformamide, TFA, CH_2Cl_2, 40 °C; (ii) NaOMe, MeOH (7%); (d) (i) iodopentane, K_2CO_3, DMF, 90 °C (61%); (ii) Li, NH_3, THF, −60 °C (70%); (e) see ref. 14; (f) valeric acid, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroxyquinoline (EEDQ), DMF, 0 °C (23%); (g) benzoic acid, EEDQ, DMF, 0 °C (23%); (h) butanesulfonyl chloride, $NaHCO_3$, DMF (36%).

and potassium carbonate, followed by the removal of the benzyl ethers under Birch reduction afforded **7**. The *N*-acyl and *N*-sulfonyl derivatives **4–6** were prepared from cyclophellitol aziridine **3**, which was prepared from **12** by Birch reduction as reported previously.[14] Reaction of **12** with 1-butanesulfonyl-chloride and sodium bicarbonate in dimethylformamide furnished sulfonamide **6**. Treatment of **12** with a pre-activated solution of valeric acid or benzoic acid with 2-ethoxy-1-ethoxy-carbonyl-1,2-dihydroquinoline (EEDQ) furnished **4** and **5**, respectively. As we noted before, acylated cyclophellitol aziridines are prone to nucleophilic ring opening and in order to suppress this unwanted process, compounds **4** and **5** were purified by HPLC using either a neutral (acetonitrile–water) or basic (25–50 mM aqueous NH_4HCO_3–acetonitrile) gradient. In contrast to the acylated cyclophellitol aziridines **4** and **5**, *N*-sulfonyl aziridine **6** and *N*-alkyl aziridine **7** proved more stable and could be purified to homogeneity using standard silica gel column chromatography.

The inhibitory potential of cyclophellitol **1** and analogues **2–7** towards the three human retaining β-glucosidases GBA, GBA2 and GBA3 was evaluated by determining their apparent IC_{50} values (Table 1). To this end, the enzymes were incubated with a concentration series of the inhibitor for 30 minutes followed by measuring the residual enzymatic activity using the fluorogenic substrate 4-methylumbelliferyl β-D-glucopyranoside.

In agreement with the literature values[4] on almond β-glucosidase, cyclophellitol thiirane **2** did not inhibit the three human retaining β-glucosidases up to 100 μM. The corresponding aziridine **3** inhibits all enzymes with equal or slightly higher potency than **1** and subtle, though persistent, differences are observed in the series **4–7** when compared to **1**. Acylation as well as alkylation, but not sulphonylation, of the aziridine results in compounds that effectively inhibit GBA3. Whereas N-sulfonylation, as in **6**, results in a small drop in GBA/GBA2 inhibitory activity, N-acylation (**4**, **5**) yields inhibitors with potency about equal to that of unsubstituted aziridine **3**. The most potent inhibitor of this series however turned out to be *N*-alkyl derivative **7**, which inhibits GBA and GBA2 10–100 fold more effectively when compared to **1**. This result is of importance since compound **7** appeared considerably more stable during its synthesis and purification, and is presumably also more stable under physiological conditions.

In conclusion, we have shown that modifications of the epoxide in cyclophellitol (**1**) can have a profound effect on the inhibitory potency. As is evident, substitution of the epoxide oxygen for sulphur is detrimental for inhibitory activity. The corresponding aziridine derivative is however a viable retaining β-glucosidase inhibitor and *N*-alkyl, *N*-acyl and *N*-sulfonyl substituents are all tolerated. A subtle though important improvement is found in changing an *N*-acyl substituent to an *N*-alkyl substituent. This modification leads to an improved inhibitor with respect to potency, but also to an intrinsically more stable compound. Apart from inhibitor design, our results may have a significant impact on the future design of activity-based probes directed to retaining β-glucosidases, and to retaining glycosidases in general. Our current activity-based retaining glycosidase probe design is based on N-acylation of aziridines. While these probes are amenable for *in vitro* and *in situ* labelling of such enzymes, their intrinsic reactivity in both slightly acidic and slightly basic aqueous media make them relatively difficult to handle. We are currently exploring the synthesis and evaluation of the corresponding *N*-alkyl activity-based probes and will report on the outcome of these studies in the near future.

Table 1 Inhibitory potency (apparent IC_{50} values in μM) of compounds **1–7** against GBA, GBA2 and GBA3

	Inhibitor						
Enzyme	**1**	**2**	**3**	**4**	**5**	**6**	**7**
GBA	0.35	>100	0.5	0.07	1.4	2.5	0.017
GBA2	2	>100	0.4	0.2	0.055	0.3	0.003
GBA3	>100	>100	>100	0.5	20	0.3	0.7

Experimental

General

All reagents and solvents were a commercial grade and used as received unless stated otherwise. THF and methylene chloride were stored over flamed-dried 3 Å molecular sieves. All reactions were performed under an inert atmosphere unless stated otherwise. Solvents used for flash chromatography were of pro analysis quality. Reactions were monitored by TLC analysis using aluminum sheets pre-coated with silica gel 60 with detection by UV-absorption (254 nm) and by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$ (25 g L^{-1}) and $(NH_4)_4Ce(SO_4)_4{\cdot}H_2O$ (10 g L^{-1}) in 10% sulphuric acid followed by charring at ~150 °C or by spraying with 20% sulphuric acid in ethanol followed by charring at ~150 °C. Column chromatography was performed using Screening Device silica gel and the indicated solvents. ^{1}H NMR, ^{13}C NMR, COSY and HSQC spectra were recorded on a Bruker DMX-400 (400/100 MHz), Bruker AV-400 (400/100 MHz) and Bruker AV-600 (600/150 MHz) spectrometer in the given solvent. Chemical shifts are reported as *δ*-values in ppm relative to the chloroform residual solvent peak or tetramethylsilane (TMS) as internal standard or the deuterated solvent signal for CD_3OD. Coupling constants are given in Hz. All given ^{13}C spectra are proton decoupled. High-resolution mass spectra were recorded with a LTQ Orbitrap (Thermo Finnigan). HPLC-MS purifications were performed on an Agilent Technologies 1200 series automated HPLC system with a Quadrupole MS 6130, equipped with a semi-preparative Gemini C18 column (Phenomenex, 250 × 10, 5μ particle size).

2,3-Di-*O*-benzyl-1,6-*epi*-cyclophellitol 9. A solution of 0.4 mM Na_2EDTA solution in H_2O (21 mL) and trifluoroacetone (9.32 mL, 104 mmol) were added to **8** (2.36 g, 6.94 mmol) in acetonitrile (6.7 mL). A mixture of oxone (21.3 g, 34.7 mmol) and $NaHCO_3$ (4.08 g, 48.6 mmol) was added to the solution over a period of 15 min at 0 °C. After stirring at 0 °C

for 3 h, the reaction mixture was diluted with H_2O, extracted with EtOAc, dried over $MgSO_4$ and concentrated under reduced pressure. Purification by silica gel column chromatography (hexanes–Et_2O, 12 : 88 → 10 : 90 and 8 : 92 → 0 : 100) furnished **10** (767 mg, 2.15 mmol, 31%) and **9** (1.14 g, 3.19 mmol, 46%) as white crystals. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.41–7.17 (m, 10H, $H_{Ar}Bn$), 4.97 (d, J = 11.2 Hz, 1H, CH_2Bn), 4.79 (dd, J = 12.0, 21.2 Hz, 2H, CH_2Bn), 4.64 (d, J = 11.2 Hz, 1H, CH_2Bn), 3.86–3.77 (m, 3H, H-2, H-8), 3.56 (t, J = 10.0 Hz, 1H, H-3), 3.41 (t, J = 10.0 Hz, 1H, H-4), 3.34–3.33 (m, 1H, H-6), 3.10 (d, J = 3.6 Hz, 1H, H-1), 2.88 (br s, 1H, OH), 2.54 (br s, 1H, OH), 2.15–2.10 (m, 1H, H-5). ^{13}C NMR (100 MHz, $CDCl_3$): δ 138.2, 137.9, 128.5, 128.4, 128.0, 127.9, 127.8, 80.8, 79.4, 75.4, 72.5, 70.2, 62.5, 54.4, 54.0, 43.5. HRMS: found 357.16989 $[M + H]^+$, calculated for $[C_{21}H_{25}O_5]$ 357.16965.

1,6-*epi*-Cyclophellitol. A catalytic amount of $Pd(OH)_2$ was added to a solution of **9** (170 mg, 0.48 mmol) in MeOH (2.5 mL). The solution was stirred under H_2 atmosphere for 6 h and next filtered over a small pad of celite and concentrated under reduced pressure. The crude product was resuspended in chloroform and filtered to yield 1,6-*epi*-cyclophellitol as white crystals (82.0 mg, 0.47 mmol, 97%). ^{1}H NMR (400 MHz, MeOD): δ 3.93–3.89 (m, 2H, H-2, H-8), 3.78 (dd, J = 5.6, 11.2 Hz, 1H, H-8), 3.46 (dd, J = 2.0, 4.4 Hz, 1H, H-6), 3.41 (dd, J = 8.4, 10.0 Hz, 1H, H-3), 3.36–3.31 (m, 2H, H-1, H-4), 2.05–2.02 (m, 1H, H-5). ^{13}C NMR (100 MHz, $CDCl_3$): δ 73.0, 71.2, 69.3, 60.3, 57.5, 55.1, 44.1. HRMS: found 177.07576 $[M + H]^+$, calculated for $[C_7H_{12}O_5]$ 177.07575.

2,3,4,8-Tetra-*O*-benzoyl-1,6-*epi*-cyclophellitol 11. 1,6-*epi*-Cyclophellitol was taken up in pyridine (2.5 mL). Benzoic anhydride (1.09 g, 4.8 mmol) and a catalytic amount of DMAP were added to the solution and the reaction mixture was stirred at 50 °C for 18 h. The mixture was quenched with 1 M HCl, extracted with EtOAc, dried over $MgSO_4$ and concentrated under reduced pressure. Purification by silica gel column chromatography (hexanes–EtOAc, 84 : 16 → 80 : 20) afforded **11** (73 mg, 0.12 mmol, 26%) as a clear oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.14–8.02 (m, 5H, $H_{Ar}Bz$), 7.86 (d, J = 7.2 Hz, 1H, $H_{Ar}Bz$), 7.78 (d, J = 7.2 Hz, 1H, $H_{Ar}Bz$), 7.65–7.59 (m, 1H, $H_{Ar}Bz$), 7.57–7.33 (m, 8H, $H_{Ar}Bz$), 7.31–7.27 (m, 2H, $H_{Ar}Bz$), 7.21 (t, J = 7.6 Hz, 2H, $H_{Ar}Bz$), 5.99 (dd, J = 9.2, 10.4 Hz, 1H, H-3), 5.82 (dd, J = 2.4, 9.2 Hz, 1H, H-2), 5.67 (t, J = 10.4 Hz, 1H, H-4), 4.67 (dd, J = 3.6, 11.6 Hz, H-8), 4.49 (dd, J = 5.6, 11.6 Hz, 1H, H-8), 3.79 (dd, J = 1.6, 3.6 Hz, 1H, H-6), 3.48 (d, J = 4.0 Hz, 1H, H-1), 3.00–2.96 (m, 1H, H-5). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.2, 166.1, 165.8, 165.6, 133.6, 133.4, 133.4, 133.3, 133.1, 133.0, 130.1, 129.9, 129.8, 129.7, 129.7, 129.5, 129.4, 129.0, 128.9, 128.8, 128.7, 128.5, 128.5, 128.4, 128.3, 128.3, 128.2, 128.1, 127.8, 72.4, 70.2, 69.3, 62.9, 54.4, 53.6, 40.7. HRMS: found 593.18069 $[M + H]^+$, calculated for $[C_{35}H_{29}O_9]$ 593.18061.

Cyclophellitol thiirane 2. *N*,*N*-Dimethylthioformamide (24.5 μL, 0.29 mmol) and trifluoroacetic acid (10.7 μL, 0.14 mmol) was added to a solution of **11** (73 mg, 0.12 mmol) in anhydrous methylene chloride (7.2 mL). After stirring for 18 h at 40 °C, the reaction mixture was concentrated under reduced pressure and the resulting crude product was dissolved in MeOH (1 mL). A catalytic amount of NaOMe was added to the solution and stirred for 5 h at ambient temperature. The reaction mixture was neutralized with Amberlite IR-120 H^+, filtered and concentrated *in vacuo*. Purification by silica column chromatography (methylene chloride–MeOH, 98 : 2 → 96 : 4) afforded **3** (3.7 mg, 19 μmol, 7.0%) as a white solid. ^{1}H NMR (400 MHz, MeOD): δ 4.09 (dd, J = 4.4, 10.4 Hz, 1H, H-8), 3.97 (d, J = 7.2 Hz, 1H, H-2), 3.53 (dd, J = 8.4, 9.2 Hz, 1H, H-8), 3.50 (dd, J = 2.4, 4.8 Hz, 1H, H-6), 3.21–3.16 (m, 2H, H-3, H-4), 3.07 (d, J = 6.4 Hz, 1H, H-1), 2.33–2.27 (m, 1H, H-5). ^{13}C NMR (100 MHz, $CDCl_3$): δ 79.9, 75.6, 68.7, 66.0, 45.9, 41.6, 41.3. HRMS: found 193.05346 $[M + H]^+$, calculated for $[C_7H_{13}O_4S]$ 193.05291.

2,3-Di-*O*-benzyl-7-pentylcyclophellitol aziridine. A solution of **12** (91 mg, 0.21 mmol), 1-iodopentane (60 μL, 0.46 mmol) and potassium carbonate (87 mg, 0.63 mmol) in DMF was stirred at 90 °C for 18 h. The reaction mixture was diluted with water and subsequently extracted with Et_2O, dried over $MgSO_4$ and concentrated under reduced pressure. Purification by silica column chromatography (methylene chloride–MeOH, 97 : 3 → 96 : 4) gave the title compound (56 mg, 0.13 mmol, 61%) as colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39–7.26 (m, 10H, $H_{Ar}Bn$), 4.97 (d, J = 11.2 Hz, 1H, CH_2Bn), 4.77 (d, J = 11.6 Hz, 1H, CH_2Bn), 4.65 (dd, J = 9.2, 11.6 Hz, 2H, CH_2Bn), 3.95 (dd, J = 6.4, 10.8 Hz, 1H, H-8), 3.87 (dd, J = 4.4, 10.8 Hz, 1H, H-8), 3.71 (d, J = 8.4 Hz, 1H, H-2), 3.53–3.45 (m, 2H, H-4, OH), 3.31 (dd, J = 8.4, 10.0 Hz, 1H, H-3), 2.78 (br s, 1H, OH), 2.27 (dt, J = 7.2, 14.8 Hz, 1H, CH_2 alkyl), 2.12–2.05 (m, 1H, CH_2 alkyl), 2.02–1.97 (m, 1H, H-5), 1.72 (dd, J = 3.6, 6.0 Hz, 1H, H-6), 1.55 (d, J = 6.0 Hz, 1H, H-1), 1.47 (q, J = 7.2 Hz, 2H, CH_2 alkyl), 1.31–1.19 (m, 4H, CH_2 alkyl), 0.90 (t, J = 6.4 Hz, 3H, CH_3 alkyl).^{13}C NMR (100 MHz, $CDCl_3$): δ 138.4, 137.8, 128.8, 128.5, 128.4, 128.0, 127.9, 127.8, 127.7, 84.1, 81.0, 74.7, 72.1, 69.6, 68.1, 65.0, 61.0, 42.5, 41.5, 40.3, 29.5, 29.0, 22.5, 14.0. HRMS: found 427.26619 $[M + H]^+$, calculated for $[C_{26}H_{36}NO_4]$ 427/26724.

7-Pentylcyclophellitol aziridine 7. Ammonia (5 mL) was condensed at −60 °C. Lithium (22 mg) was added and the mixture was stirred for 30 min at −60 °C until lithium was completely dissolved. To this solution was added a solution of 2,3-di-*O*-benzyl-7-pentylcyclophellitol aziridine (56 mg, 0.13 mmol) in THF (3.0 mL). The reaction mixture was stirred for 30 min at −60 °C and subsequently quenched with milliQ-water (2 mL). The solution was allowed to come to ambient temperature and stirred until all ammonia had evolved. Next, the solution was concentrated *in vacuo*, dissolved in milliQ-water and neutralized with Amberlite IR-120 H^+. Product bound to the resin was washed with water and subsequently eluted with a 1 M NH_4OH solution and evaporated under reduced pressure. The resulting solid was again purified on Amberlite IR-120 NH_4^+ using milliQ-water as eluent until the eluate was neutral. Evaporation of the combined eluate under reduced pressure gave crude **7**, which was purified by silica column chromatography (methylene chloride–MeOH, 92 : 8) yielding **7** (8.2 mg, 34 μmol, 26%) as a white solid. ^{1}H NMR (400 MHz, MeOD): δ 3.97 (dd, J = 4.4,

10.0 Hz, 1H, H-8), 3.62–3.54 (m, 2H, H-2, H-8), 3.08 (t, J = 9.6 Hz, 1H, H-3), 3.00 (t, J = 9.6 Hz, 1H, H-4), 2.33 (dt, J = 8.0, 11.6 Hz, 1H, CH_2 alkyl), 2.16–2.10 (m, 1H, CH_2 alkyl), 1.98 (dd, J = 3.6, 6.0 Hz, 1H, H-6), 1.89–1.83 (m, 1H, H-5), 1.63 (d, J = 6.4 Hz, H-1), 1.56 (q, J = 7.6 Hz, 2H, CH_2 alkyl), 1.32–1.27 (m, 4H, CH_2 alkyl), 0.90 (t, J = 1.6 Hz, 3H, CH_3 alkyl). ^{13}C NMR (100 MHz, MeOD): δ 79.0, 73.9, 70.1, 63.7, 62.2, 45.5, 43.0, 30.7, 30.1, 23.7, 14.4. HRMS: found 246.16987 $[M + H]^+$, calculated for $[C_{12}H_{24}NO_4]$ 246.16998.

7-(*N*-Pentoyl)-cyclophellitol aziridine 4. A pre-activated mixed anhydride solution (1 M) was prepared by dissolving EEDQ (209 mg, 0.85 mmol) and valeric acid (0.09 mL, 0.85 mmol) in DMF (0.85 mL) and the reaction mixture was stirred at ambient temperature for 2 h before use. Cyclophellitol aziridine **3** (21 mg, 0.12 mmol) was dissolved in DMF (0.6 mL) and the solution was cooled to 0 °C before 1 M pre-activated mixed anhydride solution (0.06 mL, 0.06 mmol) was added. The reaction mixture was stirred at 0 °C for 30 min and additional pre-activated solution (0.06 mL, 0.06 mmol) was added. After stirring for 30 min at 0 °C, the reaction mixture was quenched with MeOH and concentrated under reduced pressure. Purification by semi-preparative reversed phase HPLC (linear gradient: 13% → 16%, 3CV, solutions used: A: H_2O, B: acetonitrile) gave the title compound (7.1 mg, 27 μmol, 23%) as a white solid. ^{1}H NMR (400 MHz, MeOD): δ 4.05 (dd, J = 4.4, 10.4 Hz, 1H, H-8), 3.70–3.65 (m, 2H, H-2, H-8), 3.19 (dd, J = 8.0, 10.0 Hz, 1H, H-3), 3.06 (t, J = 9.6 Hz, 1H, H-4), 3.01 (dd, J = 3.2, 6.0 Hz, 1H, H-6), 2.71 (d, J = 6.0, Hz, 1H, H-1), 2.49 (dt, J = 1.2, 7.2 Hz, 2H, CH_2 alkyl), 1.99–1.93 (m, 1H, H-5), 1.62–1.55 (m, 2H, CH_2 alkyl), 1.40–1.30 (m, 2H, CH_2 alkyl), 0.93 (t, J = 7.2 Hz, CH_3 alkyl). ^{13}C NMR (100 MHz, MeOD): δ 188.6, 79.1, 73.4, 69.3, 63.5, 62.2, 45.3, 42.4, 41.0, 36.7, 28.3, 23.4, 14.2. HRMS: found 260.14926 $[M + H]^+$, calculated for $[C_{12}H_{22}NO_5]$ 260.14925.

7-(*N*-Benzoyl)-cyclophellitol aziridine 5. A solution of EEDQ (84 mg, 0.34 mmol) and benzoic acid (41 mg, 0.34 mmol) were dissolved in DMF (0.34 mL) and the solution was stirred at room temperature for 2 h. Pre-activated mixed anhydride solution (0.17 μL, 0.17 mmol) was added to cyclophellitol aziridine **3** (46 mg, 0.26 mmol) in DMF (1.0 mL) at 0 °C. After stirring for 30 min at 0 °C, an additional amount of pre-activated mixed anhydride solution (0.17 μL, 0.17 mmol) was added and the reaction mixture was stirred for 2 h at 0 °C and then quenched with MeOH (1 mL) and concentrated *in vacuo*. Purification by semi-preparative reversed HPLC (linear gradient: 10% → 16%, 3CV, solutions used A: H_2O, B: acetonitrile) and lyophilization yielding **5** (17 mg, 0.06 mmol, 23%) as a white powder. ^{1}H NMR (400 MHz, MeOD): δ 7.97 (d, J = 7.2 Hz, 2H, H_{ArBz}), 7.59 (t, J = 7.2 Hz, 1H, H_{ArBz}), 7.48 (t, J = 7.6 Hz, 2H, H_{ArBz}), 4.04–4.00 (m, 2H, H-2, H-8), 3.73 (dd, J = 8.0, 10.4 Hz, 1H, H-8), 3.31–3.22 (m, 2H, H-3, H-4), 3.19 (dd, J = 3.2, 6.0 Hz, 1H, H-6), 2.72 (d, J = 6.0 Hz, 1H, H-1), 2.08–2.00 (m, 1H, H-5). ^{13}C NMR (100 MHz, MeOD): δ 181.5, 134.3, 134.1, 134.0, 130.1, 129.7, 129.5, 128.5, 79.3, 73.5, 69.7, 63.7, 45.3, 45.1, 40.8. HRMS: found 280.11765 $[M + H^+]$, calculated for $[C_{14}H_{17}NO_4]$ 280.11795.

7-(*N*-Butylsulfonyl)-cyclophellitol aziridine 6. A solution of cyclophellitol aziridine **3** (18 mg, 0.1 mmol), sodium hydrogen carbonate (56 mg, 0.67 mmol) and 1-butanesulfonyl chloride (13 μmol, 0.1 mmol) in DMF was stirred at ambient temperature for 18 h. The reaction mixture was filtered over a small pad of celite and the filtrate was concentrated under reduced pressure. Purification by silica column chromatography (methylene chloride–MeOH, 92 : 8) afforded **6** (11 mg, 36 μmol, 36%) as a colourless oil. ^{1}H NMR (400 MHz, MeOD): δ 4.02 (dd, J = 4.4, 10.4 Hz, 1H, H-8), 3.68 (d, J = 8.0 Hz, 1H, H-2), 3.54 (t, J = 9.6 Hz, 1H, H-8), 3.32–3.23 (m, 2H, CH_2 sulfonyl and MeOD solvent peak), 3.21–3.15 (m, 2H, H-3, H-6), 3.03 (t, J = 10.0 Hz, 1H, H-4), 2.87 (d, J = 6.8 Hz, 1H, H-1), 2.03–1.96 (m, 1H, H-5), 1.87–1.79 (m, 2H, CH_2 sulfonyl), 1.53–1.44 (m, 2H, CH_2 sulfonyl), 0.95 (t, 3H, CH_3 sulfonyl). ^{13}C NMR (100 MHz, MeOD): δ 78.6, 73.0, 69.2, 63.0, 52.3, 44.5, 43.7, 26.3, 22.6, 13.9. HRMS: found 308.11279 $[M + H]^+$, calculated for $[C_{11}H_{22}NO_6S]$ 308.11623.

Enzyme activity assays – determination of IC_{50} values

The activity of GBA was assayed at 37 °C by incubating with 3.75 mM 4-methylumbelliferyl-β-D-glucopyranoside (4MU-β-D-Glc) as substrate in 150 mM McIlvain buffer, pH 5.2, containing 0.1% (w/v) BSA, 0.2% (w/v) sodium taurocholate, and 0.1% (v/v) Triton X-100. Purified recombinant GBA (Cerezyme, Genzyme, USA) was employed as enzyme source. GBA3 activity was measured at 37 °C in 150 mM McIlvain buffer, pH 6.0, containing 0.1% (w/v) BSA and 3.75 mM substrate. A human splenectomized N370S/RecNcI Gaucher spleen was homogenized by sonication in cold Nanopure water, centrifuged for 30 min at 12 000 rpm (Sorvall RC-5b, Dupont Instruments, rotor SS-34), and supernatant was used as source for GBA3. The pellet was homogenized by sonication in cold water, washed thrice by centrifugation, *vide supra*, resuspended in 50 mM potassium phosphate buffer, pH 5.8, and incubated with 5 mM conduritol B epoxide for 30 min at 4 °C before being used as GBA2 source. Activity of GBA2 was measured at 37 °C in 150 mM McIlvaine buffer, pH 5.8, containing 0.1% (w/v) BSA and 3.75 mM substrate. To determine the apparent IC_{50} value, enzyme was pre-incubated with a range of inhibitor dilutions for 30 min at 37 °C, prior to the addition of 4MU-β-D-Glc. After quenching the substrate reaction with excess NaOH-glycine (pH 10.3), fluorescence was measured with a fluorimeter LS30 (Perkin Elmer) using λ_{EX} 366 nm and λ_{EX} 445 nm.

Acknowledgements

The China Scholarship Council (CSC, to JBJ), the Netherlands Organization for Scientific Research (NWO-CW, to HSO) and the European Research Council (ERC, Advanced Grant to HSO) are acknowledged for the financial support.

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