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Synthesis and biological evaluation of 8-carboxy-cyclophellitol aziridine derivatives as β-glucuronidase inhibitors and activity-based probes


6.1 Introduction

β-Glucuronidases hydrolyze β-glucuronic acid (GlcUA)-containing carbohydrates to release GlcUA and are found in all living organisms. β-Glucuronidases are classified into two glycoside hydrolase (GH) families in human, namely, GH2 and GH79, in the Carbohydrate-Active enZymes (CAZy) database, according to their amino acid sequences and tertiary structure.\(^1\)\(^2\) β-Glucuronidases hydrolyze β-glucuronide substrates through a formal two-step double displacement mechanism, first proposed by Koshland.\(^3\) Human GH2 lysosomal β-glucuronidase cleaves GlcUA residues from the non-reducing termini of glycosaminoglycans including dermatan sulphate and heparan sulfate, and its deficiency in man causes the lysosomal storage disorder, mucopolysaccharidosis type VII (MPSVII), also known as Sly syndrome.\(^4\) The GH79 family contains a number of β-glucuronidases from mammalian, fungal and bacterial,\(^5\) such as heparanase that is responsible for hydrolysis of heparin sulfate proteoglycans. β-glucuronidase activities are often associated with inflammation processes and have also been found responsible for the promotion of cancers.\(^6\)
The research described in the previous chapters focused on the development of configurational and functional isomers of the natural product, cyclophellitol, as an activity-based retaining exo-glycosidase probe scaffold. Cyclophellitol is a potent and selective mechanism-based irreversible inhibitor of retaining β-glucosidases. Substitution of the epoxide moiety in cyclophellitol for an aziridine yields cyclophellitol aziridine, which inhibits retaining β-glucosidases in a similar fashion. Structurally, cyclophellitol and its aziridine analog are close to β-glucopyranose in configuration. They fit well in the active site pocket of retaining β-glucosidases, and undergo acid-catalyzed ring-opening following nucleophilic attack on C1 by the enzyme active site nucleophilic residue (Figure 1A) to yield a stable, covalent adduct. Both cyclophellitol and cyclophellitol aziridine have been modified to yield retaining β-glucosidase-selective activity-based probes (ABPs) by grafting reporter moieties on either the cyclophellitol C8 carbon or the cyclophellitol aziridine nitrogen.

The high potency and selectivity of cyclophellitol (aziridine), together with conserved catalytic mechanism of retaining GHs, opens up the potential to exploit selective inhibitors and ABPs for other class of retaining GHs. Indeed, by adapting the configuration of cyclophellitol aziridine to the natural substrate of target GHs, potent mechanism-based inhibitors of α-glucosidases, α/β-galactosidases, and α/β-mannosidases have been prepared in the past. More recently, cyclophellitol aziridine-based ABPs have been developed for profiling α-galactosidases (GLA) and α-L-fucosidases (FUCA) (Figure 1B). These ABPs were created by N-acylation of the aziridine nitrogen with 8-azidoctanoic acid, followed by introduction of reporter groups (biotin, fluorophores) through azide-alkyne [2+3] cycloaddition ‘click’ ligation. In line with these studies, this chapter investigates the potential to develop 8-carboxy-cyclophellitol aziridine inhibitors and probes aimed at inhibiting and labeling β-D-glucuronidases. Specifically, the synthesis and enzyme inhibition properties of compounds...
2, 3, 12 and ABPs 4-11 (Figure 2), emulating in configuration and substitution pattern glucuronic acid (1) are presented. The chapter ends with describing the elucidation of the crystal structure of selected cyclophellitol aziridine derivatives in complex to the bacterial \( \beta \)-glucuronase from *Acidobacterium capsulatum*, AcaGH79.  

**Figure 2.** Structures of \( \beta \)-D-glucuronic acid, \( \beta \)-glucuronidase inhibitors and activity-based probes subject of the research described in this chapter.

### 6.2 Results and Discussion

#### Synthesis

The synthesis procedure of 8-carboxy-cyclophellitol 1 is depicted in Scheme 1. Cyclohexene diol 13, prepared as reported before,\textsuperscript{16,17} was treated with meta-chloroperbenzoic acid in a biphasic buffer to yield epoxide 14 in a stereoselective fashion as described in the literature.\textsuperscript{17} Next, the primary alcohol in 14 was selectively oxidized by 2,2,6,6-tetramethylpiperidine-1-oxyl and (diacetoxyiodo)benzene (TEMPO/BAIB) oxidation yielding carboxylate 15. Addition of tBuOH to homogenize the biphasic (DCM/H\textsubscript{2}O) reaction mixture enhanced the rate of hydration of the intermediate aldehyde, thus ensuring efficient oxidation of the lipophilic substrate. Purification of 15 with silica gel column chromatography caused 20-40% epoxide hydrolysis. To circumvent this, 15 was purified by HPLC using 50 mM aq. NH\textsubscript{4}HCO\textsubscript{3} as the mobile phase. Hydrogenolysis with hydrogen gas and Pearlman’s catalyst followed by HPLC purification yielded 8-carboxy-cyclophellitol 2, in 4.4% overall yield based on 13.

Reagents and conditions: (a) 3-chloroperbenzoic acid (mCPBA), Na₂HPO₄ (aq., 1.0 M), 1,2-dichloroethane, 50 °C, 55%; (b) BAIB, TEMPO, DCM/tBuOH/H₂O (4:4:1, v/v/v), 0 °C, 35%; (c) H₂, Pd(OH)₂/C, MeOH, 23%.

The preparation of 8-carboxy-cyclophellitol aziridine 3 started from cyclohexane diol 13 as well (Scheme 2). Partially protected aziridine 16 was prepared following the three-step procedure for the installation described in Chapter 5 for the construction of α-glucopyranosyl-configured cyclophellitol aziridines (first installation of the trichloroacetimidate, next iodo-imination, then hydrolysis of the formed cyclic iminal and final intramolecular iodine displacement to give the aziridine) in 45% yield. Removal of the benzyl groups was accomplished by Birch reduction, as reported previously to yield cyclophellitol aziridine (17). In the first instance, oxidation of the primary alcohol in 17 with catalytic amounts of TEMPO and NaClO as co-oxidant at pH 10.5 was attempted, but the desired 8-carboxy-cyclophellitol aziridine (3) was not detected in the resulting reaction mixtures. Application of TEMPO/BAIB combination led to the formation of corresponding aldehyde (from 17) as witnessed by LC-MS analysis. However, further oxidation of this aldehyde to 3 proceeded very sluggishly and only a small amount of the target product was isolated after silica gel chromatography. Using a biphasic solution (DCM/tBuOH/H₂O) and varying the concentration of the starting material in solvents also proved ineffective (Table 1). The aziridine moiety appeared to be sensitive to the oxidation conditions used. To avoid this sensitivity the aziridine nitrogen was protected as, either the fluorenylmethyloxycarbonyl (Fmoc) group (19) or the carboxybenzyl (Cbz) group (20). Subjection of 19 or 20 to the TEMPO/BAIB conditions yielded 21 or 22, respectively, in good yields (Table 1). Birch reduction on either 21 or 22 led to the efficient and simultaneous removal of all protective groups. Cation exchange chromatography (Amberlite IR120 H⁺ resin) on the crude product obtained from subjecting Fmoc aziridine 21 to the Birch conditions did not result in the isolation of pure aziridine 3, this because of concomitant elution from the resin of Fmoc protective group remnants. However, subjecting Cbz aziridine 22 to the Birch reduction/cation exchange purification sequence gave homogeneous aziridine 3 in 79% yield (21 mg) in the final deprotection/purification sequence (16% yield over the four steps starting from 13).

Scheme 2. Synthesis of 8-carboxy-cyclophellitol aziridine 3.
Synthesis and biological evaluation of 8-carboxy-cyclophellitol aziridine derivatives

Reagents and conditions: (a) i) CCl₃CN, DBU, DCM, 0 °C; ii) I₂, NaHCO₃, H₂O; iii) 37% HCl aq., dioxane, 60 °C; iv) NaHCO₃, MeOH, 45%; (b) i) NH₃ (liq.), Li, THF, -60 °C; ii) Amberlite IR120 H⁺/NH₄⁺, 79%; (c) NaOCl, NaBr, TEMPO, H₂O; (d) Fmoc-OSu, pyridine, THF, 75%; or CbzCl, pyridine, THF, 75%; (e) BAIB, TEMPO, DCM/tBuOH/H₂O (4:4:1, v/v/v), 0 °C, 92% or 60%; (f) i) NH₃ (liq.), Li, THF, -60 °C; ii) Amberlite IR120 H⁺, 79%;

Table 1. TEMPO oxidations of cyclophellitol aziridines 16, 17, 19 and 20

<table>
<thead>
<tr>
<th>Regent</th>
<th>Solvent</th>
<th>Temperature</th>
<th>Reaction time</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 TEMPO/NaClO</td>
<td>NaBr, NaOH</td>
<td>H₂O</td>
<td>20 °C</td>
<td>0</td>
</tr>
<tr>
<td>16 TEMPO/BAIB</td>
<td>ACN/H₂O (1/1, v/v)</td>
<td>0 °C</td>
<td>24h</td>
<td>0</td>
</tr>
<tr>
<td>16 TEMPO/BAIB</td>
<td>DCM/H₂O (2/1, v/v)</td>
<td>0 °C, 20 °C</td>
<td>5h</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>16 TEMPO/BAIB</td>
<td>DCM/tBuOH/H₂O (4/4/1, v/v/v)</td>
<td>0 °C</td>
<td>22h</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>16 TEMPO/BAIB</td>
<td>DCM/tBuOH/H₂O (8/8/1, v/v/v)</td>
<td>0 °C</td>
<td>3h</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>17 TEMPO/NaClO</td>
<td>NaBr, NaOH</td>
<td>H₂O</td>
<td>20 °C</td>
<td>0</td>
</tr>
<tr>
<td>19 TEMPO/BAIB</td>
<td>DCM /H₂O (2/1, v/v)</td>
<td>0 °C</td>
<td>5h</td>
<td>48%</td>
</tr>
<tr>
<td>19 TEMPO/BAIB</td>
<td>DCM/tBuOH/H₂O (4/4/1, v/v/v)</td>
<td>0 °C</td>
<td>5h</td>
<td>92%</td>
</tr>
<tr>
<td>20 TEMPO/BAIB</td>
<td>DCM/tBuOH/H₂O (4/4/1, v/v/v)</td>
<td>0 °C</td>
<td>5h</td>
<td>60%</td>
</tr>
</tbody>
</table>

With 8-carboxy-cyclophellitol (2) and 8-carboxy-cyclophellitol aziridine (3) in hand, synthetic routes of N-alkylated and N-acylated derivatives of 3 were explored. Direct acylation or alkylation of aziridine 3 proved complicated, but oxidation of the primary alcohol in partially protected, N-alkyl/acyl aziridine 17 proved more tractable (Scheme 3). Because compound 3 was hard to be obtained and compound 24 and 26 have already been synthesized in previous report. In the first instance, N-acyl aziridine 24 was prepared by acylation of the aziridine
nitrogen in 17 with 8-azidoocctanoic acid 23 using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) as the activating agent. Selective oxidation of the primary alcohol in 24 proved to be challenging, as 24 appeared to be highly unstable in both acidic and (strong) basic media. Following the optimized procedure developed for the synthesis of 8-carboxy-cyclophellitol aziridine (3), treatment of 24 with TEMPO/BAIB at 0 °C under mild basic conditions yielded crude 4. The crude reaction mixture was lyophilized and purified by HPLC using neutral (milliQ H2O/ACN) or slightly basic (25mM aq. NH4OAc) eluents, followed again by lyophilization.

Scheme 3. Synthesis of N-substituted 8-carboxy-cyclophellitol aziridine derivatives 4-11.

Reagents and conditions: (a) EEDQ, DMF, 57%; (b) TEMPO, BAIB, H2O/MeCN (1/1), NaHCO3, 0 °C, 5%; (c) CuSO4 (1.0 M in H2O), sodium ascorbate (1.0 M in H2O), DMF, 5: 5%, 6: 16%, 8: 7%, 9: 8%, 10: 21%, 11: 8%; (d) K2CO3, DMF, 50%; (e) TEMPO, NaClO, NaBr, NaOH, H2O, 15%.

Hydrolysis of the aziridine moiety proved to complicate the synthesis (copper (I)-catalyzed [2+3] azide-alkyne click ligation to BODIPY-alkyne 28 and biotin-alkyne 30 respectively) and purification of compounds 5 and 6 as well. Although these compounds could be obtained in crude form (as judged by LC-MS and NMR spectrum), no analytically pure material could be harvested from the crude products (the compounds proved to be active though as can be seen from the biological evaluation below).

The synthesis of N-alkylated 8-carboxy-cyclophellitol aziridine derivatives proved to be much easier than the synthesis of their N-acyl counterparts. Thus, reaction of 17 with
Synthesis and biological evaluation of 8-carboxy-cyclophellitol aziridine derivatives

1-azido-8-iodooctane and K$_2$CO$_3$ in DMF afforded compound 26 in 50% yield. Selective oxidation of the primary alcohol in 26 was accomplished using TEMPO/NaClO as the oxidant at pH 10.5 to afford after HPLC purification compound 7 in 15% yield. Finally, ABPs 8-11 were prepared in moderate yields but good purity by Cu(I)-catalyzed azide alkyne click ligation with BODIPY-alkynes 27 and 28, Cy5-alkyne 29 and biotin-alkyne 30, respectively, followed by HPLC purification and lyophilization. The final products proved to be stable during lyophilization and HPLC purification conditions (50 mM NH$_4$CO$_3$ in H$_2$O), and even during LC/MS detection in the presence of 1% TFA.


Reagents and conditions: (a) DMAP, Et$_3$N, TrtCl, DCM, 3 h, 75%; (b) Na, MeOH, 3 h, 91%; (c) i: (COCl)$_2$, DMSO, DCM, -65 °C, 2 h; ii: Et$_3$N, -65 to 5 °C, 2 h; iii: NH$_4$HCO$_3$, NaBH$_3$CN, 3 Å molecular sieves, MeOH, 0 °C, 1 h, then 0 °C to rt, 20 h, 85%; (d) CbzCl, THF, Et$_3$N, rt, 20 h, 72%; (e) p-TsOH, DCM, 0 °C to rt, 3 h, 83%; (f) TEMPO, BAIB, DCM/H$_2$O (2/1, v/v), 0 °C, 5 h, 71%; (g) i) BnBr, Cs$_2$CO$_3$, DMF, 3 h, 82%; ii: H$_2$, Pd(OH)$_2$/C, AcOH/H$_2$O (4/1, v/v) 16 h, 57%.

(2S,3R,4R,5S)-2,3,4-trihydroxypipecolic acid 12 was synthesized as depicted in Scheme 4. Starting from tetrabenzyl glucose 31, intermediate diol 32 was prepared in 6 steps in 83% yield following the literature procedure. Protection of the primary hydroxyl group in 32 as the trityl ether afforded 33. After deacetylation of the C-1 hydroxyl group, Swern oxidation of the two free alcohols followed by double reductive amination gave orthogonally protected deoxynjirimycin 35 following a procedure previously developed for differently protected deoxynojirimycin derivatives. Subsequently, the amine in 35 was protected as the tert-butyloxycarbamate, after which the trityl ester was removed under acidic (TsOH) conditions. Subsequently the primary alcohol of 37 was oxidized (TEMPO/BAIB) to carboxylic acid 38 and hydrogenation over palladium(II) hydroxide on carbon in aq. acetic acid gave inhibitor 12, the analytical and spectroscopical data of which matched those reported.

Comparative and competitive ABPP on GH79 β-glucuronidase AcaGH79

Having the panel of inhibitors and probes 2-12 available, their potency (as reflected by apparent IC$_{50}$ values) in inhibiting 4MU-β-glucuronide hydrolysis by recombinant
Acidobacterium capsulatum β-glucuronidase (AcaGH79) was established first. All compounds proved to inhibit AcaGH79, although their potency (IC₅₀ values) varied considerably (Table 1). Cy5-aziridine 10 proved to be the most potent inhibitor of the set of ABPs 5, 6, 8-11 (IC₅₀ 51.8 ± 7.4 nM) with the two biotin-aziridines 6, 11 being the weakest of the series (though with an IC₅₀ of around 9.5 μM and 8.1 μM, respectively, still quite potent). Of the non-tagged covalent inhibitors, acyl aziridine 4 proved to be the most potent inhibitor, slightly less outperforming than the competitive inhibitor 12. Epoxide 2 and alkyl aziridine 7 turned out to be considerably less potent AcaGH79 inhibitors. Importantly, all ABPs excepting (at the concentrations measured) biotin aziridines 6 and 11 are able to reach subcellular β-glucuronidase at nanomolar concentrations. Additionally, the potency of alkyl aziridine 7-11 is roughly equal to the potency of acyl aziridines 4-6. From this observation combined with the observation that alkyl aziridines are much more stable and easily accessible, one may conclude that N-alkylated cyclophellitol aziridine derivatives are the reagents of choice in the development of retaining β-glucuronidase activity-based probes. The cyclophellitol and cyclophellitol aziridine derivatives bind AcaGH79 covalently and irreversibly, as evident from their second order kinetic constants (measured for compounds 2, 3, 7, 8, see Figure 3A). As well, ABP 8 effectively labels AcaGH79 both at 4 °C and 37 °C (Figure 3B). Labeling of AcaGH79 with ABP 8 can be abolished by competition with either of the compounds 2, 3, 7, 12, 9-11 or with the fluorogenic substrate, 4MU-β-glucuronide as well as by denaturing AcaGH79 prior to inclusion of the probe (Figure 3C). Competitive labeling was also obtained with acyl aziridine inhibitor 4 and ABP 5 (Figure 3D). Maximal AcaGH79 labeling with ABP 8 was observed at pH 4-5, in line with the observed maximal hydrolysis of fluorogenic substrate by AcaGH79 (Figure 3E, F).

Table 1. Potency (IC₅₀) of compounds 2-12 as inhibitors of recombinant AcaGH79 from Acidobacterium capsulatum in vitro using 4MU-β-glucuronide as the fluorogenic substrate. Data were average values of two separate experiments measured in duplicate, error ranges depict standard deviation.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀(nM)</td>
<td>4,520 ± 315.9</td>
<td>1594 ± 19.6</td>
<td>952 ± 11.2</td>
<td>1111 ± 8.4</td>
<td>9525 ± 615</td>
<td>2155 ± 1193.8</td>
<td>4356 ± 28.9</td>
<td>2949 ± 19.0</td>
<td>518 ± 7.4</td>
<td>8,084.4 ± 553.2</td>
<td>44.5 ± 3.3</td>
</tr>
</tbody>
</table>
Figure 3 A) Inhibition kinetic parameters for compounds 2, 3, 7, 8. The graph depicts the obtained $K_{observed}$ and the table shows the kinetic parameters $k_i$ and $K_i'$ estimated with one-phase exponential association. Data shown represents average of two separate experiments in duplicate, and the error bars depict standard deviation. B) Time-dependent labeling of recombinant AcaGH79 with green-BODIPY ABP 8 at 4 °C and 37 °C, respectively. C) Competitive labeling of AcaGH79 with ABP 8 by pre-incubation with 10% SDS, substrate, inhibitors 2, 3, 12, 7 or probes 9, 10, 11. D) Competitive labeling of AcaGH79 with ABP 5 by pre-incubation with acyl aziridine inhibitor 4. E) Detection of AcaGH79 labeling efficiency at various pH with ABP 8. F) AcaGH79 labeling with ABP 8 (closed squares) compared to enzymatic activity of substrate 4-methylumbelliferyl $\beta$-D-glucuronide (4MU$\beta$DGluc) hydrolysis (open circles) at various pHs.

X-ray crystallography of AcaGH79 complexed to inhibitors 3, 4 and 7

In order to provide further evidence of the covalent attachment of the $\beta$-glucuronic acid-configured ABPs to $\beta$-glucuronidase, crystal structures of AcaGH79 in complex with aziridines 4 and 7 were obtained. These structures clearly showed covalent binding of the compounds, with the aziridine ring opened by the AcaGH79 active site nucleophile, Glu287 (Figure 4A, B). Both acyl aziridine 4 and alkyl aziridine 7 adopt a $4C_1$ conformation with the aziridine rings opened across the C1-C6 bond in trans-diaxial fashion. The structure of the AcaGH79 mutant in its catalytic nucleophile (E287Q) in complex with 7 revealed (Figure 4C) unhydrolyzed 7 present in the active site and adopting a $4H_3$ conformation. This conformation is also seen for competitive inhibitor 12 in wild type AcaGH79 (Figure 4D), and is consistent with the expected $1S_3$-$4H_3$-$4C_1$ catalytic itinerary employed by $\beta$-glucuronidases.20
Figure 3. Crystal structures of AcaGH79 from *Acidobacterium capsulatum* in complex with 4, 7 and 12. A) AcaGH79-acyl aziridine 4; B) AcaGH79-alkyl aziridine 7; C) Mutant E287N AcaGH79--alkyl aziridine 7; D) AcaGH79-trihydroxypipeolic acid 12.

6.3 Conclusion

In conclusion, this chapter describes the development of potent, mechanism-based β-glucuronidase inhibitors and activity-based probes. The synthesis of N-acyl-8-carboxy-cyclophellitol aziridine proved challenging and no homogeneous pure material could be obtained. The corresponding N-alkyl derivatives however were accessible and turned out to be at least as effective in inhibiting and labeling the bacterial β-glucuronidase, AcaGH79. These results match those obtained for N-alkyl-cyclophellitol aziridine and N-acyl-cyclophellitol aziridine, the reduced counterparts of the molecules described in chapter 4 of this thesis in inhibiting and labeling retaining β-glucosidases. The crystal structure of AcaGH79 complexed to 4 and 7 together with the structure of AcaGH79 complexed to the competitive inhibitor 12 (a structure matched by that of the mutant enzyme complexed with 7) gives a clear view on the conformational trajectory by which a substrate
glucuronide is processed by retaining β-glucuronidases. The activity-based β-glucuronidase probes described here will be further evaluated, both in activity-based protein profiling and in structural studies, in the next chapter.

6.4 Experimental sections

Gene cloning, expression and protein purification

The coding sequence of the AcGH79 gene was cloned into the pET28a (Novagen) expression vector with an N-terminal His6 tag. The E287N mutant was produced by polymerase chain reaction (PCR) using the primer 5'-CTGACCCAAACGAATTCC-3' (forward primer) and 5'-GAATTCTTGGGTGCAG-3' (reverse primer). Both the wild-type and mutant proteins were overexpressed in *Escherichia coli* strain BL21 (DE3) GOLD using LB medium. The transformed cells were grown at 37 °C in LB media containing 50 µg mL⁻¹ kanamycin until the A600 nm reached 0.8. Expression of the recombinant proteins was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside for 12 h at 25 °C. The cells were harvested by centrifugation at 8000 g for 30 min and resuspended in 50 mL lysis buffer (20 mM HEPES, NaCl 200 mM, imidazole 5 mM, pH 7.0). After 20 min of sonication and 30 min of centrifugation at 12000 xg, the filtered supernatant containing His₆-AcGH79 was loaded onto a His Trap column (GE Healthcare), equilibrated with the lysis buffer. The column was washed with lysis buffer and the His₆-AcGH79 protein was eluted with the same buffer with supplement of 400 mM imidazole over a gradient of 100 mL. The fractions containing the His₆-AcGH79 were then loaded onto a Hilopect 16/60 Superdex 75 column (GE Healthcare). The fractions containing the His₆-AcGH79 were pooled and concentrated to the final concentration of 14.5 mg mL⁻¹.

Enzyme activity assays and IC₅₀ measurements for AcaGH79.

The β-D-glucuronidase activity of AcaGH79 was assayed at 37 °C by incubating with 2.5 mM 4-methylumbelliferyl-β-D-glucuronide as substrate in 150 mM McIlvaine buffer, pH 5.0, supplemented with 0.1% (w/v) BSA. To determine the apparent in *vitro* IC₅₀ value, recombinant AcaGH79 was firstly pre-incubated with a range of inhibitor dilutions for 30 min at 37 °C, prior to addition of the substrate. The enzymatic reaction was quenched by adding excess NaOH-glycine (pH 10.6), after which fluorescence of liberated 4-methylumbelliferyl was measured with a fluorimeter LS55 (Perkin Elmer) using λₑₓ 366 nm and λₑₘ₄ 445 nm. The in *situ* IC₅₀ value was determined by incubating fibroblast cell lines expressing wild-type AcaGH79, grown to confluence, with a range of inhibitor dilutions for 2 h. Hereafter, cells were washed three times with PBS and subsequently harvested by scraping in potassium phosphate buffer (25 mM K₂HPO₄-KH₂PO₄, pH 6.5, supplemented with 0.1% (v/v) Triton X-100 and protease inhibitor cocktail (Roche)). After determination of the protein concentration (BCA kit, Pierce), lysates were aliquoted and frozen at -80 °C. Residual AcaGH79 activity was measured using the aforementioned substrate assay. Data was corrected for background fluorescence, then normalized to the untreated control condition and finally curve-fitted via one phase exponential decay function (GraphPad Prism 5.0).³

Determination of inhibition constants

The kinetic parameters of inhibition were determined by adding AcaGH79 to the appropriate McIlvaine buffer, containing both substrate and various concentrations of the corresponding inhibitor. The mixtures were incubated for 0 to 60 min at 37 °C and the reactions were quenched at intervals of 5 min with excess NaOH-glycine (pH 10.6).
Inhibitory constants $k_i$ and $K'_i$ were determined by firstly calculating the $K_{\text{observed}}$ per inhibitor concentration, followed by curve-fitting the data to a one phase exponential association function (GraphPad Prism 5.0).

**In vitro** labeling and SDS-PAGE analysis and fluorescence scanning

All the labeling samples were samples resolved on 10% SDS-PAGE. Electrophoresis in sodium dodecylsulfate containing 10% polyacrylamide gels was performed as earlier described. Wet slab-gels were then scanned for ABP-emitted fluorescence using a Bio-rad ChemiDoc MP imager using green Cy2 ($\lambda_{\text{EX}}$ 470 nm, bandpass 30 nm; $\lambda_{\text{EM}}$ 530 nm, bandpass 28 nm) for 8, red Cy3 ($\lambda_{\text{EX}}$ 530 nm, bandpass 28 nm; $\lambda_{\text{EM}}$ 605 nm, bandpass 50 nm) for 5, 9, and blue Cy5 ($\lambda_{\text{EX}}$ 625 nm, bandpass 30 nm; $\lambda_{\text{EM}}$ 695 nm, bandpass 55) for 10. All samples were denatured with 5× Laemmli buffer (50% (v/v) 1.0 M Tris−HCl, pH 6.8, 50% (v/v) 100% glycerol, 10% (w/v) DTT, 10% (w/v) SDS, 0.01% (w/v) bromophenol blue), boiled for 4 min at 100 °C, and separated by gel electrophoresis on 10% (w/v) SDS-PAGE gels running continuously at 90 V for 30 min and 200 V for 50 min.

Influence of pH on ABP labeling involved pre-incubation of AcaGH79 at pH 2−8 for 30 min on ice, prior to addition of 100 nM 8, dissolved in Nanopure H₂O and incubating for 30 min at 37 °C. Assessment of 2, 3, 7, 8 labeling kinetics involved pre-cooling of 10 ng AGH79 on ice for 15 min, followed by addition of similarly cooled 100 nM 8 solution. After mixing, 8 labeling was chased for 0–60 min at either 4 °C or 37 °C, whereafter labeling was stopped by denaturation. For alkyl aziridines competitive labeling on AcaGH79, 100 ng AcaGH79 was pre-incubated with inhibitors (25 mM 4MU-β-D-glucuronide, 100 μM 2, 3, 1.0 mM 12, or 1.0 μM 7, 9, 10, 11, or boiled for 4 min in 2% (w/v) SDS, prior to labeling with 100 nM 8, dissolved in Nanopure H₂O, for 30 min at 37 °C. For acyl aziridines competitive labeling, 100 ng wild-type AcaGH79 was pre-incubated with 1 μM inhibitor 4, prior to labeling with 100 nM 5 in 150 mM McIlvaine buffer, pH 5.0, for 30 min at 37 °C.

**X-ray crystallography and structure solution**

Initial crystallization screening of native AcGH79 was conducted by sitting drop vapor diffusion method at 20 °C, by mixing 0.5 μL of the protein solution (4.5 mg mL⁻¹) and an equal volume of precipitant against 100 μL of reservoir solution composed of 0.8 M to 1.5 M sodium phosphate monobasic/postassium phosphate dibasic in the ratio of NaH₂PO₄/K₂HPO₄ = 0.5/9.5 (v/v, pH not adjusted). Some preliminary hits were obtained in several conditions and after refinement of the crystallization conditions, well diffracting crystals were obtained by mixing 0.7-0.8 μL 4.5 mg mL⁻¹ protein stock with 0.5 μL precipitant composed of 0.8 M to 1.2 M NaH₂PO₄/K₂HPO₄ = 0.5/9.5 (v/v, pH not adjusted) overnight at 20 °C, using the sitting drop vapour diffusion method. The E287Q mutant of AcGH79 was crystallized by mixing 0.3-0.5 μL of the protein solution (12 mg mL⁻¹) with 0.50 μL precipitant composed of 1.2 M to 1.5 M NaH₂PO₄/K₂HPO₄ = 1.0 : 9.0 (v/v, pH not adjusted).

All four complexes, namely wt-12, wt-4, wt-7 and E287Q-7 were produced by soaking method. The drops containing the crystals of the corresponding wt AcGH79 or E287Q were supplemented with 1.0 μL of 5.0 mM 12, 4 or 7 in the precipitant solution freshly prepared before soaking procedure for 1 h at 20 °C before fishing. 2 M lithium sulphate was used as cryoprotectant for all the crystals before being flash frozen in liquid nitrogen. Diffraction data for all the crystals were collected at 100 K on beamline i04-1 the Diamond Light Source and were processed using the xia2.
implementation of XDS and programs from CCP4 suite. Parts of the data processing statistics and structure refinement are listed in Table 1.

Table 1. The statistics of the data processing and structure refinement.

<table>
<thead>
<tr>
<th></th>
<th>Native</th>
<th>wt-4</th>
<th>wt-7</th>
<th>wt-12</th>
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<td>Cell dimensions</td>
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<tr>
<td>(a, b, c) (Å)</td>
<td>150.7, 44.9, 83.4</td>
<td>150.7, 44.8, 83.3</td>
<td>148.5, 44.9, 82.2</td>
<td>150.6, 44.7, 83.2</td>
<td>150.2, 45.1, 82.0</td>
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<tr>
<td>(\alpha, \beta, \gamma) (°)</td>
<td>90°, 115.6°, 90°</td>
<td>90°, 115.5°, 90°</td>
<td>90°, 114.8°, 90°</td>
<td>90°, 115.5°, 90°</td>
<td>90°, 115.1°, 90°</td>
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<tr>
<td>Resolution (Å)</td>
<td>42.1 (1.19)</td>
<td>66.7 (1.80)</td>
<td>42.6 (1.60)</td>
<td>42.4 (1.60)</td>
<td>28.9 (1.24)</td>
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<tr>
<td>(R_{sym}) or (R_{merge})</td>
<td>0.033 (0.443)</td>
<td>0.068 (0.540)</td>
<td>0.058 (0.664)</td>
<td>0.054 (0.594)</td>
<td>0.045 (0.489)</td>
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<tr>
<td>Completenseness (%)</td>
<td>92.0 (55.4)</td>
<td>98.0 (98.0)</td>
<td>99.5 (99.7)</td>
<td>95.8 (96.3)</td>
<td>89.8 (49.7)</td>
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<td>Redundancy</td>
<td>3.8 (2.7)</td>
<td>4.1 (4.1)</td>
<td>4.0 (3.7)</td>
<td>4.2 (4.3)</td>
<td>3.9 (2.7)</td>
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<td><strong>Refinement</strong></td>
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<tr>
<td>Resolution (Å)</td>
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<td>1.85 (1.80)</td>
<td>1.63 (1.60)</td>
<td>1.63 (1.60)</td>
<td>1.27 (1.24)</td>
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<tr>
<td>No. reflections</td>
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<td>45856 (3332)</td>
<td>64976 (3170)</td>
<td>63367 (3085)</td>
<td>126454 (5143)</td>
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<tr>
<td>(R_{work})/(R_{free})</td>
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<td>0.1967</td>
<td>0.1675</td>
<td>0.1631</td>
<td>0.1699</td>
</tr>
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</table>

Highest resolution shell is shown in parenthesis.

**General synthesis**

All reagents were of a commercial grade and were used as received unless stated otherwise. Dichloromethane (DCM), tetrahydrofuran (THF) and N,N-dimethylformamide (DMF) were stored over 4 Å molecular sieves, which were dried in vacuo before use. All reactions were performed under an argon atmosphere unless stated otherwise. Solvents used for flash column chromatography were of pro analysis quality. Reactions were monitored by TLC analysis using Merck aluminium sheets pre-coated with silica gel 60 with detection by UV absorption (254 nm) and by spraying with a solution of \((\text{NH}_4)_7\text{Mo}_7\text{O}_{24}\cdot\text{H}_2\text{O}\) (25 g/L) and \((\text{NH}_4)_4\text{Ce}({\text{SO}_4})_4\cdot\text{H}_2\text{O}\) (10 g/L) in 10% sulfuric acid followed by charring at \(~150\) °C or by spraying with an aq. solution of KMnO\(_4\) (7%) and K\(_2\)CO\(_3\) (2%) followed by charring at \(~150\) °C. Column chromatography was performed using either Baker or Screening Device silica gel 60 (0.04 - 0.063 mm) in the indicated solvents. 1H-NMR and 13C-NMR spectra were recorded on Bruker AV-850 (850/214 MHz), Bruker DMX-600 (600/150
MHz) and Bruker AV-400 (400/100 MHz) spectrometer in the given solvent. Chemical shifts are given in ppm relative to the deuterated chloroform or methanol residual solvent peak or tetramethylsilane (TMS) as internal standard. Coupling constants are given in Hz. All given $^{13}$C-NMR spectra are proton decoupled. High-resolution mass spectra were recorded with a LTQ Orbitrap (Thermo Finnigan). Optical rotations were measured on Polar automatic polarimeter (Sodium D-line, $\lambda = 589$ nm). IR spectra were recorded on a Shimadzu FT-IR 83000 spectrometer. LC-MS analysis was performed on a Surveyor HPLC system (Thermo Finnigan) equipped with a Gemini column (Gemini, 4.6 mm x 50 mm, 3 μm particle size, Phenomenex) equipped with buffers A: H2O, B: MeCN (MeCN) and C: 1% aq. TFA or 50 mM NH4HCO3 in H2O. For reversed-phase HPLC purifications an Agilent Technologies 1200 series instrument equipped with a semi preparative Gemini C18 column (10 x 250 mm) was used. The applied buffers were A: 25mM NH4OAc or 50 mM NH4HCO3 in H2O, B: MeCN.

(1R,2R,3R,4S,5R,6R)-4,5-bis(benzyloxy)-2-(hydroxymethyl)-7-oxabicyclo[4.1.0]heptan-3-ol (14): A mixture of diol $^{13}$ (3.4 g, 10 mmol, 1.0 eq.) and mCPBA (4.4 g, 18 mmol, 1.8 eq.) in 1,2-dichloroethane (166 mL), NaH2PO4 (1.0 M in H2O, 100 mL), and Na2HPO4 (1.0 M in H2O, 100 mL) was vigorously stirred at 50 °C for 18 h. The layers were separated, and the water layer was extracted with EtOAc. The combined organic layers were dried with MgSO4 and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (88%→92%, Et2O in pentane) to provide $\beta$-epoxide 14 (1.9 g, 5.4 mmol, 55%).

$^1$H-NMR (400 MHz, CDCl3): δ ppm 7.40 – 7.28 (m, 10H), 4.97 (d, $J = 11.6$ Hz, 2H), 4.83 (d, $J = 11.2$ Hz, 2H), 4.70 – 4.66 (m, 2H), 4.03 (dd, $J = 6.4$, 10.8Hz, 1H), 3.91 (dd, $J = 5.6$, 10.8Hz, 1H), 3.83(d, $J = 7.6$Hz, 1H), 3.51 – 3.46 (m, 1H), 3.42 – 3.38 (m, 1H), 3.29 – 3.28 (m, 1H), 3.17 (d, $J = 4.0$Hz, 1H), 3.01 – 2.77 (br, 1H), 2.19 – 2.13 (m, 1H); $^{13}$C-NMR (100 MHz, CDCl3): δ ppm 138.27, 137.46, 128.70, 128.64, 128.51, 128.18, 128.09, 128.01, 127.98, 83.60, 79.45, 74.94, 72.69, 68.64, 63.99, 55.01, 53.07, 43.44; HRMS: calculated for C21H25O5 [M+H+] 357.16965, found 357.16987.

(1R,2R,3R,4S,5R,6R)-4,5-bis(benzyloxy)-3-hydroxy-7-oxabicyclo[4.1.0]heptane-2-carboxylic acid (15): 2, 3-Di-O-benzylcyclophellitol 14 (33 mg, 0.10 mmol, 1.0 eq.) was dissolved in 650 µL DCM/tBuOH/H2O (4/4/1, v/v/v) mixture and was cooled down to 0˚C. TEMPO (75 mg, 19 µmol, 0.20 eq.) and BAIB (75 mg, 0.27 mmol, 2.5 eq.) were added to the solution and it was stirred at 0˚C. The reaction was monitored by TLC. The reaction was quenched after 3 h with 1.0 mL sat. Na2S2O3 solution and extracted with EtOAc. The organic layer with crude product was dried with MgSO4 and concentrated in vacuo. Crude product was purified via HPLC (linear gradient: 28%→38% B, 3 CV, solutions used A: 50 mM NH4HCO3 in H2O, B: MeCN) and concentrated in vacuo resulting in 15 (12 mg, 0.032 mmol, 35%). $^1$H-NMR (400 MHz, CD3OD): δ ppm 7.36 – 7.23 (m, 10H), 4.87 – 4.73 (m, 3H), 4.66 (d, $J = 11.5$ Hz, 1H), 3.81 – 3.74 (m, 2H), 3.54 – 3.53 (m, 1H), 3.41 – 3.36 (m, 1H), 3.19 (d, $J = 3.7$ Hz, 1H), 2.68 – 2.65 (M, 1H); $^{13}$C-NMR (100 MHz, CD3OD): δ ppm 140.36, 139.42, 129.38, 129.38, 129.15, 129.13, 128.77, 128.41, 85.29, 80.46, 75.79, 73.62, 69.24, 56.52, 54.39, 51.36; HRMS: calculated for C21H23O6 [M+Na+] 393.13086, found 393.13077.

(1R,2R,3R,4S,5R,6S)-3,4,5-trihydroxy-7-oxabicyclo[4.1.0]heptane-2-carboxylic acid (2): A mixture of Product 15 (12 mg, 0.032 mmol, 1.0 eq) and Pd(OH)2/C (3.0 mg, 20% wt. loading(dry basis)) in MeOH
(1.5 mL) was stirred at room temperature under hydrogen atmosphere for 24 h. The catalyst was then filtered off through celite and washed with MeOH. The filtrate was concentrated under reduced pressure and the crude product was purified by semi preparative reversed phase HPLC (linear gradient: 0% → 20% B, 3 CV, solutions sed A: 50mM NH₄HCO₃ in H₂O, B: MeCN) and lyophilization gave the title compound 2 (1.4 mg, 7.4 μmol, 23 %) as white solid.

1H-NMR (400 MHz, D₂O): δ ppm 3.76 (d, J = 8.5 Hz, 1H), 3.61 – 3.56 (m, 1H), 3.51 - 3.50 (m, 1H), 3.33 – 3.28 (m, 1H), 3.17 (d, J = 3.8 Hz, 1H), 2.71 – 2.68 (m, 1H);

13C-NMR (100 MHz, D₂O): δ ppm 75.59, 70.74, 67.65, 56.13, 55.79, 50.77;


(1R,2R,3R,4S,5S,6R)-4,5-bis(benzyloxy)-2-(hydroxymethyl)-7-azabicyclo[4.1.0]heptan-3-ol (16): Building block product 13 (1.4 g, 4.11 mmol, 1 eq.) was co-evaporated thrice in toluene and was subsequently dissolved in dry DCM (100 mL). The solution was cooled down to 0 °C. DBU (62 µL, 0.42 mmol, 0.1 eq.) and trichloroacetonitrile (0.495 ml, 4.94 mmol, 1.2 eq.) were added to the solution. The reaction was stirred at 0 °C and monitored with TLC. After 4 h, extra DBU (0.1 eq.) and trichloroacetonitrile (1.2 eq.) were added to the solution. After 5 h, TLC showed that the starting material was completely consumed. Subsequently, H₂O (13 mL), NaHCO₃ (3.6 g, 43 mmol, 10 eq.) and iodine (3.2 g, 13 mmol, 3.1 eq.) were added to the solution. And the reaction mixture was stirred overnight at room temperature. Afterwards, it was quenched with 10% aq. Na₂S₂O₃, concentrated in vacuo and extracted with EtOAc. The organic layer with the intermediate product was dried over MgSO₄ and concentrated in vacuo again. In the following step the intermediate was dissolved in dioxane (37 mL) and 37% HCl (aq., 12 mL). The reaction mixture was refluxed at 60 °C for 4 h. After which it was concentrated in vacuo and redissolved in 100 mL MeOH. NaHCO₃ (14 g, 165 mmol, 40 eq.) was added until pH = 7. The reaction mixture was stirred at room temperature for 3 days. The reaction mixture was concentrated in vacuo and extracted with EtOAc. The organic layer was dried with MgSO₄ and concentrated in vacuo again. The crude product was purified via silica gel column chromatography (4% → 10%, MeOH in DCM) yielding aziridine 16 (0.66 g, 1.8 mmol, 45%) as white solid. TLC: Rf 0.5 (EtOAc/EtOH, 3/2, v/v); 1H-NMR (400 MHz, CDCl₃): δ ppm 7.39 – 7.27 (m, 10H), 4.98 (d, J = 9.7 Hz, 1H), 4.78 (d, J = 9.3 Hz, 1H), 4.67 (dd, J = 11.4, 4.5 Hz, 2H), 3.99 (m, 2H), 3.75 (d, J = 9.4 Hz, 1H), 3.53 (t, J = 9.9 Hz, 1H), 3.39 – 3.34 (dd, J = 10.0, 8.1 Hz, 1H), 2.43 – 2.41 (m, 1H), 2.27 (d, J = 6.1 Hz, 1H), 2.08 – 2.02 (m, 1H); 13C-NMR (100 MHz, CDCl₃): δ ppm 138.54, 137.92, 128.66, 128.61, 128.05, 128.03, 127.92, 84.38, 81.23, 74.89, 72.37, 68.36, 64.49, 42.57, 33.22, 31.72; HRMS: calculated for C₂₁H₂₆NO₄ [M+H⁺] 356.1852, found 356.1856.

(1R,2R,3S,4R,5R,6R)-5-(hydroxymethyl)-7-azabicyclo[4.1.0]heptane-2,3,4-triol (17): Ammonia (8 mL) was condensed at -60 °C and lithium (75 mg, 11 mmol, 24 eq.) was added and stirred until all the lithium was dissolved. Then a solution of aziridine 16 (160mg, 0.45 mmol, 1 eq.) in dry THF (10 mL) was added to the solution above. The reaction mixture was stirred at -60 °C. The reaction was quenched with milliQ-H₂O (3.0 mL) after 1 h. The solution was allowed to come to room temperature and stirred until all ammonia had evaporated. Next, the solution was concentrated in vacuo, redissolved in milliQ-H₂O, and neutralized with Amberlite IR-120 H⁺. Product bound to the resin was washed with water and subsequently eluted with NH₄OH solution (1.0 M) and evaporated under reduced pressure. The resulting solid was purified by Amberlite IR-120 NH₄⁺ resin using milliQ-H₂O as eluens until the eluate was neutral. Concentration of the combined eluate under reduced pressure gave the fully deprotected aziridine 17 (51 mg, 0.30 mmol, 65%). 1H-NMR (400 MHz, D₂O): δ ppm 3.81 – 3.74 (m, 1H), 3.54 –
3.44 (m, 2H), 3.11 (t, \( J = 10.1 \text{ Hz} \), 1H), 2.88 (t, \( J = 10.1 \text{ Hz} \), 1H), 2.52 – 2.45 (m, 1H), 2.21 (d, \( J = 6.3 \text{ Hz} \), 1H), 2.01 – 1.95 (m, 1H); \(^{13}\text{C-NMR (100 MHz, D}_2\text{O): } \delta \text{ ppm 76.70, 71.67, 67.38, 61.47, 42.83, 34.78, 33.05.}

\((9\text{H-fluoren-9-yl)methyl (1R,2S,3S,4R,5R,6R)-2,3-bis(benzyloxy)-4-hydroxy-5-(hydroxymethyl)-7-azabicyclo[4.1.0]heptane-7-carboxylate (19): \)}

Aziridine \(16 \) (220 mg, 0.61 mmol, 1.0 eq.) was dissolved in THF (6.2 mL). Fmoc-OSU (160 mg, 0.62 mmol, 1.0 eq.) and pyridine (55 \( \mu \text{L}, 0.68 \text{ mmol, } 1.1 \text{ eq.} \) were added. The reaction was stirred at room temperature for 3 h, after which TLC showed the completion of the reaction. The reaction mixture was concentrated in vacuo, redissolved in EtOAc. It was washed with water and brine. The organic layer was dried with MgSO\(_4\) and concentrated in vacuo again. The crude product was purified via silica gel column chromatography (40% – 60%, EtOAc in pentane) yielding aziridine \(19 \) (269 mg, 0.47 mmol, 75%) as a white solid. TLC: \( R_f 0.25 \) (EtOAc/Pentane, 1/1, v/v);

\[^{1}\text{H-NMR (400 MHz, CDCl}_3\): } \delta \text{ ppm 7.72 (t, } \( J = 7.2 \text{ Hz} \), 2H), 7.58 – 7.53 (m, 2H), 4.87 (d, \( J = 11.3 \text{ Hz} \), 1H), 4.70 – 4.51 (m, 4H), 4.44 (d, \( J = 11.5 \text{ Hz} \), 1H), 4.19 (t, \( J = 5.3 \text{ Hz} \), 1H), 3.75 – 3.61 (m, 2H), 3.35 – 3.19 (m, 2H), 2.49 – 2.41 (m, 2H), 2.00 – 1.90 (m, 1H);

\[^{13}\text{C-NMR (100 MHz, CDCl}_3\): } \delta \text{ ppm 162.64, 143.42, 143.26, 141.50, 141.44, 141.50, 141.44, 137.31, 128.60, 128.56, 128.20, 128.08, 128.06, 128.02, 127.94, 127.32, 127.27, 124.78, 124.75, 120.13, 83.80, 79.45, 74.86, 71.87, 68.41, 67.44, 63.88, 47.28, 42.33, 40.28, 38.55, 29.79 ; HRMS: calculated for C\(_{36}\)H\(_{35}\)NO\(_6\) [M+H\(^{+}\)] 578.25371, found 578.25321.

\(\text{Benzyl(1R,2S,3S,4R,5R,6R)-2,3-bis(benzyloxy)-4-hydroxy-5-(hydroxymethyl)-7-azabicyclo[4.1.0]heptane-7-carboxylate (20): \)}

Aziridine \(16 \) (82 mg, 0.23 mmol, 1 eq.) was dissolved in THF (2.3 mL). CbzCl (39 mg, 0.23 mmol, 1.0 eq.) and pyridine (22.3 \( \mu \text{L}, 0.28 \text{ mmol, 1.2 eq.} \) were added to the solution. The reaction was stirred at room temperature for 1.5 h and was monitored by TLC. It was shown that all the starting material was converted to a higher running product. The reaction mixture was extracted with EtOAc and concentrated in vacuo. The crude product was purified via silica gel column chromatography (1% – 9% MeOH in DCM) yielding aziridine \(20 \) (85 mg, 0.17 mmol, 75%). TLC: \( R_f 0.6 \) (MeOH/DCM, 1/19, v/v);

\[\left[\alpha\right]_D^{20} +49.2 \ (c = 1, \text{ CHCl}_3); \]

\[^{1}\text{H-NMR (400 MHz, CDCl}_3\): } \delta \text{ ppm 7.40 – 7.26 (m, 15H), 5.14 – 5.13 (m, 2H), 4.96 (d, } \( J = 11.3 \text{ Hz} \), 1H), 4.83 (d, \( J = 11.5 \text{ Hz} \), 1H), 4.66 – 4.61 (m, 2H), 4.02 – 3.98 (m, 1H), 3.90 (br, 1H), 3.81 (d, 1H), 3.45 (t, \( J = 9.7 \text{ Hz} \), 1H), 3.35 (m, 1H), 2.86 – 2.79 (m, 2H), 2.12 – 2.07 (m, 1H); \[^{13}\text{C-NMR (100 MHz, CDCl}_3\): } \delta \text{ ppm 162.55, 138.26, 137.31, 135.45, 128.82, 128.67, 128.63, 128.22, 128.13, 128.00, 127.97, 83.84, 79.53, 74.93, 72.07, 68.66, 68.64, 64.20, 40.35, 38.83; HRMS: calculated for C\(_{29}\)H\(_{31}\)NO\(_6\) [M+H\(^{+}\)] 490.22241, found 490.22215.

\(\text{(1R,2S,3R,4S,5S,6R)-7-(((9\text{H-fluoren-9-yl)methoxy} \text{carbonyl})-4,5-bis(benzyloxy)-3-hydroxy-7-azabicyclo[4.1.0]heptane-2-carboxylic acid (21): } \)}

Fmoc-protected aziridine \(19 \) (172 mg, 0.30 mmol, 1 eq.) was dissolved in 1.5 mL DCM/tBuOH/H\(_2\)O (v/v/v, 4:4:1) mixture and cooled down to 0 \( ^\circ \text{C. TEMPO (9.3 mg, 60 \( \mu \text{mol, 0.2 eq.} \) and BAIB (240 mg, 0.74 mmol, 2.5 eq.) were added to the solution and it was stirred at 0 \( ^\circ \text{C. The reaction was quenched with sat. aq. Na}_2\text{S}_2\text{O}_3 \) solution (5.0 mL), extracted with EtOAc. The organic layer was dried with MgSO\(_4\) and concentrated in vacuo. The crude product was purified by silica gel column chromatography (40% – 90% EtOAc with 0.15% AcOH in Pentane) yielding aziridine \(21 \) (162 mg, 0.27 mmol, 92%) as a white solid. TLC: \( R_f 0.45 \) (EtOAc/EtOAc/AcOH, 0.98/9/0.02, v/v/v); \([\alpha]_D^{20} +18 \ (c = 0.5, \text{ CHCl}_3); \)

\(\text{IR (neat, cm}^{-1}\): 2922, 2852,
Synthesis and biological evaluation of 8-carboxy-cyclophellitol aziridine derivatives

1724, 1450, 1433, 1381, 1278, 1251, 1215, 1111, 1074, 1055, 1082, 987, 756, 698; 1H-NMR (400 MHz, CDCl₃): δ ppm 7.65 (dd, J = 7.2, 3.2 Hz, 2H), 7.55 – 7.49 (m, 2H), 7.41 – 7.24 (m, 14H), 4.82 (d, J = 11.3 Hz, 1H), 4.64 (d, J = 10.9 Hz, 2H), 4.52 – 4.40 (m, 2H), 4.33 (dd, J = 10.7, 6.2 Hz, 1H), 4.13 (t, J = 6.1 Hz, 1H), 3.81 (t, J = 10.0 Hz, 1H), 3.74 (d, J = 8.1 Hz, 1H), 3.30 (dd, J = 9.8, 8.4 Hz, 1H), 2.96 (dd, J = 5.7, 3.0 Hz, 1H), 2.80 (dd, J = 9.9, 2.8 Hz, 1H), 2.52 (d, J = 6.0 Hz, 1H)

13C-NMR (100 MHz, CDCl₃): δ ppm 174.24, 162.21, 143.63, 143.39, 141.38, 141.35, 138.23, 137.28, 128.62, 128.54, 128.24, 128.12, 127.99, 127.92, 127.32, 127.28, 125.17, 125.02, 120.09, 120.04, 83.00, 79.12, 74.98, 72.22, 68.16, 66.93, 47.22, 47.00, 39.40, 39.13; HRMS: calculated for C₃₆H₃₃NO₇ [M+H⁺] 592.23298, found 592.23273.

(1R,2S,3R,4S,5S,6R)-4,5-bis(benzyloxy)-7-((benzyloxy)carbonyl)-3-hydroxy-7-azabicyclo[4.1.0]heptane-2-carboxylic acid (22): Aziridine 20 (85 mg, 0.17 mmol, 1.0 eq.) was dissolved in DCM/tBuOH/H₂O mixture (1.2 mL, 4:4:1, v/v/v) and cooled down to 0 °C. TEMPO (5.3 mg, 34 µmol, 0.20 eq.) and BAIB (137 mg, 42 mmol, 2.5 eq.) were added to the solution and it was stirred at 0 °C for 3 h. The reaction was monitored with TLC. The reaction was quenched by sat. aq. Na₂S₂O₃ solution (3.0 mL), extracted with EtOAc. The organic layer was dried with MgSO₄ and concentrated in vacuo. The crude product was purified by silica gel column chromatography (0.5%→10% MeOH in DCM) yielding aziridine 22 (50 mg, 0.10 mmol, 60%). TLC: Rf 0.3 (MeOH/DCM, 1/19, v/v); [α]D₂₀ +56 (c = 1, CHCl₃); 1H-NMR (400 MHz, CDCl₃): δ ppm 7.46 – 6.26 (m, 15H), 5.11 – 5.02 (m, 2H), 4.89 (d, J = 10.7 Hz, 1H), 4.79 – 4.62 (m, 3H), 3.86 – 3.80 (m, 2H), 3.42 – 3.34 (m, 1H), 3.15 (br, 1H), 2.87 – 2.80 (m, 2H; 13C-NMR (100 MHz, CDCl₃): δ ppm 162.24, 138.30, 137.30, 135.55, 128.70, 128.63, 128.54, 128.42, 128.19, 128.10, 127.98, 127.94, 127.89, 83.13, 79.11, 74.99, 72.32, 68.55, 67.05, 39.58, 39.40; HRMS: calculated for C₂₉H₂₉NO₇ [M+H⁺] 504.20168, found 504.20148.

(1R,2S,3R,4S,5S,6R)-3,4,5-trihydroxy-7-azabicyclo[4.1.0]heptane-2-carboxylic acid (3): Ammonia 10 mL was condensed at -60 °C and lithium (23 mg, 3.4 mmol, 24 eq.) was dissolved in the liquid. After 30 min, aziridine 22 (50 mg, 0.14 mmol, 1.0 eq.) was dissolved in dry THF (6.0 mL) and added to the solution. The reaction mixture was stirred at - 60 °C for 0.5 h, then 15 mg extra lithium was added to the solution for stirring one more hour. The reaction was quenched with 4.0 mL milliQ-H₂O. The solution was allowed to come to room temperature and stirred until all ammonia had extracted. Next, the solution was concentrated in vacuo, redissolved in milliQ-H₂O, and neutralized with Amberlite IR-120 H⁺. Product bound to the resin was washed with water and subsequently eluted with NH₄OH solution (1.0 M) and evaporated under reduced pressure. Concentration of the combined eluate fractions under reduced pressure gave the fully deprotected aziridine 3 (21 mg, 0.11 mmol, 79%). 1H-NMR (400 MHz, D₂O): δ ppm 3.61 (d, J = 8.5 Hz, 1H), 3.51 – 3.46 (m, 1H), 3.23 – 3.19 (m, 1H), 2.62 – 2.50 (m, 2H), 2.20 – 2.18 (m, 1H); 13C-NMR (100 MHz, D₂O): δ ppm 179.42, 76.65, 72.21, 67.95, 49.86, 34.26, 33.41; HRMS: calculated for C₇H₁₁NO₅ [M+H⁺] 190.07100, found 190.07105.

8-Azido-1-((1R,2S,3R,4S,5R,6R)-2,3,4-trihydroxy-5-(hydroxymethyl)-7-azabicyclo[4.1.0]heptan-7-yl)octan-1-one (24): 8-azidoocatcanoic acid 23 (93 mg, 0.50 mmol, 1.2 eq.) and EEDQ (123 mg, 0.50 mmol, 1.2 eq.) were dissolved in DMF (0.5 mL) and stirred at room temperature for 2 h. Pre-activated mixed anhydride solution (300 µL, 0.60 eq.) was added to the deprotected aziridine 17 solution at 0 °C and stirred for 30 min. Additional pre-activated
solution (200 µL, 0.40 eq) was added. The resulting mixture was stirred at 0 °C for 3 h. The reaction was quenched by MeOH (2.0 mL) and the mixture was concentrated in vacuo. Then the crude product was purified by HPLC (linear gradient: 25%→31% B in A, 3 CV, solutions used: A: H₂O, B: MeCN). After lyophilization, pure product 24 was obtained as colorless oil (27 mg, 0.079 mmol, 18.9%).

1H-NMR (400 MHz, CD₃OD): δ ppm 4.05 (dd, J = 10.4, 4.4 Hz, 1H), 3.69 - 3.65 (m, 2H), 3.30 - 3.25 (m, 2H, and MeOD solvent signals), 3.19 (dd, J = 10.0, 8.4, 1H), 3.06 (t, J = 9.6 Hz, 1H), 3.01 (dd, J = 6.0, 2.8Hz, 1H), 2.72 (d, J = 6.0 Hz, 1H), 2.48 (t, J = 7.2Hz, 2H), 1.99 - 1.93 (m, 1H), 1.63–1.54 (m, 4H), 1.36 (s, 6H);

13C-NMR (101MHz, CD₃OD): δ ppm 188.54, 79.02, 73.37, 69.34, 63.69, 52.38, 45.2, 42.40, 41.00, 36.83, 30.09, 29.80, 27.61; IR (neat, cm⁻¹): 3341.9, 2934.6, 2860.2, 2363.2, 2343.2, 2095.8, 1671.7, 1559.8, 1418.4, 1371.6, 1278.3, 1180.9, 1088.7, 1027.3, 976.6, 668.2; HRMS: calculated for C₁₅H₂₇N₄O₅ [M+H⁺] 343.19760, found 343.19761.

(1R,2S,3R,4S,5S,6R)-7-(8-azidoctanoyl)-3,4,5-trihydroxy-7-azabicyclo[4.1.0]heptane-2-carboxylic acid (4): The primary alcohol compound 24 (89 mg, 0.26 mmol, 1.0 eq.) was dissolved in MeCN/H₂O (3.0 mL, 1/1, v/v). The solution was cooled to 0 °C, then BAIB (183 mg, 0.57 mmol, 2.2 eq.), the solution of TEMPO (8.1 mg, 0.052 mmol, 0.2 eq.) in MeCN/H₂O (200 µL, 1/1, v/v) and NaHCO₃ (88 mg, 1.0 mmol, 4.0 eq.) was added, the resulting mixture was stirred for 2 h at 0 °C. More addition of BAIB (183 mg, 0.57 mmol, 2.2 eq.), the solution of TEMPO (8.1 mg, 0.052 mmol, 0.2 eq.) in MeCN/H₂O (100 µL, 1/1, v/v) and NaHCO₃ (50 mg, 0.60 mmol, 2.4 eq.) was added and the reaction mixture stirred overnight from 0 °C to room temperature, then the reaction was quenched with 10% Na₂S₂O₃. After lyophilization, the crude product was purified by semi-preparative reversed HPLC (linear gradient: 16%→19% B in A, 3 CV, solutions used: A: 25 mM NH₄OAc in H₂O, B: MeCN) and the fraction were freeze-dried to afford the desired product as a white powder (4.2 mg, 0.012 mmol, 5%).

1H-NMR (400 MHz, CD₃OD): δ ppm 3.67 (dd, J = 13.1, 9.0 Hz, 2H), 3.27 (t, J = 7.0 Hz, 2H), 3.13 (dd, J = 5.8, 2.5 Hz, 1H), 2.66 (d, J = 5.8 Hz, 1H), 2.61 – 2.49 (m, 2H), 2.49 – 2.38 (m, 1H), 2.28 – 2.17 (m, 1H), 1.68 – 1.52 (m, 4H), 1.43 – 1.28 (m, 6H); 13C-NMR (100 MHz, CD₃OD): δ ppm 78.88, 73.55, 69.40, 52.47, 50.68, 41.71, 41.54, 36.76, 30.10, 30.02, 29.85, 27.68, 26.3; 1H-NMR (400 MHz, D₂O): δ ppm 3.78 (d, J = 8.4 Hz, 1H), 3.64 (t, J = 10.2 Hz, 1H), 3.32 – 3.25 (m, 3H), 3.17 (dd, J = 5.7, 3.3 Hz, 1H), 2.75 (d, J = 5.8 Hz, 1H), 2.64 (dd, J = 9.9, 3.3 Hz, 1H), 2.52 – 2.32 (m, 2H), 2.24 (d, J = 7.0 Hz, 1H), 1.61 – 1.48 (m, 4H), 1.29 - 1.32 (m, 6H); 13C-NMR (100 MHz, D₂O): δ ppm 190.68, 77.04, 72.20, 68.74, 51.93, 51.22, 41.13, 41.00, 36.32, 28.75, 28.64, 28.60, 26.45, 25.01; LC-MS: Rₜ 3.12 min, linear gradient 10%→90% B with 10% NH₄OAc in 15 min; ESI-MS: m/z = 357.2 (M+H)⁺; HRMS: calculated for C₁₅H₁₆N₄O₆ [M+H⁺] 357.17686, found: 357.17687.

(1R,2S,3R,4S,5S,6R)-7-(8-(4-(4-(5,5-difluoro-3,7-bis(4-methoxyphenyl)-5H-4,5-4,5-4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)butyl)-1H-1,2,3-triazol-1-yl)octanoyl)-3,4,5-trihydroxy-7-azabicyclo[4.1.0]heptane-2-carboxylic acid (5): Acyl Azido-aziridine compound 4 (5.2 mg, 0.015 mmol, 1.0 eq.) was dissolved in DMF (0.60 ml), red BODIPY-alkyne (8.5 mg, 0.017 mmol, 1.2 eq.), CuSO₄ (1.0 M in H₂O, 10 µL, 0.010 mmol, 0.60 eq.) and sodium ascorbate (1.0 M in H₂O, 11 µL, 0.011 mmol, 0.70 eq.) were added to the solution under argon atmosphere, and the mixture was stirred at room temperature for 2 h, the reaction was checked with LC-MS within the elution system of 10% NH₄OAc. The reaction mixture was lyophilization and then the crude product was purified by semi-preparative reversed HPLC (linear gradient: 42%→48% B in A, 3 CV, solutions used...
A: 25 mM NH₄OAc in H₂O, B: MeCN and the fractions were freeze-dried to afford the desired product 5 as a purple powder (1.2 mg, 0.0014 mmol, 9.5%). ¹H-NMR (500 MHz, CD₃OD): δ ppm 7.87 – 7.81 (m, 4H), 7.74 – 7.70 (m, 2H), 7.67 – 7.61 (m, 2H), 7.45 (d, J = 4.4 Hz, 2H), 6.99 – 6.95 (m, 4H), 6.69 (d, J = 4.4 Hz, 2H), 4.33 (t, J = 7.1 Hz, 2H), 3.85 (s, 7H), 3.81 (d, J = 13.3 Hz, 4H), 3.08 (t, J = 5.1 Hz, 2H), 2.82 – 2.73 (m, 3H), 2.24 – 2.09 (m, 5H), 2.04 (s, 4H), 1.91 – 1.83 (m, 8H), 1.66 – 1.50 (m, 6H), 1.39 – 1.22 (m, 20H); LC-MS: Rf 7.51 min, linear gradient 0%→90% B with 10% NH₄OAc in 15 min; ESI-MS: m/z = 841.6 (M+H⁺); HRMS: calculated for C₄₅H₆₂BF₃N₄O₆ [M+H⁺]⁺ 841.39023, found: 841.38982.

(1R,2S,3R,4S,5S,6R)-3,4,5-trihydroxy-7-(8-((6-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)(methyl)-1H,1,2,3-triazol-1-yl)octanoyl)-7-azabicyclo[4.1.0]heptane-2,3,4-trialcohol (26): Unprotected aziridine 17 (163 mg, 0.93 mmol, 1.0 eq.) was dissolved in DMF (4.0 mL). 1-Azido-8-iodooctane 25 (379 mg, 1.3 mmol, 1.5 eq.) and K₂CO₃ (448 mg, 4.0 mmol, 4.3 eq.) were added to the solution and the reaction mixture was stirred at 80 °C under refluxing conditions for 24 h. The crude product was purified by silica gel column chromatography (MeOH in DCM, 2%→20%) yielding 26 as light yellow oil (150 mg, 0.46 mmol, 49%). TLC: Rf 0.39 (DCM/MeOH, 5/1, v/v); [α]D 20 +50 (c = 1, MeOH); ¹H-NMR (400 MHz, CD₃OD): δ ppm 4.00 (dd, J = 10.1, 4.6 Hz, 1H), 3.66 – 3.59 (m, 2H), 3.28 (t, J = 6.8 Hz, 2H), 3.15 – 3.02 (m, 2H), 2.40 – 2.33 (m, 1H), 2.18 – 2.11 (m, 1H), 2.01 – 1.98 (m, 1H), 1.93 – 1.86 (m, 1H), 1.66 (d, J = 6.3 Hz, 1H), 1.62 – 1.54 (m, 4H), 1.42 – 1.29 (m, 8H); ¹³C-NMR (100 MHz, CD₃OD): δ ppm 78.92, 73.79, 70.15, 63.76, 62.05, 52.40, 45.36, 45.34, 42.98, 30.45, 30.24, 30.14, 29.85, 28.25, 27.72; IR (neat, cm⁻¹): 3315, 2926, 2095, 1464, 1348, 1256, 1096, 1020, 818; LC-MS: Rf 4.52 min, linear gradient 10%→90% B in 12.5 min; ESI-MS: m/z = 329.2 (M+H⁺); HRMS: calculated for C₁₅H₂₂N₂O₄ [M+H⁺]⁺ 329.21833, found: 329.21809.
(1R,2S,3R,4S,5S,6R)-7-(8-(azidooctyl)-3,4,5-trihydroxy-7-azabicyclo[4.1.0]heptane-2-carboxylic acid (7): Compound 26 (77 mg, 0.23 mmol, 1.0 eq.), TEMPO (0.23 mg, 0.0015 mmol, 0.0065 eq.), and NaBr (9.5 mg, 0.092 mmol, 0.40 eq.) were dissolved in water (5.0 mL) at 2 °C. A 13% sodium hypochlorite solution (0.24 mL, 0.51 mmol, 2.2 eq.) was added dropwise to the mixture, which was adjusted to pH 10.5 by addingaq. NaOH (0.50 M). The reaction was conducted at 2 °C and checked with LC-MS with an elution system of 10% aq. NH₄OAc. Upon completion, the reaction was quenched by adding 96% EtOH (1.0 mL) and the pH was adjusted to 7 by adding aq. 0.5 M HCl. The volatiles were removed by lyophilization and then the crude product was purified by semi-preparative reverse phase HPLC (linear gradient: 19%→26% B in A, 3 CV, solutions used A: 50 mM NH₄HCO₃ in H₂O, B: MeCN), the fractions were concentrated and lyophilized to afford product 7 as a white powder (12.0 mg, 0.035 mmol, 15%). ¹H-NMR (850 MHz, CD₃OD): δ ppm 3.63 (d, J = 8.2 Hz, 1H), 3.62 – 3.57 (m, 1H), 3.27 (t, J = 6.9 Hz, 2H), 3.18 – 3.16 (m, 1H), 2.41 – 2.38 (m, 1H), 2.35 – 2.30 (m, 1H), 2.18 – 2.10 (m, 2H), 1.62 – 1.55 (m, 4H), 1.53 (d, J = 6.2 Hz, 1H), 1.43 – 1.27 (m, 8H); ¹³C-NMR (214 MHz, CD₃OD): δ ppm 78.89, 74.25, 70.09, 62.24, 52.47, 50.87, 44.64, 43.67, 36.30, 30.53, 30.38, 30.21, 29.91, 28.39, 27.80; ¹H-NMR (400 MHz, CD₂OD): δ ppm 179.81, 76.44, 71.91, 68.53, 59.87, 51.23, 50.17, 42.90, 42.35, 28.42, 28.34, 28.11, 27.91, 26.31, 25.86; LC-MS: Rₜ 4.41 min, linear gradient 10%→90% B in 12.5 min; ESI-MS: m/z = 343.2 (M+H)⁺; HRMS: calculated for C₁₅H₂₆N₂O₅ [M+H]⁺ 343.19760, found: 343.19777.

(1R,2S,3R,4S,5S,6R)-7-(8-(4-(4-(5,5-difluoro-1,3,7,9-tetramethyl-5H-4l4,5l4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)butyl)-1H-1,2,3-triazol-1-yl)octyl)-3,4,5-trihydroxy-7-azabicyclo[4.1.0]heptane-2-carboxylic acid (8): Azide compound 7 (5.0 mg, 0.015 mmol, 1.0 eq.) was dissolved in DMF (0.5 mL) in an 1 mL Eppendorf tube, green BODIPY-alkyne 27 (6.9 mg, 0.017 mmol, 1.2 eq.), CuSO₄ (1.0 M in H₂O, 8.8 µL, 0.0088 mmol, 0.60 eq) and sodium ascorbate (1.0 M in H₂O, 9.5 µL, 0.0095 mmol, 0.65 eq.) were added to the solution under argon atmosphere, and the mixture was stirred at room temperature overnight. The reaction was checked by LC-MS with an elution system of 10% aq. NH₄OAc. Upon completion, the reaction was quenched by adding 96% EtOH (1.0 mL) and then the crude product was purified by semi-preparative reverse phase HPLC (linear gradient: 35%→44% B in A, 3 CV, solutions used A: 25 mM NH₄HCO₃ in H₂O, B: MeCN), the fractions were concentrated and lyophilized giving 8 as an orange powder (2.2 mg, 0.0032 mmol, 22%). ¹H-NMR (850 MHz, CD₃OD): δ ppm 7.90 (s, 2H), 7.75 (s, 1H), 6.11 (s, 2H), 4.34 (t, J = 7.0 Hz, 2H), 3.63 – 3.58 (m, 2H), 3.18 – 3.12 (m, 1H), 3.05 – 3.01 (m, 2H), 2.78 (t, J = 7.3 Hz, 2H), 2.49 – 2.45 (m, 1H), 2.43 (s, 6H), 2.38 (s, 6H), 2.22 – 2.18 (m, 2H), 2.11 – 2.08 (m, 1H), 1.92 – 1.88 (m, 5H), 1.88 – 1.83 (m, 2H), 1.69 – 1.63 (m, 2H), 1.57 – 1.48 (m, 2H), 1.36 – 1.21 (m, 8H); ¹³C-NMR (214 MHz, CD₃OD): δ ppm 154.92, 148.51, 147.88, 142.17, 132.57, 132.41, 122.59, 78.66, 74.02, 69.83, 62.02, 51.23, 44.79, 43.39, 32.31, 31.24, 30.87, 30.36, 29.87, 29.09, 28.19, 27.34, 25.91, 16.50, 14.43; LC-MS: Rₜ 6.24 min, linear gradient 10%→90% B, 12.5 min; ESI-MS: m/z = 671.2 (M+H)⁺; HRMS: calculated for C₂₄H₃₆BF₃N₆O₅ [M+H]⁺ 671.39043, found: 671.39001.

(1R,2S,3R,4S,5S,6R)-7-(8-[(4-(4-(5,5-difluoro-3,7-bis(4-methoxyphenyl)-5H-4l4,5l4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)butyl)-1H-1,2,3-triazol-1-yl)octyl]-3,4,5-trihydroxy-7-azabicyclo[4.1.0]heptane-2-carboxylic acid (9):
Azide compound 7 (6.0 mg, 0.018 mmol, 1.0 eq.) was dissolved in DMF (0.6 mL), red BODIPY-alkyne 28 (10 mg, 0.021 mmol, 1.2 eq.), CuSO4 (1.0 M in H2O, 10 µL, 0.011 mmol, 0.60 eq) and sodium ascorbate (1.0 M in H2O, 11 µL, 0.012 mmol, 0.65 eq.) were added to the solution under argon atmosphere, and the mixture was stirred at room temperature overnight, the reaction was checked by LC-MS with an elution system of 10% aq. NH4OAc. The volatiles were concentrated in vacuo and then the crude product was purified by semi-preparative reversed HPLC (linear gradient: 43%→50% B in A, 12 min, solutions used A: 25 mM NH4HCO3 in H2O, B: MeCN) and the fractions were concentrated and lyophilized to afford product 9 as a purple powder (1.9 mg, 0.0023 mmol, 13%).

1H-NMR (850 MHz, CD3OD): δ ppm 7.87 – 7.83 (m, 4H), 7.71 (s, 1H), 7.44 (d, J = 4.3 Hz, 2H), 6.99 – 6.95 (m, 4H), 6.69 (d, J = 4.3 Hz, 2H), 4.33 (t, J = 7.1 Hz, 2H), 3.85 (s, 6H), 3.65 – 3.58 (m, 2H), 3.18 – 3.16 (t, J = 9.2 Hz, 1H), 3.08 – 3.06 (m, 2H), 2.80 – 2.78 (m, 2H), 2.39 – 2.38 (m, 1H), 2.30 – 2.25 (m, 1H), 2.13 – 2.11 (m, 1H), 1.89 – 1.82 (m, 6H), 1.51 (d, J = 6.2 Hz, 1H), 1.34 – 1.20 (m, 11H);

13C-NMR (214 MHz, CD3OD): δ ppm 162.18, 158.78, 148.59, 146.79, 137.49, 132.16, 128.44, 126.52, 123.26, 114.61, 78.88, 74.21, 70.08, 62.19, 55.82, 51.27, 50.83, 44.62, 43.65, 34.22, 31.28, 30.99, 30.42, 30.38, 30.22, 29.91, 27.40, 25.82; LC-MS: Rt 7.00 min; linear gradient 10%→90% B in 12.5 min; ESI-MS: m/z = 827.3 (M+H)+; HRMS: calculated for C44H53BF2N6O7 [M+H+] 827.41172, found: 827.41098.

1-(6-(((1-(8-((1R,2S,3R,4S,5S,6R)-2-carboxy-3,4,5-trihydroxy-7-azabicyclo[4.1.0]heptan-7-yl)octyl)-1H-1,2,3-triazol-4-yl)methyl)amino)-6-oxohexyl)-3,3-dimethyl-2-((1E,3E)-5-(E)-1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3H-indol-1-ium chloride (10):

Azide compound 7 (6.0 mg, 0.017 mmol, 1.0 eq.) was dissolved in DMF (0.6 mL), Cy-5 alkyne 29 (12 mg, 0.021 mmol, 1.2 eq.), CuSO4 (1.0 M in H2O, 10 µL, 0.011 mmol, 0.6 eq) and sodium ascorbate (1.0 M in H2O, 11 µL, 0.012 mmol, 0.65 eq.) were added to the solution under argon atmosphere, and the mixture was stirred at room temperature overnight, the reaction was checked by LC-MS with an elution system of 10% aq. NH4OAc. The volatiles were concentrated in vacuo and then the crude product was purified by semi-preparative reversed HPLC (linear gradient: 40%→50% B in A, 12 min, solutions used A: 25 mM NH4HCO3 in H2O, B: MeCN) and the fractions were concentrated and lyophilized to afford product 10 as a dark blue powder (2.5 mg, 0.0029 mmol, 17%).

1H-NMR (850 MHz, CD3OD): δ ppm 8.26 – 8.22 (m, 2H), 7.85 (s, 1H), 7.49 – 7.48 (m, 1.3 Hz, 2H), 7.42 – 7.39 (m, 2H), 7.31 – 7.28 (m, 1H), 7.27 – 7.25 (m, 1H), 6.62 (t, J = 12.4 Hz, 1H), 6.29 – 6.27 (m, 2H), 4.41 (s, 2H), 4.36 (t, J = 7.0 Hz, 2H), 4.08 (t, J = 7.6 Hz, 2H), 3.63 – 3.58 (m, 3H), 3.16 (t, J = 9.2 Hz, 1H), 2.40 – 2.35 (m, 1H), 2.25 (t, J = 7.3 Hz, 2H), 2.16 – 2.09 (m, 2H), 1.89 – 1.83 (m, 8H), 1.84 – 1.78 (m, 2H), 1.72 – 1.68 (m, 10H), 1.56 – 1.42 (m, 7H), 1.34 – 1.22 (m, 10H);

13C-NMR (214 MHz, CD3OD): δ ppm 175.75, 175.36, 174.62, 155.53, 155.48, 146.14, 144.26, 143.53, 142.63, 142.52, 129.79, 129.73, 126.65, 126.25, 126.22, 126.18, 123.41, 123.27, 112.05, 111.85, 104.43, 104.28, 78.91, 74.23, 70.11, 62.14, 51.35, 50.81, 50.54, 50.50, 44.77, 44.61, 43.66, 36.47, 35.59, 31.53, 31.25, 30.30, 30.19, 29.86, 28.20, 28.12, 27.95, 27.81, 27.33, 27.30, 26.40, 24.18; LC-MS: Rf 6.29 min, linear gradient 10%→90% B in 12.5 min; ESI-MS: m/z = 862.5 (M+H)+; HRMS: calculated for C66H80BF2N6O7 [M+H+] 863.53038, found: 863.52618.
(1R,2S,3R,4S,5S,6R)-3,4,5-trihydroxy-7-(8-(4-((6-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexanamido)methyl)-1H-1,2,3-triazol-1-yl)octyl)-7-azabicyclo[4.1.0]heptane-2-carboxylic acid (11): Azide compound 7 (9.2 mg, 0.027 mmol, 1.0 eq.) was dissolved in DMF (0.8 mL) in eppi tube, Biotin-alkyne 30 (12.6 mg, 0.032 mmol, 1.2 eq.), CuSO$_4$ (1.0 M in H$_2$O, 10 µL, 0.011 mmol, 0.60 eq.) and sodium ascorbate (1.0 M in H$_2$O, 11 µL, 0.012 mmol, 0.65 eq.) were added to the solution under argon atmosphere, and the mixture was stirred at room temperature overnight, the reaction was checked with LC-MS with an elution system of 10% aq. NH$_4$OAc. The volatiles were concentrated in vacuo and then the crude product was purified by semi-preparative reversed HPLC (linear gradient: 40%→50% B in A, 12 min, solutions used A: 25 mM NH$_4$HCO$_3$ in H$_2$O, B: MeCN) and the fractions were concentrated and lyophilized to afford product 11 as a white powder (2.6 mg, 0.0035 mmol, 13%).

$^1$H-NMR (600 MHz, CD$_3$OD): δ ppm 7.87 (s, 1H), 4.49 (dd, $J = 7.9$, 4.9 Hz, 1H), 4.46 – 4.34 (m, 4H), 4.31 (dd, $J = 7.9$, 4.4 Hz, 1H), 3.70 - 3.54 (m, 2H), 3.24 – 3.12 (m, 4H), 2.96 – 2.89 (m, 1H), 2.70 (d, $J = 12.7$ Hz, 1H), 2.43 (br, 1H), 2.34 - 21 (m, 6H), 1.98 – 1.81 (m, 3H), 1.78 – 1.47 (m, 11H), 1.44 (q, $J = 7.5$ Hz, 2H), 1.39 – 1.24 (m, 10H);

$^{13}$C-NMR (150 MHz, CD$_3$OD): δ ppm 176.00, 175.98, 166.14, 124.20, 79.21, 78.79, 74.15, 70.06, 69.31, 63.68, 63.38, 62.13, 61.62, 57.03, 51.39, 44.75, 43.60, 41.06, 40.20, 36.82, 36.75, 35.62, 31.30, 30.40, 30.25, 30.12, 29.96, 29.79, 29.50, 28.25, 27.56, 27.20, 26.94, 26.70, 26.54;

LC-MS: R$_t$ 4.06 min, linear gradient 10-90% B in 12.5 min; ESI-MS: $m/z$ = 737.3 (M+H$^+$); HRMS: calculated for C$_{34}$H$_{56}$N$_8$O$_8$S [M+H$^+$] 737.40146, found: 737.40167.

(2S,3R,4R,5R)-2,3,4-tris(benzyloxy)-5,6-dihydroxyhexyl acetate (32): The Intermediate 32 (20.6 g, 41.7 mmol) was prepared via previous reported synthesis, in 6 steps from compound 31 in 83% total yield. [α]$_D$ +11.8 (c = 1, CHCl$_3$);

$^1$H-NMR (400 MHz, CDCl$_3$) δ ppm 7.35 – 7.24 (m, 15H), 4.70 – 4.55 (m, 6H), 4.31 (dd, $J = 7.9$, 4.4 Hz, 1H), 4.23 – 4.18 (m, 1H), 3.96 – 3.92 (m, 1H), 3.84 – 3.81 (m, 1H), 3.76 – 3.71 (m, 3H), 3.67 – 3.63 (m, 1H), 1.97 (s, 3H);

$^{13}$C-NMR (100 MHz, CDCl$_3$): δ ppm 170.75, 137.80, 137.66, 137.49, 128.80, 128.60, 128.58, 128.56, 128.22, 128.20, 128.07, 128.06, 78.04, 77.08, 76.73, 76.42, 73.94, 73.77, 73.36, 71.81, 63.63, 63.53, 20.96; IR (neat, cm$^{-1}$): 3447, 2876, 1738, 1497, 1454, 1368, 1231, 1026, 733, 696; HRMS: calculated for C$_{29}$H$_{34}$NO$_7$ [M+Na$^+$] 517.21967, found: 517.21919.

(2S,3R,4R,5R)-2,3,4-tris(benzyloxy)-5-hydroxy-6-(trityloxy)hexyl acetate (33): Product 32 (20 g, 41.7 mmol) was dissolved in DCM (500 mL). Then DMAP (0.25 g, 2.1 mmol, 0.05 eq.), Et$_3$N (10 mL, 72 mmol, 1.7 eq) and triphenylmethyl chloride (15 g, 46 mmol, 1.3 eq.) were added. The reaction was then stirred for 3 h at room temperature. Afterwards aq. sat. NaHCO$_3$ solution (600 mL) was added to quench the reaction. The product was extracted from the water layer with EtOAc. The combined organic layers were dried over MgSO$_4$, filtered and concentrated under reduced pressure. The crude mixture was purified by silica gel column chromatography (10%→20%, EtOAc in pentane) providing product 33 (23 g, 31 mmol, 75 %). [α]$_D$ +6 (c = 1, CHCl$_3$); $^1$H-NMR (400 MHz, CDCl$_3$): δ ppm 7.45 – 7.41 (m, 6H), 7.29 – 7.19 (m, 22H), 7.13 – 7.10 (m, 2H), 4.65 – 4.56 (m, 3H), 4.48 – 4.44 (m, 3H), 4.38 (dd, $J = 12.0$, 3.6 Hz, 1H), 4.15 – 4.04 (m, 2H), 3.94 – 3.85 (m, 2H), 3.77 – 3.74 (m, 1H), 3.36 – 3.29 (m, 2H), 3.01 (d, $J = 5.6$ Hz, 1H), 1.90 (s, 3H); $^{13}$C-NMR (100 MHz, CDCl$_3$): δ ppm 170.77, 143.86, 138.09, 137.86, 137.82, 128.78, 128.51, 128.46, 128.36, 128.25, 128.13, 127.95, 127.83, 127.74, 127.18, 86.75,
Synthesis and biological evaluation of 8-carboxy-cyclophellitol aziridine derivatives

78.35, 77.11, 76.81, 74.31, 73.15, 72.86, 70.69, 64.74, 64.12, 20.95; IR (neat, cm⁻¹): 3030, 2876, 2347, 1738, 1449, 1231, 1067, 737, 696, 633; HRMS: calculated for C₄₈H₄₈O₇ [M+Na⁺] 759.32922, found: 759.32905.

(2S,3R,4R,5R)-2,3,4-tris(benzyloxy)-6-(trityloxy)hexane-1,5-diol (34): Product 33 (21 g, 29 mmol, 1.0 eq.) was co-evaporated with toluene and then dissolved in dry MeOH (500 mL). Thereafter catalytic amount of Na (0.60 g) was added. The mixture was stirred for 3 h at room temperature and then concentrated at reduced pressure. The residue was dissolved in H₂O (300 mL). The product was extracted from the water layer with EtOAc. The combined organic layers were washed with brine (300 mL), dried over MgSO₄, filtered and concentrated under reduced pressure. Finally the reaction mixture was purified by silica gel column chromatography (12% → 50%, EtOAc in pentane) giving product 34 (18.56 g, 26.71 mmol, 91%). [α]D₂₀ -4 (c = 1, CHCl₃); ¹H-NMR (400 MHz, CDCl₃): δ ppm 7.72 – 7.67 (m, 6H), 7.51 – 7.35 (m, 22H), 7.32 – 7.27 (m, 2H), 4.95 – 4.71 (m, 4H), 4.69 – 4.63 (m, 2H), 4.33 (br, 1H), 4.17 – 4.07 (m, 2H), 4.06 – 3.99 (m, 1H), 3.98 – 3.89 (m, 1H), 3.85 – 3.75 (m, 1H), 3.69 – 3.62 (m, 1H), 3.61 – 3.45 (m, 2H), 2.76 (br, 1H); ¹³C-NMR (100 MHz, CDCl₃): δ ppm 143.71, 138.09, 137.75, 137.60, 129.06, 128.75, 128.61, 128.31, 128.22, 128.17, 128.04, 127.80, 127.62, 126.97, 125.97, 86.53, 79.39, 79.01, 76.91, 74.30, 72.81, 72.75, 70.63, 64.58, 61.64; IR (neat, cm⁻¹): 2932, 1736, 1449, 1371, 1240, 1043, 745, 696; HRMS: calculated for C₄₆H₄₆NO₆ [M+Na⁺] 717.31866, found: 717.31864.

(2R,3R,4S)-2,3,4-tris(benzyloxy)-5-oxo-6-(trityloxy)hexanal: COCl₂ (8.7 mL, 101 mmol, 4.0 eq.) was dissolved in DCM (92 mL) and cooled to -78 °C. Then DMSO (9.0 mL, 126 mmol, 5.0 eq.) in DCM (54 mL) was added dropwise over 10 min. The mixture was stirred for 40 min while kept below -70 °C. Then a solution of 34 (17 g, 25 mmol, 1.0 eq.) in DCM (50 mL) was added dropwise over 15 min. After this the mixture was kept below -65 °C for 2 h while stirring, followed by dropwise addition of Et₃N (42 mL, 302 mmol, 12 eq.) over 10 min. The mixture was then warmed to -5 °C over 2 h and concentrated under reduced pressure.

(2R,3R,4R,5S)-3,4,5-tris(benzyloxy)-2-((trityloxy)methyl)piperidine (35): Crude product aldehyde (1.0 eq.) was co-evaporated with toluene thrice after which it was dissolved in MeOH (500 mL). Subsequently NH₄HCO₂ (32 g, 504 mmol, 20 eq.) was added. The solution was then cooled to 0 °C and stirred for 20 min. Activated molecular sieves (250 g, 4 Å) were added and the mixture was stirred for another 20 min. Then NaBH₃CN (6.3 g, 101 mmol, 4.0 eq.) was added. The mixture was cooled to 0 °C and stirred for 1 hour, after which the cooling was removed and the mixture was stirred for another 20 h. It was then filtered and concentrated under reduced pressure. The residue was dissolved in EtOAc (300 mL). The solution was then washed with aq., sat. NaHCO₃ (300 mL). The product was extracted from the water layer with EtOAc. Subsequently the combined organic layers were dried over MgSO₄, filtered and concentrated under reduced pressure. At last the mixture was purified by silica gel column chromatography (11% → 25%, EtOAc in pentane) giving product 35 (14 g, 21 mmol, 85 % over 2 steps). ¹H-NMR (400 MHz, CDCl₃): δ ppm 7.51 – 7.20 (m, 28H), 6.90 – 6.88 (m, 2H), 4.97 (d, J = 10.8 Hz, 1H), 4.81 – 4.67 (m, 4H), 4.22 (d, J = 10.8 Hz, 1H), 3.58 – 3.50 (m, 3H), 3.41 – 3.28 (m, 2H), 3.24 – 3.15 (m, 1H), 2.79 – 2.71 (m, 1H), 2.62 – 2.50 (m, 1H), 1.98 (br, 1H); ¹³C-NMR (100 MHz, CDCl₃): δ ppm 143.79, 138.93, 138.67, 138.21, 128.83, 128.51, 128.47, 128.26, 128.11, 128.05, 127.92, 127.85, 127.76, 127.66, 127.58, 127.16, 87.51, 86.65, 75.89, 80.93, 80.38, 75.10, 72.90,
Benzyl (2R,3R,4R,5S)-3,4,5-tris(benzyloxy)-2-((trityloxy)methyl)piperidine-1-carboxylate (36):

Product 35 (160 mg, 0.24 mmol, 1.0 eq.) was dissolved in 3 mL THF, Et3N (33 μL, 0.24 mmol, 1.0 eq.) and CbzCl (82 mg, 0.48 mmol, 2.0 eq.) were added into the solution, then the mixture was stirred at room temperature for 20 h. The reaction was diluted by EtOAc, washed by sat. aq. NaHCO3 and brine. The organic layer was dried over MgSO4, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (5%→30%, EtOAc in pentane) giving in product 36 (140 mg, 0.17 mmol, 72%).

1H-NMR (400 MHz, CDCl3): δ ppm 7.65–7.13 (m, 35H), 5.20 (br, 2H), 4.87 (d, J = 11.1 Hz, 2H), 4.78–4.67 (m, 2H), 3.91–3.80 (m, 2H), 3.72–3.40 (m, 3H); 13C-NMR (100 MHz, CDCl3): δ ppm 143.90, 141.13, 138.17, 138.05, 136.56, 128.96, 128.88, 128.68, 128.42, 128.31, 127.97, 127.92, 127.88, 127.82, 127.72, 127.67, 127.64, 127.51, 127.08, 126.94, 126.11, 86.70, 82.09, 78.31, 74.77, 73.90, 73.07, 70.53, 67.35, 65.18, 61.89, 56.42, 29.75; HRMS: calculated for C54H51NO6 [M+H]+ 810.37891, found: 810.37956.

Benzyl (2R,3R,4R,5S)-3,4,5-tris(benzyloxy)-2-(hydroxymethyl)piperidine-1-carboxylate (37):

Product 36 (140 mg, 0.17 mmol, 1.0 eq.) was dissolved in DCM/MeOH (2.0 mL, 1/1, v/v), followed by addition of p-toluenesulfonic acid monohydrate (6.6 mg, 0.035 mmol, 0.20 eq.). After stirring overnight at room temperature, the reaction was quenched with Et3N. The reaction was diluted by EtOAc, washed by brine. The combined organic layers were dried over MgSO4, filtered and concentrated under reduced pressure. The mixture was then purified by silica gel column chromatography (10%→50%, EtOAc in pentane) to afford product 37 (80 mg, 0.14 mmol, 83%). [α]D20 + 2.2 (c = 1, CHCl3); 1H-NMR (400 MHz, CDCl3): δ ppm 7.37–7.24 (m, 20H), 5.14 (s, 2H), 4.86–4.58 (m, 5H), 4.53 (d, J = 11.8 Hz, 1H), 4.02–3.85 (m, 3H), 3.78–3.67 (m, 5H); 13C-NMR (100 MHz, CDCl3): δ ppm 156.37, 138.13, 138.03, 136.38, 128.63, 128.56, 128.52, 128.49, 128.20, 128.06, 128.03, 127.92, 127.88, 127.80, 77.53, 75.52, 73.37, 71.35, 67.63, 61.48; HRMS: calculated for C35H37NO6 [M+H]+ 568.26936, found: 568.26952.

(2S,3R,4R,5S)-3,4,5-tris(benzyloxy)-1-((benzyloxy)carbonyl)piperidine-2-carboxylic acid (38):

The primary alcohol compound 37 (80 mg, 0.14 mmol, 1.0 eq.) was dissolved in DCM/H2O (1.5 mL, 2/1, v/v), then BAIB (112.7 mg, 0.35 mmol, 2.5 eq.) and TEMPO (4.4 mg, 0.028 mmol, 0.20 eq.) were added in the solution, the resulting mixture was stirred for 5 h at room temperature. The reaction was quenched with 10% Na2S2O3, was extracted by EtOAc and washed by brine. The combined organic layers were dried over MgSO4, filtered and concentrated under reduced pressure. The mixture was then purified by silica gel column chromatography (25%→50%, EtOAc in pentane with 1% AcOH) to afford product 38 (58 mg, 0.10 mmol, 71%). 1H-NMR (400 MHz, CDCl3): δ ppm 10.85 (br, 1H), 7.44–7.21 (m, 20H), 5.47–5.07 (m, 3H), 4.93–4.51 (m, 5H), 4.46–4.14 (m, 3H), 3.90–3.56 (m, 3H); 13C-NMR (100 MHz, CDCl3): δ ppm 174.90, 174.74, 156.91, 156.17, 138.06, 137.58, 137.38, 136.29, 128.91, 128.67, 128.41, 128.38, 128.26, 128.00, 127.90, 127.77, 127.71, 127.39, 126.09, 77.48, 77.16, 76.84, 74.17, 73.75, 73.38, 71.64, 71.51, 70.72, 67.71, 55.95, 55.05, 40.74, 39.97; HRMS: calculated for C35H35NO7 [M+H]+ 582.24863, found: 582.24883.
Dibenzyl (25R,3R,4R,5S)-3,4,5-tris(benzyloxy)piperidine-1,2-dicarboxylate: Product 38 (180 mg, 0.32 mmol, 1.0 eq.) was dissolved in DCM/H$_2$O (3.0 mL, 2/1, v/v) and cooled to 0 °C. TEMPO (10 mg, 0.064 mmol, 0.20 eq.) and BAIB (255 mg, 0.79 mmol, 2.5 eq.) were added at 0 °C. After 6 h stirring, the reaction mixture was quenched by 10% aq. Na$_2$S$_2$O$_3$ solution and extracted by DCM, being successively washed with water and brine, dried over MgSO$_4$ and concentrated under reduced pressure. The residue was co-evaporated twice with toluene. To a stirred solution of the residual syrup and benzyl bromide (76 μL, 0.63 mmol, 2.0 eq.) in dry DMF (5.0 mL) was added cesium carbonate (103 mg, 0.32 mmol, 1.0 eq.) at room temperature. The mixture was stirred for 3 h, and poured into water, before the mixture was extracted with Et$_2$O. The extract was washed by water and brine, dried over MgSO$_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (10 % → 30 %, EtOAc in pentane) to give Bn protected 38 (160 mg, 0.24 mmol, 75% over two steps).

[$\alpha$]$_D^{20}$ +19 (c = 1, CHCl$_3$); $^1$H-NMR (400 MHz, DMSO-d$_6$): δ ppm 7.34–7.22 (m, 25H), 5.14–5.07 (m, 3H), 4.99–4.95 (m, 2H), 4.62–4.47 (m, 6H), 4.25–4.24 (m, 1H), 4.03–3.99 (m, 1H), 3.87–3.85 (m, 1H), 3.76–3.72 (m, 1H), 3.64–3.60 (m, 1H), 3.09 (s, 1H);

$^{13}$C-NMR (100 MHz, DMSO-d$_6$): δ ppm 168.46, 155.21, 138.01, 137.44, 136.24, 135.07, 128.20, 128.04, 127.87, 127.81, 127.63, 127.57, 127.54, 127.28, 127.20, 127.12, 127.06, 126.94, 126.90, 126.73, 74.51, 70.89, 70.56, 69.90, 66.21, 66.03, 55.94, 40.26.

(2S,3R,4R,5S)-3,4,5-trihydroxypiperidine-2-carboxylic acid (12): A mixture of Product Bn-protected 38 (160 mg, 0.24 mmol, 1.0 eq.) and Pd(OH)$_2$/C (50 mg, 20% wt. loading(dry basis)) in 80% AcOH in H$_2$O (5.0 mL) was stirred at room temperature under a hydrogen atmosphere for 24 h. The catalyst was then filtered off and washed with MeOH. The filtrate and washings were combined and concentrated under reduced pressure to give semi-crystalline solid. Recrystallization from water ethanol gave final product 12 (24 mg, 0.14 mmol, 57%) as crystalline solid.

[$^1$H-NMR (400 MHz, D$_2$O): δ ppm 3.75–3.67 (m, 1H), 3.62 (t, J = 9.6 Hz, 1H), 3.47 (t, J = 9.0 Hz, 1H), 3.41–3.34 (m, 2H), 2.81 (t, J = 11.8 Hz, 1H), 1.88 (s, 1H); $^{13}$C-NMR (100 MHz, D$_2$O): δ ppm 173.10, 76.05, 70.77, 67.73, 61.71, 45.70; HRMS: calculated for C$_6$H$_{11}$NO$_5$ [M+H$^+$] 178.07100, found: 178.07105.

6.5 References


