C-Alkyl 5-membered ring imino sugars as new potent cytotoxic glucosylceramide synthase inhibitors

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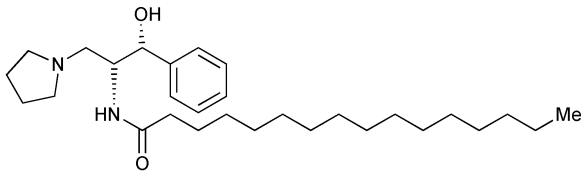
The stereoselective preparation of novel *C*-alkyl 5-membered ring imino sugars and their biological evaluation with regard to GCS inhibition and cytotoxicity in a murine melanoma model are reported.

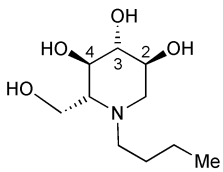
Glucosylceramide synthase (GCS, EC 2.4.1.80) catalyses the transfer of glucose to the C-1 hydroxyl of ceramide, the first step in the biosynthesis of glucosylceramide-based glycosphingolipids (GSLs). Slowing glucosylceramide production already constitutes a relevant approach against lysosomal storage disorders characterized by inherited deficiencies in GSL catabolism.[1] This strategy, the so called substrate deprivation therapy, resulted in the commercialisation of Zavesca® (*N*-butyl-1-deoxynojirimycin) for the treatment of mild to moderate type I Gaucher disease. Notably, GSLs have been involved in many cellular processes including cell–cell communication, cell adhesion, differentiation, proliferation and oncogenic transformation.[2] Overexpression of GCS observed in cancer cells has been related to the ineffective host immune response and to tumour progression and/or metastasis.[3] Moreover, by reducing the accumulation of the pro-apoptotic ceramide, GCS could prevent cancer cell death in response to some chemotherapeutic agents.[4] Conversely, GCS inhibition may resensitize multidrug resistant (MDR) cancer cells to antineoplastic agents, suggesting that an elevated GCS activity participates in a new MDR mechanism.[5] These observations have led to the concept that GCS may represent a key enzymatic target in anti-cancer chemotherapy.[6]

Known synthetic low molecular mass GCS inhibitors belong to two main classes. The "P drug" family encompasses highly lipophilic D-*threo* sphingolipid analogues based on a 1-phenyl-2-alkoylamino-3-amino-1-propanol pattern (Fig. 1).[7] The *N*-butyl-1-deoxynojirimycin (NB-DNJ), and analogues thereof, represent the second category of GCS inhibitors.[8] Derived from imino sugars, these non-cytotoxic derivatives typically retain their glucosidase inhibition potency, which represents a limitation to the therapeutic use of NB-DNJ.

Whereas the *N*-alkyl imino sugars such as NB-DNJ have been extensively studied, examples of their *C*-alkyl congeners are less common and concern 1-substituted derivatives.[9] Compain and Martin have prepared series of *N*-alkyl-1-deoxynojirimycin analogues bearing additional *C*-branched lipophilic chains in an attempt to enhance GCS inhibition through better ceramide mimicry.[10] Potent β-glucocerebrosidase inhibitors based on a 6-alkyl isofagomine scaffold have also been described by Fan.[11] Interestingly, the potential of 5-membered ring imino sugars in the context of sphingolipid metabolism remains almost unexplored.[12]



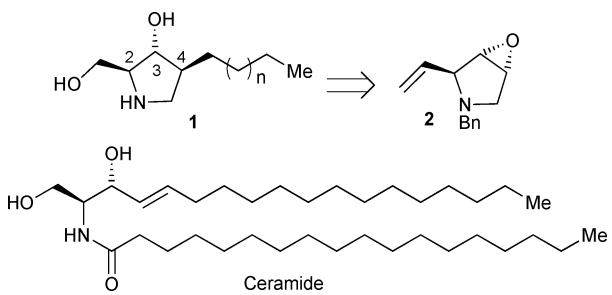
D-*threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4)



N-butyl-1-deoxynojirimycin (NB-DNJ)

Fig. 1 Structure of two GCS inhibitors.

We anticipated that a pyrrolidine-based imino sugar could represent a suitable molecular scaffold to elaborate GCS inhibitor candidates. In the course of the development of a stereoselective route towards imino sugars, we gained access to the versatile epoxypyrrolidine intermediate **2**, which appeared to be a suitable precursor of novel *C*-alkyl 5-membered ring imino sugars (Scheme 1).[13] We decided to take advantage of a regioselective epoxide opening reaction to achieve branching of the aliphatic chain in the 4-position, yielding all-*trans* trisubstituted pyrrolidines. Further oxidative manipulation of the vinyl moiety would allow access to a proper imino sugar framework through formation of an hydroxymethyl group at C-2. The structural and stereochemical homology between the aminodiol moiety of the



Scheme 1 Structure of the ceramide, of the GCS inhibitor candidate **1** and of its synthetic precursor.

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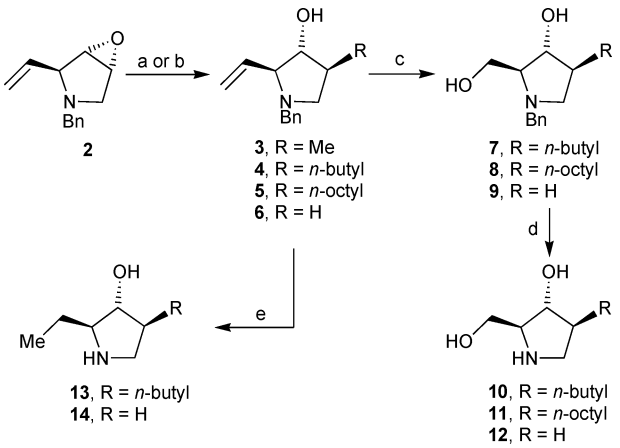
targeted pyrrolidines and that of the sphingosine backbone of ceramide further prompted us to initiate this study.

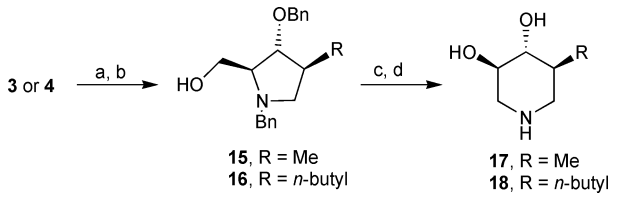
We thus report here the stereoselective preparation and the biological evaluation in regard with GCS inhibition and cytotoxicity of *C*-alkyl 5-membered ring imino sugars of type **1**.

Introduction of the *n*-alkyl residue was achieved *via* treatment of epoxide **2** with the corresponding Gilman-like cuprate (Scheme 2). Remarkably, the expected adducts were isolated as a unique regio- and diastereoisomer. Thus a methyl, *n*-butyl and *n*-octyl residue could be readily branched in 60 to 77% yields. In order to prepare analogues lacking the lipophilic chain, unsubstituted intermediate **6** was also prepared in 95% yield by totally regiocontrolled C-4 hydride delivery. This level of regioselectivity is worth noting with regard to the relatively modest steric bulk generated by the vinyl moiety. Setting up of the hydroxymethyl appendage was smoothly accomplished using a metal-free oxidative cleavage. Thus, ozonolysis of olefins **4**–**6** was carried out after protection of the amino group by salification. Immediate reduction of the resulting sensitive amino aldehydes afforded the expected intermediates **7**–**9** in 52 to 70% overall yields. A final hydrogenolysis gave the targeted pyrrolidines **10**–**12** in good yields. Treatment of intermediate **4** and **6** under same conditions delivered derivatives **13** and **14** bearing an ethyl residue in place of the hydroxymethyl group.



Scheme 2 Synthesis of the tested pyrrolidines. *Reagents and conditions*: (a) Me_2CuLi, or *n*-Bu_2CuLi, or *n*-Oct_2CuLi, Et_2O, −20 °C, **3**, 77%; **4**, 63%; **5**, 60%; (b) $LiAlH_4$, Et_2O, 0 °C, **6**, 95%; (c) (i) HCl, MeOH, 0 °C; (ii) O_3, MeOH, −78 °C; (iii) $NaBH_4$, MeOH, −78 to −10 °C, **7**, 70%; **8**, 52%; **9**, 68%; (d) 7–8 bar H_2, Pd/C, conc. HCl, MeOH, **10**, 90%; **11**, 52%; **12**, quant. brsm at 82% conversion; (e) 7–8 bars H_2, Pd/C, MeOH, **13**, quant.; **14**, 40%.

In order to highlight the eventual relevance of the pyrrolidinic scaffold of our GCS inhibitor candidates, we decided to compare them to their direct 6-membered ring analogues. Having in hands 2-hydroxymethyl pyrrolidines, we thus focused our attention on the stereospecific ring expansion reaction developed by Cossy.[14] Although precedents are scarce, such a process should allow access to all-*trans* 3-alkyl-4,5-dihydroxy piperidines (Scheme 3). Interestingly, these structures could also be viewed as relevant 2-deoxy-2-alkyl DNJ analogues. Indeed, it has been proposed, on the basis of Butters' ceramide mimicry model, that an alkyl chain at the 2-position of NB-DNJ would simulate the hydrophobic portion of the sphingosine framework (Fig. 1).[10] In



Scheme 3 Synthesis of the tested piperidines. *Reagents and conditions*: (a) BnBr, NaH, NaI, 4 Å MS, DMF, R = Me, 85%; R = *n*-butyl, 84%; (b) (i) HCl, MeOH, 0 °C; (ii) O_3, MeOH, −78 °C; (iii) $NaBH_4$, MeOH, −78 °C to −10 °C, **15**, 63%; **16**, 63%; (c) (i) TFAA, *p*-dioxane; (ii) Et_3N, reflux; (iii) NaOH 2.5 M, R = Me, 91% brsm at 85% conversion; R = *n*-butyl, 78% brsm at 86% conversion; (d) 7–8 bar H_2, Pd/C, conc. HCl, MeOH, **17**, 71%; **18**, 78%.

this event, intermediates **3** and **4** were first benzylated before being converted to primary alcohols **15** and **16** *via* ozonolysis. These 3,4-disubstituted precursors initially proved to be poorly reactive when treated with trifluoroacetic anhydride and Et_3N. However, after careful optimisation of the reaction conditions, the expected enlarged products could be secured in good yields. In this respect, the use of *p*-dioxane as solvent proved decisive. The deprotected piperidines **17** and **18** were finally obtained after hydrogenolysis of the benzyl groups.

5-Membered ring imino sugars **8** and **10**–**14** were tested on a mouse melanoma B16 cell line (Fig. 2).[15] Indeed, melanoma is largely considered to be a radiation- and chemotherapy-refractory neoplasm. As observed after incubating intact living cells with a fluorescent ceramide substrate, not only pyrrolidine **10** (at 50 μM) exerted a 34% GCS inhibition but also this potency was comparable to that of NB-DNJ under the same conditions (49% inhibition).[16] In order to distinguish the contribution to activity of the different structural features of imino sugar **10**, we then tested a series of analogues. The primary hydroxyl group appeared crucial as its replacement by a methyl residue proved unfavourable, giving around 6% inhibition for **13**. The observed drop in potency (16% inhibition) for the C-4 unsubstituted compound **12** clearly indicated the importance of the lipophilic portion of inhibitor **10**. Compound **14**, lacking both the primary hydroxyl group and the

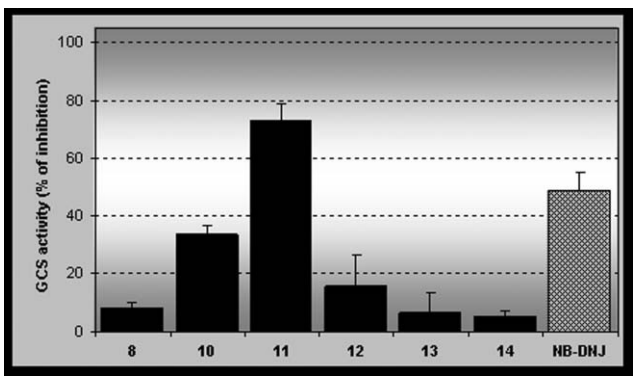


Fig. 2 Glucosylceramide synthase inhibition. Melanoma cells were pre-incubated overnight with the indicated compounds (10 μM for **8** and **11**, 50 μM for **10**, **12**–**14** and NB-DNJ). NBD-C6-ceramide (5 μM) was added and after 2 h lipids were extracted. The extent of NBD-C6-glucosylceramide formation was then assessed. Data are expressed as a percentage of the values of B16 untreated cells.

C-alkyl chain, was also shown to be inactive. Notably, introduction of an *n*-octyl residue, instead of an *n*-butyl, was accompanied by a dramatic enhancement in potency, reaching 73% GCS inhibition (at only 10 μM) for **11**. Finally, the detrimental influence of the substitution at the nitrogen atom was suggested by the comparison of **11** with its *N*-benzyl analogue **8** (10% inhibition at 10 μM).

Remarkably, the pyrrolidinic framework of these *C*-alkyl imino sugars revealed a determinant for their activity, since the piperidines **17** and **18** only displayed a marginal GCS inhibition.

We next investigated the potential cytotoxic effect of our new GCS inhibitors on the B16 cell line (Fig. 3). Compound **10** exhibited dose-dependent cytotoxicity, reaching 50% activity at 25 μM. Additional findings, namely caspase activation[17] and an elevation of intracellular ceramide concentration (not shown),[18] suggest that derivative **10** triggered a ceramide-mediated apoptotic cell death. Notably, cytotoxicity was raised to 70% at 5 μM with the more potent inhibitor **11**.

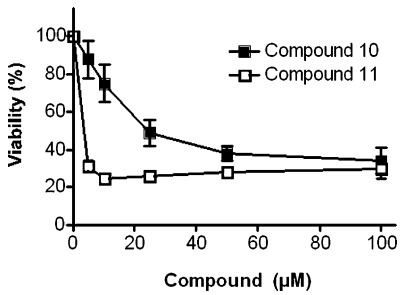


Fig. 3 Sensitivity of melanoma cells to compounds **10** or **11**. Melanoma B16 cells were seeded in 24-well plates and at 50% confluency, compounds **10** or **11** were added at the indicated concentrations. After 48 h, cell viability was estimated by assessing the cellular MTT conversion capacity. Data are expressed as a percentage of the values of untreated cells. Shown are the means ± SE ($n = 2$–5).

The present results showed that imino sugars **10** and **11** displayed an activity profile contrasting with that of the non-cytotoxic deoxynojirimycin-like GCS inhibitors.[19] This might be related to the branching position of the aliphatic residue on the imino sugar scaffold, which has been shown to influence the inhibition of sphingolipid glucosidic processing. This structural feature might somehow broaden their inhibition pattern and hence modulate their overall activity on cancer cells.

We thus described a straightforward and enantioselective access to 4-alkyl pyrrolidine-based imino sugars, relying on a regio- and stereocontrolled epoxide opening reaction, as well as their conversion into six-membered ring imino sugars.

The novel *C*-alkyl 5-membered ring imino sugars behaved as potent GCS inhibitors, displaying furthermore a marked cytotoxic behaviour in a murine melanoma model. The close resemblance between these structures and the sphingosine backbone of sphingolipids suggests that they might act as ceramide mimics. Studies aiming at elucidation of their mode of enzymatic interaction are currently pursued. The present work should also open up new prospects for the development of chemotherapeutic anti-cancer agents potentially acting through alterations of ceramide metabolism.[20]

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