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Synthesis and biological evaluation of novel PDMP analogues

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Abstract—A new series of hybrid PDMP analogues, based both on PDMP and styryl analogues of natural ceramide, has been synthesized from D-serine. The synthetic route was developed such that future introduction of different aryl groups is straightforward. Biological evaluation, both in vitro on rat liver Golgi fractions as well as in HEK-293 and COS-7 cells, revealed two lead compounds with comparable inhibitory potency as PDMP, which could be elaborated to more potent inhibitors.

1. Introduction

A plethora of biological effects has been assigned to sphingolipids (SLs) over the last two decades. Whereas SLs initially were regarded as inert structural components of cell membranes, it has now become clear that they play an important role in the regulation of a myriad of cellular processes including cellular differentiation, growth, adhesion, senescence, apoptosis and signal transduction.[1] It is obvious that disruption of this fragile cellular equilibrium, for example by impaired lysosomal degradation of SLs, could have severe pathophysiological consequences. Lysosomal storage diseases, such as Gaucher and Tay-Sachs diseases, are caused by the defective catabolism of glycosphingolipids (GSLs), resulting in substrate accumulation.[2]

Since glucosylceramide (GlcCer; **1**; Fig. 1) acts as a hub for the synthesis of more complex GSLs, it has been suggested[3] that partial reduction of the biosynthesis of GlcCer might offer a valuable strategy for treatment of storage diseases such as Gaucher disease and other sphingolipid storage diseases.

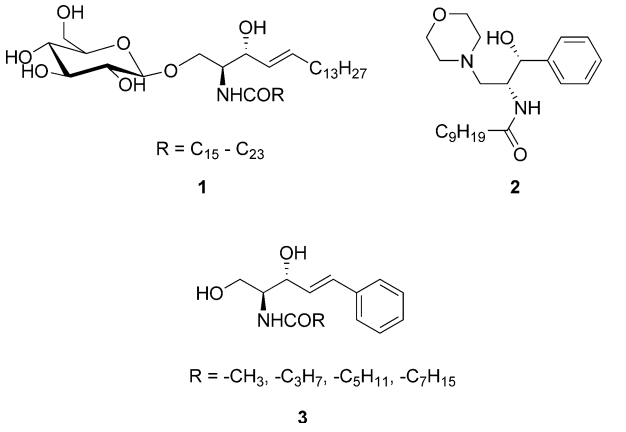

Figure 1. Structures of GlcCer (**1**), D-*threo*-PDMP (**2**) and *E*-styryl ceramides (**3**).

PDMP[4] (D-*threo*-(1*R*,2*R*)-phenyl-2-decanoylamino-3-morpholino-1-propanol; **2**) and related compounds[5] have been developed as potent inhibitors of GlcCer synthase. Surprisingly, only the D-*threo* isomer specifically inhibits GlcCer synthase.[6] Furthermore, it was shown that elongation of the acyl chain from decanoyl to palmitoyl[5a] and introduction of electron donating aromatic substituents[5b] drastically enhanced the inhibitory capacity. Moreover, a pyrrolidino head group was proposed to be the best mimic of the sugar transition state.

In our approach, we aimed at the synthesis of hybrid structures based on styryl analogues (**3**) of natural

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ceramides. It was indeed previously shown that such analogues are recognised by GlcCer synthase[7] and subsequently metabolised to the glucosylated form. Our primary concern was whether these hybrid analogues would still exhibit biological activity.

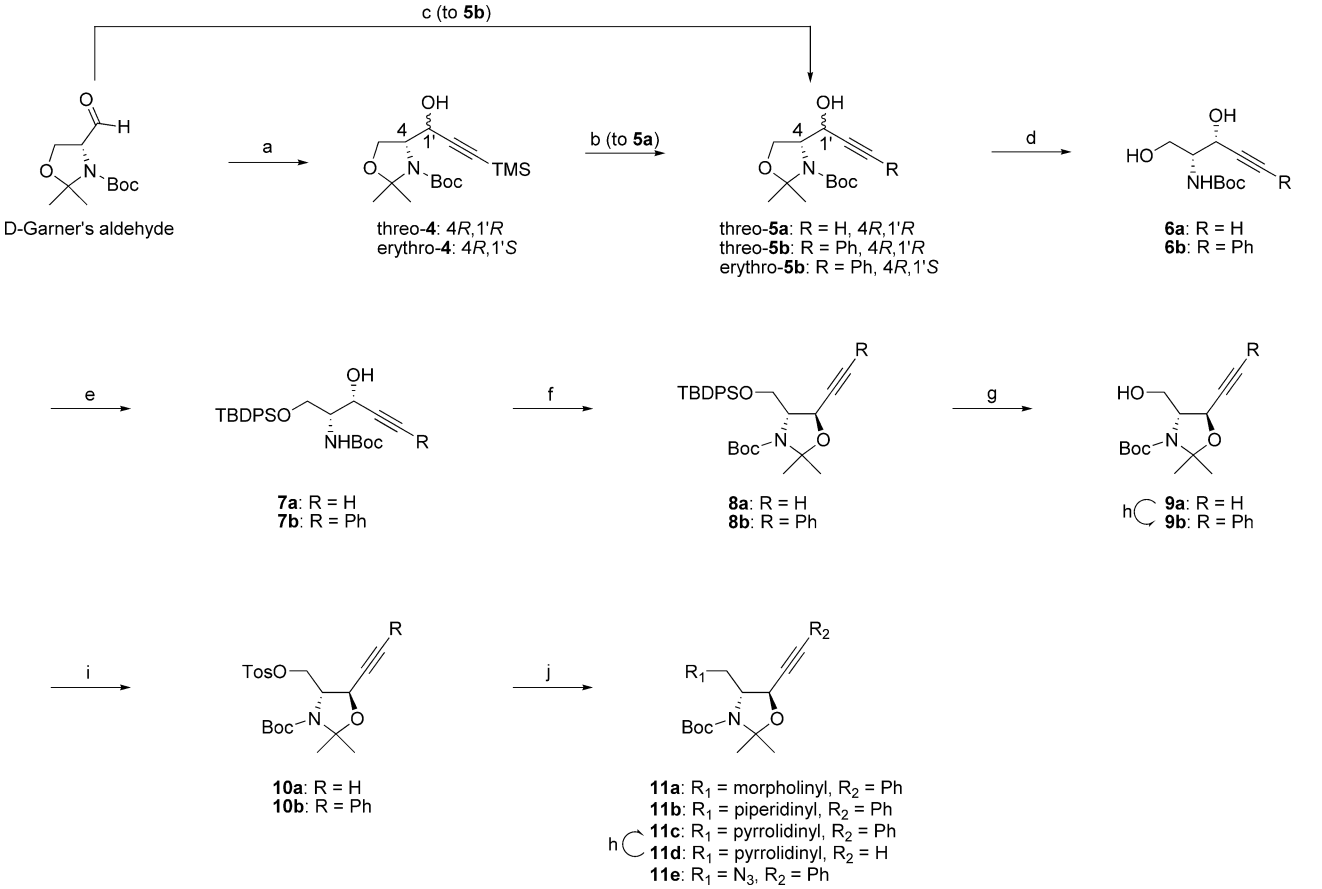
Our strategy focused mainly on the elaboration of a synthetic route towards a single advanced intermediate for introduction of the aryl moiety by Sonogashira coupling between a terminal alkyne and an appropriate aryl iodide, thereby avoiding reworking of the entire synthetic scheme for each compound. Throughout the synthetic scheme, we gained access to a number of structural analogues which could provide more insight into the structure–activity relationship of this class of compounds.

2. Results and discussion

2.1. Chemistry

The known Garner aldehyde (derived in five steps from D-serine[8]) served as a chiral building block since it allows good control of stereochemistry in nucleophilic additions and it has shown to be configurationally stable.[9] Indeed, addition of the appropriate lithium acetylide to the aldehyde under chelating conditions ($ZnBr_2$) yielded predominantly *threo*-adducts **4** and **5b** (41% and 44% isolated yields, respectively; *threo*:*erythro* 9:1; Scheme 1).[10] Removal of the TMS group from *threo*-**4** with TBAF produced terminal alkyne *threo*-**5a** (93%). Subsequent opening of the oxazolidine ring with 90% acetic acid or *p*-TsOH in MeOH followed by selective silylation of the primary alcohol gave the protected sphingosine analogues **7a** (83% from *threo*-**5a**) and **7b** (59% from *threo*-**5b**). Since it had become clear from preliminary experiments that a double amino-protective strategy would be crucial for successful elaboration to the desired compounds, acid mediated oxazolidine ring formation followed by desilylation allowed access to alcohols **9a** (94% from **7a**) and **9b** (76% from **7b**). Sonogashira coupling of terminal alkyne **9a** with iodobenzene gave alcohol **9b** in excellent yield (99%).[11]

Although we succeeded in converging both synthetic pathways at this point, it would still be more convenient to introduce the aromatic ring at a later stage to avoid the separate introduction of amine substituents for each individual aryl analogue. Therefore, both primary alcohols **9a** and **9b** were converted to the respective tosylates **10a** (94%) and **10b** (64%). The lower yield of **10b** might be ascribed to decomposition during work-up. Indeed,



Scheme 1. Reagents and conditions: (a) lithium trimethylsilylacetylide, $ZnBr_2$, Et_2O, −78 °C to rt, overnight; (b) TBAF, THF, rt, 1 h; (c) lithiumphenylacetylide, $ZnBr_2$, −78 °C to rt, overnight; (d) 90% acetic acid, 60 °C, 5 h or *p*-TsOH, MeOH, rt, 36 h; (e) TBDPSCl, imidazole, 4-DMAP, DMF, rt, 16 h; (f) *p*-TsOH, Me_2CO, 2,2-dimethoxypropane, reflux, 6 h; (g) TBAF, THF, rt, 1 h; (h) $PdCl_2(PPh_3)_2$, iodobenzene, piperidine, 70 °C, overnight; (i) *p*-TsCl, 4-DMAP, pyridine, 0 °C to rt, 35 h; (j) secondary amine, DMF, 45 °C, 72 h.

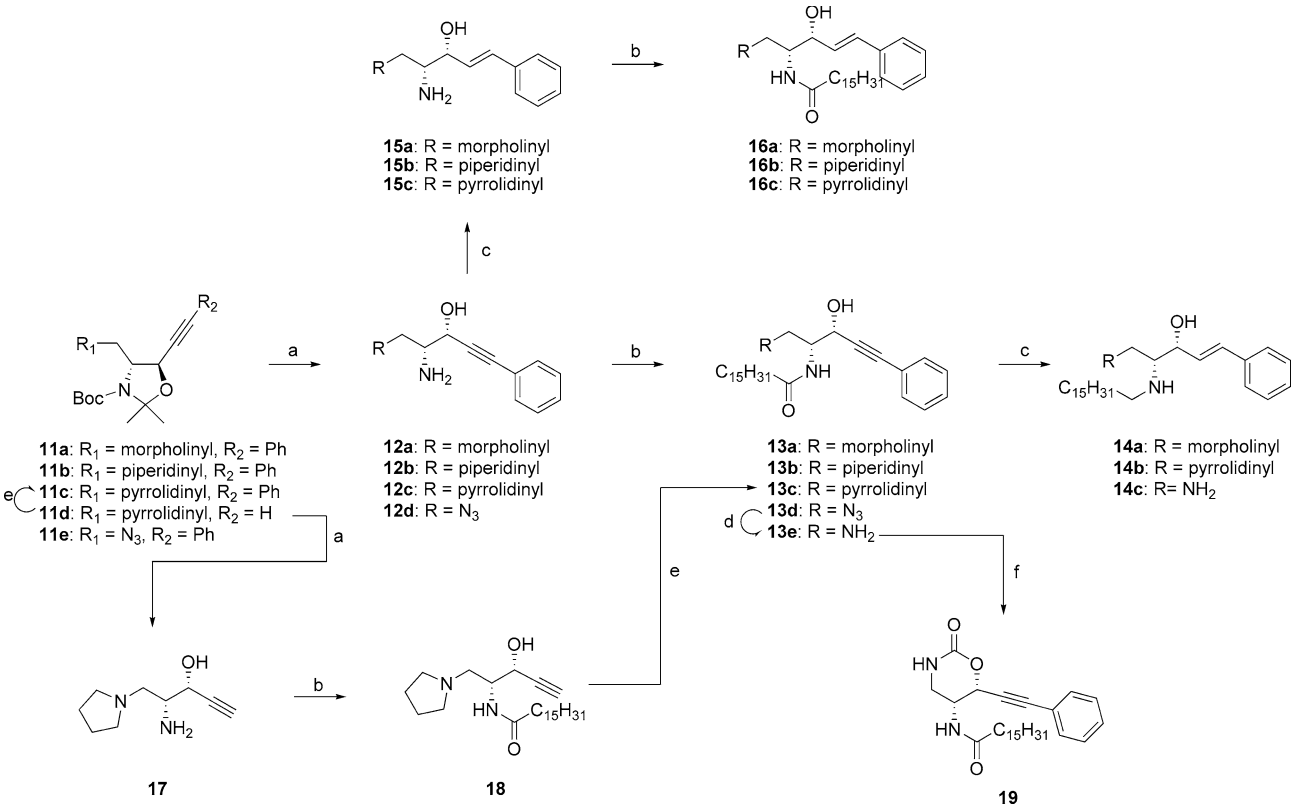
the reaction mixture became more complex when solvent removal was carried out at 40–50 °C. In contrast, when the temperature was strictly kept below 35 °C, few side products were observed. The nucleophilic nature of the *tert*-Boc group,[12] susceptible to formation of a bicyclic oxazolidine–oxazolidinone,[13] might be responsible for this phenomenon.

Treatment of **10a** and **10b** with the appropriate nucleophile in DMF at elevated temperatures gave access to key intermediates **11a–e** (62–99%). Sonogashira coupling of **11d** with iodobenzene proceeded smoothly to produce **11c** (93%).

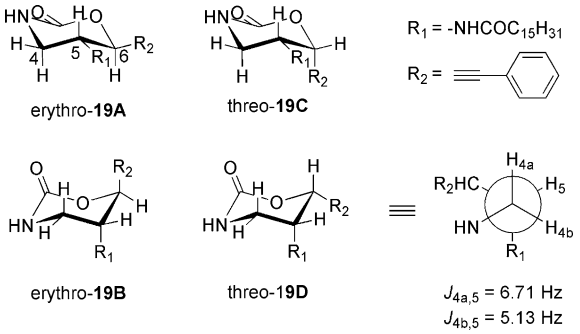
Acid mediated deprotection of **11a–e** followed by acylation gave alkyne analogues **13a–d** (48–89%) and **18** (60%; Scheme 2). Unfortunately, Sonogashira coupling of **18** with iodobenzene showed to be a bridge too far since it failed to give alkyne **13c** in satisfactory yields (32%). Therefore, key intermediate **11d** should be regarded as a solid base for future introduction of alternative aryl substituents. Reduction of **13d** under Staudinger conditions gave amine **13e** in excellent yield (97%). Since treatment of **13a** and **13b** with Na/NH_3 produced complex mixtures, we opted to reduce the alkyne with Red-Al®, although we were aware that controversial results[14] had been obtained in the presence of amides. Unfortunately, upon treatment of amides **13a**, **13b** and **13e** with Red-Al® at −78 °C, the corresponding *E*-styryl ceramines **14a** (60%), **14b** (quant.) and **14c** (45%) were isolated as the sole reaction products. Nonetheless, comparison of the biological activities of these amines with the amide counterparts could provide insight into the role of the amide function in binding to GlcCer synthase since no D-*threo* ceramines have been evaluated to date as potential inhibitors.

In order to circumvent the reduction of the amide group, amines **12a–c** were first treated with Red-Al® at −78 °C followed by acylation with *p*-nitrophenylpalmitoate to give access to PDMP analogues **16a–c** (84%, 78% and 71%, respectively).

Stereochemical assignment of the *threo* configuration was achieved by treatment of amine **13e** with triphosgene yielding oxazolidinone **19** (88%). Based on the small coupling constant $^3J_{5,6} = 3.42$ Hz, an axial–axial orientation can be excluded (Scheme 3, conformer *erythro*-**19A**). The remaining question was whether C(5)*H* was in axial or equatorial position. The values of the coupling constants $^3J_{4a,5} = 6.71$ Hz and $^3J_{4b,5} = 5.13$ Hz indicate a pseudo-axial–axial orientation. Selective irradiation of C(6)*H* ($\delta = 5.40$ ppm) and NOEDIF observation showed an increase of C(5)*H* (5.0%) and a weaker, but still significant increase of C(4)H_b (2%). Only conformer *threo*-**19D** would give a NOE contact between C(6)*H* and C(4)H_b indicating a cis-relationship of the substituents on C(5) and C(6). Performing these experiments at higher temperature (60 °C) did not affect



Scheme 2. Reagents and conditions: (a) 3 N HCl, MeOH, 50 °C, 12 h; (b) *p*-nitrophenylpalmitate, HOBT, pyridine, 50 °C, 48 h; (c) Red-Al®, Et_2O, −78 °C to rt, overnight; (d) PPh_3, THF, rt for 30 min then H_2O, rt, 48 h; (e) $PdCl_2(PPh_3)_2$, iodobenzene, piperidine, 70 °C, overnight; (f) triphosgene, TEA, CH_2Cl_2, 0 °C to rt, 1 h.



Scheme 3. Possible conformers of *erythro*/*threo*-**19**.

the coupling constant, demonstrating the presence of a single conformer.

2.2. Biological evaluation

In a preliminary, exploratory in vitro assay, using a short acyl chain analogue of ceramide, *N*-[6-[(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]hexanoyl]D-*erythro*-sphingosine (C6-NBD-D-*erythro*-ceramide), as substrate for GlcCer synthase (Fig. 2), compounds **13a**, **13b**, **13d**, **13e**, **14a–c**, **16a–c**, **18** and PDMP were evaluated as potential inhibitors of GlcCer synthase in rat liver Golgi membrane homogenates. Moreover, specificity of inhibition towards GlcCer synthase was assessed by monitoring sphingomyelin (SM) synthesis from C6-NBD-D-*erythro*-ceramide.[15] Indeed, toxicity of PDMP analogues has been associated with increased intracellular ceramide (Cer) levels.[5b] Inhibition of sphingomyelin synthase (SM synthase),[16] as well as different mechanisms, has been proposed[5a,5b] to cause this phenomena.

Interestingly, almost all compounds showed some inhibition of GlcCer synthesis (Fig. 2A). Comparison of the inhibitory activities of the morpholino series **13a**, **14a** and **16a** clearly shows that the presence of an amide carbonyl increases inhibitory activity. Moreover, alkyne **13a** and *E*-alkene **16a** seem to be equally potent. In contrast, piperidine analogues **13b** and **16b** show a small difference in favour of the *E*-alkene analogue.

Surprisingly, analogue **18** revealed that the presence of the aromatic ring was not a prerequisite for inhibitory activity. Furthermore, terminal alkyne **18** demonstrated comparable potency with respect to its aromatic *E*-alkene counterpart **16c**. Data for ceramine **14b** confirmed the necessity of the amide for distinct inhibitory activity although this analogue still reduced GlcCer synthesis by 65% at 25 μM. By switching to non-cyclic nitrogen substituents as in compounds **13d**, **13e** and **14c**, the inhibitory activity drastically dropped, thereby clearly indicating the requirement of cyclic amines as sugar transition state mimics, as previously assumed.[5a] In agreement with published results for PDMP analogues,[5a] data for **16a–c** demonstrated that a pyrrolidine substituent on –C(1) is undoubtedly favourable over a morpholino or piperidino head group.

Most analogues showed a concentration-dependent decrease in SM synthesis similar to PDMP, as depicted in Figure 2B. However, treatment with 25 or 50 μM concentrations did not significantly affect SM synthesis, except for **13d**, which induced a substantial decrease in SM synthesis. When higher molar concentrations were applied, a significant decrease in SM synthesis was noticed, except for **13b**, which showed an increase in SM at all inhibitor concentrations.

An interesting feature was noticed in the assays of compounds **13e** and **14c**. Apart from the expected SLs, a new 'upper band' was observed on TLC with an R_f which was slightly larger than the R_f of C6-NBD-ceramide. It is difficult to speculate on the nature of this metabolite, but based on its apolar behaviour, one could assume that acylation on C(1) by 1-*O*-acyl transferase[17] might have occurred, thereby yielding a compound with larger R_f than C6-NBD-ceramide. Further investigation will be necessary to reveal this metabolite's identity.

In a subsequent set of assays, the biological profile of the most potent inhibitors, **16c** and **18**, was examined in detail both in vitro on rat liver Golgi fractions and in HEK-293 cells (Fig. 3). Both compounds were equally as potent as PDMP in inhibiting GlcCer synthase in Golgi fractions, as depicted in Figure 3A. The calculated IC_{50} values from these experiments for PDMP, **16c** and **18** are 5.33, 5.44 and 4.23 μM, respectively. The value for PDMP is in good agreement with published data

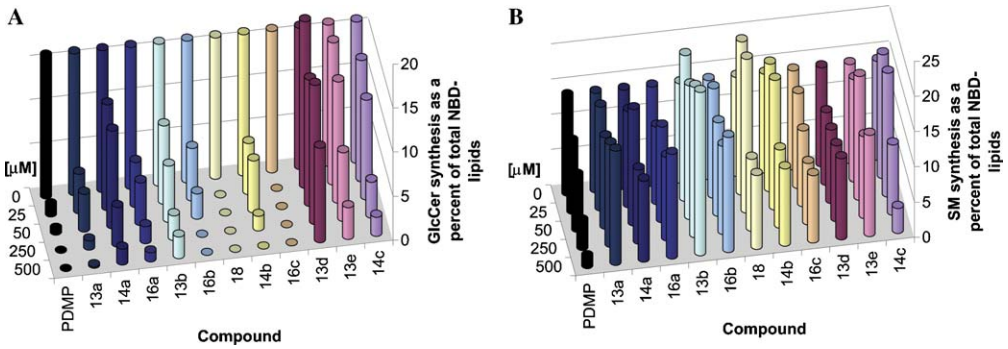


Figure 2. Effects of inhibitors on GlcCer (A) and SM (B) synthesis assayed in vitro in Golgi fractions. The reactions were carried out in the presence or absence of varying concentrations of inhibitors.

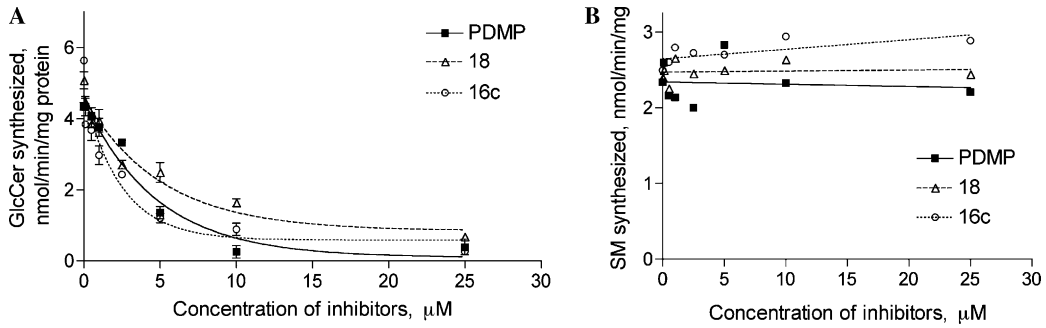


Figure 3. Effects of analogues **18** and **16c** on GlcCer (A) and SM (B) synthesis measured in vitro in Golgi fractions. The reactions were carried out in the presence and absence of various inhibitor concentrations.

(5 μM).[6] Within the concentration range of this assay, neither analogues affected SM synthesis (Fig. 3B).

Living cell inhibition was assessed by incubating HEK-293 cells for 3 h in the presence of 10, 25 and 50 μM of analogues **16c** and **18**, together with C6-NBD-ceramide (Fig. 4A). Again, both compounds inhibited GlcCer synthesis to the same extent as PDMP. Data concerning SM synthesis in HEK-293 cells correlate well with findings in vitro (Fig. 4B). Even at 50 μM no effect on SM synthesis was observed.

We next examined de novo synthesis of other SLs, using ^{3}H-serine as a precursor, in HEK-293 (Fig. 5) and COS-7 cells (data not shown), following pre-treatment with the inhibitors. GlcCer synthesis was moderately inhibited in both cell lines. While values for **16c** (62% of control) were comparable to PDMP (52% of control), **18** (37% of control) proved to be somewhat more effective. Lactosylceramide (LacCer) levels slightly decreased upon treatment with both PDMP and **16c** (48% of control), whereas no effect could be observed upon incubation with **18** (118% of control). Cer levels substantially increased (up to twofold) upon treatment with the inhibitors. In contrast, SM levels were only very slightly affected by both **16c** (81% of control) and **18** (118% of control) at this concentration. These findings indicate that inhibition of SM synthesis is not responsible for the observed ceramide accumulation in these cell lines. Therefore, other ceramide salvage pathways must be involved. The only way to disclose the true nature of the specific enzyme(s) involved in ceramide accumulation is by rigorously monitoring cellular levels of all known SLs.

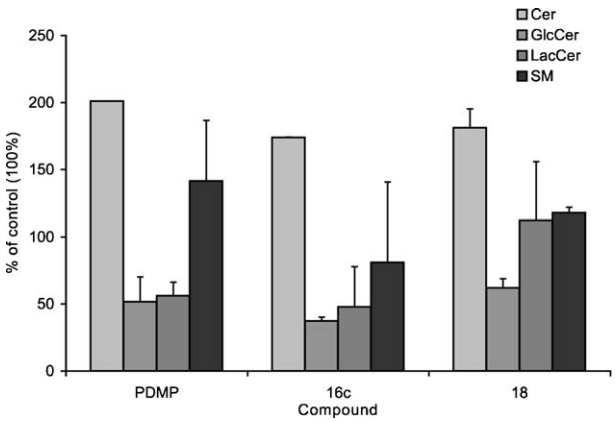


Figure 5. Effects of **18** and **16c** on de novo sphingolipid synthesis assayed using L-[3-^{3}H]-serine in HEK-293 cells. Analyses were performed after 3 h incubation following 1 h pre-treatment with 50 μM of the inhibitors.

3. Conclusion

We have developed a flexible synthetic route towards a new series of hybrid PDMP analogues. A key reaction in our approach was the Sonogashira coupling between an aryl iodide and a terminal alkyne intermediate. The influence of the synthesized compounds on GlcCer and SM synthesis was evaluated both in vitro and in living cells. Optimal inhibitory activity was observed when a pyrrolidine head group was combined with an amide-linked fatty acid. Interestingly, by replacing the *E*-styrene moiety by a terminal alkyne an equally potent analogue was obtained. This simplified terminal alkyne

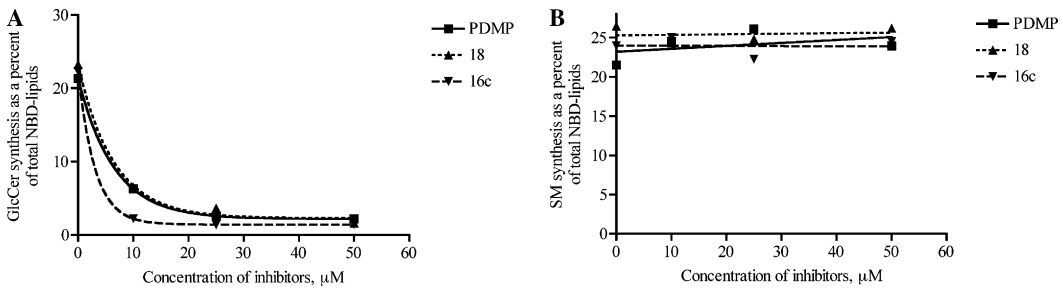


Figure 4. Effects of analogues **18** and **16c** on GlcCer (A) and SM (B) synthesis assayed in HEK-293 cells using C6-NBD-ceramide. The reactions were carried out in the presence and absence of various inhibitor concentrations.

PDMP analogue provides a solid lead for future introduction of different aryl groups in the search for PDMP analogues with enhanced biological activity.

4. Experimental

4.1. Chemistry

4.1.1. General. All reactions were carried out under inert (N_2) atmosphere. Precoated Macherey-Nagel (Düren, Germany) silica gel F_{254} plates were used for TLC and spots were examined under UV light at 254 nm and/or revealed by sulfuric acid–anisaldehyde spray or phosphomolybdic acid spray. All purifications were performed with ICN silica gel (63–200 μM, ICN, Asse Relegem, Belgium). NMR spectra were obtained with a Varian Mercury 300 or 500 spectrometer (Varian, Palo Alto, California, USA). Chemical shifts are given in parts per million (δ) relative to residual solvent peak. All signals assigned to amino and hydroxyl groups were exchangeable with D_2O. Numbering for ^1H assignment is based on the IUPAC name of the compounds. Structural assignment was confirmed with COSY, HMQC and/or NOEDIF if necessary. Exact mass measurements were performed on a quadrupole/orthogonal-acceleration time-of-flight (Q/oaTOF) tandem mass spectrometer (qTof2, Micromass, Manchester, UK) equipped with a standard electrospray ionisation (ESI) interface. Samples were infused in a 2-propanol/water (1:1) mixture at 3 μL/min. Optical rotations were measured with a Perkin-Elmer 241 polarimeter.

4.1.2. (*R*)-*tert*-Butyl 4-((*R*)-1′-hydroxy-3′-(trimethylsilyl)prop-2′-ynyl)-2,2-dimethyloxazolidine-3-carboxylate (*threo*-4). To a solution of trimethylsilylacetylene (12.53 mL, 88 mmol, 1.66 equiv) in anhydrous Et_2O (450 mL) at −78 °C, *n*-BuLi (50 mL of 1.6 M in hexanes, 80 mmol, 1.51 equiv) was added dropwise. The mixture was stirred for 1 h at 0 °C and 1 h at room temperature and was subsequently cooled to 0 °C. After addition of $ZnBr_2$ (23.89 g, 106.1 mmol, 2 equiv), the reaction mixture was stirred at room temperature for 1 h and subsequently cooled to −78 °C. D-Garner's aldehyde (12.16 g, 53.07 mmol) was dissolved in anhydrous Et_2O (50 mL), cooled to −78 °C and added dropwise to the above solution. The mixture was allowed to reach room temperature overnight and after cooling to 0 °C, saturated NH_4Cl (100 mL) was added in one portion. The aqueous layer was extracted with Et_2O (2× 100 mL). The combined organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (hexanes/EtOAc 9:1) affording *threo*-**4** (7.167 g, 41%) and a mixture of *erythro*/*threo*-**4** (4.736 g, 27%), both as a slightly yellow oil. ^1H (300 MHz; DMSO-d_6) δ: 0.11 (s, 9H, $(CH_3)_3Si$), 1.35–1.52 (m, 15H, 2× $-CH_3$ and *tert*-butyl), 3.75–3.85 (m, 1H, –C(4)*H*), 3.95 (td, 1H, J = 3.51 and 9.08 Hz, –C(5)H_a), 4.05 (dd, 1H, J = 5.28 and 8.21 Hz, –C(5)H_b), 4.68 (m, 1H, –C(4)C*H*OH), 5.77 (m, 1H, –C(4)CHO*H*); ^{13}C (75 MHz; DMSO-d_6) δ: −0.30, −0.20, 23.51, 24.77, 25.54, 26.40, 27.87, 60.12, 60.65, 61.56, 63.65, 64.09, 79.24, 79.58, 93.58, 93.94, 106.10, 106.27, 151.88, 151.19; exact mass (ESI-MS) calculated for $C_{16}H_{30}NO_4Si$ $[M+H]^+$: 328.1944, found: 328.1943.

4.1.3. (*R*)-*tert*-Butyl 4-((*R*)-1′-hydroxyprop-2′-ynyl)-2,2-dimethyloxazolidine-3-carboxylate (5a). TBAF (38.5 mL of a 1 M solution in THF, 38.5 mmol, 1.2 equiv) was added to a solution of *threo*-**4** (10.5 g, 32.05 mmol) in THF (10 mL). The solution was stirred for 1 h at room temperature and the solvent was subsequently removed under reduced pressure. The residue was dissolved in EtOAc (50 mL) and washed with saturated $NaHCO_3$ (50 mL). The aqueous layer was extracted with EtOAc (2× 50 mL). The combined organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (hexanes/EtOAc 4:1) yielding *threo*-**5a** (7.61 g, 93%) as a white solid. ^1H (300 MHz; DMSO-d_6) δ: 1.35–1.42 (m, 12H, $-CH_3$ and *tert*-butyl), 1.50 (s, 3H, $-CH_3$), 3.20 (d, 1H, J = 4.4 Hz, alkyne *H*), 3.81 (m, 1H, –C(4)*H*), 3.94 (m, 1H, –C(5)H_a), 4.02 (dd, 1H, J = 2.64 and 9.09 Hz, –C(5)H_b), 4.61 (br s, 1H, –C(4)C*H*OH), 5.71 (m, 1H, –C(4)CHO*H*); ^{13}C (75 MHz; DMSO-d_6) δ: 23.17, 24.49, 25.77, 26.58, 60.18, 60.66, 60.96, 63.39, 63.81, 75.59, 75.77, 79.23, 79.65, 83.50, 93.63, 93.94, 151.31, 152.00; exact mass (ESI-MS) calculated for $C_{13}H_{21}NO_4Na$ $[M+Na]^+$: 278.1368, found: 278.1364.

4.1.4. (*R*)-*tert*-Butyl 4-((*R*)-1′-hydroxy-3′-phenylprop-2′-ynyl)-2,2-dimethyloxazolidine-3-carboxylate (*threo*-5b). To a stirred and cooled (0 °C) solution of lithium phenylacetylide (42.7 mL of a 1 M solution in THF, 42.7 mmol, 2 equiv) in anhydrous Et_2O (200 mL), $ZnBr_2$ (10.11 g, 44.88 mmol, 2.1 equiv) was added and the mixture was stirred for 1 h at 0 °C and 1 h at room temperature and was subsequently cooled −78 °C. D-Garner's aldehyde (4.90 g, 21.37 mmol) was dissolved in anhydrous Et_2O (25 mL), the resulting solution cooled to −78 °C and added dropwise to the above solution. The mixture was allowed to reach room temperature overnight and after cooling to 0 °C, treated with satd NH_4Cl (50 mL). After separation of the phases, the aqueous layer was extracted with Et_2O (2× 100 mL) and the combined organic phase was dried over $MgSO_4$. After removal of the solvent under reduced pressure, the residue was purified by flash chromatography (hexanes/EtOAc 9:1–85:15) affording *threo*-**5b** (3.125 g; 44%) and a mixture of *erythro*- and *threo*-**5b** (2.014 g, 28%), both as a yellow oil. ^1H (300 MHz; DMSO-d_6) δ: 1.46–1.49 (m, 12H, $-CH_3$ and *tert*-butyl), 1.50 (s, 3H, $-CH_3$), 3.85–4.06 (m, 2H, –C(4)*H* and –C(5)H_a), 4.14 (dd, 1H, J = 2.35 and 9.09 Hz, –C(5)H_b), 4.82–4.92 (m, 1H, –C(4)C*H*OH), 5.88 (m, 1H, –C(4)CHO*H*), 7.35–7.40 (m, 5H, arom. *H*); ^{13}C (75 MHz; DMSO-d_6) δ: 25.78, 27.59, 60.68, 61.11, 63.65, 78.93, 83.84, 89.59, 93.34, 122.12, 127.87, 128.06, 131.02, 151.44; exact mass (ESI-MS) calculated for $C_{19}H_{26}NO_4$ $[M+H]^+$: 332.1862, found: 332.1864.

4.1.5. *tert*-Butyl (2*R*,3*R*)-1,3-dihydroxypent-4-yn-2-ylcarbamate (6a). A solution of *threo*-**5a** (7.57 g, 29.65 mmol) in 90% acetic acid was stirred for 5 h at 60 °C. The solvent was removed under reduced pressure and the

residue was co-evaporated twice with isooctane (25 mL). The residue was purified by flash chromatography (hexanes/EtOAc 1:1) yielding **6a** (5.935 g, 93%) as a slightly yellow, viscous oil. ^{1}H (300 MHz; DMSO-d_6) δ: 1.36 (s, 9H, *tert*-butyl), 3.21 (d, 1H, J = 2.06 Hz, alkyne *H*), 3.32–3.52 (m, 3H, –C(1)H_2 and –C(2)*H*), 4.28–4.35 (m, 1H, –C(3)*H*), 4.61 (t, 1H, J = 5.57 Hz, –C(1)O*H*), 5.38 (d, 1H, J = 6.45 Hz, –C(3)O*H*), 6.24 (d, 1H, J = 7.63 Hz, –N*H*); ^{13}C (75 MHz; DMSO-d_6) δ: 28.20, 56.98, 59.62, 59.95, 75.03, 77.80, 84.25, 155.41; exact mass (ESI-MS) calculated for $C_{10}H_{17}NO_4Na$ $[M+Na]^+$: 238.1055, found: 238.1047.

4.1.6. *tert*-Butyl (2*R*,3*R*)-1,3-dihydroxy-5-phenylpent-4-yn-2-ylcarbamate (6b). To a solution of *threo*-**5b** (5.91 g, 17.83 mmol) in MeOH (70 mL), *p*-TsOH·H_2O (339 mg, 1.783 mmol, 0.1 equiv) was added, and the resulting solution was stirred for 36 h at room temperature. TEA (3 mL) was added to the cooled (0 °C) solution, and the solvent was removed in vacuo. The residue was dissolved in EtOAc (100 mL) and the resulting solution extracted with satd $NaHCO_3$ (2× 25 mL) and brine (25 mL). After drying over $MgSO_4$, the solvent was removed under reduced pressure, and the residue was purified by column chromatography (hexanes/EtOAc 3:2) producing **6b** (3.64 g, 70%) as a white foam. ^{1}H (300 MHz; DMSO-d_6) δ: 1.38 (s, 9H, *tert*-butyl), 3.25–3.68 (m, 3H, –C(1)H_2 and –C(2)*H*), 4.56 (dd, 1H, J = 3.82 and 6.75 Hz, –C(3)*H*), 4.64 (t, 1H, J = 5.57 Hz, –C(1)O*H*), 5.47 (d, 1H, J = 6.45 Hz, –C(3)O*H*), 6.36 (d, 1H, J = 8.50 Hz, –N*H*), 7.32–7.42 (m, 5H, arom. *H*); exact mass (ESI-MS) calculated for $C_{16}H_{22}NO_4$ $[M+H]^+$: 292.1549, found: 292.1545.

Typical procedure for silylation of **6a** *and* **6b**. TBDPSCl (9.5 mmol) was added dropwise to a cooled solution (0 °C) of **6a** or **6b** (10 mmol), imidazole (30 mmol) and 4-DMAP (cat.) in anhydrous DMF (20 mL). The mixture was stirred overnight and the solvent was subsequently removed under reduced pressure. The residue was partitioned between Et_2O (25 mL) and satd $NaHCO_3$ (12 mL). After separation of the phases, the organic layer was washed with satd $NaHCO_3$ (12 mL) and brine (12 mL). The organic phase was dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (hexanes/EtOAc 9:1) affording **7a** and **7b** as very viscous, slightly yellow oils.

4.1.7. *tert*-Butyl (2*R*,3*R*)-1-(*tert*-butyldiphenylsilyloxy)-3-hydroxypent-4-yn-2-ylcarbamate (7a). Yield: 10.8 g (89%). ^{1}H (300 MHz; DMSO-d_6) δ: 0.97 (s, 9H, *tert*-butyl silyl), 1.37 (s, 9H, *tert*-butyl), 3.25 (d, 1H, J = 2.05 Hz, alkyn *H*), 3.60–3.82 (m, 3H, –C(1)H_2 and –C(2)*H*), 4.39–4.47 (m, 1H, –C(3)*H*), 5.49 (d, 1H, J = 6.74 Hz, –C(3)O*H*), 6.41 (d, 1H, J = 8.21 Hz, –N*H*), 7.35–7.44 (m, 4H, arom. *H*), 7.59–7.65 (m, 6H, arom. *H*); ^{13}C (75 MHz; DMSO-d_6) δ: 18.82, 26.53, 28.19, 56.90, 60.04, 62.56, 75.30, 77.77, 83.89, 127.78, 129.77, 133.01, 135.02, 155.39; exact mass (ESI-MS) calculated for $C_{26}H_{35}NO_4SiNa$ $[M+Na]^+$: 476.2233, found: 476.2234.

4.1.8. *tert*-Butyl (2*R*,3*R*)-3-hydroxy-1-(*tert*-butyldiphenylsilyloxy)-5-phenylpent-4-yn-2-ylcarbamate (7b). Yield: 4.82 g (84%). ^{1}H (300 MHz; DMSO-d_6) δ: 1.07 (s, 9H, *tert*-butyl silyl), 1.37 (s, 9H, *tert*-butyl), 3.66–3.73 (m, 1H, –C(1)H_a), 3.76–3.92 (m, 2H, –C(1)H_b and C(2)*H*), 4.56–4.67 (m, 1H, –C(3)*H*), 5.48–5.64 (m, 1H, –C(3)O*H*), 6.56 (d, 1H, J = 7.92 Hz, –N*H*), 7.28–7.48 (m, 10H, arom. *H*), 7.58–7.67 (m, 5H, arom. *H*); ^{13}C (75 MHz; DMSO-d_6) δ: 18.81, 26.54, 28.20, 57.10, 60.86, 62.83, 77.75, 84.00, 89.80, 122.24, 127.77, 128.50, 129.76, 131.27, 133.00, 133.03, 135.01, 155.46; exact mass (ESI-MS) calculated for $C_{32}H_{40}SiNO_4$ $[M+H]^+$: 530.2727, found: 530.2721.

4.1.9. General procedure for the preparation of oxazolidines 8a and 8b. To a solution of **7a** or **7b** (10 mmol) in a mixture of acetone/2,2-dimethoxypropane (2.6:1, 40 mL), *p*-TsOH.H_2O (5 mol %) was added in one portion and the reaction was refluxed for 6 h. Removal of the solvent under reduced pressure, followed by flash chromatography (hexanes:EtOAc 95:5), afforded **8a** and **8b** as colourless oils.

4.1.9.1. (4*S*,5*R*)-*tert*-Butyl 5-ethynyl-4-((*tert*-butyldiphenylsilyloxy)methyl)-2,2-dimethyloxazolidine-3-carboxylate (8a). Yield: 9.95 g (98%) ^{1}H (300 MHz; DMSO-d_6) δ: 0.96 (s, 9H, *tert*-butyl silyl), 1.15–1.44 (m, 12H, *tert*-butyl and –CH_3), 1.62 (s, 3H, –CH_3), 3.48–3.85 (m, 3H, alkyne *H* and –C(4)CH_2), 3.90–4.05 (m, 1H, –C(4)*H*), 4.82–4.97 (m, 1H, –C(5)*H*), 7.35–7.63 (m, 10H, arom. *H*); ^{13}C (75 MHz; DMSO-d_6) δ: 18.77, 26.50, 26.84, 27.24, 27.77, 61.12, 62.29, 64.27, 64.51, 65.33, 65.94, 77.03, 79.48, 79.79, 82.43, 82.92, 94.72, 95.27, 127.91, 129.98, 132.43, 134.99, 150.53, 151.01; exact mass (ESI-MS) calculated for $C_{35}H_{45}SiO_4N$ $[M+Na]^+$: 516.2546, found: 516.2554.

4.1.9.2. (4*R*,5*R*)-*tert*-Butyl 4-((*tert*-butyldiphenylsilyloxy)-methyl)-2,2-dimethyl-5-(2-phenylethynyl) oxazolidine-3-carboxylate (8b). Yield: 4.22 g (84%). ^{1}H (300 MHz; DMSO-d_6) δ: 0.95–1.05 (s, 9H, *tert*-butyl silyl), 1.18–1.50 (m, 12H, *tert*-butyl and –CH_3), 1.67 (s, 3H, –CH_3), 3.60–3.92 (m, 2H, –C(4)*H* and –C(4)CH_a), 3.98–4.10 (m, 1H, –C(4)CH_b), 5.08–5.20 (m, 1H, C(5)*H*), 7.35–7.50 (m, 10H, arom. *H*), 7.58–7.65 (m, 5H, arom. *H*); ^{13}C (75 MHz; DMSO-d_6) δ: 18.79, 26.55, 27.82, 61.32, 62.17, 64.26, 64.51, 65.23, 65.82, 77.03, 79.33, 79.64, 87.37, 87.82, 95.78, 96.02, 121.36, 127.92, 128.75, 129.09, 130.00, 131.33, 132.52, 135.07, 150.55, 151.03; exact mass (ESI-MS) calculated for $C_{35}H_{45}SiO_4N$ $[M+H]^+$: 570.3040, found: 570.3043.

4.1.10. General procedure for the preparation of alcohols 9a and 9b. To a solution of **8a** or **8b** (10 mmol) in THF (50 mL), TBAF (15 mmol) was added in one portion, and the solution was stirred for 75 min at room temperature. The solvent was subsequently removed under reduced pressure, and the residue was dissolved in EtOAc (100 mL). The organic layer was washed with saturated $NaHCO_3$ (3× 25 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes:EtOAc 4:1) yielding **9a** and **9b** as white solids.

4.1.10.1. (4*R*,5*R*)-*tert*-Butyl 5-ethynyl-4-(hydroxymethyl)-2,2-dimethyloxazolidine-3-carboxylate (9a). Yield: 4.95 g (96%). ^{1}H (300 MHz; DMSO-d_6) δ: 1.40 (s, 9H, *tert*-butyl), 1.60–1.63 (m, 6H, 2× –CH_3), 3.15–3.26 (m, 1H, –C(4)CH_a), 3.45–3.53 (m, 1H, –C(4)CH_b), 3.58 (d, 1H,J = 2.35, alkyne *H*), 3.76–3.90 (m, 1H, –C(4)*H*), 4.72–4.78 (m, 1H, –C(5)*H*), 5.05–5.15 (m, 1H, –C(4)CH$_2$O*H*); ^{13}C (75 MHz; DMSO-d_6) δ: 25.51, 26.84, 27.19, 28.01, 59.50, 60.24, 65.05, 65.32, 65.48, 65.89, 76.79, 79.42, 79.88, 83.60, 94.60, 94.95, 150.74, 151.07; exact mass (ESI-MS) calculated for $C_{13}H_{21}NO_4$Na [M+Na]$^+$: 278.1368, found: 278.1364.

4.1.10.2. (4*R*,5*R*)-*tert*-Butyl 4-(hydroxymethyl)-2,2-dimethyl-5-(2-phenylethynyl)oxazolidine-3-carboxylate (9b). Yield: 2.22 g (91%). ^{1}H (300 MHz; DMSO-d_6) δ: 1.41 (m, 9H, *tert*-butyl), 1.44 (s, 3H, –CH_3), 1.68 (s, 3H, –CH_3), 3.24–3.36 (m, 1H, –C(4)CH_a), 3.51–3.62 (m, 1H, –C(4)CH_b), 3.88–4.20 (m, 1H, –C(4)*H*), 5.02 (d, 1H, J = 1.53 Hz, –C(5)*H*), 5.06–5.17 (br s, 1H, –C(4)CH$_2$O*H*), 7.34–7.45 (m, 5H, arom. *H*); ^{13}C (75 MHz; DMSO-d_6) δ: 25.61, 27.06, 27.94, 59.51, 60.25, 65.16, 66.17, 66.49, 79.42, 79.81, 84.87, 89.01, 94.53, 94.78, 121.49, 128.70, 128.94, 131.21, 157.75; exact mass (ESI-MS) calculated for $C_{19}H_{26}O_4N$ [M+H]$^+$: 332.1862, found: 332.1865.

Conversion of **9a** *to* **9b**. To a solution of **9a** (651 mg, 2.55 mmol) and $PdCl_2(PPh_3)_2$ (36 mg, 51 μmol, 2 mol %) in piperidine (5 mL), iodobenzene (429 μL, 3.82 mmol, 1.5 equiv) was added and the mixture was stirred overnight at 70 °C. The solvent was subsequently removed under reduced pressure and the residue was purified by column chromatography rendering (hexanes/EtOAc 4:1) **9b** (841 mg, 99%) as a white solid.

4.1.11. General procedure for the preparation of tosylates 10a and 10b. Solid *p*-TsCl (3 mmol) was added to an ice-cold solution of **9a** or **9b** (1 mmol) in anhydrous pyridine (1 mL) and after stirring for 35 h at room temperature, the reaction mixture was evaporated to dryness at high vacuum (<35 °C for **10a**, 40 and −50 °C for **10b**). The residue was purified by column chromatography (hexanes/EtOAc 85:15) to yield **10a** and **10b** as white solids.

4.1.11.1. ((4*R*,5*R*)-3-(*tert*-Butoxycarbonyl)-5-ethynyl-2,2-dimethyloxazolidin-4-yl)methyl 4-methylbenzenesulfonate (10a). Yield: 1.85 g (94%). ^{1}H (300 MHz; DMSO-d_6) δ: 1.34 (s, 9H, *tert*-butyl), 1.58–1.61 (m, 6H, 2× –CH_3), 2.41 (s, 3H, tosyl –CH_3), 3.64 (d, 1H, J = 2.34 Hz, alkyne *H*), 4.00–4.10 (m, 3H, –C(4)CH_2 and –C(4)*H*), 4.70–4.75 (br s, 1H, –C(5)*H*), 7.47 (d, 2H, J = 8.21 Hz, arom. *H*), 7.78 (d, 2H, J = 8.21 Hz, arom. *H*); ^{13}C (75 MHz; DMSO-d_6) δ: 20.77, 25.40, 26.67, 27.77, 61.52, 61.79, 65.37, 65.92, 67.26, 68.11, 77.52, 80.13, 80.44, 82.40, 95.22, 95.54, 127.65, 130.30, 131.85, 145.29, 150.26, 150.87; exact mass (ESI-MS) calculated for $C_{20}H_{27}NO_6S$Na [M+Na]$^+$: 432.1457, found: 432.1453.

4.1.11.2. ((4*R*,5*R*)-3-(*tert*-Butoxycarbonyl)-2,2-dimethyl-5-(2-phenylethynyl)oxazolidin-4-yl)methyl 4-methylbenzenesulfonate (10b). Yield: 128 mg (64%). ^{1}H (300 MHz; DMSO-d_6) δ: 1.33 (s, 9H, *tert*-butyl), 1.41 (s, 3H, –CH_3), 1.65 (s, 3H, –CH_3), 2.38 (s, 3H, tosyl –CH_3), 4.04–4.22 (m, 3H, –C(4)CH_2 and –C(4)*H*), 4.93–5.20 (m, 1H, –C(5)*H*), 7.34–7.44 (m, 5H, arom. *H*), 7.47 (d, 2H, J = 8.21 Hz, arom. *H* tosyl), 7.78 (d, 2H, J = 8.21 Hz, arom. *H* tosyl); ^{13}C (75 MHz; DMSO-d_6) δ: 21.12, 25.56, 25.62, 26.88, 27.17, 27.83, 61.53, 61.85, 66.07, 66.63, 67.35, 68.20, 80.18, 80.48, 85.55, 87.84, 95.16, 95.58, 121.21, 127.74, 128.84, 130.36, 131.37, 131.88, 145.37, 150.37, 150.97; exact mass (ESI-MS) calculated for $C_{26}H_{32}O_6NS$ [M+H]$^+$: 486.1950 found: 486.1956.

4.1.12. General procedure for the preparation of 11a–d. The secondary amine (4 mmol) was added to a solution of **10a**–**b** (0.25 mmol) in anhydrous DMF (3 mL) and the mixture was heated at 45 °C for 72 h. The residue, resulting from removal of the solvent in vacuo, was purified by column chromatography (hexanes/EtOAc 4:1 for **11a** and **11b**, CH_2Cl_2/MeOH 99:1 for **11c** and **11d**) yielding **11a**–**d** as slightly brown solids.

4.1.12.1. (4*R*,5*R*)-*tert*-Butyl 2,2-dimethyl-4-(morpholinomethyl)-5-(2-phenylethynyl)oxazolidine-3-carboxylate (11a). Yield: 65 mg (62%). ^{1}H (300 MHz; DMSO-d_6) δ: 1.43 (s, 9H, *tert*-butyl), 1.48 (s, 3H, –CH_3), 1.71 (s, 3H, –CH_3), 2.32–2.46 (m, 4H, CH_2–N–CH_2 morpholine), 2.50–2.62 (m, 2H, –C(4)CH_2), 3.50–3.65 (m, 4H, CH_2–O–CH_2 morpholine), 3.98–4.18 (m, 1H, –C(4)*H*), 4.96–5.05 (m, 1H, –C(5)*H*), 7.36–7.47 (m, 5H, arom. *H*); ^{13}C (75 MHz; DMSO-d_6) δ: 25.77, 27.41, 28.04, 53.70, 60.20, 61.06, 66.26, 68.24, 79.65, 82.07, 85.08, 94.87, 121.52, 128.85, 129.13, 131.36, 150.60; exact mass (ESI-MS) calculated for $C_{23}H_{33}O_4N_2$ [M+H]$^+$: 401.2440, found: 401.2439.

4.1.12.2. (4*R*,5*R*)-*tert*-Butyl 2,2-dimethyl-5-(2-phenylethynyl)-4-((piperidin-1-yl)methyl)oxazolidine-3-carboxylate (11b). Yield: 347 mg (85%). ^{1}H (300 MHz; DMSO-d_6) δ: 1.30–1.54 (m, 18H, *tert*-butyl, –CH_3, CH_2–CH_2–CH_2 piperidine), 1.68–1.72 (s, 3H, –CH_3), 2.23–2.60 (m, 6H, –C(4)CH_2 and CH_2–N–CH_2 piperidine), 4.92–4.16 (br s, 1H, –C(4)*H*), 4.82–5.04 (br s, 1H, –C(5)*H*), 7.34–7.45 (m, 5H, arom. *H*); ^{13}C (75 MHz; DMSO-d_6) δ: 23.84, 25.57, 27.02, 27.38, 27.97, 28.97, 54.55, 60.37, 61.60, 68.24, 79.51, 84.97, 89.31, 94.87, 121.49, 128.77, 129.04, 131.28, 150.59; exact mass (ESI-MS) calculated for $C_{24}H_{35}O_3N_2$ [M+H]$^+$: 399.2648, found: 399.2640.

4.1.12.3. (4*R*,5*R*)-*tert*-Butyl 2,2-dimethyl-5-(2-phenylethynyl)-4-((pyrrolidin-1-yl)methyl)oxazolidine-3-carboxylate (11c). Yield: 187 mg (82%). ^{1}H (300 MHz; DMSO-d_6) δ: 1.40 (s, 9H, *tert*-butyl), 1.44 (s, 3H, –CH_3), 1.64–1.74 (m, 7H, CH$_2$–CH_2–CH_2–CH$_2$ pyrrolidine and –CH_3), 2.20–2.90 (br s, 5H, CH_2–N–CH_2 pyrrolidine and –C(4)CH_a), 3.23–3.34 (m, 1H, –C(4)CH_b), 3.95–4.21 (m, 1H, –C(4)*H*), 4.82–5.20 (br s, 1H, –C(5)*H*), 7.32–7.44 (m, 5H, arom. *H*); ^{13}C (75 MHz; DMSO-d_6) δ: 23.12, 25.70, 27.43, 27.97, 53.80, 56.25, 57.58, 61.82, 62.47, 67.33, 67.96, 79.57, 79.95, 85.06, 89.14, 94.63, 94.93, 121.52, 128.76, 129.04, 131.28, 150.61, 151.00; exact mass (ESI-MS) calculated for $C_{23}H_{33}O_3N_2$ [M+H]$^+$: 385.2491, found: 385.2487.

Sonogashira coupling of **11d** *with iodobenzene.* An identical procedure as for the conversion of **9a** to **9b** gave **11c** (290 mg, 93%) as a slightly brown solid.

4.1.12.4. (4*R*,5*R*)-*tert*-Butyl 5-ethynyl-2,2-dimethyl-4-((pyrrolidin-1-yl)methyl)oxazolidine-3-carboxylate (11d). Yield: 1.18 g (89%). ^{1}H (300 MHz; DMSO-d_6) δ: 1.48–1.52 (m, 12H, *tert*-butyl and –CH_3), 1.60–1.70 (m, 7H, –CH_3 and CH$_2$–CH_2–CH_2–CH$_2$ pyrrolidine), 2.30–2.60 (m, 6H, CH_2–N–CH_2 pyrrolidine and –C(4)CH_2), 3.57 (d, 1H, J = 2.34 Hz, alkyne *H*), 3.86–4.00 (m, 1H, –C(4)*H*), 4.68–4.73 (br s, 1H, –C(5)*H*); ^{13}C (75 MHz; DMSO-d_6) δ: 23.16, 25.51, 27.49, 27.96, 53.71, 57.62, 62.59, 67.23, 76.77, 79.48, 83.72, 94.97, 150.62; exact mass (ESI-MS) calculated for $C_{17}H_{29}O_3N_2$ [M+H]$^+$: 309.2178, found: 309.2180.

4.1.12.5. (4*R*,5*R*)-*tert*-Butyl 4-(azidomethyl)-2,2-dimethyl-5-(2-phenylethynyl)oxazolidine-3-carboxylate (11e). NaN_3 (268 mg, 4.12 mmol, 10 equiv) was added to a solution of **10b** (200 mg, 0.412 mmol) in anhydrous DMF (10 mL) and the mixture was heated at 45 °C for 72 h. After removal of the solvent under reduced pressure, the residue was purified by flash chromatography (hexanes/EtOAc 97:3) rendering **11e** (145 mg, 99%) as a white solid. ^{1}H (300 MHz; DMSO-d_6) δ: 1.43 (s, 9H, *tert*-butyl), 1.50 (s, 3H, –CH_3), 1.69 (s, 3H, –CH_3), 3.45–3.78 (m, 2H, –C(4)CH_2), 4.00–4.14 (br s, 1H, –C(4)*H*), 4.96 (d, 1H, J = 2.93 Hz, –C(5)*H*), 7.34–7.46 (m, 5H, arom. *H*); ^{13}C (75 MHz; DMSO-d_6) δ: 25.37, 26.89, 27.83, 49.97, 51.15, 62.56, 66.69, 67.28, 85.56, 87.56, 95.37, 121.30, 128.68, 129.08, 131.31, 150.48; exact mass (ESI-MS) calculated for $C_{19}H_{25}N_4O_3$ [M+H]$^+$: 357,1927, found: 357,1929.

4.1.13. General procedure for the preparation of 13a–d and 18. A solution of oxazolidines **11a–e** (0.8 mmol) in a mixture of MeOH/3 N HCl (1:2, 30 mL) was heated at 50 °C for 12 h and the solvent was subsequently removed under reduced pressure. The residue was covered with chloroform (3× 20 mL) and the volatiles evaporated thereby quantitatively affording crude amines **12a–d** and **17** as their hydrochloride salts which were used without further purification.

To a cooled solution (0 °C) of crude amines **12a–d** and **17** (0.8 mmol) and HOBT (10 mol %) in anhydrous pyridine (25 mL), *p*-nitrophenylpalmitate (0.8 mmol) dissolved in anhydrous DMF (5 mL) was added dropwise and the mixture was heated for 48 h at 50 °C. The solvent was subsequently removed under reduced pressure and the residue was purified by column chromatography (hexanes/EtOAc/TEA 65:34:1 for **13a–c** and **18**; hexanes/EtOAc 7:3 for **13d**) producing **13a–d** and **18** as colourless solids.

4.1.13.1. *N*-((2*R*,3*R*)-3-Hydroxy-1-morpholino-5-phenylpent-4-yn-2-yl)palmitamide (13a). Yield: 232 mg (67%). ^{1}H (300 MHz; $CDCl_3$-d_1) δ: 0.81 (t, 3H, J = 6.52 Hz, –CH_3 acyl), 1.10–1.30 (m, 24H, acyl *H*), 1.50–1.63 (m, 2H, –COCH$_2$–CH_2–C$_{13}$H$_{27}$), 2.14 (t, 2H, J = 7.50 Hz, –CO–CH_2–C$_{14}$H$_{29}$), 2.45–2.80 (m, 5H, –C(1)H_a and CH_2–N–CH_2 morpholine), 2.58 (dd, 1H, J = 10.81 and 11.57 Hz, –C(1)H_b), 3.63–3.74 (m, 4H, CH_2–O–CH_2 morpholine), 4.41–4.54 (m, 1H, –C(2)*H*), 4.72 (d, 1H, J = 3.73 Hz, –C(3)*H*), 5.55–5.90 (m, 1H, –N*H*), 7.31–7.40 (m, 5H, arom. *H*); ^{13}C (75 MHz; $CDCl_3$-d_1) δ: 13.12, 13.17, 20.06, 21.68, 24.62, 28.19, 28.34, 28.45, 28.63, 28.67, 30.90, 35.72, 46.00, 52.88, 58.61, 65.25, 65.55, 85.58, 86.08, 121.06, 127.39, 127.79, 130.78, 172.18; exact mass (ESI-MS) calculated for $C_{31}H_{51}O_3N_2$ [M+H]$^+$: 499.3899, found: 499.3902.

4.1.13.2. *N*-((2*R*,3*R*)-3-Hydroxy-5-phenyl-1-(piperidin-1-yl)pent-4-yn-2-yl)palmitamide (13b). Yield: 227 mg (48%). ^{1}H (300 MHz; DMSO-d_6) δ: 0.83 (t, 3H, J = 0.83 Hz, –CH_3 acyl), 1.12–1.29 (m, 24H, acyl *H*), 1.30–1.40 (m, 2H, –COCH$_2$–CH_2–C$_{13}$H$_{27}$), 1.41–1.53 (m, 6H, CH_2–CH_2–CH_2 piperidine), 2.09 (t, 2H, J = 6.98 Hz, –CO–CH_2–C$_{14}$H$_{29}$), 2.32–2.45 (m, 5H, –C(1)H_a, CH_2–N–CH_2 piperidine), 2.65 (dd, 1H, J = 6.74 and 12.61 Hz, –C(1)H_b), 4.04–4.14 (m, 1H, –C(2)*H*), 4.56 (d, 1H, J = 3.52 Hz, –C(3)*H*), 5.80–6.20 (br s, 1H, –C(3)O*H*), 7.31–7.42 (m, 5H, arom. *H*), 7.60–7.65 (d, 1H, J = 8.21 Hz, –N*H*); ^{13}C (75 MHz; DMSO-d_6) δ: 13.93, 22.08, 23.76, 25.52, 28.48, 28.69, 28.86, 28.93, 28.99, 29.02, 31.28, 35.38, 50.08, 54.23, 58.11, 62.20, 83.74, 90.04, 122.51, 128.37, 128.49, 131.35, 172.25; exact mass (ESI-MS) calculated for $C_{32}H_{53}O_2N_2$ [M+H]$^+$: 497.4107, found: 497.4101.

4.1.13.3. *N*-((2*R*,3*R*)-3-Hydroxy-5-phenyl-1-(pyrrolidin-1-yl)pent-4-yn-2-yl)palmitamide (13c). Yield: 129 mg (45%). ^{1}H (300 MHz; DMSO-d_6) δ: 0.83 (t, 3H, J = 7.03 Hz, –CH_3 acyl), 1.12–1.28 (m, 24H, acyl *H*), 1.42–1.51 (m, 2H, –COCH$_2$–CH_2–C$_{13}$H$_{27}$), 1.62–1.68 (m, 4H, CH_2–CH_2 pyrrolidine), 2.09 (dt, 2H, J = 2.93 and 7.04 Hz, –CO–CH_2–C$_{14}$H$_{29}$), 2.41–2.53 (m, 5H, –C(1)H_a and CH_2–N–CH_2 pyrrolidine), 2.76 (dd, 1H, J = 5.57 and 12.02 Hz, –C(1)H_b), 4.05 (ddd, 1H, J = 3.81, 7.92 and 11.43 Hz, –C(2)*H*), 4.57 (d, 1H, J = 3.81 Hz, –C(3)*H*), 5.75–5.87 (br s, 1H, –OH), 7.31–7.40 (m, 5H, arom. *H*), 7.60 (d, 1H, J = 8.50 Hz, –N*H*); ^{13}C (75 MHz; DMSO-d_6) δ: 13.88, 22.02, 23.13, 25,47, 28.45, 28.63, 28.80, 28.88, 28.96, 31.22, 35.35, 51.94, 53.72, 55.11, 62.00, 86.57, 90.12, 122.53, 128.28, 128.43, 131.29, 172.19; exact mass (ESI-MS) calculated for $C_{31}H_{51}O_2N_2$ [M+H]$^+$: 483.3951, found: 483.3953.

Sonogashira coupling of **18** *with iodobenzene.* An identical procedure as for the conversion of **9a** to **9b** gave **13c** (19 mg, 32%) as a colourless solid.

4.1.13.4. *N*-((2*R*,3*R*)-1-Azido-3-hydroxy-5-phenylpent-4-yn-2-yl)palmitamide (13d). Yield: 351 mg (62%). ^{1}H (300 MHz; DMSO-d_6) δ: 0.83 (t, 3H, J = 6.65 Hz, –CH_3 acyl), 1.10–1.30 (m, 24H, acyl *H*), 1.42–1.54 (m, 2H, –COCH$_2$–CH_2–C$_{13}$H$_{27}$), 2.12 (dt, 2H, J = 2.64 and 7.04 Hz, –CO–CH_2–C$_{14}$H$_{29}$), 3.45 (dd, 1H, J = 8.79 and 12.60 Hz, –C(1)H_a), 3.53 (dd, 1H, J = 4.69 and 12.60 Hz, –C(1)H_b), 4.02–4.12 (m, 1H, –C(2)*H*), 4.53 (app. t, 1H, J = 4.83 Hz, –C(3)*H*), 5.89 (d, 1H, J = 5.57 Hz, –C(3)O*H*), 7.32–7.44 (m, 5H, arom. *H*), 7.96 (d, 1H, J = 8.50 Hz, –N*H*); ^{13}C (75 MHz; DMSO-d_6) δ: 13.93, 13.93, 22.08, 25.34, 28.53, 28.69, 28.83, 28.91, 28.98, 29.01, 31.28, 35.41, 50.11, 53.23, 54.90, 61.43, 84.25, 88.89, 122.21, 128.51, 128.56, 131.40,

172.65; exact mass (ESI-MS) calculated for $C_{27}H_{43}O_2N_4$ $[M+H]^+$: 455.3386, found: 455.3380.

4.1.13.5. *N*-((2*R*,3*R*)-3-Hydroxy-1-(pyrrolidin-1-yl)pent-4-yn-2-yl)palmitamide (18). Yield: 71 mg (60%). ^{1}H (300 MHz; DMSO-d_6) δ: 0.82 (t, 3H, J = 6.74 Hz, –CH_3 acyl), 1.12–1.30 (m, 24H, acyl *H*), 1.38–1.50 (m, 2H, –COCH$_2$–CH_2–C$_{13}$H$_{27}$), 1.58–1.66 (m, 4H, –CH_2–CH_2 pyrrolidine), 2.06 (t, 2H, J = 7.62 Hz, –CO–CH_2–C$_{14}$H$_{29}$), 2.37–2.45 (m, 5H, –C(1)H_a and CH_2–N–CH_2 pyrrolidine), 2.68 (dd, 1H, J = 5.86 and 12.02, –C(1)H_b), 3.18 (d, 1H, J = 2.34 Hz, alkyne *H*), 3.86–3.96 (m, 1H, –C(2)*H*), 4.32 (dd, 1H, J = 1.76 and 3.82 Hz, –C(3)*H*), 7.54 (d, 1H, J = 8.21 Hz, –N*H*); ^{13}C (75 MHz; DMSO-d_6; hydrochloride salt of **18**) δ: 14.36, 23.33, 23.68, 23.89, 26.31, 26.47, 30.00, 30.18, 30.25, 30.42, 30.61, 32.64, 36.79, 51.38, 52.98, 53.63, 55.13, 63.15, 86.00, 88.42, 174.27; exact mass (ESI-MS) calculated for $C_{25}H_{47}O_2N_2$ $[M+H]^+$: 407.3638, found: 407.3632.

4.1.13.6. *N*-((2*R*,3*R*)-1-Amino-3-hydroxy-5-phenylpent-4-yn-2-yl)palmitamide (13e). To a solution of **13d** (51.4 mg, 0.113 mmol) in THF (250 μL), PPh_3 (59 mg, 0.226 mmol, 2 equiv) was added. After stirring for 10 min at room temperature, H_2O (250 μL) was added and stirring was continued for 48 h. After removal of the solvent under reduced pressure, the residue was purified by flash chromatography (CH_2Cl_2/MeOH (10% 6 N NH_3 in MeOH) 9:1) affording **13e** (47 mg, 97%) as a white solid. ^{1}H (300 MHz; DMSO-d_6) δ: 0.83 (t, 3H, –CH_3 acyl), 1.11–1.28 (m, 24H, acyl *H*), 1.41–1.53 (m, 2H, –COCH$_2$–CH_2–C$_{13}$H$_{27}$), 2.02–2.19 (m, 2H, –CO–CH_2–C$_{14}$H$_{29}$), 2.68 (dd, 1H, J = 7.62 and 12.90 Hz, –C(1)H_a), 2.85 (dd, 1H, J = 6.16 and 12.90 Hz, –C(1)H_b), 3.10–3.50 (br s, 2H, –NH_2), 3.76–3.88 (m, 1H, –C(2)*H*), 4.64 (d, 1H, J = 4.11 Hz, –C(3)*H*), 7.31–7.42 (m, 5H, arom. *H*), 7.61 (d, 1H, J = 8.50 Hz, –N*H*); ^{13}C (75 MHz; DMSO-d_6) δ: 13.95, 22.08, 25.51, 28.57, 28.69, 28.86, 28.93, 29.02, 31.28, 38.96, 54.90, 55.25, 61.74, 83.74, 90.13, 122.53, 128.36, 128.50, 131.31, 172.60; exact mass (ESI-MS) calculated for $C_{27}H_{45}O_2N_2$ $[M+H]^+$: 429.3481, found: 429.3483.

General procedure for the reduction of **13a**, **13c** *and* **13e**. Red-Al® (4 mmol) was added to a cooled (−78 °C) solution of **13a**, **13c** or **13e** (0.4 mmol) in anhydrous Et_2O (10 mL), and the resulting mixture was stirred overnight at room temperature. After quenching the reaction (0 °C) by addition of MeOH (10 mL), satd disodiumtartrate (20 mL) was added, and the mixture was stirred for an additional 4 h. Et_2O (25 mL) and satd $NaHCO_3$ (25 mL) were added, the aqueous layer was extracted with Et_2O (3× 25 mL) and the combined organic phase was dried over $MgSO_4$. Flash chromatography (hexanes/EtOAc/TEA 60:39:1 for **14a** and **14b**, CH_2Cl_2/MeOH (5% 6 N NH_3 in MeOH) 4:1 for **14c**) afforded **14a**–**c** as colourless oils.

4.1.13.7. (*E*,3*R*,4*R*)-4-(Hexadecylamino)-5-morpholino-1-phenylpent-1-en-3-ol (14a). Yield: 115 mg (60%). ^{1}H (300 MHz; DMSO-d_6 + D_2O) δ: 0.83 (t, 3H, J = 6.70 Hz, –CH_3 alkyl), 1.12–1.42 (m, 28H, alkyl *H*), 2.19 (dd, 1H, J = 7.62 and 12.31 Hz, –C(5)H_a), 2.24–2.42 (m, 5H, CH_2–N–CH_2 morpholine and –C(5)H_b), 2.58 (dt, 2H, J = 2.35 and 7.04 Hz, –NH–CH_2–C$_{15}$H$_{31}$), 2.68–2.75 (m, 1H, –C(4)*H*), 3.52 (t, 4H, J = 4.69 Hz, CH_2–O–CH_2 morpholine), 4.17 (app. t, 1H, J = 3.81 Hz, –C(3)*H*), 6.38 (dd, 1H, J = 5.28 and 15.83 Hz, –C(2)*H*), 6.53 (d, 1H, J = 15.83 Hz, –C(1)*H*), 7.15–7.40 (m, 5H, arom); ^{13}C (75 MHz; DMSO-d_6) δ: 13.94, 22.08, 26.64, 28.68, 28.86, 28.99, 29.74, 31.28, 47.74, 53.70, 58.77, 59.19, 66.32, 71.36, 126.07, 127.04, 128.43, 128.55, 132.05, 137.06; exact mass (ESI-MS) calculated for $C_{31}H_{55}O_2N_2$ $[M+H]^+$: 487.4264, found: 487.4268.

4.1.13.8. (*E*,3*R*,4*R*)-4-(Hexadecylamino)-1-phenyl-5-(pyrrolidin-1-yl)pent-1-en-3-ol (14b). Yield: 130 mg (100%). ^{1}H (300 MHz; $CDCl_3$-d_1) δ: 0.88 (t, 3H, J = 6.66 Hz, –CH_3 alkyl), 1.41–1.51 (m, 26H, alkyl *H*), 1.32–1.42 (m, 2H, –NHCH$_2$–CH_2–C$_{14}$H$_{29}$), 1.75–1.83 (m, 4H, CH_2–CH_2 pyrrolidine), 2.56–2.77 (m, 8H, –C(5)H_2, –NH–CH_2–C$_{15}$H$_{31}$ and CH_2–N–CH_2 pyrrolidine), 2.93 (dt, 1H, J = 4.17 and 6.99 Hz, –C(4)*H*), 4.16 (app. t, 1H, J = 4.90 Hz, –C(3)*H*), 6.30 (dd, 1H, J = 5.43 and 15.93 Hz, –C(2)*H*), 6.71 (dd, 1H, J = 1.03 and 15.92 Hz, –C(1)*H*), 7.20–7.43 (m, 5H, arom. *H*); ^{13}C (75 MHz; $CDCl_3$-d_1) δ: 14.12, 22.69, 23.59, 27.19, 29.36, 29.49, 29.61, 29.69, 30.23, 31.92, 48.63, 54.51, 57.69, 59.53, 73.20, 126.64, 127.35, 128.50, 130.49, 130.66, 137.11; exact mass (ESI-MS) calculated for $C_{31}H_{55}ON_2$ $[M+H]^+$: 471.4314, found: 471.4308.

4.1.13.9. (*E*,3*R*,4*R*)-5-Amino-4-(hexadecylamino)-1-phenylpent-1-en-3-ol (14c). Yield: 136 mg (45%). ^{1}H (300 MHz; DMSO-d_6 + D_2O) δ: 0.83 (t, 3H, J = 6.73 Hz, –CH_3 alkyl), 1.15–1.30 (m, 26H, alkyl *H*), 1.33–1.40 (m, 2H, –NHCH$_2$–CH_2–C$_{14}$H$_{29}$), 2.36–2.73 (m, 5H, –C(5)H_2, –NH–CH_2–C$_{15}$H$_{31}$ and –C(4)*H*), 4.35 (app. t, 1H, J = 4.06 Hz, –C(3)*H*), 6.33 (dd, 1H, J = 5.61 and 16.00 Hz, –C(2)*H*), 6.55 (d, 1H, J = 16.14 Hz, –C(1)*H*), 7.16–7.42 (m, 5H, arom. *H*); ^{13}C (75 MHz; DMSO-d_6) δ: 13.94, 22.08, 26.78, 28.69, 29.07, 30.07, 31.28, 37.02, 47.38, 57.84, 64.91, 71.55, 126.10, 127.09, 128.54, 128.74, 132.13, 137.03; exact mass (ESI-MS) calculated for $C_{27}H_{49}ON_2$ $[M+H]^+$: 417.3845, found: 417.3844.

4.1.14. General procedure for the preparation of ceramides 16a–c. Applying an identical procedure as described for the reduction of amides **13a**, **13c** and **13e** with Red-Al® quantitatively produced crude *e*-alkene amines **15a**–**c** as their hydrochloride salts, which were used without further purification.

An identical procedure as for the acylation of amines **12a**–**d** and **17** with palmitoyl chloride afforded ceramides **16a**–**c** as colourless oils.

4.1.14.1. *N*-((*E*,2*R*,3*R*)-3-Hydroxy-1-morpholino-5-phenylpent-4-en-2-yl)palmitamide (16a). Yield: 201 mg (84%). ^{1}H (300 MHz; DMSO-d_6) δ: 0.84 (t, 3H, J = 6.70 Hz, –CH_3 acyl), 1.09–1.30 (m, 24H, acyl *H*), 1.36–1.48 (m, 2H, –COCH$_2$–CH_2–C$_{13}$H$_{27}$), 2.04 (t, 2H, J = 7.47 Hz, –CO–CH_2–C$_{14}$H$_{29}$), 2.21–2.42 (m, 5H,

–C(1)H_a and CH_2–N–CH_2 morpholine), 2.54–2.64 (m, 1H, –C(1)H_b), 3.48–3.54 (m, 4H, CH_2–O–CH_2 morpholine), 4.00–4.12 (m, 1H, –C(2)*H*), 4.24–4.32 (m, 1H, –C(3)*H*), 5.21 (d, 1H, J = 4.69 Hz, –C(3)O*H*) 6.23 (dd, 1H, J = 4.98 and 15.83 Hz, –C(4)*H*), 6.47–6.56 (dd, 1H, J = 1.18 and 15.83 Hz, –C(5)*H*), 7.11–7.37 (m, 5H, arom. *H*), 7.46 (d, 1H, J = 9.09 Hz, –N*H*); ^{13}C (75 MHz; DMSO-d_6) δ: 14.02, 22.16, 25.61, 28.55, 28.77, 29.91, 28.99, 29.09, 35.49, 49.86, 53.56, 58.63, 66.29, 70.71, 126.18, 127.07, 128.16, 128.19, 128.45, 128.81, 131.29, 136.99, 172.02; exact mass (ESI-MS) calculated for $C_{31}H_{53}O_3N_2$ $[M+H]^+$: 501.4056, found: 501.4058.

4.1.14.2. *N*-((*E*,2*R*,3*R*)-3-Hydroxy-5-phenyl-1-(piperidin-1-yl)pent-4-en-2-yl)palmitamide (16b). Yield: 217 mg (78%). ^{1}H (300 MHz; DMSO-d_6) δ: 0.83 (app. t, 3H, J = 6.78 Hz, –CH_3 acyl), 1.09–1.30 (m, 32H, acyl chain and CH_2–CH_2–CH_2 piperidine), 2.04 (t, 2H, J = 7.03 Hz, –CO–CH_2–$C_{14}H_{29}$), 2.16–2.25 (dd, 1H, J = 4.69 and 12.02 Hz, –C(1)H_a), 2.25–2.38 (m, 4H, –CH_2–N–CH_2 piperidine), 2.42–2.50 (m, 1H, –C(1)H_b), 4.00–4.10 (m, 1H, –C(2)*H*), 4.24–4.30 (m, 1H, –C(3)*H*), 5.20 -5.40 (br s, 1H –C(3)O*H*) 6.23 (dd, 1H, J = 4.99 and 15.83 Hz, –C(4)*H*), 6.51 (dd, 1H, J = 1.17 and 15.83 Hz, –C(5)*H*), 7.05–7.38 (m, 5H, arom. *H*), 7.43 (d, 1H, J = 8.79 Hz, –N*H*); ^{13}C (75 MHz; DMSO-d_6) δ: 13.94, 22.09, 23.99, 25.54, 25.68, 28.49, 28.70, 28.85, 28.93, 28.99, 29.03, 31.29, 31.79, 35.46, 50.01, 54.31, 58.92, 70.91, 126.12, 127.06, 128.16, 128.21, 128.46, 128.78, 131.39, 137.05, 171.91; exact mass (ESI-MS) calculated for $C_{32}H_{55}O_2N_2$ $[M+H]^+$: 499.4263, found: 499.4258.

4.1.14.3. *N*-((*E*,2*R*,3*R*)-3-Hydroxy-5-phenyl-1-(pyrrolidin-1-yl)pent-4-en-2-yl)palmitamide (16c). Yield: 175 mg (71%). ^{1}H (300 MHz; DMSO-d_6) δ: 0.79–0.88 (app. t, 3H, –CH_3 acyl), 1.09–1.30 (m, 24H, acyl chain), 1.34–1.48 (m, 2H, –COCH_2–CH_2–$C_{13}H_{27}$), 1.58–1.69 (m, 4H, CH_2–CH_2 pyrrolidine), 2.04 (dt, 2H, J = 3.52 and 7.04 Hz, –CO–CH_2–$C_{14}H_{29}$), 2.33 (dd, 1H, J = 7.62 and 12.02 Hz, –C(1)H_a), 2.38–2.46 (m, 4H, CH_2–N–CH_2 pyrrolidine), 2.65 (dd, 1H, J = 6.45 and 12.02 Hz, –C(1)H_b), 3.96–4.07 (m, 1H, –C(2)*H*), 4.26–4.33 (m, 1H, –C(3)*H*), 6.24 (dd, 1H, J = 4.98 and 15.83 Hz, –C(4)*H*), 6.52 (dd, 1H, J = 1.18 and 15.84 Hz, –C(5)*H*), 7.10–7.38 (m, 5H, arom.), 7.45 (d, 1H, J = 8.80 Hz, –N*H*); ^{13}C (75 MHz; DMSO-d_6) δ: 13.94, 22.08, 23.15, 25.52, 28.48, 28.69, 28.83, 28.91, 28.99, 29.02, 31.28, 35.44, 51.77, 53.75, 55.85, 70.73, 126.11, 127.05, 128.16, 128.73, 128.45, 128.73, 131.37, 137.04, 171.97; Exact mass (ESI-MS) calculated for $C_{31}H_{53}O_2N_2$ $[M+H]^+$: 485.4107, found: 485.4109

4.1.15. *N*-((5*R*,6*R*)-2-Oxo-6-(2-phenylethynyl)-1,3-oxazinan-5-yl)palmitamide (19). To a cooled solution (0 °C) of **13e** (64 mg, 149 μmol) and TEA (83 μL, 596 μmol, 4 equiv) in anhydrous CH_2Cl_2 (10 mL), triphosgene (46.5 mg, 156 μmol, 1.05 equiv) dissolved in CH_2Cl_2 (1 mL) was added dropwise and the resulting solution was stirred for 1 h at room temperature. After removal of the solvent under reduced pressure, the mixture was purified by column chromatography (EtOAc) affording **19** (60 mg, 88%) as a white solid. ^{1}H (500 MHz; DMSO-d_6) δ: 0.85 (t, 3H, J = 7.00 Hz, –CH_3 acyl), 1.1–1.3 (m, 24H, acyl *H*), 1.48 (m, 2H, –COCH_2–CH_2–$C_{13}H_{27}$), 2.16 (dt, 1H, J = 7.33 and 13.91 Hz, –CO–CH_a–$C_{14}H_{29}$), 2.20 (dt, 1H, J = 7.32 and 13.91 Hz, –CO–CH_b–$C_{14}H_{29}$), 3.19 (ddd, 1H, J = 2.19, 6.71 and 11.60 Hz –C(4)H_a), 3.40 (ddd, 1H, J = 2.07, 5.13 and 11.60 Hz, –C(4)H_b), 4.36 (dddd, 1H, J = 3.66, 5.13, 6.59, and 7.81 Hz, –C(5)*H*), 5.41 (d, 1H, J = 3.42 Hz, –C(6)*H*), 7.40 (obsolete, –C(3)N*H*), 7.35–7.50 (m, 5H, arom. *H*), 8.33 (d, 1H, J = 7.82 Hz, –C(3)N*H*); ^{13}C (125 MHz; DMSO-d_6) δ: 13.99, 22.17, 25.53, 28.67, 28.79, 28.94, 28.96, 29.11, 31.37, 35.18, 41.99, 43.13, 68.12, 83.86, 86.81, 121.32, 128.67, 129.27, 131.72, 151.08, 172.98; exact mass (ESI-MS) calculated for $C_{28}H_{43}N_2O_3$ $[M+H]^+$: 455.3274 found: 455.3275.

4.2. In vitro analysis of GlcCer synthase

In vitro analysis of GlcCer synthase was performed in rat Golgi membranes using *N*-[6-[(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]hexanoyl]D-*erythro*-sphingosine (C6-NBD-D-*erythro*-Cer). C6-D-*erythro*-NBD ceramide was synthesized by N-acylation of sphingosine using the NHS-ester of NBD-hexanoic acid (Molecular Probes).[18]

A Golgi fraction was isolated from rat liver by the method of Dominguez et al.[19] as follows. Rat liver was homogenized in ice-cold 0.25 M sucrose, 50 mM Tris–HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$ and 4.5 mM $CaCl_2$ (STKCM buffer) using a motorized Potter–Elvehjem homogenizer. After centrifugation at 400*g* for 10 min, supernatants were adjusted to 0.2 M sucrose in STKCM buffer and underlayed beneath a discontinuous gradient of 0.9 and 0.4 M sucrose in STKCM buffer. After centrifugation at 83,000g_{av} for 3 h in a SW 32 rotor at 4 °C, Golgi fractions were collected at the 0.4/0.9 M sucrose interface.

The in vitro reaction mixture contained a rat liver Golgi fraction (1.6 μg of protein), UDP-glucose (5 mM), C6-NBD-D-*erythro*-ceramide (5 μM), $MnCl_2$ (5 mM) and protease inhibitors in a total volume of 1 mL of TK buffer (50 mM Tris–HCl and 25 mM KCl, pH 7.4). The reactions were allowed to proceed for 20 min at 37 °C and were terminated by addition of 3 mL of chloroform/methanol (1:2 v/v). Lipids were extracted[20] and separated by thin-layer chromatography using chloroform/methanol/9.8 mM $CaCl_2$ (60:35:8, v/v/v) as the developing solvent. C_6-NBD-sphingolipids were identified using authentic standards. C_6-NBD-fluorescence was quantified by Quantity One software after exposing the TLC plates using a Fluor-S Max spectrometer (BioRad).

4.3. Analysis of GlcCer synthase activity in living cells

Human embryonic kidney HEK-293 cells were grown to 80–90% confluency. C6-NBD-D-*erythro*-ceramide was added directly to the culture dishes, together with 10,

25 and 50 μM of inhibitors. Cells were incubated for 3 h at 37 °C in a 5% CO_2 incubator. At the end of the incubation, dishes were washed with PBS, and cells removed by scraping with a rubber stirring rod into ice-cold water. Lipids were extracted,[20] separated and quantified as above.

4.4. ^{3}H-serine labelling

Dishes, containing HEK-293 and COS-7 cells, were incubated for 1 h with 50 mM of compounds **18** and **16c**, followed by addition of L-[3-^{3}H]-serine (Amersham) (30 μCi in 3 mL medium) for another 3 h. At the end of the incubation, dishes were washed with PBS, cells removed by scraping with a rubber stirring rod into ice-cold water, and lipids extracted.[20] Phospholipids were degraded by mild alkaline hydrolysis with methanolic NaOH (100 mM) for 2 h at 37 °C. Lipid extracts were desalted by Sephadex G-25 (superfine, Sigma)[21] and separated by TLC using chloroform/methanol/9.8 mM $CaCl_2$ (60:35:8, v/v/v) as the developing solvent. Sphingolipids were visualized by spraying with cupric sulfate, followed by brief charring. Ceramide, glucosylceramide, lactosylceramide and sphingomyelin were identified using authentic standards. Corresponding bands were scraped from the TLC plates, radioactivity was recovered from silica in 1 mL of methanol, followed by addition of Ultima Gold scintillation cocktail. Radioactivity was determined by liquid scintillation counting.

References and notes

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