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Design, synthesis and activity as acid ceramidase inhibitors of 2-oxooctanoyl and *N*-oleoylethanolamine analogues

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Abstract

The synthesis of novel *N*-acylethanolamines and their use as inhibitors of the aCDase is reported here. The compounds are either 2-oxooctanamides or oleamides of sphingosine analogs featuring a 3-hydroxy-4,5-hexadecenyl tail replaced by ether or thioether moieties. It appears that, within the 2-oxooctanamide family, the C3–OH group of the sphingosine molecule is required for inhibition both *in vitro* and in cultured cells. Furthermore, although the (*E*)-4 double bond is not essential for inhibitory activity, the (*E*) configuration is required, since the analogue with a (*Z*)-4 unsaturation was not inhibitory. None of the oleamides inhibited the aCDase *in vitro*. Conversely, with the exception of *N*-oleoylethanolamine and its analogs with *S*-decyl and *S*-hexadecyl substituents, all the synthesized oleamides inhibited the aCDase in cultured cells, although with a relatively low potency. We conclude that novel aCDase inhibitors can evolve from *N*-acylation of sphingoid bases with electron deficient-acyl groups. In contrast, chemical modification of the *N*-oleoylsphingosine backbone does not seem to offer an appropriate strategy to obtain aCDase inhibitors.

Keywords: Sphingolipids; Ceramide; Farber disease; Ceramidase; Inhibitor

1. Introduction

Stress stimuli and a number of extracellular agents affect the cell fate by producing a transient elevation of the endogenous levels of ceramide. Exposure of cells to radiation or chemotherapy is associated with increased ceramide production due to the enhancement of the de novo synthesis pathway, sphingomyelin catabolism, or both. Ceramide can be metabolized into less toxic forms by glycosylation, phosphorylation, or

Abbreviations: aCDase, acid ceramidase; HRMS, high resolution-mass spectrometry; IR, infrared; NOE, *N*-oleoylethanolamine; NMR, nuclear magnetic resonance; nCDase, neutral ceramidase

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hydrolysis to sphingosine, which is in turn phosphorylated to the anti-apoptotic sphingosine-1-phosphate. Several investigations have demonstrated that inhibition of ceramide metabolization improve cancer chemotherapy. For example, Reynolds et al. (2004) showed that the combination of the synthetic retinoid fenretinide with inhibitors of either sphingosine kinase or glucosylceramide synthase increase antitumor activity in preclinical models with minimal toxicity for non-malignant cells. Moreover, blocking the conversion of ceramide

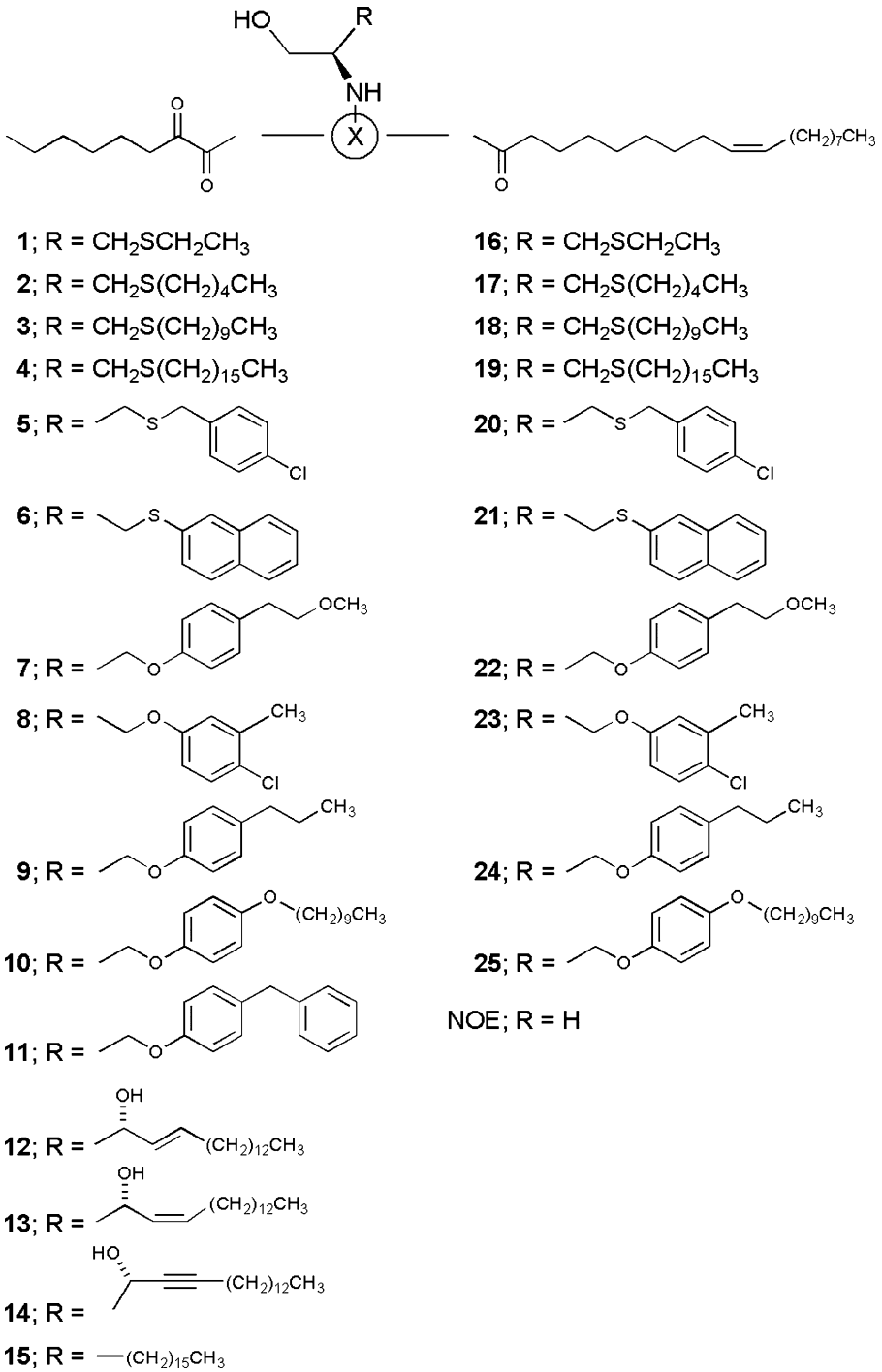


Fig. 1. Structures of NOE, 2-oxooctanamides and oleoylamides used in this work.

to glucosylceramide increases adriamicyn resistant cell sensitivity to antitumoral agents (Lavie et al., 1997; Lucci et al., 1999). In addition, inhibition of the acid ceramidase (aCDase) prevents tumor growth and metastasis of aggressive human colon cancer cell lines to the liver (Selzner et al., 2001), sensitizes prostate tumors to the effects of radiation (Samsel et al., 2004), and induces apoptosis in human melanoma (Raisova et al., 2002) and prostate cancer cells (Eto et al., 2006). Furthermore, human aCDase is overexpressed in prostate cancer and this enzyme may play an important role in prostate carcinogenesis (Raisova et al., 2002). Strelow et al. (2000) reported that overexpression of aCDase protects from tumor necrosis factor-induced cell death. This data supports a role of aCDase in signalling processes by regulating the lysosomal pool of ceramide. It also strengthens the notion that aCDase inhibitors are potential antiproliferative and cytostatic drugs useful for cancer chemotherapy.

Ceramidases hydrolyze the ceramide amide bond yielding sphingosine and fatty acids. According to their activity pH optimum, ceramidases are classified into acid (aCDase), neutral (nCDase) (El Bawab et al., 1999, 2000; Tani et al., 2000a,b; Yoshimura et al., 2002, 2004) and alkaline (Yada et al., 1995; Okino et al., 1998, 1999; Mao et al., 2000a,b, 2001). The aCDase is localized in the lysosomes. Acid ceramidase deficiencies cause Farber's disease or lipogranulomatosis (Bernardo et al., 1995; Koch et al., 1996; Bar et al., 2001), a rare recessive autosomal sphingolipidosis for which there is not known treatment. In many sphingolipidosis variants, the mutant enzymes retain catalytic activity, but are prone to fold into improper conformations, which impair their transport into the native cellular compartment for function. The misfolded proteins are retained in the endoplasmic reticulum and undergo premature degradation (Berg-Fussman et al., 1993). The use of small molecules as chemical chaperones that bind to the mutant protein and templates its correct folding for further trafficking and function is well documented (Morello et al., 2000). In this context, competitive enzyme inhibitors have been used as active-site-specific chaperones in lipid storage disorders (Fan et al., 1999; Asano et al., 2000; Sawkar et al., 2002; Lin et al., 2004), producing an increase in the residual enzyme activity, which has a significant impact on the disease outgrowth. In the light of these evidences, aCDase inhibitors deserve attention not only for their potential as chemotherapeutic drugs but also as putative chemical chaperones for the treatment of Farber disease.

The aCDase inhibitors reported to date are scarce and include B13 compound ((1*R*,2*R*)-2-(*N*-tetradecanoylamino)-1-(4-nitrophenyl)-1,3-propanediol), which has exhibited interesting activities in cancer chemotherapy (Selzner et al., 2001; Raisova et al., 2002; Samsel et al., 2004) and its *N*-tetradecyl analog AD2646 (Granot et al., 2006). Furthermore, *N*-oleoylethanolamine (NOE) has been extensively used as aCDase inhibitor in basic studies (Castillo and Teegarden, 2001; Friedrichs et al., 2002; Batra et al., 2004), but its low potency and poor selectivity preclude it from any therapeutic use. Finally, a 2-oxooctanamide analog of the dihydroceramide desaturase inhibitor GT11 (Triola et al., 2001) has been recently reported (Bedia et al., 2005) as an efficient mechanism-based inhibitor aCDase.

Based on the above precedents, the usefulness of the oleamide and 2-oxooctanamide moieties as suitable frameworks for the synthesis of novel aCDase inhibitors has been explored in this article. Specifically, we report on the results obtained with a small combinatorial library of aminoethanols *N*-acylated with 2-oxooctanoyl or oleoyl groups and branched at the C2 position of the aminoethanol core with a variety of R substituents (Fig. 1).

2. Experimental procedures

2.1. General

Solvents were distilled prior to use and dried by standard methods (Perrin and Armarego, 1988). Unless otherwise indicated, NMR spectra were recorded in $CDCl_3$ at 300 MHz for ^1H and 75 MHz for ^{13}C. Chemical shifts are reported in delta units (δ), parts per million (ppm) relative to the singlet at 7.24 ppm of $CDCl_3$ for ^1H and to the centre line of the triplet at 77.0 ppm of $CDCl_3$ for ^{13}C. IR spectra were measured in film. $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}.

2.2. Synthesis of compounds

2.2.1. 4(S)-2,2-Dimethyl-4-(p-tolylsulfonyloxymethyl) oxazolidine-3-carboxylic acid tert-butyl ester (**26**)

A solution of 2,2-dimethyl-4-(hydroxymethyl) oxazolidine-3-carboxylic acid *tert*-butyl ester (Garner and Park, 1987) (2.24 g, 9.70 mmol) in CH_2Cl_2 was added dropwise to a mixture of dimethylaminopyridine (572 mg, 4.85 mmol) and TsCl (3.70 g, 19.4 mmol) in CH_2Cl_2, followed by triethylamine (2.70 ml, 19.4 mmol) addition. After overnight stirring at room temperature, the reaction mixture was washed with 1N $KHSO_4$ (3× 30 ml), and brine (3× 30 ml). The organic

layer was dried and the solvent was removed at reduced pressure. The crude residue was purified by flash chromatography (7:1 *n*-hexane/ethyl acetate) providing tosylate 26 (3.35 g, 8.73 mmol, 90%) as a white solid: mp 107–109 °C; lit (Porte et al., 1998) 108–109 °C; TLC $R_f = 0.32$; ^{1}H NMR: δ 7.78 (d, $J = 8.0$ Hz, 2H), 7.25 (m, 2H), 4.20–3.60 (m, 5H), 2.41 (s, 3H), 1.45 (s, 3H), 1.42 (s, 3H), 1.39 (s, 9H); ^{13}C NMR: δ 152.9, 142.0, 137.3, 130.5, 127.7, 126.9, 94.4, 81.6, 67.0, 64.5, 56.2, 28.4, 27.1, 23.6, 21.2; IR: 3030, 1710, 1400, 1370, 1355, 1150 cm^{-1}; $[\alpha]_D = -78.3$ ($c = 2.0$, $CHCl_3$, 20 °C); lit (Porte et al., 1998) −79.2 ($c = 2.01$, $CHCl_3$, 25 °C).

2.3. General procedure for the reaction of tosylate **26** with alcohols and thiols

A solution of the corresponding alcohol or thiol (1.0 mmol) in dimethylformamide (1.0 ml) was added dropwise to a suspension of NaH (42 mg of a 60% dispersion in mineral oil, equivalent to 25 mg, 1.04 mmol) in dimethylformamide (2.0 ml) at room temperature. After ceasing gas evolution, the resulting mixture was transferred to a solution of tosylate **26** (200 mg, 0.52 mmol) in dimethylformamide (3.0 ml). The reaction mixture was stirred at 40 °C for 1 h, cooled to room temperature, and treated with 1N HCl (5 ml). The organic phase was extracted with diethyl ether (3× 20 ml), washed with brine, dried and evaporated to dryness to yield an oil, which was purified by flash chromatography to produce compounds **27**–**37**.

2.3.1. 4(S)-4-Ethylthiomethyl-2,2-dimethyloxazolidine-3-carboxylic acid tert-butyl ester (**27**)

Yield 98% after flash chromatography on 30:1 hexanes/ethyl acetate ($R_f = 0.27$); ^{1}H NMR: 3.86 (m, 3H), 2.82 (m, 2H), 2.50 (m, 3H), 1.52 (m, 3H), 1.42 (m, 14H), 1.22 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR: 152.0, 150.5, 94.1, 93.9, 80.1, 80.0, 66.4, 66.2, 56.5, 56.3, 34.0, 32.7, 28.3, 27.9, 27.6, 26.0, 24.1, 22.5, 14.5; IR: 2950, 1738, 1354 cm^{-1}; $[\alpha]_D = -40.4$ ($c = 4.5$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{13}H_{25}NO_3S$ $[M + Na]^+$: 298.1453; found: 298.1457.

2.3.2. 4(S)-2,2-Dimethyl-4-(pentylthiomethyl) oxazolidine-3-carboxylic acid tert-butyl ester (**28**)

Yield 99% after flash chromatography on 45:1 hexanes/ethyl acetate ($R_f = 0.27$); ^{1}H NMR: 3.92 (m, 3H), 2.82 (m, 2H), 2.50 (m, 2H), 1.54 (m, 3H), 1.44 (m, 18H), 0.84 (t, $J = 6.6$, 3H); ^{13}C NMR: 152.1, 151.9, 94.0, 93.9, 80.2, 80.0, 66.1, 66.0, 57.5, 57.3, 34.5, 33.9, 32.2, 31.0, 29.6, 29.4, 28.5, 28.3, 27.5, 26.9, 24.4, 23.0, 22.1, 14.0; the spectrum shows rotamers; IR: 2950, 1740, 1352 cm^{-1}; $[\alpha]_D = -74.9$ ($c = 0.5$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{16}H_{31}NO_3S$ $[M + Na]^+$: 340.1923; found: 340.1916.

2.3.3. 4(S)-4-Decylthiomethyl-2,2-dimethyloxazolidine-3-carboxylic acid tert-butyl ester (**29**)

Yield 82% after flash chromatography on hexanes ($R_f = 0.27$); ^{1}H NMR: 3.90 (m, 3H), 2.90 (m, 2H), 2.45 (m, 2H), 1.57 (m, 3H), 1.42 (m, 14H), 1.22 (m, 14H), 0.84 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR: 150.4, 150.6, 93.0, 93.9, 79.9, 79.7, 66.1, 65.9, 57.9, 57.6, 34.4, 33.7, 32.2, 31.7, 29.8, 29.6, 29.4, 29.2, 29.0, 28.6, 28.4, 28.3, 27.5, 26.8, 24.2, 23.0, 22.4, 14.0; IR: 2960, 1730, 1350 cm^{-1}; $[\alpha]_D = -61.8$ ($c = 0.5$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{21}H_{41}NO_3S$ $[M + Na]^+$: 410.2705; found: 410.2721.

2.3.4. 4(S)-4-Hexadecylthiomethyl-2,2-dimethyloxazolidine-3-carboxylic acid tert-butyl ester (**30**)

Yield 88% after flash chromatography on hexanes ($R_f = 0.3$); ^{1}H NMR: 3.87 (m, 3H), 2.84 (m, 2H), 2.45 (m, 3H), 1.54 (m, 4H), 1.46 (m, 12H), 1.23 (m, 26H), 0.84 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR: 152.0, 151.8, 95.1, 94.9, 80.1, 79.9, 66.4, 66.3, 57.3, 57.2, 34.4, 34.3, 32.1, 31.8, 29.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.1, 28.7, 28.4, 28.3, 27.5, 26.8, 24.2, 23.0, 22.6, 14.0; IR: 2954, 1738, 1355 cm^{-1}; $[\alpha]_D = -42.0$ ($c = 1.0$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{27}H_{53}NO_3S$ $[M + Na]^+$: 494.3644; found: 494.3631.

2.3.5. 4(S)-4-(4-Chlorobenzylthiomethyl)-2,2-dimethyloxazolidine-3-carboxylic acid tert-butyl ester (**31**)

Yield 98% after flash chromatography on 25:1 hexanes/ethyl acetate ($R_f = 0.34$); mp 74 °C; ^{1}H NMR: 7.25 (m, 5H), 4.20–3.80 (m, 3H), 3.69 (s, 2H), 2.79 (m, 1H), 2.49 (m, 1H), 1.58 (s, 3H), 1.52 (s, 3H), 1.48 (s, 9H), 1.44 (s, 3H), 1.40 (s, 3H); the spectrum shows rotamers; ^{13}C NMR: 151.9, 151. 7, 137.0, 136.6, 132.7, 132.6, 130.7, 129.9, 128.5, 128.4, 94.1, 93.6, 80.2, 79.8, 66.4, 56.9, 56.7, 36.0, 35.3, 34.6, 33.1, 28.3, 27.5, 26.7, 24.2, 23.0; IR: 2954, 1717, 1656, 1592 1355 cm^{-1}; $[\alpha]_D = -128.9$ ($c = 1.0$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{18}H_{26}ClNO_3S$ $[M + Na]^+$: 394.1220; found: 394.1235.

*2.3.6. 4(S)-2,2-Dimethyl-4-(2-naphthylthiomethyl)oxazolidine-3-carboxylic acid tert-butyl ester (**32**)*

Yield 95% after flash chromatography on 30:1 hexanes/ethyl acetate ($R_f = 0.35$); mp 75 °C; ^{1}H NMR: 8.04 (s, 1H), 7.90–7.76 (m, 3H), 7.46 (m, 3H), 4.20–3.90 (m, 3H), 3.66 (m, 1H), 3.42 (m, 1H), 2.90 (m, 2H), 1.64 (s, 3H), 1.62 (s, 3H), 1.52 (s, 9H), 1.48 (s, 3H), 1.46 (s, 3H), 1.38 (s, 9H); ^{13}C NMR: 152.0, 151.4, 134.0, 133.6, 133.0, 132.2, 132.0, 131.4, 128.8, 128.6, 128.4, 127.8, 127.6, 127.5, 127.3, 127.2, 126.6, 126.4, 126.2, 126.0, 125.3, 125.1, 94.3, 93.8, 80.5, 80.1, 66.0, 56.5, 35.9, 33.4, 28.4, 27.8, 26.9, 24.2, 23.0; IR: 2954, 1738, 1717, 1592 1535 cm^{-1}; $[\alpha]_D = +126.5$ ($c = 1.0$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{21}H_{27}NO_3S$ $[M + Na]^+$: 396.1610; found: 396.1621.

*2.3.7. 4(R)-4-((4-(2-Methoxyethyl)phenoxy)methyl)-2,2-dimethyloxazolidine-3-carboxylic acid tert-butyl ester (**33**)*

Yield 71% after flash chromatography on 30:1 hexanes/ethyl acetate ($R_f = 0.23$); ^{1}H NMR: 7.13 (m, 4H), 6.87 (m, 4H), 4.26 (m, 1H), 4.18 (m, 2H), 4.08 (m, 3H), 3.98 (m, 2H), 3.81 (m, 2H), 3.55 (t, $J = 6.6$ Hz, 4H), 3.34 (s, 6H), 2.81 (t, $J = 6.6$ Hz, 4H), 1.61 (s, 3H), 1.56 (s, 3H), 1.48 (s, 30H); the spectrum shows rotamers; ^{13}C NMR: 156.9, 156.8, 152.1, 151.6, 131.4, 131.0, 129.6, 114.5, 114.4, 93.9, 93.4, 80.3, 80.1, 73.7, 66.5, 66.0, 65.3, 65.1, 58.5, 55.9, 55.6, 35.1, 29.6, 28.4, 28.3, 27.4, 26.7, 24.2, 22.9; IR: 2978, 2934, 1698, 1508, 1388, 1244, 1171, 1113 cm^{-1}; $[\alpha]_D = -40.4$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{20}H_{31}NO_5$ $[M + Na]^+$: 388.2100; found: 388.2090.

*2.3.8. 4(R)-4-((4-Benzylphenoxy)methyl)-2,2-dimethyloxazolidine-3-carboxylic acid tert-butyl ester (**34**)*

Yield 84% after flash chromatography on 25:1 hexanes/ethyl acetate ($R_f = 0.23$); ^{1}H NMR: 7.27 (m, 2H), 7.18 (m, 2H), 7.09 (m, 2H), 6.87 (m, 2H), 4.28 (m, 1H), 4.18 (m, 2H), 4.09 (m, 4H), 3.99 (m, 2H), 1.62 (s, 3H), 1.56 (s, 3H), 1.49 (s, 15H), 1.48 (s, 15H); the spectrum shows rotamers; ^{13}C NMR: 156.7, 156.6, 152.2, 151.6, 141.5, 141.4, 133.6, 133.3, 129.8, 128.7, 128.3, 125.8, 114.5, 93.9, 93.4, 80.3, 80.1, 66.6, 66.0, 65.3, 65.1, 55.9, 55.7, 40.9, 29.6, 28.4, 28.3, 27.4, 26.7, 24.2, 23.0; the spectrum shows rotamers; IR: 2977, 2368, 2328, 1702, 1612, 1507, 1388, 1365, 1232, 1167, 1082, 1036 cm^{-1}; $[\alpha]_D = -50.1$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{24}H_{31}NO_4$ $[M + Na]^+$: 420.2151; found: 420.2159.

*2.3.9. 4(R)-4-((4-Chloro-3-methylphenoxy)methyl)-2,2-dimethyloxazolidine-3-carboxylic acid tert-butyl ester (**35**)*

Yield 76% after flash chromatography on 30:1 hexanes/ethyl acetate ($R_f = 0.23$); ^{1}H NMR: 7.21 (d, $J = 8.8$ Hz, 2H), 6.79 (m, 2H), 6.72 (m, 2H), 4.25 (m, 1H), 4.15 (m, 2H), 4.05 (m, 3H), 3.99 (m, 2H), 3.81 (m, 2H), 2.32 (s, 6H), 1.62 (m, 6H), 1.49 (m, 24H); the spectrum shows rotamers; ^{13}C NMR: 156.9, 156.8, 152.3, 152.2, 137.0, 136.8, 129.4, 125.8, 117.1, 113.0, 112.8, 94.0, 93.4, 80.5, 80.1, 66.8, 66.2, 65.2, 65.0, 55.9, 55.7, 29.6, 28.4, 28.3, 27.4, 26.7, 24.1, 22.9, 20.1; the spectrum shows rotamers; IR: 2965, 2366, 1694, 1596, 1479, 1426, 1386, 1240, 1171, 1084, 1039 cm^{-1}; $[\alpha]_D = -47.4$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{18}H_{26}ClNO_4$ $[M + Na]^+$: 378.1448; found: 378.1457.

*2.3.10. 4(R)-2,2-Dimethyl-4-((4-propylphenoxy)methyl)oxazolidine-3-carboxylic acid tert-butyl ester (**36**)*

Yield 83% after flash chromatography on 40:1 hexanes/ethyl acetate ($R_f = 0.23$); ^{1}H NMR: 7.07 (d, $J = 8.2$ Hz, 4H), 6.86 (m, 4H), 4.28 (m, 1H), 4.18 (m, 2H), 4.10 (m, 4H), 3.99 (m, 3H), 3.82 (t, $J = 7.7$ Hz, 4H), 2.52 (m, 4H), 1.62 (s, 3H), 1.56 (s, 3H), 1.49 (s, 30H), 0.92 (t, $J = 7.7$ Hz, 6H); the spectrum shows rotamers; ^{13}C NMR: 156.5, 156.3, 152.1, 151.8, 135.1, 134.9, 129.2, 114.3, 114.2, 94.0, 93.4, 80.3, 80.0, 66.6, 66.0, 65.3, 65.1, 56.0, 55.7, 37.0, 29.6, 28.4, 28.3, 27.4, 26.7, 24.6, 24.2, 22.9, 13.6; the spectrum shows rotamers; IR: 2977, 2370, 1703, 1507, 1365, 1232, 1167, 1082 cm^{-1}; $[\alpha]_D = -37.3$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{20}H_{31}NO_4$ $[M + Na]^+$: 372.2151; found: 372.2144.

*2.3.11. 4(R)-4-((4-(Decyloxy)phenoxy)methyl)-2,2-dimethyloxazolidine-3-carboxylic acid tert-butyl ester (**37**)*

Yield 75% after flash chromatography on 30:1 hexanes/ethyl acetate ($R_f = 0.33$); ^{1}H NMR (500 MHz): 6.88 (m, 4H), 6.83 (m, 4H), 4.28 (m, 2H), 4.17 (m, 2H), 4.12 (m, 2H), 4.07 (m, 2H), 4.00 (m, 2H), 3.91 (m, 4H), 3.80 (m, 2H), 1.76 (m, 4H), 1.63 (s, 3H), 1.51 (s, 12H), 1.28 (m, 28H), 0.89 (t, $J = 6.3$ Hz, 6H); the spectrum shows rotamers; ^{13}C NMR (125 MHz): 153.5, 153.3, 152.5, 152.4, 152.2, 151.6, 115.4, 115.3, 93.9, 93.4, 80.3, 80.0, 68.5, 67.3, 66.6, 65.3, 65.1, 56.0, 55.8, 31.8, 29.6, 29.5, 29.4, 29.3, 29.2, 28.4, 28.3, 27.4, 26.7, 25.9, 24.2, 23.0, 22.6, 14.0; the spectrum shows rotamers; IR: 2926, 2852, 2370, 2341, 1704, 1510, 1468, 1389, 1365, 1232, 1082, 825 cm^{-1}; $[\alpha]_D = -36.8$ ($c = 1$, $CHCl_3$,

20 °C); HRMS—calculated for $C_{27}H_{45}NO_5$ $[M+Na]^+$: 486.3196; found: 486.3186.

2.4. General method for the synthesis of α-ketoamides **12–15**

To a solution of the corresponding sphingoid aminoalcohol (0.1 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.15 mmol) and 1-hydroxybenzotriazole hydrate (0.15 mmol) in tetrahydrofurane was added a solution of the required carboxylic acid (0.15 mmol) in CH_2Cl_2 (2 ml). The reaction mixture was stirred at room temperature for 12 h, diluted with CH_2Cl_2 (10 ml), washed with brine and dried. Solvent was removed under vacuum and purification of the residue by flash chromatography eluting with a mixture of CH_2Cl_2–MeOH (97:3) furnished pure compounds **12–15** as oils.

2.4.1. (E)-(1′S,2′R)-N-(2-Hydroxy-1-hydroxymethyl-3-heptadecenyl)-2-oxooctanamide (**12**)

Yield 71%; ^{1}H NMR: 7.69 (1H, $J=8.7$ Hz, NH); 5.78 (dt, 1H, $J=15.3$ Hz, $J'=6.9$ Hz), 5.52 (dd, 1H, $J=15.1$ Hz, $J'=5.1$ Hz), 4.32 (t, 1H, 5.1 Hz, H3), 3.98 (dd, 1H, $J=11.4$ Hz, $J'=3.9$ Hz, H1), 3.86 (m, 1H, H2), 3.71 (dd, 1H, $J=11.4$, $J'=3.3$ Hz, H1), 2.90 (t, 2H, $J=7.2$ Hz), 2.03 (dd, 2H, $J=13.2$, $J'=6.3$ Hz), 1.59 (m, 4H), 1.2–1.4 (m, 30 H), 0.87 (t, 6H, $J=6.9$ Hz); ^{13}C NMR: 198.8, 160.3, 134.8, 128.3, 74.0, 61.8, 54.4, 36.8, 32.2, 31.9, 31.5, 29.6, 29.5, 29.4, 29.3, 29.1, 29.0, 22.6, 28.7, 23.1, 22.4, 14.1, 13.9; IR: 3264, 2923, 1662, 1461, 1373, 1036 cm^{-1}; $[\alpha]_D=+7.53$ ($c=0.62$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{26}H_{49}NO_4$ $[M+Na]^+$: 462.3560; found: 462.3550.

2.4.2. (Z)-(1′S,2′R)-N-(2-Hydroxy-1-hydroxymethyl-3-heptadecenyl)-2-oxooctanamide (**13**)

Yield 78%; ^{1}H NMR: 0.87 (t, 6H, $J=6.9$ Hz), 7.62 (1H, $J=8.1$ Hz, NH); 5.59 (m, 1H), 5.48 (dd, 1H, $J=9.9$ Hz, $J'=8.4$ Hz), 4.69 (m, 1H, H3), 4.04 (dd, 1H, $J=11.1$ Hz, $J'=3.3$ Hz, H1), 3.81 (m, 1H, H2), 3.74 (dd, 1H, $J=11.4$ Hz, $J'=3.3$ Hz, H1), 2.91 (t, 2H, $J=7.2$ Hz), 2.65 (bs, 1H, OH), 2.42 (bs, 1H, OH), 2.1 (m, 2H), 1.61 (m, 4H), 1.2–1.4 (m, 30 H); ^{13}C NMR: 198.8, 160.3, 135.1, 128.1, 69.2, 62.0, 54.7, 36.8, 31.9, 31.5, 29.6, 26.8, 29.5, 29.4, 29.3, 29.2, 28.7, 28.2, 27.9, 27.8, 23.1, 22.6, 22.4, 17.5, 14.1, 13.9; IR: 3365, 2933, 1658, 1462, 1022.7 cm^{-1}; $[\alpha]_D=-5.21$ ($c=0.69$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{26}H_{49}NO_4$ $[M+Na]^+$: 462.3560; found: 462.3546.

2.4.3. (1′S,2′)-N-(2-Hydroxy-1-hydroxymethyl-3-heptadecynyl)-2-oxooctanamide (**14**)

Yield 74%; ^{1}H NMR: 7.66 (1H, $J=8.4$ Hz, NH); 4.63 (m, 1H, H3), 4.13 (dd, 1H, $J=11.4$ Hz, $J'=3.9$ Hz, H1), 4.0 (m, 1H, H2), 3.77 (dd, 1H, $J=11.1$ Hz, $J'=4.2$ Hz, H1), 2.93 (t, 2H, $J=8.1$ Hz), 2.67 (bs, 1H, OH), 2.21 (dt, 2H, $J=6.9$ Hz, $J'=2.1$ Hz,), 1.60 (t, 2H, $J=7.2$ Hz), 1.50 (t, 2H, $J=7.2$ Hz), 1.2–1.4 (m, 30 H), 0.87 (t, 6H, $J=6.9$ Hz); ^{13}C NMR: 198.6. 160.5, 122.3, 88.8, 62.2, 64.0, 54.8, 36.8, 31.9, 28.9, 31.5, 28.7, 29.6, 29.4, 28.4, 29.3, 29.1, 22.6, 22.4, 18.6, 23.0, 13.9, 14.1; IR: 3312, 2923, 1719, 1665, 1536, 1463, 1384, 1064 cm^{-1}; $[\alpha]_D=-1.3$ ($c=0.63$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{26}H_{47}NO_4$ $[M+Na]^+$: 460.3403; found: 460.3417.

2.4.4. (1′S,2′R)-N-(2-Hydroxy-1-hydroxymethylheptadecyl)-2-oxooctanamide (**15**)

Yield 69%; ^{1}H NMR: 7.72 (1H, $J=7.2$ Hz, NH); 4.0 (d, 1H, $J=9.3$ Hz), 3.79 (m, 3H), 2.93 (t, 2H, $J=7.5$ Hz), 2.61 (bs, 1H, OH), 2.53 (bs, 1H, OH), 1.58 (m, 2H), 1.2–1.4 (m, 30 H), 0.87 (t, 6H, $J=6.3$ Hz); ^{13}C NMR: 198.6, 160.5, 29.6, 29.5, 73.7, 61.9, 53.8, 36.8, 31.9, 34.4, 31.5, 28.7, 25.8, 29.4, 29.3, 22.6, 22.4, 23.1, 14.1, 14.0; IR: 3290, 2934, 1658, 1532, 1462, 1068 cm^{-1}; $[\alpha]_D=+4.54$ ($c=0.35$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{26}H_{51}NO_4$ $[M+Na]^+$: 464.3716; found: 464.3725.

2.5. General method for the synthesis of **1–11** and **16–25** by deprotection-acylation of **27–37**

A solution of the starting oxazolidines **27–37** (1.0 mmol) in CH_2Cl_2 (2.0 ml) was treated with trifluoroacetic acid (1.0 ml of a 20% solution in CH_2Cl_2). The reaction mixture was stirred for 5 min at room temperature and the solvent removed under vacuum to afford crude aminoalcohols, as their trifluoroacetate salts, which were submitted to acylation without further purification. A solution of the crude trifluoroacetate in CH_2Cl_2 (2.0 ml) was treated with triethylamine (2.5 mmol), followed by dropwise addition of a solution of the corresponding carboxylic acid (1.8 mmol), 1-hydroxybenzotriazole hydrate (1.8 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.8 mmol) in CH_2Cl_2 (2.0 ml). After stirring for 16 h at room temperature, the organic phase was washed with 3× 10 ml of 1N HCl solution and brine (3× 10 ml). After the usual work-up, the crude residue was taken up in CH_2Cl_2 (5.0 ml), treated with Amberlite A-21 (20 equiv., $L=4.7$ mequiv./g), and stirred at room tem-

perature for 3 h. Filtration and evaporation produced an oily residue, which was purified by flash chromatography.

*2.5.1. (2′S)-N-(1-(Ethylthio)-3-hydroxypropan-2-yl)-2-oxooctanamide (**1**)*

Yield 67% after flash chromatography on 3:1 hexanes/acetone ($R_f = 0.35$); ^{1}H NMR: 7.42 (broad, 1H), 4.04 (m, 1H), 3.88 (dd, $J = 11.1$ Hz, $J' = 4.5$ Hz, 1H), 3.76 (dd, $J = 11.1$ Hz, $J' = 4.0$ Hz, 1H), 2.91 (t, $J = 7.2$ Hz, 2H), 2.77 (m, 2H), 2.58 (q, $J = 7.4$ Hz, 2H), 1.62 (m, 2H), 1.29 (m, 8H), 0.88 (m, 6H); ^{13}C NMR: 199.0, 160.4, 63.4, 51.0, 36.9, 32.5, 31.7, 28.9, 26.7, 23.3, 22.6, 14.9, 14.2; IR: 3320, 2011, 2852, 2386, 1725, 1658, 1534, 1465, 1040 cm^{-1}; $[\alpha]_D = +5.9$ ($c = 1.0$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{13}H_{25}NO_3S$ $[M + Na]^+$: 298.1453; found: 298.1447.

*2.5.2. (2′S)-N-(1-Hydroxy-3-(pentylthio)propan-2-yl)-2-oxooctanamide (**2**)*

Yield 60% after flash chromatography on 4:1 hexanes/acetone ($R_f = 0.33$); ^{1}H NMR: 7.42 (broad, 1H), 4.03 (m, 1H), 3.86 (dd, $J = 11.3$ Hz, $J' = 4.4$ Hz, 1H), 3.74 (dd, $J = 11.3$ Hz, $J' = 3.7$ Hz, 1H), 2.90 (t, $J = 7.1$ Hz, 2H), 2.77 (dd, $J = 13.6$ Hz, $J' = 6.9$ Hz, 1H), 2.73 (dd, $J = 13.6$ Hz, $J' = 6.5$ Hz, 1H), 1.58 (m, 4H), 1.30 (m, 12H), 0.89 (m, 6H); ^{13}C NMR: 199.0, 160.4, 63.4, 51.0, 36.9, 33.0, 32.9, 31.7, 31.1, 29.4, 28.9, 23.3, 22.6, 22.4, 14.2, 14.1; IR: 3120, 2856, 1710, 1663. 1535, 1475, 1035 cm^{-1}; $[\alpha]_D = +2.2$ ($c = 1.0$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{16}H_{31}NO_3S$ $[M + Na]^+$: 340.1922; found: 340.1912.

*2.5.3. (2′S)-N-(1-(Decylthio)-3-hydroxypropan-2-yl)-2-oxooctanamide (**3**)*

Yield 63% after flash chromatography on 4:1 hexanes/acetone ($R_f = 0.38$); ^{1}H NMR: 7.41 (broad, 1H), 4.05 (m, 1H), 3.89 (m, 1H), 3.79 (m, 1H), 2.93 (t, $J = 7.3$ Hz, 2H), 2.79 (dd, $J = 13.6$ Hz, $J' = 5.6$ Hz, 1H), 2.74 (dd, $J = 13.6$ Hz, $J' = 6.4$ Hz, 1H), 2.56 (t, $J = 7.4$ Hz, 2H), 2.24 (broad, 1H), 1.58 (m, 8H), 1.32 (m, 16H), 0.89 (t, $J = 7.1$ Hz, 6H); ^{13}C NMR: 198.7, 160.1, 63.2, 50.7, 36.6, 32.7, 32.6, 31.8, 31.4, 29.6, 29.5, 29.4, 29.2, 29.1, 28.7, 28.6, 23.0, 22.5, 22.3, 14.0, 13.9; IR: 3324, 2915, 2853, 1715, 1654, 1534, 1470, 1044 cm^{-1}; $[\alpha]_D = +8.5$ ($c = 1.0$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{21}H_{41}NO_3S$ $[M + Na]^+$: 410.2705; found: 410.2715.

*2.5.4. (2′S)-N-(1-(Hexadecylthio)-3-hydroxypropan-2-yl)-2-oxooctanamide (**4**)*

Yield 42% after flash chromatography on 3:1 hexanes/ethyl acetate ($R_f = 0.35$); ^{1}H NMR: 7.41 (broad, 1H), 4.03 (m, 1H), 3.87 (dd, $J = 11.1$ Hz, $J' = 4.6$ Hz, 1H), 3.75 (dd, $J = 11.1$ Hz, $J' = 4.0$ Hz, 1H), 2.91 (t, $J = 7.1$ Hz, 2H), 2.78 (dd, $J = 13.5$ Hz, $J' = 6.7$ Hz, 1H), 2.71 (dd, $J = 13.5$ Hz, $J' = 6.4$ Hz, 1H), 2.54 (t, $J = 7.1$ Hz, 2H), 2.17 (broad, 1H), 1.60 (m, 4H), 1.25 (m, 32H), 0.87 (m, 6H); ^{13}C NMR: 198.7, 160.1, 63.2, 50.7, 36.6, 32.7, 32.6, 31.8, 31.4, 29.5, 29.5, 29.5, 29.4, 29.4, 29.2, 29.1, 28.7, 28.6, 23.0, 22.5, 22.3, 14.0, 13.9; IR: 3315, 2925, 2853, 2375, 1717, 1656, 1521, 1466, 1033 cm^{-1}; $[\alpha]_D = +9.0$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{27}H_{53}NO_3S$ $[M + Na]^+$: 494.3644; found: 494.3649.

*2.5.5. (2′S)-N-(1-(4-Chlorobenzylthio)-3-hydroxypropan-2-yl)-2-oxooctanamide (**5**)*

Yield 69% after flash chromatography on 3:1 hexanes/acetone ($R_f = 0.33$); ^{1}H NMR: 4.02 (m, 1H), 3.82 (dd, $J = 11.1$ Hz, $J' = 4.4$ Hz, 1H), 3.70 (dd, $J = 11.1$ Hz, $J' = 4.0$ Hz, 1H), 3.70 (s, 2H), 2.90 (t, $J = 7.6$ Hz, 2H), 2.62 (m, 2H), 2.17 (broad, 1H), 1.60 (m, 2H), 1.29 (m, 8H), 0.88 (t, $J = 6.7$ Hz, 3H); ^{13}C NMR: 199.0, 160.4, 136.5, 130.5, 128.9, 63.2, 50.5, 37.0, 35.9, 32.0, 31.7, 28.9, 23.3, 22.6, 14.2; IR: 3344, 2923, 2861, 2388, 1721, 1673, 1521, 1093, 1042 cm^{-1}; $[\alpha]_D = +9.0$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{18}H_{26}ClNO_3S$ $[M + Na]^+$: 394.1220; found: 394.1232.

*2.5.6. (2′S)-N-(1-Hydroxy-3-(2-naphylthio)propan-2-yl)-2-oxooctanamide (**6**)*

Yield 61% after flash chromatography on 3:1 hexanes/acetone ($R_f = 0.35$); ^{1}H NMR: 7.89 (s, 1H), 7.78 (m, 3H), 7.46 (m, 3H), 4.11 (m, 1H), 3.93 (m, 1H), 3.75 (m, 1H), 3.31 (dd, $J = 13.9$ Hz, $J' = 6.2$ Hz, 1H), 3.24 (dd, $J = 13.9$ Hz, $J' = 7.1$ Hz, 1H), 2.81 (t, $J = 6.3$ Hz, 2H), 1.65 (broad, s, 1H), 1.51 (m, 2H), 1.27 (m, 8H), 0.88 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR: 198.8, 160.3, 133.9, 132.5, 132.1, 129.0, 128.0, 127.9, 127.5, 127.4, 126.9, 126.2, 62.9, 51.2, 36.9, 34.2, 31.7, 31.1, 28.9, 23.2, 22.6, 14.2; IR: 3341, 2928, 2387, 1718, 1675, 1043, 810 cm^{-1}; $[\alpha]_D = +30.3$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{21}H_{27}NO_3S$ $[M + Na]^+$: 396.1610; found: 396.1512.

*2.5.7. (2′S)-N-(1-Hydroxy-3-(4-(2-methoxyethyl) phenoxy)propan-2-yl)-2-oxooctanamide (**7**)*

Yield 58% after flash chromatography on 3:1 hexanes/acetone ($R_f = 0.35$); ^{1}H NMR: 7.55 (broad, 1H),

7.53 (broad, 1H), 7.15 (d, $J = 8.7$ Hz, 2H), 6.84 (d, $J = 8.7$ Hz, 2H), 4.26 (m, 1H), 4.16 (dd, $J = 9.5$ Hz, $J' = 4.2$ Hz, 1H), 4.08 (dd, $J = 9.6$ Hz, $J' = 5.0$ Hz, 1H), 3.95 (dd, $J = 11.2$ Hz, $J' = 4.5$ Hz, 1H), 3.84 (dd, $J = 11.2$ Hz, $J' = 4.5$ Hz, 1H), 3.56 (t, $J = 7.1$ Hz, 2H), 3.34 (s, 3H), 2.92 (t, $J = 7.2$ Hz, 2H), 2.82 (t, $J = 7.0$ Hz, 2H), 1.61 (m, 2H), 1.30 (m, 6H), 0.88 (t, $J = 7.2$ Hz, 3H); ^{13}C NMR: 198.9, 160.5, 156.7, 132.2, 130.1, 114.6, 73.9, 67.0, 62.4, 58.9, 50.7, 37.0, 35.5, 31.7, 28.9, 23.3, 22.6, 14.2; IR: 3335, 2928, 2862, 2367, 1712, 1688, 1513, 1469, 1240, 1115 cm^{-1}; $[\alpha]_D = -2.8$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{20}H_{31}NO_5$ $[M + Na]^+$: 388.2100; found: 388.2107.

*2.5.8. (2′S)-N-(1-(4-Chloro-3-methylphenoxy)-3-hydroxypropan-2-yl)-2-oxooctanamide (**8**)*

Yield 59% after flash chromatography on 3:1 hexanes/acetone ($R_f = 0.32$); ^{1}H NMR: 7.51 (broad, 1H), 7.23 (d, $J = 8.5$ Hz, 1H), 6.79 (d, $J = 2.7$ Hz, 1H), 6.69 (dd, $J = 8.7$ Hz, $J' = 3.5$ Hz, 1H), 4.26 (m, 1H), 4.15 (dd, $J = 9.5$ Hz, $J' = 4.3$ Hz, 1H), 4.06 (dd, $J = 9.4$ Hz, $J' = 5.1$ Hz, 1H), 3.96 (m, 1H), 3.84 (m, 1H), 2.92 (t, $J = 7.2$ Hz, 2H), 2.33 (s, 3H), 2.23 (broad, 1H), 1.59 (m, 2H), 1.30 (m, 6H), 0.88 (t, $J = 7.2$ Hz, 3H); ^{13}C NMR: 198.9, 160.5, 156.8, 137.5, 130.0, 129.9, 126.9, 117.3, 113.2, 66.9, 62.1, 50.6, 37.0, 31.7, 28.9, 23.3, 22.6, 20.5, 14.2; IR: 3329, 2957, 2919, 2856, 2349, 1747, 1661, 1573, 1482, 1295, 1177, 1067 cm^{-1}; $[\alpha]_D = +5.2$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{18}H_{26}ClNO_4$ $[M + Na]^+$: 378.1449; found: 378.1456.

*2.5.9. (2′S)-N-(1-Hydroxy-3-(4-propylphenoxy)propan-2-yl)-2-oxooctanamide (**9**)*

Yield 67% after flash chromatography on 3:1 hexanes/acetone; mp 55–56 °C; ($R_f = 0.34$); ^{1}H NMR: 7.54 (broad, 1H), 7.11 (d, $J = 8.6$ Hz, 2H), 6.84 (d, $J = 8.6$ Hz, 2H), 4.27 (m, 1H), 4.18 (dd, $J = 9.5$ Hz, $J' = 4.2$ Hz, 1H), 4.10 (dd, $J = 9.5$ Hz, $J' = 4.9$ Hz, 1H), 3.98 (m, 1H), 3.85 (m, 1H), 2.93 (t, $J = 7.3$ Hz, 2H), 2.53 (t, $J = 7.4$ Hz, 2H), 2.30 (broad, 1H), 1.63 (m, 4H), 1.32 (m, 6H), 0.93 (t, $J = 7.3$ Hz, 3H), 0.89 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR: 198.9, 160.5, 156.3, 136.0, 129.7, 114.4, 67.1, 62.5, 50.7, 37.3, 37.0, 31.7, 28.9, 24.9, 23.3, 22.6, 14.2, 13.9; IR: 3590, 2932, 2396, 1738, 1684, 1510, 1373, 1216, 1119, 1072, 975 cm^{-1}; $[\alpha]_D = -3.81$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{20}H_{31}NO_4$ $[M + Na]^+$: 372.2151; found: 372.2142.

*2.5.10. (2′S)-N-(1-(4-(Decyloxy)phenoxy)-3-hydroxypropan-2-yl)-2-oxooctanamide (**10**)*

Yield 71% after flash chromatography on 3:1 hexanes/acetone; mp 87–88 °C; ($R_f = 0.29$); ^{1}H NMR (500 MHz): 7.54 (broad, 1H), 6.83 (m, 4H), 4.24 (m, 1H), 4.13 (dd, $J = 9.5$ Hz, $J' = 4.2$ Hz, 1H), 4.04 (dd, $J = 9.6$ Hz, $J' = 4.9$ Hz, 1H), 3.95 (dd, $J = 11.4$ Hz, $J' = 4.5$ Hz, 1H), 3.89 (t, $J = 6.6$ Hz, 2H), 3.83 (dd, $J = 11.2$ Hz, $J' = 5.0$ Hz, 1H), 2.92 (t, $J = 7.5$ Hz, 2H), 1.74 (m, 2H), 1.60 (m, 2H), 1.43 (m, 2H), 1.29 (m, 18H), 0.87 (t, $J = 7.1$ Hz, 6H); ^{13}C NMR (125 MHz): 198.9, 160.5, 154.1, 152.2, 115.7, 115.6, 68.8, 67.8, 62.5, 50.7, 37.0, 32.1, 31.7, 29.8, 29.7, 29.6, 29.5, 28.9, 26.2, 23.3, 22.9, 22.6, 14.3, 14.2; IR: 3271, 2920, 2847, 1707, 1659, 1507, 1242, 1049, 829 cm^{-1}; $[\alpha]_D = -10.2$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{27}H_{45}NO_5$ $[M + Na]^+$: 486.3195; found: 486.3204.

*2.5.11. (2′S)-N-(1-(4-Benzylphenoxy)-3-hydroxypropan-2-yl)-2-oxooctanamide (**11**)*

Yield 59% after flash chromatography on 3:1 hexanes/acetone; mp 81–82 °C; ($R_f = 0.31$); ^{1}H NMR: 7.53 (broad, 1H), 7.30 (m, 2H), 7.21 (m, 1H), 7.18 (m, 1H), 7.12 (d, $J = 8.6$ Hz, 2H), 6.84 (d, $J = 8.6$ Hz, 2H), 4.27 (m, 1H), 4.17 (dd, $J = 9.6$ Hz, $J' = 4.2$ Hz, 1H), 4.09 (dd, $J = 9.5$ Hz, $J' = 5.0$ Hz, 1H), 3.96 (m, 1H), 3.93 (s, 2H), 3.84 (m, 1H), 2.92 (t, $J = 7.3$ Hz, 2H), 2.28 (broad, 1H), 1.59 (m, 2H), 1.31 (m, 6H), 0.89 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR: 198.9, 160.5, 141.5, 134.4, 130.2, 129.0, 128.6, 126.2, 114.7, 67.0, 62.4, 50.6, 41.2, 37.0, 31.7, 28.9, 23.3, 22.6, 14.2; IR: 3322, 2923, 2359, 2340, 1721, 1652, 1506, 1244, 1079 cm^{-1}; $[\alpha]_D = -2.8$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{24}H_{31}NO_4$ $[M + Na]^+$: 420.2151; found: 420.2164.

*2.5.12. (2′S,9E)-N-(1-(Ethylthio)-3-hydroxypropan-2-yl)octadec-9-enamide (**16**)*

Yield 63% after flash chromatography on 3:1 hexanes/ethyl acetate ($R_f = 0.33$); ^{1}H NMR: 6.03 (broad, 1H), 5.36 (m, 2H), 4.06 (m, 1H), 3.84 (dd, $J = 11.2$ Hz, $J' = 5.0$ Hz, 1H), 3.72 (dd, $J = 11.3$ Hz, $J' = 3.5$ Hz, 1H), 2.79 (dd, $J = 13.6$ Hz, $J' = 6.5$ Hz, 1H), 2.71 (dd, $J = 13.6$ Hz, $J' = 6.7$ Hz, 1H), 2.59 (q, $J = 7.4$ Hz, 2H), 2.23 (t, $J = 7.5$ Hz, 2H), 2.02 (m, 2H), 1.64 (m, 2H), 1.32 (m, 17H), 0.89 (t, $J = 6.7$ Hz, 3H); ^{13}C NMR: 173.8, 129.9, 129.6, 64.4, 50.6, 36.6, 32.5, 31.7, 29.7, 29.6, 29.6, 29.4, 29.2, 29.1, 29.0, 28.9, 28.8, 27.1, 27.0, 26.2, 25.5, 22.5, 14.6, 14.0; IR: 3371, 2924, 2855, 2382, 1652, 1551, 1463, 1371 cm^{-1}; $[\alpha]_D = +2.0$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{23}H_{45}NO_2S$ $[M + Na]^+$: 422.3069; found: 422.3060.

*2.5.13. (2′S,9E)-N-(1-Hydroxy-3-(pentylthio)propan-2-yl)octadec-9-enamide (**17**)*

Yield 61% after flash chromatography on 3:1 hexanes/ethyl acetate ($R_f = 0.31$); ^{1}H NMR: 6.06 (broad,

1H), 5.34 (m, 2H), 4.03 (m, 1H), 3.80 (dd, $J = 11.0$ Hz, $J' = 4.7$ Hz, 1H), 3.69 (dd, $J = 11.4$ Hz, $J' = 3.6$ Hz, 1H), 3.02 (broad, 1H), 2.75 (dd, $J = 13.5$ Hz, $J' = 6.5$ Hz, 1H), 2.67 (dd, $J = 13.5$ Hz, $J' = 6.7$ Hz, 1H), 2.53 (t, $J = 7.3$ Hz, 2H), 2.21 (t, $J = 7.3$ Hz, 2H), 2.00 (m, 2H), 1.60 (m, 4H), 1.28 (m, 18H), 0.88 (m, 6H); ^{13}C NMR: 173.8, 129.9, 129.6, 64.3, 50.6, 36.6, 32.9, 32.3, 31.8, 30.8, 29.6, 29.6, 29.4, 29.2, 29.1, 29.1, 29.0, 27.1, 27.0, 25.5, 22.1, 14.0, 13.8; IR: 3305, 2925, 2856, 1646, 1542, 1463, 1035 cm^{-1}; $[\alpha]_D = +10.3$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{26}H_{51}NO_2S$ $[M + Na]^+$: 464.3539; found: 464.3557.

2.5.14. (2′S,9E)-N-(1-Hydroxy-3-(decylthio)propan-2-yl)octadec-9-enamide (**18**)

Yield 58% after flash chromatography on 4:1 hexanes/ethyl acetate; mp 53 °C; ($R_f = 0.29$); ^{1}H NMR (500 MHz): 6.05 (broad, 1H), 5.34 (m, 2H), 4.03 (m, 1H), 3.80 (dd, $J = 11.0$ Hz, $J' = 4.4$ Hz, 1H), 3.69 (m, 1H), 2.99 (broad, 1H), 2.75 (dd, $J = 13.6$ Hz, $J' = 6.6$ Hz, 1H), 2.67 (dd, $J = 13.5$ Hz, $J' = 6.8$ Hz, 1H), 2.53 (t, $J = 7.3$ Hz, 2H), 2.21 (t, $J = 7.3$ Hz, 2H), 2.00 (m, 2H), 1.63 (m, 4H), 1.29 (m, 26H), 0.87 (t, $J = 6.5$ Hz, 6H); ^{13}C NMR (125 MHz): 173.8, 129.9, 129.6, 64.4, 50.6, 36.6, 33.0, 32.3, 31.8, 29.6, 29.6, 29.4, 29.2, 29.1, 29.1, 29.0, 28.7, 27.1, 27.0, 25.5, 22.5, 14.0; IR: 3401, 3311, 2922, 2848, 1636, 1534, 1461, 1078 cm^{-1}; $[\alpha]_D = +9.7$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{31}H_{61}NO_2S$ $[M + Na]^+$: 534.4321; found: 534.4335.

2.5.15. (2′S,9E)-N-(1-Hydroxy-3-(hexadecylthio)propan-2-yl)octadec-9-enamide (**19**)

Yield 60% after flash chromatography on 4:1 hexanes/ethyl acetate ($R_f = 0.27$); ^{1}H NMR (500 MHz): 6.05 (broad, 1H), 5.35 (m, 2H), 4.02 (m, 1H), 3.80 (dd, $J = 11.5$ Hz, $J' = 5.4$ Hz, 1H), 3.69 (m, 1H), 3.00 (broad, 1H), 2.75 (dd, $J = 13.5$ Hz, $J' = 6.5$ Hz, 1H), 2.67 (dd, $J = 13.5$ Hz, $J' = 6.7$ Hz, 1H), 2.53 (t, $J = 7.3$ Hz, 2H), 2.21 (t, $J = 7.4$ Hz, 2H), 2.00 (m, 2H), 1.60 (m, 4H), 1.28 (m, 42H), 0.87 (t, $J = 6.5$ Hz, 6H); ^{13}C NMR (125 MHz): 173.8, 129.9, 129.6, 64.3, 50.6, 36.6, 33.0, 32.4, 31.8, 31.8, 29.6, 29.5, 29.5, 29.4, 29.4, 29.2, 29.2, 29.1, 29.1, 29.0, 28.7, 27.1, 27.0, 25.5, 22.5, 14.0; IR: 3317, 2922, 2854, 1640, 1557, 1467, 1045 cm^{-1}; $[\alpha]_D = +5.6$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{37}H_{73}NO_2S$ $[M + Na]^+$: 618.5260; found: 618.5241.

2.5.16. (2′S,9E)-N-(1-(4-Chlorobenzylthio)-3-hydroxypropan-2-yl)octadec-9-enamide (**20**)

Yield 66% after flash chromatography on 3:1 hexanes/ethyl acetate; mp 48 °C; ($R_f = 0.31$); ^{1}H NMR: 7.30 (m, 4H), 5.98 (broad, 1H), 5.36 (m, 2H), 4.06 (m, 1H), 3.76 (dd, $J = 11.1$ Hz, $J' = 4.5$ Hz, 1H), 3.70 (s, 2H), 3.66 (dd, $J = 11.1$ Hz, $J' = 3.8$ Hz, 1H), 2.64 (dd, $J = 13.6$ Hz, $J' = 6.8$ Hz, 1H), 2.60 (dd, $J = 13.6$ Hz, $J' = 6.6$ Hz, 1H), 2.18 (t, $J = 7.5$ Hz, 2H), 2.01 (m, 2H), 1.62 (m, 2H), 1.30 (m, 14H), 0.89 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR: 174.0, 136.7, 133.2, 130.5, 129.9, 63.9, 50.4, 37.0, 35.8, 32.3, 32.1, 29.9, 29.9, 29.7, 29.5, 29.5, 29.4, 29.3, 27.4, 27.4, 25.9, 22.9, 14.3; IR: 3300, 2927, 2853, 2361, 1643, 1544, 1494, 100, 1019 cm^{-1}; $[\alpha]_D = +7.7$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{28}H_{46}ClNO_2S$ $[M + Na]^+$: 518.2836; found: 518.2819.

2.5.17. (2′S,9E)-N-(1-Hydroxy-3-(2-naphylthio)propan-2-yl)octadec-9-enamide (**21**)

Yield 66% after flash chromatography on 3:1 hexanes/ethyl acetate; mp 71–72 °C; ($R_f = 0.33$); ^{1}H NMR: 7.90 (s, 1H), 7.79 (m, 3H), 7.48 (m, 3H), 6.04 (broad, 1H), 5.36 (m, 2H), 4.15 (m, 1H), 3.90 (dd, $J = 11.2$ Hz, $J' = 4.5$ Hz, 1H), 3.73 (dd, $J = 11.1$ Hz, $J' = 3.8$ Hz, 1H), 3.30 (dd, $J = 13.9$ Hz, $J' = 6.2$ Hz, 1H), 3.25 (dd, $J = 13.9$ Hz, $J' = 6.8$ Hz, 1H), 2.12 (t, $J = 7.4$ Hz, 2H), 2.02 (m, 2H), 1.56 (m, 2H), 1.28 (m, 14H), 0.89 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR: 174.0, 133.9, 132.9, 132.1, 130.2, 129.9, 128.9, 127.9, 127.5, 127.4, 127.3, 126.9, 126.1, 63.8, 51.1, 36.9, 34.4, 32.1, 30.0, 29.9, 29.7, 29.5, 29.4, 29.3, 27.4, 27.4, 25.8, 22.9, 14.3; IR: 3310, 2918, 2853, 1628, 1557, 1466, 812, 741 cm^{-1}; $[\alpha]_D = +22.3$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{31}H_{47}NO_2S$ $[M + Na]^+$: 520.3225; found: 520.3210.

2.5.18. (2′S,9E)-N-(1-Hydroxy-3-(4-(2-methoxyethyl)phenoxy)propan-2-yl)octadec-9-enamide (**22**)

Yield 53% after flash chromatography on 3:1 hexanes/ethyl acetate ($R_f = 0.29$); ^{1}H NMR: 7.14 (d, $J = 8.4$ Hz, 2H), 6.84 (d, $J = 8.4$ Hz, 2H), 6.16 (broad, 1H), 5.33 (m, 2H), 4.30 (m, 1H), 4.10 (dd, $J = 9.8$ Hz, $J' = 4.7$ Hz, 1H), 4.07 (dd, $J = 9.4$ Hz, $J' = 4.5$ Hz, 1H), 3.93 (dd, $J = 11.1$ Hz, $J' = 4.0$ Hz, 1H), 3.78 (dd, $J = 11.7$ Hz, $J' = 5.3$ Hz, 1H), 3.55 (t, $J = 7.0$ Hz, 2H), 3.34 (s, 3H), 2.82 (t, $J = 6.9$ Hz, 2H), 2.35 (t, $J = 7.4$ Hz, 2H), 2.22 (t, $J = 7.9$ Hz, 2H), 2.00 (m, 2H), 1.63 (m, 2H), 1.27 (m, 12H), 0.87 (t, $J = 6.4$ Hz, 3H); ^{13}C NMR: 178.4, 163.5, 135.3, 130.1, 114.6, 73.9, 63.5, 58.9, 50.6, 36.9, 35.5, 33.4, 32.1, 29.9, 29.9, 29.7, 29.5, 29.4, 29.3, 29.0, 27.4, 25.8, 22.9, 14.3; IR: 3310, 2922, 2851, 2383, 2304, 1659, 1513, 1464, 1243 cm^{-1}; $[\alpha]_D = -2.4$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{30}H_{51}NO_4$ $[M + Na]^+$: 512.3716; found: 512.3726.

*2.5.19. (2′S,9E)-N-(1-(4-Chloro-3-methylphenoxy)-3-hydroxypropan-2-yl)octadec-9-enamide (**23**)*

Yield 53% after flash chromatography on 3:1 hexanes/ethyl acetate ($R_f = 0.28$); ^{1}H NMR: 7.23 (d, $J = 8.7$ Hz, 1H), 6.79 (d, $J = 2.9$ Hz, 1H), 6.69 (dd, $J = 8.7$ Hz, $J' = 3.0$ Hz, 1H), 6.07 (d, 1H), 5.34 (m, 2H), 4.27 (m, 1H), 4.12 (dd, $J = 9.5$ Hz, $J' = 4.2$ Hz, 1H), 4.06 (dd, $J = 9.5$ Hz, $J' = 5.0$ Hz, 1H), 3.93 (dd, $J = 11.2$ Hz, $J' = 4.4$ Hz, 1H), 3.77 (dd, $J = 10.9$ Hz, $J' = 4.3$ Hz, 1H), 2.59 (broad, 1H), 2.33 (s, 3H), 2.22 (t, $J = 7.3$ Hz, 2H), 2.00 (m, 2H), 1.62 (m, 2H), 1.26 (m, 14H), 0.87 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR: 173.6, 156.6, 137.1, 129.9, 129.6, 129.6, 126.4, 116.9, 112.9, 67.4, 62.7, 50.2, 36.6, 31.8, 29.6, 29.6, 29.4, 29.2, 29.1, 29.1, 29.0, 27.1, 27.0, 25.5, 22.5, 20.2, 14.0; IR: 3311, 2928, 2851, 2363, 2339, 1621, 1547, 1486, 1462, 1241, 1079 cm^{-1}; $[\alpha]_D = -6.9$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{28}H_{46}ClNO_3$ $[M + Na]^+$: 502.3064; found: 502.3045.

*2.5.20. (2′S,9E)-N-(1-Hydroxy-3-(4-propylphenoxy) propan-2-yl)octadec-9-enamide (**24**)*

Yield 64% after flash chromatography on 3:1 hexanes/ethyl acetate; mp 110–111 °C; ($R_f = 0.30$); ^{1}H NMR: 7.08 (d, $J = 8.7$ Hz, 2H), 6.81 (d, $J = 8.7$ Hz, 2H), 6.22 (broad, 1H), 5.34 (m, 2H), 4.28 (m, 1H), 4.13 (dd, $J = 9.6$ Hz, $J' = 4.1$ Hz, 1H), 4.07 (dd, $J = 9.5$ Hz, $J' = 4.5$ Hz, 1H), 3.93 (dd, $J = 11.3$ Hz, $J' = 4.5$ Hz, 1H), 3.78 (dd, $J = 11.3$ Hz, $J' = 4.7$ Hz, 1H), 2.52 (t, $J = 7.4$ Hz, 2H), 2.22 (t, $J = 7.4$ Hz, 2H), 1.99 (m, 2H), 1.60 (m, 2H), 1.27 (m, 22H), 0.91 (t, $J = 7.3$ Hz, 3H), 0.87 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR: 174.4, 156.4, 136.0, 129.7, 126.7, 114.4, 112.1, 67.8, 63.4, 50.7, 37.3, 36.9, 32.1, 30.0, 29.9, 29.7, 29.5, 29.4, 29.4, 29.3, 27.4, 27.3, 25.9, 24.9, 22.9, 14.3, 13.9; IR: 3305, 2924, 2853, 2357, 1655, 1503, 1235 cm^{-1}; $[\alpha]_D = -3.9$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{30}H_{51}NO_3$ $[M + Na]^+$: 496.3767; found: 496.3756.

*2.5.21. (2′S,9E)-N-(1-(4-(Decyloxy)phenoxy)-3-hydroxypropan-2-yl)octadec-9-enamide (**25**)*

Yield 67% after flash chromatography on 3:1 hexanes/ethyl acetate ($R_f = 0.32$); mp 95–96 °C; ^{1}H NMR: 6.82 (m, 4H), 6.16 (broad, 1H), 5.34 (m, 2H), 4.25 (m, 1H), 4.09 (dd, $J = 9.5$ Hz, $J' = 4.1$ Hz, 1H), 4.04 (dd, $J = 9.6$ Hz, $J' = 4.7$ Hz, 1H), 3.93 (m, 1H), 3.88 (t, $J = 6.6$ Hz, 2H), 3.77 (m, 1H), 2.87 (broad, 1H), 2.21 (t, $J = 7.3$ Hz, 2H), 2.00 (m, 2H), 1.74 (q, $J = 6.8$ Hz, 2H), 1.63 (m, 2H), 1.43 (m, 2H), 1.28 (m, 26H), 0.87 (t, $J = 6.7$ Hz, 6H); ^{13}C NMR: 174.0, 154.0, 152.3, 130.2, 129.9, 115.6, 115.6, 68.8, 68.4, 63.3, 50.7, 36.9, 32.1, 29.9, 29.9, 29.8, 29.7, 29.7, 29.6, 29.5, 29.5, 29.4, 29.4, 29.3, 27.4, 27.3, 26.2, 25.9, 22.9, 14.3; IR: 3323, 2953, 2921, 2851, 1642, 1562, 1508, 1460, 1240, 1051, 1016 cm^{-1}; $[\alpha]_D = -10.1$ ($c = 1$, $CHCl_3$, 20 °C); HRMS—calculated for $C_{37}H_{65}NO_4$ $[M + Na]^+$: 610.4811; found: 610.4832.

2.6. Biological activity

2.6.1. Inhibition of ceramidases in vitro

Mitochondria and lysosomes were used as the nCDase and aCDase sources, respectively, and were prepared from rat liver as reported (El Bawab et al., 2002; Bedia et al., 2005). The effect of the compounds on the aCDase activity was determined in 96-well plates using a novel ceramide analog as substrate (Bedia et al., submitted for publication). To each well, the following was added: 20 mM AcOH/AcONa buffer pH 4.5 containing 0.25% Triton X-100 (43 μl), test compound (1.2 μl of a 2.5 mM solution in ethanol, 3 nmol), substrate (1.2 μl of a 2.5 mM solution in ethanol, 3 nmol) and protein suspension (30 μl/well, 5 μg/μl, 150 μg/well). After 3 h at 37 °C, the reactions were stopped with methanol (75 μl/well) and 100 mM glycine–NaOH buffer pH 10.6 (30 μl/well). To each well was then sequentially added freshly prepared solutions of $NaIO_4$ (25 μl, 10 mg/ml) and bovine serum albumin (25 μl, 2 mg/ml) in 0.1 M phosphate buffer, pH 8. After 16 h in the dark, the fluorescence released was measured at excitation and emission wavelengths of 355 and 460 nm, respectively. Enzyme activities were determined by comparison of the experimental fluorescence values with a calibration curve prepared with umbelliferone, at final concentrations in the range of 0–3.2 μM. The effect of the compounds on the nCDase activity were determined following the same procedure, but using 25 mM Tris–HCl buffer pH 7.4, 0.1 mg of mitochondrial protein and a 1 h incubation period. Reaction was stopped with 75 μl/well of methanol (no glycine buffer was added).

2.6.2. Inhibition of aCDase in cell culture

Fibroblasts from a Farber patient (Moh. pAS cell line) transformed to overexpress the aCDase were used. The cells were routinely grown in a humidified 5% CO_2 atmosphere at 37 °C in Dulbecco's modified Eagel medium containing L-glutamine (2 mmol/l), penicillin (100 U/ml), streptomycin (100 μg/ml) and heat-inactivated fetal calf serum (10%). Twenty-four hours before the experiment, cells were seeded (1/3 dilution from a confluent culture in a 25 cm^2 flask) in 96-well plates (100 μl cell suspension/well). The medium was

removed and 100 μl of the same medium containing 16 μM of both the novel substrate and test compound (substrate only in controls) were added. The plates were placed back in the incubator and, after 4 h, methanol (50 μl/well), $NaIO_4$ (100 μl/well, 10 mg/ml in 0.1 M phosphate buffer pH 8.0) and BSA (50 μl/well, 2 mg/ml in 0.1 M phosphate buffer pH 8.0) were sequentially added. The fluorescence was measured after 16 h in the dark as indicated above.

3. Results

3.1. Synthesis

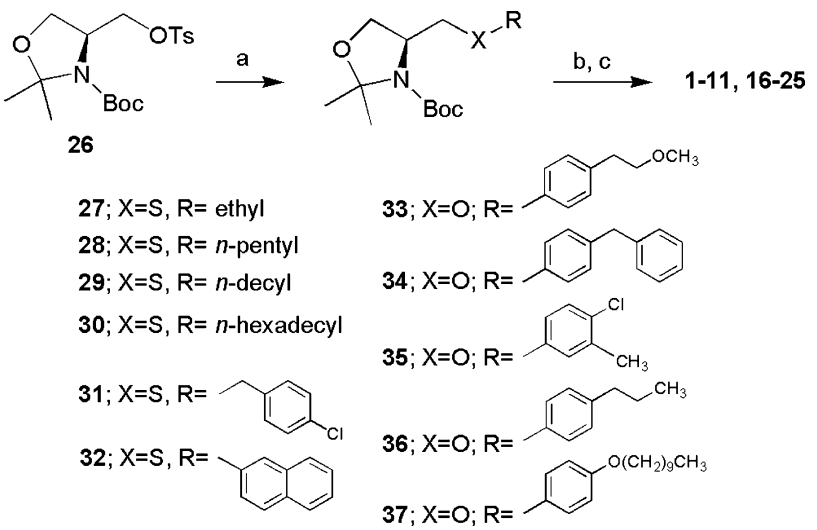
Compounds **1–11** and **16–25** were synthesized from tosylate **26** (Scheme 1), obtained by a slight modification of a reported procedure (Garner and Park, 1987; Porte et al., 1998). Nucleophilic displacement of the tosyloxy group in **26** by a series of selected thiolates or alkoxides afforded the corresponding intermediates **27–37** in excellent yields. Acetonide hydrolysis and *N*-Boc removal was carried out simultaneously by treatment with a solution of trifluoroacetic acid in CH_2Cl_2 to furnish the corresponding trifluoroacetate salts, which were submitted to coupling with the required carboxylic acid. Amides **12–15** were obtained from direct coupling of 2-oxooctanoic acid or oleic acid with the corresponding sphingoid base, which was either commercially available or had been obtained previously in our laboratories (Triola et al., 2003).

3.2. Library diversity retrospective assessment

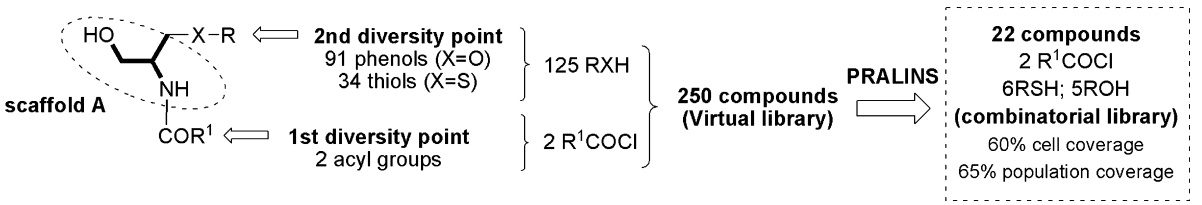
The design of a chemical library implies the proper choice of building blocks in order to guarantee the maximum diversity of the library members. This is especially important when small libraries are considered for preliminary exploratory studies.

In order to assess the chemical diversity covered by the synthesized combinatorial subset, a virtual combinatorial library of 250 compounds was enumerated and the coverage of the synthesized full-array was compared to the best-optimized full-array selection.

This virtual combinatorial library is a two-component library resulting from the derivatization of scaffold A (Scheme 2) using two acyl groups (*N*-(2-oxoamide) and *N*-oleoyl) for the first diversity point and 125 R groups for the second diversity point. This last set of virtual



Scheme 1. (a) ROH or RSH/NaH/DMF, 40 °C, 1 h; (b) TFA/CH_2Cl_2, 5 min; (c) 1: RCOOH, HOBt-EDC/CH_2Cl_2; 2: Amberlite A-21, CH_2Cl_2.



Scheme 2. *In silico* design and analysis of the combinatorial library used in this study.

reactants consists of 91 phenols and 34 thiols, which were selected by filtering a set of compounds extracted from standard chemical product catalogues with drug-likeness criteria. Obviously, the corresponding 11 R groups (6 thioethers and 5 ethers) included in the 2 × 11 synthesized combinatorial subset, compounds (**1**–**11** and **16**–**25**), were also present in this list of 125 virtual reactants.

After enumeration with MOE program (2004), energy minimization was carried out with force field MMFF94 with 0.01 kcal/mol gradient for convergence. A set of 100 standard 2D and 3D descriptors was calculated, among them physical-chemistry, spatial, topological indices and information content indices are included. Finally, dimensionality of the data was reduced by principal component analysis (PCA) down to eight components that account for 90% of the variance. Diversity assessment was measured by cell-based methods, in terms of both space coverage (number of cells covered by selected subset/number of total cells) and population coverage (total number of compounds included in represented cells/total number of molecules in library). In order to partition the space, ward clustering was chosen as it yields homogenous groups. Hierarchical tree level was stopped at 22 clusters that match the selection size. On the other hand, simulated annealing was employed to optimize a full-array selection recruited at the same ward-clustered space.

Hence, the synthesized subset represents up to 60% of cell coverage (65% population coverage), compared to the maximum full-array optimal value found of 84% (94% population). PRALINS, Program for Rational Analysis of Libraries *in silico* (Pascual et al., 2003) carried out the diversity selection and evaluation.

3.3. Inhibition of ceramidases

The screening of the compounds as ceramidase inhibitors was carried out in 96-well plates using a fluorimetric procedure recently developed in our laboratories, which is of use both *in vitro* and in cultured cells (Bedia et al., submitted for publication). This assay is based on the method developed by Reymond and co-workers (Badalassi et al., 2000) and uses a novel ceramide analog as substrate. The free base arising from ceramidase hydrolysis is chemically oxidized with $NaIO_4$ to give an aldehyde that releases a highly fluorescent umbelliferone upon β-elimination. Fluorescence intensity is thus proportional to the enzyme hydrolytic activity. This fluorogenic substrate is hydrolyzed by both aCDase and nCDase with similar efficiencies, but it is not a substrate of either anandamide hydrolase or acid *N*-palmitoylethanolamine hydrolase (Bedia et al., submitted for publication).

Using this procedure, the activity of the compounds synthesized as aCDase inhibitors was first investigated *in vitro* (Fig. 2). In rat liver preparations incubated with equimolar concentrations of substrate and test compound (40 μM), α-ketoamides **1**, **2**, **4**, **8** and **10** had a slight, but statistically significant inhibitory activity (around 20% inhibition). On the other hand, the *N*-2-oxooctanamides of sphingosine (**12**), sphinganine (**15**) and the acetylenic base (**14**) brought about a 25–50% inhibition of the aCDase. Compound **12** seemed to be the most potent, whereas its (*Z*)-isomer **13** was inactive in the same experimental conditions. On the other hand, neither NOE nor the *N*-oleoylethanolamine derivatives **16**–**25** inhibited aCDase activity *in vitro*. Finally none of the above amides inhibited the nCDase at equimolar concentrations (40 μM) with respect to the substrate, whereas 40 μM sphingosine, which was used as a positive control (Usta et al., 2001), caused a 64% inhibition (data not shown).

Using the same substrate and procedure, the activity of the compounds was then investigated in cell culture using fibroblasts from a Farber disease patient (Moh. pAS cell line) transduced to overexpress aCDase. Preliminary experiments evidenced that the fluorogenic sub-

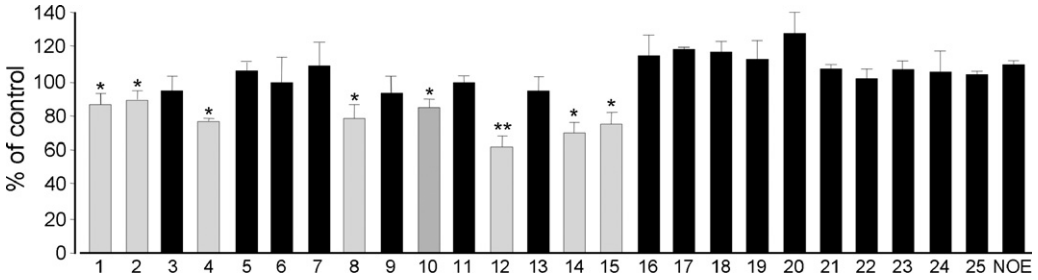


Fig. 2. Effect of compounds on aCDase *in vitro*. The experiments were performed as described in Section 2. Bars represent the mean ± S.D. of three to six replicates. Grey bars indicate statistically significant differences with respect to controls (unpaired two-tailed *t*-test, $^{*}p \leq 0.05$; $^{**}p \leq 0.0005$; black bars, not significant).

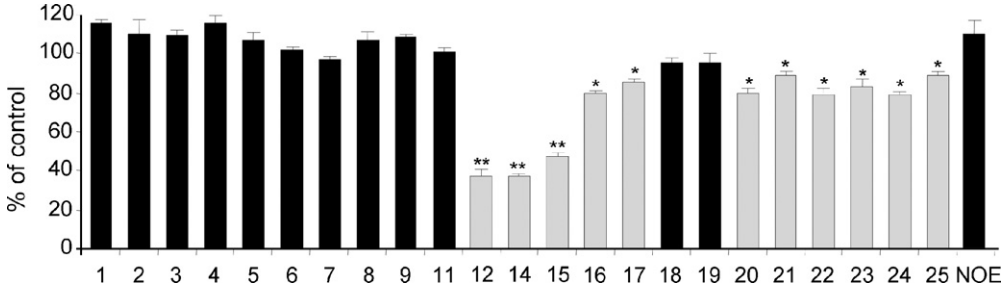


Fig. 3. Effect of compounds on aCDase in cultured Farber fibroblasts (Moh. pAS cell line) overexpressing the aCDase. The experiments were performed as described in Section 2. Bars represent the mean ± S.D. of three replicates. Grey bars indicate statistically significant differences with respect to controls (unpaired two-tailed *t*-test, $^{*}p \leq 0.05$; $^{**}p \leq 0.001$; black bars, not significant). Compounds **10** and **13** were cytotoxic in the assay conditions and their activity on aCDase could not be determined.

strate was not cytotoxic at concentrations up to 32 μM for 24 h. Likewise, no cytotoxicity was produced by the analogs (20 μM, 16 h), except for compounds **10** and **13**, which decreased cell viability to 94% and 42%, respectively. Amongst the non-toxic compounds, 2-oxooctanamides **1**–**11** did not inhibit the aCDase activity in cell culture, whereas this enzyme activity was reduced to approximately 40% with respect to controls in the presence of the α-ketoamides of sphingosine (**12**), sphinganine (**15**) and the acetylenic base (**14**) (Fig. 3). On the other hand, very low, but significant inhibitory activity was elicited by the *N*-oleoyl subset, except for thioethers **18** and **19**, having a C10 and C16 straight chain substituent at the sulphur atom, respectively, and NOE, which had no effect.

4. Discussion

In the light of their therapeutic potential, the search for potent and selective aCDase inhibitors is of interest. In a previous article (Bedia et al., 2005) we reported on the aCDase inhibitory activity of α-ketoamides of a sphingosine-like long chain base. This result was not unexpected, since aCDase is a cysteine hydrolase (Tsuboi et al., 2005) and several authors have reported that α-ketoamides are active-site directed inhibitors of serine and cysteine hydrolases, including fatty acid amide hydrolases (Koutek et al., 1994; De Petrocellis et al., 1997; Deutsch et al., 1997; Bisogno et al., 1998; Boger et al., 1999, 2000; Vandevoorde et al., 2003). Inhibition by these compounds occurs by reaction of the electron deficient α-carbonyl group with the active-site nucleophilic amino acid to form an hemiacetal intermediate, which mimics the reaction transition state.

Since the α-ketoamide function appeared to be a promising moiety leading to aCDase inhibitors, the synthesis of a small α-ketoamide library was undertaken. The common scaffold consisted on a simplified sphingoid base moiety devoid of the C3 stereogenic centre and either a sulphur or an oxygen atom bridge, which was introduced as a $–CH_2–$ bioisosteric unit to ease the preparation by parallel synthetic methodology. The skeletal variations on the sphingoid backbone were properly chosen in order to maximize diversity as found with PRALINS. Finally, the 2-oxooctanoyl group was selected to discriminate between the aCDase and nCDase (Bedia et al., 2005), since the latter requires longer chain *N*-acyl groups for recognition (Usta et al., 2001). Amongst the resultant *N*-2-oxooctanoyl derivatives, compounds **1**, **2**, **4**, **8** and **10** inhibited the aCDase *in vitro* (Fig. 2), although with low potency. However, no compound was inhibitory in cultured cells (Fig. 3), in which **10**, a moderate aCDase inhibitor *in vitro*, exhibited a remarkable cytotoxicity. These results, compared to the potency of the previously reported α-ketoamides (Bedia et al., 2005), suggested that the sphingoid base C3–OH was necessary for enhanced inhibitory potency. To confirm this issue, the 2-oxooctanamides of sphingosine (**12**), its (*Z*)-isomer (**13**), sphinganine (**15**), and the 4,5-acetylenic long chain base (**14**) were synthesized and their activities were investigated. Compounds **12**, **14** and **15** inhibited the aCDase both *in vitro* and in cultured cells with higher effectiveness than compounds **1**–**11**. Interestingly, the 2-oxooctanamide of (*Z*)-sphingosine (**13**) was not active *in vitro* (Fig. 2), which suggests that the *Z* geometry of the sphingoid base double bond lowers the affinity of the compound for the enzyme. This compound exhibited a remarkable cytotoxicity and its effect on aCDase in cultured cells could not be determined. The mechanisms underlying the toxicity of both **13** and **10** are currently under investigation. In the case of **10**, it is possible that an accumulation of ceramide because of aCDase inhibition will contribute to cell death. This does not explain the toxicity of **13**, since it did not inhibit either aCDase or nCDase.

Although nCDase is a serine protease (Galadari et al., 2005) and therefore, potentially sensitive to α-ketoamides, none of the compounds **1**–**15** inhibited this enzyme. As discussed in a previous article (Bedia et al., 2005), assuming that ceramidase inhibition by α-ketoamides occurs at the enzyme active centre (Koutek et al., 1994; De Petrocellis et al., 1997; Deutsch et al., 1997; Bisogno et al., 1998; Boger et al., 1999, 2000; Vandevoorde et al., 2003), and considering the structural requirement of nCDase for substrates with long chain *N*-acyl groups (Usta et al., 2001), the α-oxooctanamide unit is too short for the appropriate binding needed for inhibition. Besides the presence of the *N*-2-oxooctanoyl group, the absence of the C3–OH function in compounds **1**–**11** may further reduce their enzyme affinity, since this secondary hydroxyl group is also important for nCDase activity (Usta et al., 2001). Additional affinity decrease may be also involved in the lack of effect of compounds **13**–**15**, since the presence of an (*E*)-4,5 double bond in the substrate is required for efficient nCDase hydrolysis (Usta et al., 2001).

As mentioned in Section 1, NOE has been reported as an inhibitor of aCDase. Nevertheless, it is quite weak, unselective, and inhibition in cell culture does not seem reproducible. Since *N*-oleoylsphingosine is an aCDase substrate (Al et al., 1989), it appears that removal of the 3-hydroxy-4,5-hexadecenyl tail converts a substrate into a poor inhibitor. Thus, we envisaged that better aCDase inhibitors might be obtained if the tail is not removed, but structurally modified. To explore this possibility, the same amine precursors of the α-ketoamide library were *N*-acylated with oleic acid to obtain a family of NOE analogues substituted at C2. Neither NOE nor any of its analogs inhibited the aCDase or the nCDase activity *in vitro*. Likewise, NOE, **18** and **19** were not active on aCDase in cell culture. Conversely, all the other compounds of this series inhibited the aCDase in cultured cells, although with a rather low but significant potency. The lack of activity of NOE, **18** and **19** in the cell model used here may indicate that these compounds do not have the needed hydrophobicity for activity (i.e. to cross the cell membrane). Accordingly, while the estimated $C\log P$ of the active compounds lies within the range 8.5–10.5, that of NOE is two units lower ($C\log P$ 6.57) and those of **18** and **19**, having the longer chains at C2, are around 2 and 5 units higher, respectively ($C\log P$: **18**, 12.66; **19**, 15.83). The different activity exhibited by the *N*-oleoylethanolamine family *in vitro* and in cell culture may indicate that inhibition *in vitro* requires higher concentration than in cultured cells. Unfortunately, this hypothesis could not be confirmed, since higher concentrations of the *N*-oleoyl derivatives were above the critical micelle concentrations. Another possible explanation is that inhibition is not caused by the oleamides, but by the corresponding amines resulting from hydrolysis mediated by fatty acid amide hydrolases. However, the results obtained in the experiments with amines putatively formed upon hydrolysis of oleamides **17** and **19**–**22** do not support this hypothesis. Thus, only the free base corresponding to **19**, which was not inhibitory in cells, was slightly inhibitory *in vitro* (mean % of control ± S.D.: 78.0 ± 6.0; significantly different at $p \leq 0.005$, unpaired two-tailed *t*-test) whereas the other amines, corresponding to oleamides effective in cells (**17** and **20**–**22**), did not inhibit the aCDase *in vitro* (data not shown). Finally, it is also possible that the observed inhibition by oleamides in intact cells is secondary to the interaction of the compounds with their real intracellular target.

The ineffectiveness of these compounds on the nCDase can be explained, as mentioned above for the α-ketoamides, invoking a lack of affinity for the enzyme arising from the absence of the C3–OH group. Nevertheless, this explanation is valid for compounds acting on the enzyme active-site, which is not known for NOE.

5. Concluding remarks

Acylation of a sphingoid base amino group with electron deficient-containing moieties, such as 2-oxoacyl groups, appears to be a suitable strategy to obtain ceramidase inhibitors. The use of short chain acylating units confers specificity for the aCDase in front of the neutral enzyme. Although the C3–OH group seems to be necessary for inhibition, the double bond at C4 is not a strict requirement for inhibitory activity. On the other hand, chemical modification of the *N*-oleoylsphingosine backbone does not seem to be an appropriate approach to find aCDase inhibitors.

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