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Synthesis of a novel fluorescent ceramide analogue and its use in the characterization of recombinant ceramidase from *Pseudomonas aeruginosa* PA01

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Abstract

Ceramidase (CDase) hydrolyses the *N*-acyl linkage of the sphingolipid ceramide. We synthesized the non-fluorescent ceramide analogue *(4E,2S,3R)*-2-*N*-(10-pyrenedecanoyl)-1,3,17-trihydroxy-17-(3,5-dinitrobenzoyl)-4-heptadecene (**10**) that becomes fluorescent upon hydrolysis of its *N*-acyl bond. This novel substrate was used to study several kinetic aspects of the recombinant CDase from the pathogenic bacterium *Pseudomonas aeruginosa* PA01. Maximum CDase activity was observed above 1.5 μM substrate, with an apparent K_m of 0.5 ± 0.1 μM and a turnover of 5.5 min^{-1}. CDase activity depends on divalent cations without a strong specificity. CDase is inhibited by sphingosine and by several sphingosine analogues. The lack of inhibition by several mammalian CDase inhibitors such as D-*erythro*-MAPP, L-*erythro*-MAPP or *N*-oleoylethanolamine points to a novel active site and/or substrate binding region. The CDase assay described here offers the opportunity to develop and screen for specific bacterial CDase inhibitors of pharmaceutical interest.

Keywords: Ceramidase; Fluorescent assay; Kinetics; Inhibition

1. Introduction

Sphingolipids such as gangliosides, cerebrosides, sphingomyelin, and ceramide are significant constituents of eukaryotic cells, and occur mainly in the plasma membrane, the Golgi apparatus, and lysosomes. Furthermore, sphingolipids are highly bioactive compounds. They are involved in the regulation of cell growth, the induction of cell differentiation and apoptosis (for

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reviews see Liu et al., 1999; Mathias et al., 1998; Sharma and Shi, 1999; Spiegel and Merrill, 1996).

Ceramidase (CDase) is an enzyme that acts at the lipid–water interface like lipases do, and hydrolyses the *N*-acyl bond in ceramide to yield a free fatty acid and free sphingosine. CDases have been reported in eukaryotes and prokaryotes with acid (Bernardo et al., 1995; Koch et al., 1996; Li et al., 1998, 1999; Spence et al., 1986), neutral (Tani et al., 2000) and alkaline (Bawab et al., 2000; Mao et al., 2000; Nilsson and Duan, 1999; Okino et al., 1998, 1999; Wertz and Downing, 1990; Yada et al., 1995) pH optima.

In mammals CDase plays an important role in the control of the cellular ceramide content, and in the regulation of intracellular signal transduction. Acid CDase has been detected in rat brain, kidney, liver and spleen, human kidney and cerebellum, leukocytes, spleen, and plasma. Neutral and alkaline CDases have been found in pig intestinal mucosa, rat organs, human cerebellum and fibroblasts, pig lens epithelium, human leukocytes, and porcine epidermis (Hassler and Bell, 1993).

The lysosomal acid CDase is the best known human CDase. The enzyme is activated by anionic phospholipids and sphingolipid activator proteins (Linke et al., 2001). The hereditary deficiency of lysosomal acid CDase, such as in the lethal Farber disease (Bar et al., 2001), leads to the accumulation of ceramide in the lysosomes.

Ceramide and CDase activities have thusfar been determined with the much debated diglyceride kinase assay (Hofmann, 1999; Perry and Hannun, 1999 and references therein), with radioactive (Misutake et al., 1997), and with fluorescent assays (Tani et al., 1998, 1999). These discontinuous assays either have a low reliability, or are laborious and include a separation step. There is a need for a rapid CDase assay that allows to detect and follow the enzymatic reaction continuously in real time.

To meet these requirements we synthesized a novel quenched fluorescent ceramide analogue which becomes fluorescent when it is hydrolyzed by CDase, and demonstrated its usefulness in the characterization of the alkaline CDase from *Pseudomonas aeruginosa* PA01 (Nieuwenhuizen et al., to be published elsewhere).

2. Experimental procedures

1-Bromo-12-dodecanol, 2,3-dihydropyran, Red-Al (3.5 M in toluene), and butyllithium (BuLi, 1.6 M in hexane) were from Aldrich. Lithium acetylide ethylene diamine complex (95%), Amberlyst 15, Triton X-100, triethylamine, L(−)- and D(+)-norephenedrin were from Fluka. Cyclohexanone, 3,5-dinitrobenzoylchloride, *p*-toluene sulfonic acid and dicyclohexylcarbodiimide (DCC) were from Acros. 10-pyrene decanoic acid was from Molecular Probes. (1*S*,2*R*)-D-*erythro*-2-(*N*-myristoylamino)-1-phenyl-1-propanol (D-*erythro*-MAPP), L-*erythro*-MAPP and *N*-oleoylethanolamine were from CalBiochem. D/L-sphinganine, D-*erythro*-sphingosine, 2-[*N*-morpholino]-ethanesulfonic acid (MES), 3-[*N*-morpholino]-propanesulfonic acid (MOPS), tris(hydroxymethyl) aminoethane (Tris), boric acid and bovine serum albumin were from Sigma. L-*erythro*-sphingosine was from Larodan.

Protein concentrations were determined with a Bio-Rad DC (Detergent-Compatible) Protein Assay (BioRad) with bovine serum albumin as standard.

Hexanes, ethylacetate (EtOAc), methanol (MeOH), diethylether (Et_2O), and chloroform ($CHCl_3$) were distilled prior to use. Toluene and dichloromethane (CH_2Cl_2) were dried by distillation from phosphorpentoxide (P_2O_5). Tetrahydrofuran (THF) and Et_2O were dried by distillation under a N_2 atmosphere from $LiAlH_4$ (5 g l^{-1}) directly prior to use. Dimethylsulfoxide (DMSO), pyridin and hexamethylphosphortriamide (HMPT) were dried over 3 Å molsieves.

Column Chromatography was performed with Merck Kieselgel 60 (230–400 mesh, ASTM). TLC analyses were performed on Merck Kieselgel F254 DC-Fertig plates.

^{1}H NMR spectra were recorded with a Bruker AC 300 (300 MHz) spectrometer in $CDCl_3$ at 300 K. ^{1}H chemical shifts are given in ppm (δ) relative to internal TMS.

High Resolution Fast Atom Bombardment was carried out using a JEOL JMS SX/SX 102A four-sector mass spectrometer coupled to a MS-MP 9021D/UDP data system.

The samples were mixed with 3-nitrobenzylalcohol and were introduced via the direct insertion probe into the ion source. During the High Resolution FABMS measurements a resolving power of 5000 (10% valley definition) was used. B/E scantype mass spectra were acquired with CA (Collision Activation) in the first field free region of the instrument, using Ar as collision gas. The pressure of the collision gas was adjusted to obtain a 50% reduction of the beam. Resolution of the instrument was maintained at 1000 throughout the experiments.

CDase was produced in *Escherichia coli* (BL 21) and isolated from inclusion bodies as described (Nieuwenhuizen et al., to be published elsewhere).

2.1. Synthesis of the quenched fluorescent ceramide analogue **10**

The synthesis of ceramide analogue **10** was performed as outlined in Fig. 2.

2.1.1. 1,1-Dimethylethyl (S)-4-formyl-2,2-dimethyl-3-oxazolidinecarboxylate (**3**)

Compound **3** was synthesized in four steps from L-serine as described (Garner and Min Park, 1987).

^{1}H NMR ($CDCl_3$) δ: 1.49–1.52 (m, 15H), 4.11 (m, 2H), 4.41 (m, H), 9.58 (bd, H, $J = 16.3$ Hz).

2.1.1.1. 1-(2-Tetrahydropyranyl) tetradecyl-13-yn (**2**).

1-Bromo-12-dodecanol (2.5 g, 9.4 mmol), 1,2-dihydropyran (4 ml, 38 mmol), and a catalytic amount of *p*-toluenesulfonic acid were dissolved in dry toluene (20 ml), and stirred for 1 h at room temperature. Triethylamine (1 ml) was added, and the reaction mixture was concentrated in vacuo. The residue was dissolved in dry DMSO (50 ml), cooled on ice, and lithium acetylide ethylene diamine complex (1.5 g, 16 mmol) was carefully added. The suspension was allowed to warm to room temperature and stirred for 4 h. The reaction mixture was then poured into a saturated $NaHCO_3$ solution (300 ml) and extracted with $CHCl_3$ (5×100 ml). The combined organic phases were subsequently washed with saturated NaCl (200 ml), water (200 ml), and dried (Na_2SO_4), and then concentrated in vacuo. Column chromatography (silica, hexanes/EtOAc, 95/5, v/v) yielded pure **2** as a colorless oil (2.20 g, 7.5 mmol, 80%).

^{1}H NMR ($CDCl_3$) δ: 1.22–1.45 (m, 16H), 1.46–1.62 (m, 8H), 1.63–1.88 (m, 2H), 1.93 (t, H, $J = 2.6$ Hz), 2.17 (dt, 2H, $J = 6.9$ Hz, $J = 2.6$ Hz), 3.35 (m, H), 3.51 (m, H), 3.72 (m, H), 3.87 (m, H), 4.57 (m, H).

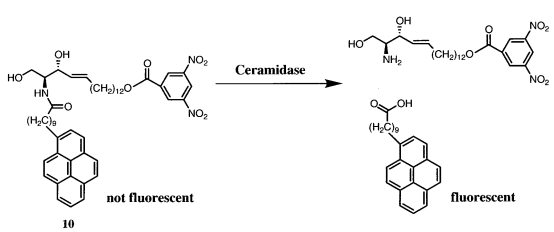

Fig. 1. The hydrolysis of CDase analogue **10** by CDase.

2.1.1.2. tert-Butyl(4S, 1′R)-2,2-dimethyl-4-[1′-hydroxy-(15′-(2-tetrahydropyranyl)pentadec)-2′-ynyl] oxazolidine-3-carboxylate (**4**).

Compound **4** was synthesized as described (Herold, 1988) with some modifications.

To a solution of compound **2** (1.82 g, 6.2 mmol) in dry THF (50 ml, -78 °C, under N_2) BuLi (1.6 M in hexane, 3.8 ml, 6.1 mmol) was added via cannula. After 30 min dry HMPT (2.8 ml, 16 mmol) was added. A solution of compound **3** (0.90 g, 3.9 mmol) in dry THF (5 ml) was added dropwise. The reaction mixture was stirred for 2 h at -78 °C. The reaction mixture was then allowed to warm to -20 °C, and saturated NH_4Cl (200 ml) and EtOAc (100 ml) were added. The mixture was stirred for 18 h at room temperature. After phase separation, the water phase was extracted with EtOAc (5×100 ml). The combined organic phases were subsequently washed with saturated NaCl (100 ml), water (100 ml), dried (Na_2SO_4) and concentrated in vacuo. Column chromatography (silica, hexane/EtOAc/triethylamine, 800/200/1, v/v/v) yielded pure **4** (1.00 g, 1.90 mmol, 49%) as a colorless oil. The *erythro* conformation of **4** was confirmed by the 11.2 Hz coupling in compound **7** (vide infra) indicating a trans coupling of protons a and b (see Fig. 2).

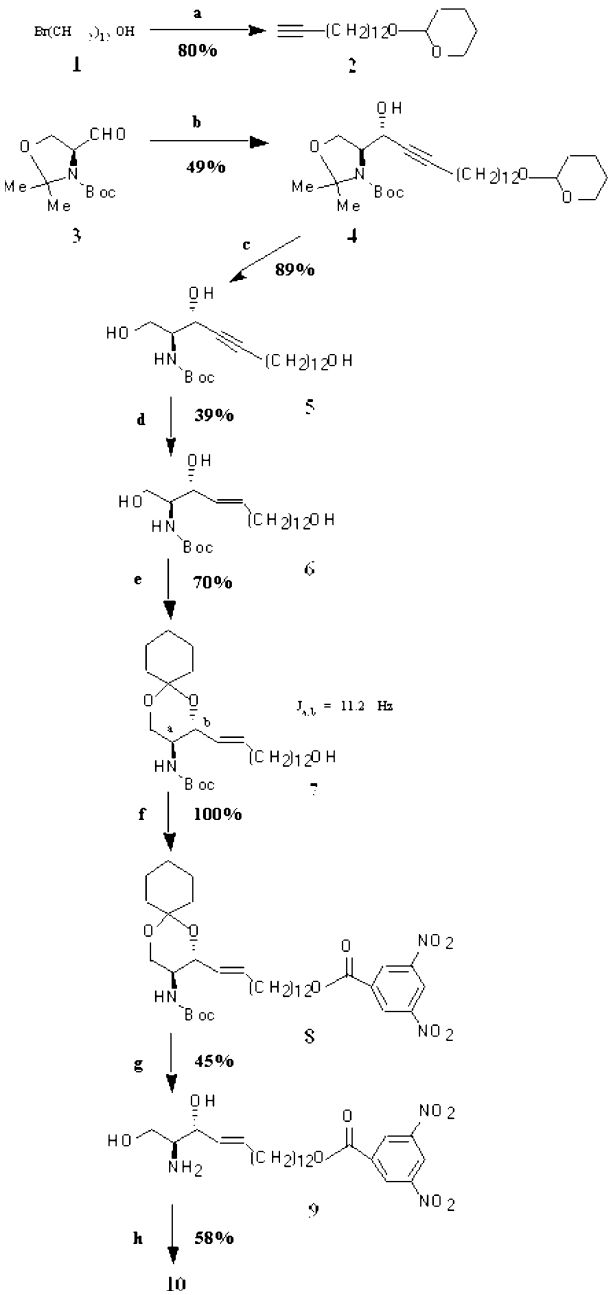

Fig. 2. Synthesis of ceramide analogue **10**. (a) I; 1,2 dihydropyran and *p*-toluene sulfonic acid in toluene; II, lithium acetylide ethylene diamine complex in DMSO. (b) BuLi, HMPT in THF, −78 °C. (c) Amberlyst 15, MeOH. (d) Red-Al, Et_2O, 0 °C. (e) Cyclohexanone, *p*-toluenesulfonic acid in refluxing toluene. (f) 3,5-Dinitrobenzoyl chloride in CH_2Cl_2 and pyridin. (g) I; HCl in EtOAc, II; Amberlyst 15 in MeOH. (h) 10-Pyrene decanoic acid anhydride in THF.

^{1}H NMR ($CDCl_3$) δ:1.19–1.78 (m, 40H), 1.82 (m, 2H), 2.19 (dt, H, $J = 6.6$ Hz, $J = 1.8$ Hz), 3.39 (m, H), 3.48 (m, H), 3.72 (m, H), 3.75 (m, H), 3.90 (m, H), 4.10 (m, 3H), 4.45–4.71 (bs, H), 4.61 (m, H).

*2.1.1.3. tert-Butyl(1S, 2R)-N-[2-hydroxy-1-(hydroxymethyl)-3-hexadecynyl-16-ol]carbamate (**5**).* Compound **4** (1.00 g, 1.9 mmol) was dissolved in MeOH (20 ml), Amberlyst 15 (500 mg) was added, and the mixture was stirred for 55 h at room temperature. After filtration (G4 glass filter) and concentration in vacuo, compound **5** (690 mg, 1.7 mmol, 89%) was obtained as a light yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.21–1.69 (m, 29H), 2.22 (dt, 2H, $J = 7.0$ Hz, $J = 1.9$ Hz), 2.41 (bs, H), 2.87 (bs, H), 3.64 (t, 2H, $J = 6.6$ Hz), 3.76 (bd, 2H, $J = 8.5$ Hz), 4.10 (m, H), 4.60 (s, H), 5.30 (bs, H), NH not visible.

*2.1.1.4. tert-Butyl(3E, 1S, 2R)-N-[2-hydroxy-1-(hydroxymethyl)-3-hexadecenyl-16-ol]carbamate (**6**).* To an ice cold solution of compound **5** (690 mg, 1.7 mmol) in dry Et_2O (20 ml), Red-Al (3.5 M in toluene, 7 ml, 22.5 mmol) was added dropwise under a N_2 atmosphere. The reaction mixture was stirred for 30 min. The reaction mixture was then stirred for 24 h at room temperature. The clear reaction mixture was cooled on ice, and MeOH (10 ml) was added slowly. Then saturated sodium–potassium tartrate (150 ml) was added, and the mixture was stirred for 1 h at room temperature. After phase separation, the water phase was extracted with Et_2O (4×50 ml). The combined organic phases were subsequently washed with saturated sodium–potassium tartrate (3×50 ml), saturated NaCl (2×50 ml), dried (Na_2SO_4), and concentrated in vacuo. Column chromatography (silica, hexanes/EtOAc, 1/1, v/v) and repeated crystallization from hexanes/EtOAc (9/1, v/v) yielded pure **6** as a white solid (263 mg, 0.66 mmol, 39%, melting point (m.p.) 66–67 °C).

^{1}H NMR ($CDCl_3$) δ: 1.20–1.65 (m, 29H), 2.06 (m, 2H), 2.51 (bs, 2H), 3.64 (t, 2H, $J = 6.6$ Hz), 3.70 (m, 2H), 3.93 (dd, H, $J = 3.7$ Hz, $J = 11.3$ Hz), 4.32 (s, H), 5.27 (bs, H), 5.53 (dd, H, $J = 6.6$ Hz, $J = 15.4$ Hz), 5.78 (dt, H, $J = 6.6$ Hz, $J = 15.4$ Hz), NH not visible.

*2.1.1.5. tert-Butyl(3E, 1S, 2R)-N-[1,2-O-cyclohexylidene-2-hydroxy-1-(hydroxymethyl)-3-hexadecenyl-16-ol]carbamate (**7**).* Compound **6** (263 mg, 0.66 mmol), cyclohexanone (0.82 ml, 0.79 mmol) and a catalytic amount of *p*-toluenesulfonic acid were dissolved in dry toluene (50 ml). The reaction mixture was refluxed for 30 min, and the reaction was completed by distillation of 20 ml solvent and water. Triethylamine (1 ml) was added, and the reaction mixture was concentrated in vacuo. Column chromatography (silica, hexanes/EtOAc/triethylamine, 800/200/1, v/v/v) yielded pure **7** (226 mg, 0.46 mmol, 70%) as a colorless oil.

^{1}H NMR ($CDCl_3$) δ: 1.15–1.62 (m, 35H), 1.80 (m, 2H), 1.98 (m, 4H), 3.41 (m, H), 3.58 (t, 2H, $J = 6.6$ Hz), 3.62 (m, H), 3.89 (dd, H, $J = 5.4$ Hz, $J = 11.2$), 4.02 (bs, H), 4.45 (d, H, $J = 8.5$ Hz), 5.39 (dd. H, $J = 7.4$ Hz, $J = 15.4$ Hz), 5.70 (dt, H, $J = 6.6$ Hz, $J = 15.4$ Hz), NH not visible.

*2.1.1.6. tert-Butyl(3E, 1S, 2R)-N-[1,2-O-cyclohexylidene-2-hydroxy-1-(hydroxymethyl)-3-hexadecenyl-16-(3,5-dinitrobenzoyl)]carbamate (**8**).* A solution of 3,5-dinitrobenzoylchloride (117 mg, 0.51 mmol) in dry CH_2Cl_2 (10 ml) was added dropwise to a stirred solution of compound **7** (226 mg, 0.46 mmol) and dry pyridine (1 ml) in dry CH_2Cl_2 (20 ml). The reaction was stirred for 18 h at room temperature, and was then concentrated in vacuo. Column chromatography (silica, hexanes/EtOAc, 8/2, v/v) yielded pure **8** (321 mg, 0.46 mmol, 100%) as a light yellow oil.

^{1}H NMR ($CDCl_3$) δ: 1.12–1.60 (m, 37H), 1.79 (m, 2H), 2.01 (m, 2H), 3.42 (m, H), 3.60 (m, H), 3.88 (dd, H, $J = 5.4$ Hz, $J = 11.2$ Hz), 4.02 (bs, H), 4.31 (bs, H), 4.39 (t, 2H, $J = 6.8$ Hz), 5.39 (dd, H, $J = 7.3$ Hz, $J = 15.4$ Hz), 5.71 (dt, H, $J = 6.7$ Hz, $J = 15.4$ Hz), 9.11 (m, 2H), 9.17 (m, H).

*2.1.1.7. (4E, 2S,3R)-2-Amino-1,3,17-trihydroxy-17(3,5-dinitrobenzoyl)-4-heptadecene (**9**).* Compound **8** (321 mg, 0.46 mmol) was dissolved in 0.42 M HCl in EtOAc (30 ml, 12.6 mmol) and the mixture was stirred for 24 h at room temperature (Gibson et al., 1994). The removal of the *tert*-butyloxycarbonyl (BOC) group was followed via TLC (silica, hexanes/EtOAc, 1/1, v/v) and ninhydrin staining. The reaction mixture was concentrated in vacuo, the residue was dissolved in MeOH (10 ml), Amberlyst 15 (500 mg) was added, and the mixture was stirred for 24 h at room temperature. The removal of the cyclohexylidene group was checked with TLC ($CHCl_3$/MeOH, 8/2, v/v). After filtration (G4 glass filter), concentration in vacuo, and column chromatography (silica, $CHCl_3$/MeOH, 8/2, v/v), compound **9** (100 mg, 0.21 mmol, 45%) was obtained as a waxy white solid.

^{1}H NMR ($CDCl_3$) δ: 1.16–1.52 (m, 20H), 1.79 (m, 2H), 2.01 (m, 2H), 3.27 (bs, H), 3.54 (bs, 2H), 3.81 (bd, 2H, $J = 4.5$ Hz), 4.42 (s, H), 4.44 (t, 2H, $J = 6.8$ Hz), 5.46 (dd, H, $J = 6.2$ Hz, $J = 15.4$ Hz), 8.81 (dt, H, $J = 15.4$ Hz, $J = 6.7$ Hz), 9.14 (m, 2H), 9.21 (m, H).

*2.1.1.8. (4E, 2S,3R)-2-N-(10-Pyrenedecanoyl)-1,3,17-trihydroxy-17-(3,5-dinitrobenzoyl)-4-heptadecene (**10**).* 10-Pyrene decanoic acid (22 mg, 0.06 mmol) and DCC (400 mg, 1.9 mmol) were dissolved in dry THF (10 ml) and stirred at room temperature for 1 h in the dark. The anhydride was added dropwise to a solution of compound **9** in dry THF (5 ml, 29 mg, 0.06 mmol) at room temperature over a 1 h period. The reaction mixture was stirred for 18 h in the dark at room temperature. After addition of water (1 ml), the reaction mixture was stirred for 15 min and concentrated in vacuo. Traces of water were coevaporated with MeOH. Column chromatography (silica, $CHCl_3$/MeOH, 99/1, v/v) yielded **10** (29 mg, 35 μmol, 58%, R_F 0.12) as a yellow wax.

^{1}H NMR ($CDCl_3$) δ: 1.16–1.69 (m, 30 H), 1.79 (m, 4H), 2.01 (m, 2H), 2.22 (t, 2H, $J = 7.5$ Hz), 2.87 (bs, 2H), 3.31 (t, 2H, $J = 7.8$ Hz), 3.69 (bd, H, $J = 9.2$ Hz), 3.91 (m, 2H), 4.29 (bs, H), 4.40 (t, 2H, $J = 6.9$ Hz), 5.52 (dd, H, $J = 15.4$ Hz, $J = 6.5$ Hz), 5.76 (dt, H, $J = 15.4$ Hz, $J = 6.6$ Hz), 6.25 (d, H, $J = 7.3$ Hz), 7.84 (d, H, $J = 7.8$ Hz), 7.98 (m, 3H), 8.01–8.14 (m, 4H), 8.23 (d, H, $J = 9.2$ Hz), 8.96 (m, 2H), 9.04 (m, H).

FAB-MS:. The cationized molecular ion $[M + H]^+$ at m/z 850.4635 is in good agreement with the theoretical m/z value of 850.4643. Fragmentation of the molecular ion yielded ions at m/z 832.47 and m/z 814.47 and are assigned to frag-

ments $[M–H_2O]^+$ and $[M–2H_2O]^+$, respectively. Ions at m/z 355.15, 371.17 and 414.27 are assigned to $[C(O)–(CH_2)_9–pyrene]^+$, $[H_2N–C(O)–(CH_2)_9–pyrene]^+$ and $[HO–CH_2–CH–NH–C(O)–(CH_2)_9–pyrene]^+$, respectively.

2.2. Characterization of CDase

Some kinetic aspects of recombinant CDase were studied using the quenched fluorescent ceramide analogue **10** as a substrate. All experiments were performed at 30 °C using a Photon Technology International Fluorimeter equipped with a LPS-220 Lamp Power Supply, a 810-Photomultiplier Detector System, a MD-5020 Motordrive, and a Dell Dimensions M166a computer with FELIX 1.11 (PTI) software. Excitation was done at 346 (± 2 nm), and the reaction was followed by monitoring the emission at 378 (± 2 nm). The substrate (5 μl, 0.4 mM in MeOH, 2 μM) was added to the buffer (1 ml) and the mixture was allowed to equilibrate for 5 min. Then CDase (5 μl, 0.06 mg ml^{-1}, 4.3 nM) was added, and the reaction was allowed to proceed for 20 min. The slope of the increase in fluorescence versus time was used to assess the CDase activity. Above pH 9.5 the enzymatic hydrolysis was corrected for the auto hydrolysis of the 3,5-dinitrobenoyl ester.

An average molecular weight of 624 for Triton X-100 was used to calculate its concentration.

Curve fitting was done with KALEIDAGRAPH 3.0.2 software (Macintosh version).

The effect of the Triton X-100 concentration on the CDase reaction was measured in 50 mM Tris–HCl, 4 mM $CaCl_2$, pH 8.5.

The pH dependence of the CDase activity was tested in 50 mM buffers (MES, MOPS, Tris, and Borate), at 4 mM $CaCl_2$ and 1.6 mM Triton X-100, in the pH range from pH 5.5 to 11.0.

The influence of the Ca^{2+}, Ba^{2+}, and Mg^{2+} on the CDase activity was tested in 50 mM Tris–HCl, 1.6 mM Triton X-100, pH 8.5. The influence of the Zn^{2+} concentration was measured in 50 mM MES, 1.6 mM Triton X-100, pH 6.5.

The effect of the CDase concentration on the reaction rate tested with 20 μM substrate in 50 mM Tris–HCl, 1.6 mM Triton X-100, 4 mM $CaCl_2$, pH 8.5.

2.2.1. Inhibitor assay

The CDase inhibitors D-*e*-MAPP, L-*e*-MAPP, *N*-oleoylethanolamine, L(−)-norephenedrin, D-(+)-norephenedrin, D/L-sphinganine, and D-*erythro*-sphingosine and L-*erythro*-sphingosine were dissolved in ethanol. In all inhibitor assays, the final concentration of organic solvent was 0.5% (v/v).

3. Results

3.1. Synthesis of ceramide analogue *10*

Here we describe the synthesis of a quenched fluorescent ceramide analogue **10** (Fig. 1). The 3,5-dinitrobenzoyl group is an efficient intramolecular quencher of the fluorescence of the pyrene group. Upon hydrolysis of the *N*-acyl bond by CDase the average distance between the quenching and fluorescent groups increases, giving rise to a strong increase of the pyrene fluorescence, thus allowing the real time detection of the CDase reaction.

In Fig. 2 the reaction scheme for the synthesis of ceramide analogue **10** is shown. Compound **2** was synthesized from bromo alcohol **1** in 80% yield, in a two-step synthesis with 2,3-dihydropyran and lithiumacetylide. Acetylenic compound **2** was then coupled with BuLi to aldehyde **3**, which is a frequently used intermediate in the sphingolipid synthesis (Azuma et al., 2000; Duffin et al., 2000; Garner and Min Park, 1987; Garner et al., 1988; Herold, 1988; Yin et al., 1998). The coupling was done in the presence of HMPT to direct the coupling to the *erythro* form. Diacetal **4** was obtained in 49% yield (>95% *erythro*, vide infra). The selective removal of the acetal protective groups with the use of the acidic cation exchange resin Amberlyst 15 in MeOH yielded triol **5** in 89% yield. The partial reduction of **5** with Red-Al yielded compound **6** only in 39% yield after extensive recrystallisation to remove traces of unreduced **5**. In order to achieve the selective coupling of the 3,5-dinitrobenzoyl group to the ω hydroxyl group, the free neighboring hydroxyl groups were selectively protected with cyclohexanone, which afforded acetal **7** in 70%

yield. The *erythro* conformation was confirmed by the 11.2 Hz coupling indicating a trans coupling of protons a and b (see Fig. 2). The use of acetone instead of cyclohexanone gave a 50/50 mixture of the desired acetal and the oxazolide. The presence of the less flexible and more bulky ring structure probably prevents the formation of the undesired oxazolide. The reaction of **7** with 3,5-dinitrobenzoyl chloride gave compound **8** in quantitative yield. The selective removal of the BOC group was achieved with dry HCl in EtOAc. The acetal was then removed with the use of Amberlyst 15 in MeOH. Unfortunately the ester bond was also partially hydrolyzed under these conditions, and intermediate **9** was obtained in 45% yield. Finally target compound **10** was synthesized in 58% yield, in a reaction of 10-pyrene decanoic acid anhydride with **9**, making use of the higher reactivity of the $-NH_2$ compared with the –OH groups. We successfully synthesized ceramide analogue **10** in 3% overall yield starting from intermediate **3**.

3.2. Kinetics of recombinant CDase from P. aeruginosa PA01

First we tested the influence of the Triton X-100 concentration on the CDase hydrolysis reaction at pH 8.5 in the presence of Ca^{2+} (4 mM), using the quenched fluorescent ceramide analogue **10** (20 μM) at a fixed CDase concentration (4.3 nM). As expected a steep rise in activity was observed at the critical micellar concentration (~ 0.8 mM) of Triton X-100, and the maximum reaction rate was observed at 1.6 mM Triton X-100. All further experiments were, therefore, conducted at that detergent concentration.

CDase did not hydrolyze the 3,5-dinitrobenzoyl ester linkage in the substrate, even after 24 h incubations, as was shown by TLC analyses. Only one fluorescent product was formed which coeluted with the pyrene fatty acid, indicating the selective cleavage of the amide bond by CDase.

The pH dependence of the CDase hydrolysis reaction at a fixed CDase concentration (4.3 nM), and substrate (20 μM) is shown in Fig. 3. The recombinant CDase is active over a broad pH range, and shows more than 90% of its maximum activity between pH 7.5 and 9.5. Note that CDase is more active in MOPS buffer than in MES or Tris at the same pH. To avoid possible alkaline hydrolysis of the ester bond in the substrate, all

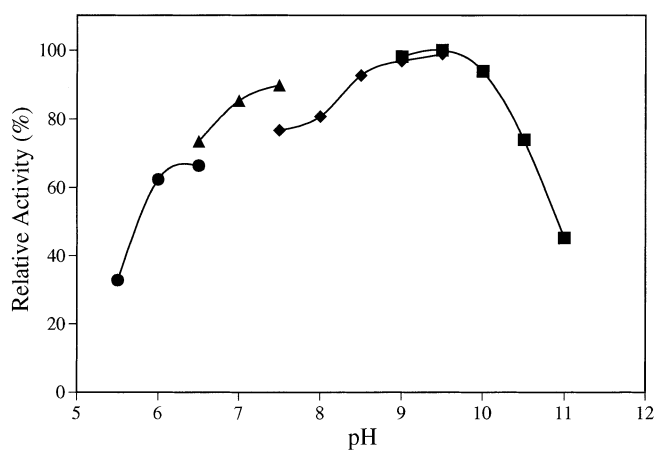

Fig. 3. pH-dependence of the CDase reaction with 4.3 nM CDase in 50 mM buffer with 1.6 mM Triton X-100, 4 mM $CaCl_2$, 20 μM substrate at 30 °C. ● MES, ▲ MOPS, ◆ Tris, ■ borate. The relative reaction rates were determined as described in Section 2.

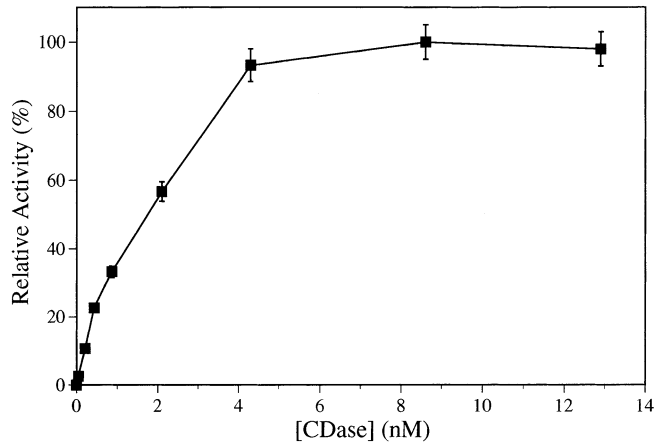

Fig. 4. Effect of the CDase concentration on the reaction rate. Reaction conditions: 50 mM Tris–HCl pH 8.5, 4 mM $CaCl_2$, 20 μM substrate, 30 °C. The relative reaction rates were determined as described in Section 2.

further experiments were performed at pH 8.5 (see Section 2).

The CDase reaction rate at pH 8.5 and 20 μM substrate depends on the CDase concentration, but maximum activity is reached at 4.3 nM CDase. This kinetic behavior compares well with that of lipases (Fig. 4).

When the substrate concentration was varied at a fixed enzyme concentration (4.3 nM) at pH 8.5, maximum CDase activity was observed above 1.5 μM substrate, with an apparent K_m of 0.5 ± 0.1 μM.

The ion-dependence of the CDase reaction was tested at pH 8.5 with 2 μM substrate and 4.3 nM CDase. The enzyme displays optimal activity at Ca^{2+} concentrations above 7 μM, with a K_d value of 1.2 ± 0.4 μM. Ba^{2+} and Mg^{2+} can replace Ca^{2+} as cofactors for CDase and have K_d values of 8.5 ± 0.2 and 9.8 ± 0.3 μM, respectively. The maximum activity of CDase in the presence of Mg^{2+} is only 36% of that in the presence of optimal Ca^{2+} or Ba^{2+} concentrations. The effect of Zn^{2+} was measured at pH 6.5 because of the low solubility of the Zn^{2+} ion at higher pH values. CDase was as active with Zn^{2+} as with Ca^{2+} at pH 6.5. The K_d for the reaction in the presence of Zn^{2+} is 1.0 ± 0.1 μM.

The turnover of CDase (2.1 nM) with the substrate (2 μM) in 50 mM Tris–HCl, 1.6 mM Triton X-100, 4 mM $CaCl_2$, pH 8.5 was found to be 5.5 min^{-1} (specific activity of 78 nmol min^{-1} mg^{-1}) from a fluorescence calibration curve with free 10-pyrene decanoic acid in the same buffer. This value compares well with the turnover of ^{14}C-*N*-palmitoyl sphingosine of 10.9 min^{-1} (155 nmol min^{-1} mg^{-1}) (Nieuwenhuizen et al., to be published elsewhere).

The inhibiting potential of several ceramide and sphingosine analogues was tested at pH 8.5 with 2 μM substrate, 1.6 mM Triton X-100 and 4 mM $CaCl_2$.

The known inhibitor of human alkaline CDase D-*erythro*-MAPP (Bielawska et al., 1996) nor L-*erythro*-MAPP inhibit CDase from *P. aeruginosa* PA01 even at 20 μM (i.e. ten times the substrate concentration). *N*-oleoylethanolamine, an acid CDase inhibitor (Spinedi et al., 1999 and references therein), also did not affect the CDase activity at 20 μM. CDase activity was not affected by L(−)- or D(+)-norephenidrin at 20 μM. D/L-sphinganine, the racemic saturated sphingosine analogue, reduced the CDase activity to 17% at 20 μM. The aparent K_i for D/L-sphinganine was 3.3 ± 0.3 μM under the test conditions.

At 20 μM D-*erythro*-sphingosine and L-*erythro*-sphingosine reduced the CDase activity to 3 and 80%, respectively. The apparent K_i for D-*erythro*-sphingosine was 4.0 ± 0.4 μM under the test conditions. The apparent K_i for L-*erythro*-sphingosine was not determined.

CDase has a strong chiral preference for the binding of D-*erythro*-sphingosine compared with the L-enantiomer, as is reflected in the different inhibiting power of both enentiomers. It is expected that D-sphinganine, like D-*erythro*-sphingosine, is the most powerful inhibiting enantiomer. Since we tested the racemic sphinganine mixture and hence the concentration of the inhibiting species is lower, this could indicate that D-sphinganine is a stronger CDase inhibitor than D-*erythro*-sphingosine.

4. Discussion

The existing CDase assays make use of radioactive ceramide or a fluorescent ceramide analogue, and depend on a separation protocol to determine the ceramide hydrolysis (Misutake et al., 1997; Tani et al., 1999). Another assay to determine the ceramide concentration depends on the diglyceride kinase assay, which is questioned as a reliable assay (Hofmann, 1999; Perry and Hannun, 1999). In contrast, our novel CDase assay does not depend on a laborious separation step nor does it require the error-prone use of a second enzyme.

In the present study we describe the synthesis of a novel quenched fluorescent CDase substrate. The versatile synthesis route enables the production of different ceramide analogues with different chiral centers, depending on the chirality of starting compound **3** and the reaction conditions in step b (see Fig. 2) (Garner et al., 1988; Herold, 1988). We chose to synthesize the D-*erythro* form because its chirality is identical with that of natural ceramide.

Ceramide analogue **10** enables the real time measurement of CDase activity and is a valuable tool in the characterization of CDases. It allows for rapid testing of assay conditions and the effect of different cofactors much faster, and more reliably than with the existing CDase assays.

The ester bond between the 3,5-dinitrobenzoyl group and the sphingosine moiety is unstable at high pH, and may be susceptible to hydrolysis by esterases which may be present in more complex systems (i.e. intact cells). Therefore, we plan to synthesize ceramide analogues in which the quenching moiety is linked via a more stable ether or sulfonic ester linkage.

Our novel assay allows for the high-throughput screening of modulators of CDase activity with therapeutic potential.

The metabolism of macrophage phospholipids produce key mediators of inflammation and major second messengers that modulate inflammatory responses during sepsis. In a recent report (Lo et al., 1999) the effect of ceramide and sphingosine on LPS activated rabbit alveolar macrophages is described. The authors found that ceramide did not have any effect on TNF production or TNF mRNA levels with or without LPS activation of the macrophages, whereas sphingosine inhibited TNF production and TNF mRNA expression in LPS stimulated macrophages. The LPS induced NFκB activity was reduced by sphingosine. Thus sphingosine, in contrast to ceramide, down-regulates macrophage activation induced by LPS stimulation.

These data may indicate that the CDase product sphingosine helps the pathogenic *Pseudomonas* species to escape macrophages, and that CDase may be regarded as a virulence factor in combination with sphingomyelinase.

Since the *Pseudomonas* CDase is not inhibited by the known mammalian CDase inhibitors, D-*erythro* MAPP (Bielawska et al., 1996) or *N*-oleoyl ethanolamine (Spinedi et al., 1999), and because the bacterial enzyme is the only divalent cation-dependent CDase known to date, it is highly likely that bacterial CDase has a different enzymatic mechanism than its mammalian counterparts. This finding together with our assay offers the opportunity to screen for, and develop, specific inhibitors for bacterial CDase that do not interfere with the mammalian sphingolipid metabolism, and which may help to fight *Pseudomonas* infections.

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