Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 3159–3164

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SYNTHESIS AND APOPTOGENIC ACTIVITY OF FLUORINATED CERAMIDE AND DIHYDROCERAMIDE ANALOGUES

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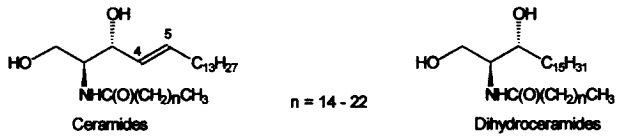
Received 16 July 1999; accepted 4 October 1999

Abstract: Short-chain 3-fluoro-(dihydro)ceramide analogues are synthesized from L-serine using diethylaminosulfur trifluoride (DAST) as fluorinating agent. The apoptogenic activity of these compounds was measured in three different cell lines and compared with their hydroxylated counterparts.

Introduction

Ceramides (Figure 1), generated from sphingomyeline, act as second messengers and play an important role in apoptosis.[1] Dihydroceramides, which lack the 4,5-*trans* carbon-carbon double bond, are biologically inactive.[2] Cytokines[3] (such as TNF-α, interleukine-1, γ-interferon), chemotherapeutic agents (e.g. daunorubicine[4]) and ionizing radiation[5] induce apoptosis by increasing the intracellular ceramide concentrations. In addition, exogenously administered short-chain, cell-permeable ceramides are able to induce apoptosis, thus mimicking such inducing effects. Reasoning that isosteric and isoelectronic substitution of a hydroxyl group by a fluorine atom is often associated with interesting bio-activities,[6] we have synthesized new series of fluorinated, cell-permeable (dihydro)ceramide analogues. The potential of these compounds to induce apoptosis in Molt cells, K-422 cells and peripheral blood lymphocytes was assessed by flow cytometry.

Figure 1



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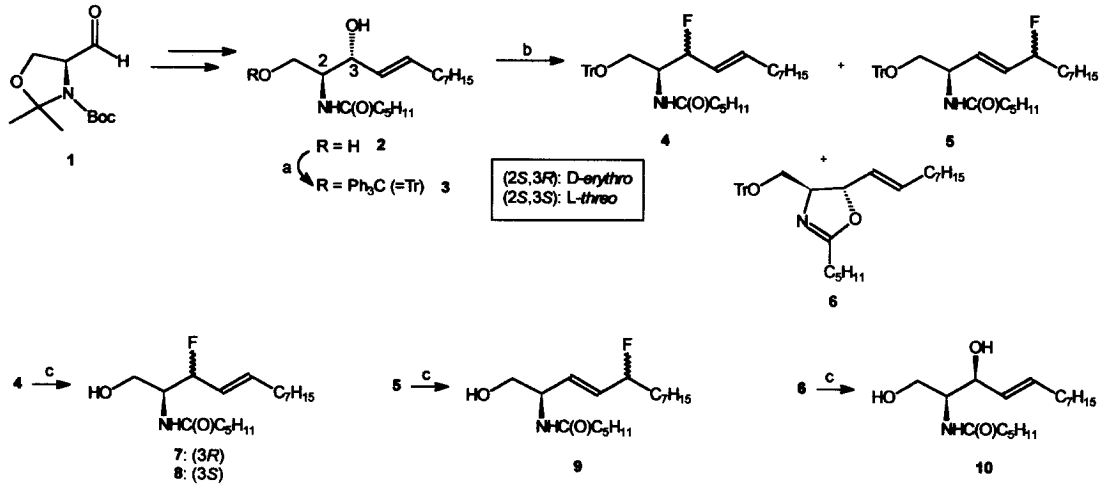
0960-894X/99/$ - see front matter
PII: S0960-894X(99)00553-3

Synthesis

We pursued a synthetic sequence leading to the D-*erythro* and L-*threo* fluorinated ceramide analogues **7** and **8** and to the L-*threo* fluorinated dihydroceramides **16**, **17** and **24**, based on L-serine as chiral pool molecule and diethylaminosulfur trifluoride (DAST) as fluorinating agent.[7] Modification of a previous strategy developed by Herold[8] accessed the D-*erythro*-ceramide analogue **2** (Scheme 1) with a shortened sphingoid backbone. Diastereoselective addition of the lithium salt of 1-nonyne to the Garner aldehyde **1** (prepared from L-serine according to a literature procedure[9]) in the presence of HMPA, followed by Birch-reduction and *N*-acylation, afforded the ceramide analogue **2**.[10]

Scheme 1



a: TrCl, pyridine, 100 °C, 90 %; b: DAST, CH_2Cl_2, -78 °C, 4: 28 %, 5: 57 %, 6: 14 %; c: Amberlyst 15, CH_3OH, 7 and 8: 50 %, 9: 65 %, 10: 20 %.

After protection of the primary hydroxyl group in **2** as a trityl ether (Scheme 1), **3** was treated with 1.5 equivalents of DAST in CH_2Cl_2 at –78 °C to give compounds **4**, **5** and **6** in a ratio of 2/4/1. NMR analysis showed that **4** and **5** each prevailed as epimeric mixtures, which could not be separated at this stage. Fluorides **5** are obviously formed by a SN_2'-type substitution of the hydroxyl group in **3**.[11] Based on the observation that D-*erythro*- and L-*threo*-epimers can be distinguished by the upfield resonance of *H*-C(3) in the ^{1}H-NMR spectrum of the *erythro* (3*R*) compound,[8] it was deduced that the *threo*-fluoride **4** was predominantly formed (*threo*/*erythro* ≈ 2.5/1).[12]

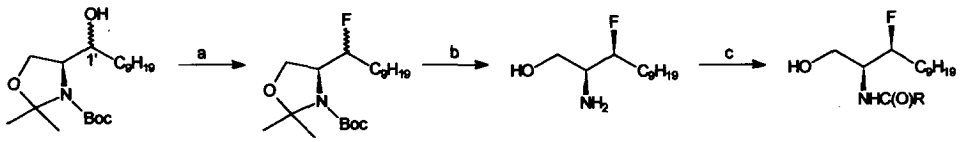
Deprotection of compounds **4** and **5** using Amberlyst 15 gave epimers **7** and **8**[13] and an inseparable epimeric mixture (ratio ≈ 1/1) of **9** respectively. Hydrolysis of the oxazoline derivative **6** furnished the L-*threo*-ceramide **10**, indicating that inversion of the configuration at C-3 had occurred during cyclization from **3**. Thus, both fluorinated ceramides **7** and **8** were obtained in a pure form for the first time.

Surprisingly, reaction of DAST with the saturated congener of **2**[14] (after tritylation of the primary hydroxyl group) afforded only the oxazoline derivative in a quantitative yield, while no trace of fluorinated compounds

could be detected. Therefore, it was decided to introduce the fluorine atom at a stage where this neighbouring group (imidol) participation was obviated. Fluorination of the *erythro*-alcohol **11** (synthesized from the Garner aldehyde **1**[14]) using DAST led to the *threo*-fluoride **13** (SN_2-mechanism) as a single product (Scheme 2). Cleavage of the oxazolidine and deprotection of the *tert*-Boc-group with trifluoroacetic acid yielded the L-*threo* sphinganine (dihydrosphingosine) analogue **15**. *N*-Acylation of **15** using acetyl chloride and hexanoyl chloride gave rise to the 3-fluorinated L-*threo* dihydroceramides **16** and **17**, respectively.[15]

Fluorination of the *threo*-alcohol **12** with DAST afforded the *erythro*-compound **14**, albeit in very low and impractical yield (5 %). The low reactivity of *threo*-alcohol **12** is most probably due to an intramolecular hydrogen bonding between the urethane carbonyl and the secondary hydroxyl group.[16]

Scheme 2

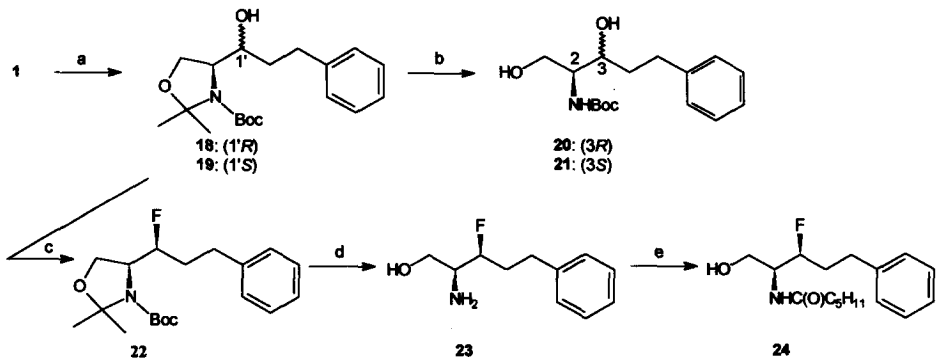


11: (1'*R*) **12**: (1'*S*) **13**: (1'*S*) **14**: (1'*R*) **15** **16**: R = CH_3 **17**: R = C_5H_{11}

a: DAST, CH_2Cl_2, -78 °C, **13**: 81 %, **14**: 5 %; b: TFA/H_2O 3:1, rt, 82 %; c: RC(O)Cl, THF, 50 % aq. NaOAc, rt, **16**: 83 %, **17**: 88 %.

The synthesis of a dihydroceramide analogue containing an aromatic residue started with the Grignard addition of phenethylmagnesium chloride to the Garner aldehyde **1**, affording a mixture of the epimeric alcohols **18** and **19** (Scheme 3).

Scheme 3



18: (1'*R*) **19**: (1'*S*) **20**: (3*R*) **21**: (3*S*) **22** **23** **24**

a: $C_6H_5(CH_2)_2MgCl$, THF, -78 °C to rt, 73 %; b: TosOH, CH_3OH, rt, 64 %; c: DAST, CH_2Cl_2, -78 °C, 26 %; d: TFA/H_2O 3:1, rt, 76 %; e: $C_5H_{11}C(O)Cl$, THF, 50 % aq. NaOAc, rt, 77 %.

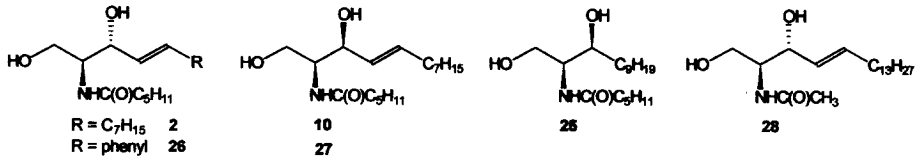
Separation was not feasible at this stage and overlap of the ^{1}H-NMR signals led to uncertainty in the determination of the epimeric composition. Cleavage of the oxazolidine with TosOH afforded the *N-tert*-Boc-protected sphinganine analogues **20** and **21**. The *erythro*/*threo* ratio of 1:2 was obtained by integration of the *NH*-signals (δ = 5.35 ppm (*erythro*); δ = 5.25 ppm (*threo*)). Preferential formation of the *threo*-alcohol **19** can

be rationalized in terms of a chelated Cram model, in which the Grignard reagent functions as a chelating Lewis acid.[17]

The mixture of epimeric alcohols **18** and **19** was used further in the fluorination reaction. Surprisingly, only one epimer was formed, as judged from NMR. Apparently, the *erythro*-alcohol **18** underwent substitution at a much faster rate than the *threo*-alcohol **19**, yielding *threo*-fluoride **22**. Deprotection gave **23**, which was acylated with hexanoyl chloride to yield the 3-fluoro-dihydroceramide **24**.[18]

The synthesis of the hydroxylated ceramide analogues **2**, **10**, **26** and **27** has been described previously,[10] whereas the synthesis of the hydroxylated dihydroceramide analogue **25** will be reported in due course[14] (Figure 2).

Figure 2



Biological Evaluation and Discussion

To study the influence of the isosteric substitution of a hydroxyl group for a fluorine atom, the apoptogenic activity of the fluorinated analogues was compared with that of their hydroxylated counterparts **2**, **10** and **25**.

Two cell lines (Molt cells and K-422 cells) and peripheral blood lymphocytes, isolated from two healthy volunteers, were treated with 30 μM of a (dihydro)ceramide analogue. Compound **28** (a well known inducer of apoptosis[2]) was used as a positive control. After an incubation period of 24 h, the number of apoptotic cells was measured by flow cytometry, using fluoresceine-isothiocyanate labeled Annexin V and 7-amino-actinomycin D as vital dye (Table 1).[19]

As can be seen from Table 1, shortening of the sphingoid base backbone from C_{18} to C_{12} (compound **2**) led to a drastic decrease in apoptogenic activity, which shows the significance of having a sufficient number of carbon atoms in the sphingoid base backbone. Isosteric replacement of the secondary hydroxylgroup of the short-chain ceramide analogues **2** and **10** for a fluorine atom yielded compounds **7** and **8**, respectively, which have an increased apoptogenic activity in the two cell lines and peripheral blood lymphocytes. Noteworthy is that L-*threo* ceramide **10** is more apoptogenic than its D-*erythro* epimer **2** in K-422 cells and peripheral blood lymphocytes. By introducing a 3-fluoro substituent, this difference between *erythro* (**7**) and *threo* (**8**) compounds is less pronounced.

It is commonly believed that only ceramides, containing a 4,5-*trans* carbon-carbon double bond, induce apoptosis.[2] In sharp contrast, we found that introduction of a fluorine-substituent (compound **17**) for a hydroxyl group into the dihydroceramide-analogue **25** dramatically increased the apoptogenic activity in Molt cells and K-422 cells. Conversely, in peripheral blood lymphocytes, a decrease in the number of apoptotic cells was

noted. To further explore the structure-activity relationship related to dihydroceramide **17**, we shortened the *N*-acyl chain from C_6 to C_2 (compound **16**). As the percentage of apoptotic cells decreased, the significance of *N*-acyl chain length was highlighted. To probe the prerequisite of the sphingoid base backbone for exhibiting apoptogenic activity, we synthesized the analogue **24**, carrying an aromatic residue. Loss of the apoptogenic potential, which was also observed for compounds **26** and **27** (data not shown), confirmed that apoptosis is triggered by the presence of an alkyl hydrocarbon chain.

Table 1: Percentage of apoptotic Molt cells, K-422 cells and peripheral blood lymphocytes (PBL), 24 h after treatment with 30 μM of a (dihydro)ceramide analogue.[a]

COMPOUND	MOLT CELLS	K-422 CELLS	PBL
2	3.5 ± 3.5	12.5 ± 2.7	12.0 ± 12.0
7	11.0 ± 3.1	34.7 ± 7.4	40.0 ± 4.3
8	13.7 ± 5.9	51.2 ± 8.5	47.7 ± 12.9
10	1.0 ± 0.6	32.7 ± 3.9	33.5 ± 12.5
16	3.0 ± 2.5	7.3 ± 2.4	3.0 ± 0.0
17	31.6 ± 6.1	32.0 ± 7.9	46.0 ± 12.5
24	2.0 ± 0.0	2.5 ± 2.5	0.5 ± 0.5
25	9.0 ± 3.5	15.0 ± 6.5	57.0 ± 10.2
28	51.0 ± 9.2	27.7 ± 6.5	48.8 ± 12.5

[a] Each value represents the mean ± SEM of at least two independent experiments.

Conclusion

We established a scheme for the synthesis of new, short-chain 3-fluoro-ceramides (D-*erythro*, as well as L-*threo* epimers) and L-*threo* 3-fluoro-dihydroceramides. The Garner aldehyde **1** served as a suitable starting material, while DAST proved to be an efficient fluorinating agent.

The failure of cells to undergo apoptotic cell death might be involved in the pathogenesis of cancer.[20] As shown in this paper, we were able to modify (dihydro)ceramides in such way that an increased apoptogenic activity in K-422 cancer cells was obtained. Unfortunately, at the same time, the apoptogenic potential of the fluorinated compounds also increased in Molt cells and peripheral blood lymphocytes. Further exploring the structure-activity relationship of these compounds could possibly lead to the development of (dihydro)ceramides as a new class of anti-cancer agents, that selectively induce apoptosis in tumor cells.

Acknowledgments

This work was supported by doctoral fellowships offered by the Special Research Fund of the University of Gent (BOZF 01101497, to S. D. J.) and the Flemish Institute for the Promotion of Scientific-Technological

Research in Industry (IWT, Brussels, Belgium, to I. V. O.). We thank Prof. P. Van Veldhoven for helpful discussions.

References and Notes

(1) Mathias, S.; Peña, L.A. & Kolesnick, R.N. *Biochem. J.* **1998**, *335*, 465-480.
(2) Bielawska, A.; Crane, H.M.; Liotta, D.; Obeid, L.M. & Hannun, Y.A. *J. Biol. Chem.* **1993**, *268*, 26226-26232.
(3) Kolesnick, R. & Golde, D.W. *Cell* **1994**, *77*, 325-328.
(4) Bose, R.; Verheij, M.; Haimovitz-Friedman, A.; Scotto, K.; Fuks, Z. & Kolesnick, R. *Cell* **1995**, *82*, 405-414.
(5) Haimovitz-Friedman, A.; Kan, C.C.; Ehleiter, D.; Persaud, R.S.; McLoughin, M.; Fuks, Z. & Kolesnick, R.N. *J. Exp. Med.* **1994**, *180*, 525-535.
(6) Welch, J.T. *Tetrahedron* **1987**, *43*, 3123-3197.
(7) Middleton, W.J. *J. Org. Chem.* **1975**, *40*, 574-578.
(8) Herold, P. *Helv. Chim. Acta* **1988**, *77*, 354-361.
(9) Garner, P. & Park, J.M. *J. Org. Chem.* **1987**, *52*, 2361-2364.
(10) Van Overmeire, I.; Boldin, S.A.; Dumont, F.; Van Calenbergh, S.; Slegers, G.; De Keukeleire, D.; Futerman, A.H. & Herdewijn, P. *J. Med. Chem.* **1999**, *42*, 2697-2705.
(11) Kozikowski, A.P. & Wu, J.P. *Tetrahedron Lett.* **1990**, *31*, 4309-4312. These authors observed a similar allylic rearrangement in the fluorination of sphingosine, using 2-chloro-1,1,2-trifluorotriethylamine.
(12) The diastereoselectivity was determined from the HPLC chromatogram of **4** (silica, EtOAc/hexane 1/9).
(13) Compounds **7** and **8** were obtained in pure form after preparative HPLC on silica (CH_3OH/CH_2Cl_2, 1:99). ^{1}H-NMR data of **7** and **8** (500 MHz, $CDCl_3$): (2*S*,3*R*,4*E*)-2-Hexanoylamino-3-fluoro-4-dodecen-1-ol (**7**): δ 0.86-0.93 (6 H, m, 2 CH_3), 1.20-1.43 (14 H, m, 7 CH_2), 1.60-1.67 (2 H, m, CH_2), 2.04-2.11 (2 H, m, 2H-C(6)), 2.21 (2 H, dt, J = 7.7 Hz and 2 Hz, C(O)CH_2), 3.73 (1 H, dd, J = 11.5 Hz and 3.5 Hz, H_a-C(1)), 3.92 (1 H, dd, J = 11.2 Hz and 4.1 Hz, H_b-C(1)), 4.07-4.17 (1 H, m, $^3J_{H,F}$ = 21 Hz, H-C(2)), 4.97 (0.5 H, t, J = 5.9 Hz, H-C(3)), 5.06 (0.5 H, t, J = 6 Hz, $^2J_{H,F}$ = 48.2 Hz, H-C(3)), 5.51-5.60 (1 H, m, H-C(5)), 5.84-5.92 (1 H, m, H-C(4)), 6.00 (1 H, d, J = 8 Hz, NH) ppm; (2*S*,3*S*,4*E*)-2-Hexanoylamino-3-fluoro-4-dodecen-1-ol (**8**): δ 0.87-0.97 (6 H, m, 2 CH_3), 1.20-1.45 (14 H, m, 7 CH_2), 1.60-1.67 (2 H, m, CH_2), 2.02-2.11 (2 H, m, 2H-C(6)), 2.23 (2 H, t, J = 7.5 Hz, C(O)CH_2), 3.70-3.80 (2 H, m, 2H-C(1)), 4.04-4.09 (0.5 H, m, H-C(2)), 4.10-4.15 (0.5 H, m, $^3J_{H,F}$ = 23.8 Hz, H-C(2)), 5.07 (0.5 H, dd, J = 6.5 Hz and 3.5 Hz, H-C(3)), 5.16 (0.5 H, dd, J = 6.5 Hz and 3.5 Hz, $^2J_{H,F}$ = 47.7 Hz, H-C(3)), 5.46-5.55 (1 H, m, H-C(5)), 5.80-5.95 (2 H, m, NH and H-C(4)) ppm.
(14) De Jonghe, S.; Van Overmeire, I.; Poulton, S.; Hendrix, C.; Busson, R.; Van Calenbergh, S.; De Keukeleire, D.; Spiegel, S. & Herdewijn, P. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3175-3180.
(15) ^{1}H-NMR data of **17** (500 MHz, $CDCl_3$) (2*S*,3*S*)-2-Hexanoylamino-3-fluoro-dodecan-1-ol: δ 0.85-0.91 (6 H, m, 2 CH_3), 1.25-1.35 (16 H, m, 8 CH_2), 1.55-1.65 (6 H, m, 3 CH_2), 2.15 (1 H, br s, OH), 2.25 (2 H, t, J = 7.5 Hz, C(O)CH_2), 3.72 (1 H, dd, J = 11.5 Hz and 3 Hz, H_a-C(1)), 3.92 (1 H, dd, J = 11.7 Hz and 4 Hz, H_b-C(1)), 4.02-4.12 (1 H, m, $^3J_{H,F}$ = 22 Hz, H-C(2)), 4.51-4.56 (0.5 H, m, H-C(3)), 4.61-4.66 (0.5 H, m, $^2J_{H,F}$ = 49.4 Hz, H-C(3)), 6.18 (1 H, d, J = 8 Hz, NH) ppm.
(16) Williams, L.; Zhang, Z.; Shao, F.; Carroll, P.J. & Joullié, M.M. *Tetrahedron* **1996**, *52*, 11673-11694.
(17) Villard, R.; Fotiadu, F. & Buono, G. *Tetrahedron: Asymm.* **1998**, *9*, 607-611.
(18) ^{1}H-NMR data of **24** (500 MHz, DMSO-d_6) (2*S*,3*S*)-2-Hexanoylamino-3-fluoro-5-phenyl-pentan-1-ol: δ 0.83 (3 H, t, J = 6.9 Hz, CH_3), 1.18-1.30 (4 H, m, 2 CH_2), 1.43-1.53 (2 H, m, C(O)CH_2CH_2), 1.80-1.95 (2 H, m, 2H-C(4)), 2.05-2.13 (2 H, m, C(O)CH_2), 2.58-2.65 (1 H, m, H_a-C(5)), 2.72-2.80 (1 H, m, H_b-C(5)), 3.43-3.51 (2 H, m, 2H-C(1)), 3.95-4.04 (1 H, m, H-C(2)), 4.43-4.48 (0.5 H, m, H-C(3)), 4.53-4.58 (0.5 H, m, $^2J_{H,F}$ = 48.4 Hz, H-C(3)), 4.75 (1 H, t, J = 5.5 Hz, OH), 7.18 (3 H, d, J = 7 Hz, arom H), 7.27 (2 H, t, J = 7.6 Hz, arom H), 7.70 (1 H, d, J = 9 Hz, NH) ppm.
(19) Koopman, G.; Reutelingsperger, C.P.M.; Kuijten, G.A.M.; Keehnen, R.M.J.; Pals, S.T. & van Oers, M.H.J. *Blood* **1994**, *84*, 1415-1420.
(20) Thompson, C.B. *Science* **1995**, *267*, 1456-1462.