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Versatile Synthesis of Phenoxydiazirine-Based Fatty Acid Analogues and Photoreactive Galactosylceramide

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Abstract—A versatile synthesis of diazirine-based photoreactive fatty acid analogues is reported. The key step is phenoxy alkylation of diazirine with halo alkyl acid esters. The conditions described will be acceptable for the synthesis of various alkyl-length derivatives. The fatty acid derivatives are acceptors for reverse reactions of sphingolipid ceramide *N*-deacylase (SCDase), which catalyzes the condensation of psychosine and fatty acids to form photoreactive galactosylceramide. The photoreactive galactosylceramide can also be prepared with chemical synthesis, condensation of psychosine and fatty acid succinimidyl ester, and is recognized with anti-GarCer antibody both before and after irradiation.

Photoaffinity labeling is a well-established method to elucidate ligand–biomolecule interactions.[1–5] Many investigators consider carbene precursors, especially 3-phenyl-3-trifluoromethyl diazirine, as the photoreactive groups of choice, because of the specific character of the photolabeling reaction.[4,6] Photoaffinity labeling with fatty acid analogues will reveal hydrophobic interactions between biomolecule and lipids,[7] but the complicated synthesis of the 3-phenyl-3-trifluoromethyl diazirinyl three-membered ring has resulted in fewer applications of it in biomolecular studies than those of other photophors (azide and benzophenone). The synthesis of photoreactive fatty acid analogues presents a similar problem. A few routes for synthesis of diazirine-based fatty acid analogues were reported,[8,9] but the fatty acid equivalents should be introduced before construction of diazirinyl three-membered ring. We have been elucidating approaches for the post-functionalization of the 3-phenyl-3-trifluoromethyl diazirinyl photophor.[10–13] In this paper, we describe a versatile and easy approach for synthesis of diazirine-based fatty acid analogues by phenoxy alkylation, introduction to sphingolipid, and the biological properties of the photoreactive compounds.

To archive versatile synthesis of photoreactive fatty acids, *m*-methoxy diazirine **1**,[10] which is available for large scale preparation (over 0.5 mol), was demethylated with BBr_3 to afford **2**. The phenolic compound was subjected to the phenoxy alkylation condition[11] with methyl 11-bromoundecanoate. The reaction proceeded smoothly at 60 °C to afford phenoxydiazirinyl fatty acid methyl ester **3** with yield of 70%. The reaction with ethyl 5-bromovalerate afforded the shorter carbon-length fatty-acid derivative **6** under the same condition. These esters were easily hydrolyzed to afford the corresponding acids **4** and **7**. These synthetic routes will be more convenient to synthesis variable carbon length diazirine-based photoreactive fatty acid analogues than previous routes. The carboxylic acid **4** was converted to succinimidyl ester **5** with *N*-hydroxysuccinimide and water-soluble carbodiimide in CH_3CN in moderate yield (86%). The compound **5** reacted with psychosine **8**, lyso-galactosylceramide, in the anhydrous triethylamine and DMF to afford photoreactive galactosylceramide analogues **9** with 74% yield (Fig. 1).[14] The synthetic routes will enable us to condense variable length photoreactive fatty acid and various *N*-deacylated sphingolipids (sphingosine, lysosphingomyelin, lysoganglioside, etc.) to synthesis of various photoreactive sphingolipid derivatives.

It is recently reported that sphingolipid ceramide *N*-deacylase (SCDase) catalyze the hydrolytic break down

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of *N*-acyl linkage between fatty acids and sphingosine bases, and its reverse reaction, condensation of sphingolipid and fatty acid, under different detergent and pH conditions.[15–18] To elucidate the biological properties of phenoxy diazirinyl fatty acid, the compound **4** was subjected to enzymatic condensation of psychosine **8** with reverse reaction of SCDase. The same amount of psychosine and photoreactive fatty acid **4** (10 nmol) were incubated with SCDase (10 μU) at 37 °C for 48 h. One fifth of the fatty acid was converted to photoreactive galactosylceramide **9**. The results indicated that the enzyme recognized phenoxy diazirinyl fatty acid **4** as substrate. It is first time that the enzyme utilized the photoreactive fatty acid derivative to produce sphingolipids. The compounds **4** will be useful to elucidate hydrophobic interaction between fatty acid and those utilized enzymes.

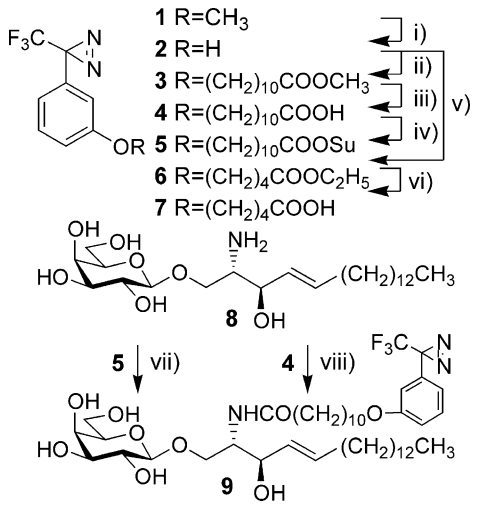

Figure 1. Synthesis of diazirine based fatty acids and galactosylceramide: (i) BBr_3, CH_2Cl_2, 0 °C, 85%; (ii) $Br(CH_2)_{10}COOCH_3$, $(n\text{-}Bu)_4NI$, DMF, 60 °C, 19 h, 70%; (iii) NaOH, CH_3OH, rt, 2 h, 90%; (iv) HOSu, EDC–HCl, CH_3CN, rt, 3 h, 86%; (v) $Br(CH_2)_4COOC_2H_5$, $(n\text{-}Bu)_4NI$, DMF, 60 °C, 12 h, 60%; (vi) NaOH, C_2H_5OH, rt, 2 h, 95%; (vii) DMF, TEA, rt, 10 h, 74%; (viii) SCDase, 0.1% Triton X-100, phosphate buffer pH 7.0, 37 °C, 48 h, 20%.

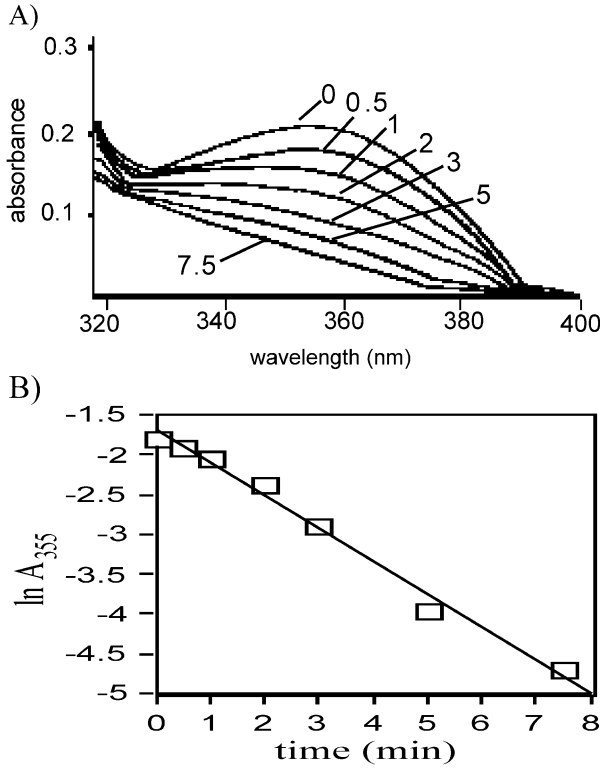

Figure 2. Photolysis of a synthetic galactosylceramide analogue **7**: (A) UV spectra of the photolysis reaction mixture at times (in min) indicated with numbers; (B) the decay of the absorbance at 355 nm as a function of time of photolysis in a semi-log representation.

The photoreactive galactosylceramide analogue **9** was applied to irradiation experiments to ensure photoactivation abilities. The compound **9** in methanol/chloroform = 1:2 (0.7 mM) was placed at distance 2 cm from black light lamp (15 W) at 0 °C. The spectrogram at each interval was measured. The diazirine specific broad adsorption at 355 nm was smoothly decreased during the irradiation (Fig. 2A). The half-life of the compound in this condition was calculated as 1.7 min from semi-log plot (Fig. 2B). Photoreactive fatty acid derivatives **4** also smoothly decomposed in the same condition ($t_{1/2}$ was calculated as 1.2 min in $CHCl_3$).

Several sphingolipids have antigen activities used to detect localization of these compounds in the cell. Anti-galactosylceramide antibody is of great interest due to its usefulness as oligodendroglial markers. We performed an antigenicity test of photoreactive galactosylceramide **9** against rabbit anti-galactosylceramide antibody.[20] The synthetic galactosylceramide analogues, one is un-irradiated and the other one is irradiated for 30 min, and enzymatic synthesis reaction mixture (Fig. 3 lanes 1, 2 and 3, respectively) were developed on TLC. The developed sphingolipids were transferred to PVDF membrane (far-eastern blotting) in a manner identical with literature.[19] The membrane was subjected to

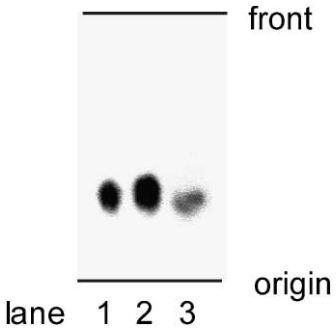

Figure 3. Immunodetection of photoreactive galactosylceramide analogues, blotted to PVDF membrane, by rabbit anti-galactosylceramide antibody. TLC plate was developed with $CHCl_3$/methanol = 6:1, then transferred to PVDF membrane in a manner identical with literature. The membrane was blocked for 1 h in 5% skim milk in 0.1% Tween 20, phosphate-buffered saline pH 7.4 (T-PBS), washed twice with T-PBS for 5 min and immersed in rabbit anti-galactosylceramide antibody (1:50 dilution of the manufacturer's solution) for 2 h. After washing six times with T-PBS for 5 min, the membrane was immersed in goat anti-rabbit IgG peroxidase conjugate (1:25,000 dilution of the manufacturer's solution) for 1 h, then washed with T-PBS six times. The immuno-treated membrane was subjected to chemiluminescence detection and exposed to film for 0.5 min. Lane 1: chemically synthesized compounds **9**; lane 2: photolyzed compound **9** in $CHCl_3$/methanol = 2:1; lane 3: enzymatic reaction mixture of psychosine and fatty acid **4** with SCDase.

immunodetection with rabbit anti-galactosylceramide antibody and goat anti-rabbit IgG peroxidase conjugate, then treated with chemiluminescence reagent to detect galactosylceramide analogues. Both un-irradiated and irradiated diazirine based galactosylceramide analogue was recognized by the rabbit anti-galactosylceramide antibody. It is first time photoactivated galactosylceramide was recognized by anti-galactosylceramide antibody. Furthermore, the enzymatic reaction mixture of fatty acid **4** and psychosine afforded chemiluminescence signal. We subjected psychosine, lesser antigen for the antibody, for far-eastern blotting and immunodetection, but no chemiluminescence signals was afforded. It can be presumed that psychosine was removed from PVDF membrane during the immunodetection process. It is considered that lipophilicity of fatty acid moiety should be important in this manner. The results indicate that small amounts of enzymatic reaction are detectable with this method and without a radioisotope precursor.

Specific tags should be needed to manipulate photolabeled components from the labeled mixture. We have previously reported that combination of photoaffinity labeling and avidin–biotin technology (photoaffinity biotinylation) is a useful non-radioisotopic method to detect and retrieve the labeled components from a complicated mixture.[10,13,21–25] Here we show that blotted and irradiated photoreactive galacytosylceramide was recognized by a specific antibody. This indicates that the technique of antigen–antibody interaction for label reagent itself will also be useful to apply to isolation of labeled components. The galactosylceramide is closely related to Krebbe's disease[26] and metachromatic leukodystrophy.[27] The diazirine-based photoreactive galactosylceramide should be useful for investigating the molecular mechanisms in biological functions.

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References and Notes

1. Bayley, H. In *Laboratory Techniques in Biochemistry and Molecular Biology: Photogenerated Reagents in Biochemistry and Molecular Biology*; Work, T. S., Burdon, R. H., Eds.; Elsevier: Amsterdam, 1983; p 25.
2. Brunner, J. *Annu. Rev. Biochem.* **1993**, *62*, 483.
3. Kotzyba-Hilbert, F.; Kapfer, I.; Goeldner, M. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1296.
4. Hatanaka, Y.; Nakayama, H.; Kanaoka, Y. *Rev. Heteroatom. Chem.* **1996**, *14*, 213.
5. Dorm, G.; Prestwich, G. D. *Trends Biotechnol.* **2000**, *18*, 64.
6. Gillingham, A. K.; Koumanov, F.; Hashimoto, M.; Holman, G. D. Detection and Analysis of Glucose Transporters Using Photolabelling Techniques. In *Membrane Transport: A Practical Approach*; Baldwin S. A. Ed.; Oxford University Press: Oxford, 2000; p 193.
7. Knoll, F.; Kolter, T.; Sandohoff, K. *Methods Enzymol.* **1999**, *311*, 568.
8. Weber, T.; Brunner, J. *J. Am. Chem. Soc.* **1995**, *117*, 3084.
9. Li, G.; Bittman, R. *Tetrahedron Lett.* **2000**, *41*, 6737.
10. Hatanaka, Y.; Hashimoto, M.; Kurihara, H.; Nakayama, H.; Kanaoka, Y. *J. Org. Chem.* **1994**, *59*, 383.
11. Hatanaka, Y.; Hashimoto, M.; Kanaoka, Y. *Bioorg. Med. Chem.* **1994**, *2*, 1367.
12. Hashimoto, M.; Kanaoka, Y.; Hatanaka, Y. *Heterocycles* **1997**, *46*, 119.
13. Hashimoto, M.; Hatanaka, Y.; Nabeta, K. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2481.
14. Compound **3**: EI-MS *m*/*z* 372 (M^+-N_2); ^{1}H NMR ($CDCl_3$) δ 7.43 (1H, dd, *J* = 8.6, 7.6 Hz), 7.07 (1H, d, *J* = 8.6 Hz), 6.90 (1H, d, *J* = 7.6 Hz), 6.82 (1H, s), 4.08 (2H, t, *J* = 6.4 Hz), 3.81 (3H, s), 2.45 (2H, t, *J* = 7.5 Hz), 1.90 (2H, m), 1.78 (2H, m), 1.72–1.45 (12H, m).
Compound **4**: EI-MS *m*/*z* 358 (M^+-N_2); ^{1}H NMR ($CDCl_3$) δ 7.19 (1H, dd, *J* = 8.3, 7.6 Hz), 6.84 (1H, d, *J* = 8.3 Hz), 6.67 (1H, d, *J* = 7.6 Hz), 6.59 (1H, s), 3.85 (2H, t, *J* = 6.4 Hz), 2.27 (2H, t, *J* = 7.4 Hz), 1.69 (2H, m), 1.56 (2H, m), 1.36–1.23 (12H, m).
Compound **5**: EI-MS *m*/*z* 456 (M^+-N_2); ^{1}H NMR ($CDCl_3$) δ 7.21 (1H, dd, *J* = 8.2, 7.9 Hz), 6.86 (1H, d, *J* = 8.2 Hz), 6.68 (1H, d, *J* = 7.9 Hz), 6.61 (1H, s), 3.87 (2H, t, *J* = 6.4 Hz), 2.77 (4H, s), 2.54 (2H, t, *J* = 7.4 Hz), 1.68 (4H, m), 1.42–1.25 (12H, m).
Compound **9**: FAB-MS *m*/*z* 830 ($[M+H]^+$); ^{1}H NMR (CD_3OD) δ 7.40 (1H, dd, *J* = 8.3, 7.9 Hz), 7.06 (1H, d, *J* = 8.3 Hz), 6.82 (1H, d, *J* = 7.9 Hz), 6.72 (1H, s), 5.73 (1H, m), 5.48 (1H, m), 4.26 (1H, t, *J* = 7.3 Hz), 4.00 (2H, t, *J* = 6.4 Hz), 3.90–3.30 (10H, m), 2.21 (2H, m), 2.06 (2H, m), 1.84 (2H, m), 1.62–1.31 (12H, m), 0.93 (3H, m).
15. Ito, M.; Kurita, T.; Kita, K. *J. Biol. Chem.* **1995**, *270*, 24370.
16. Mitsutake, S.; Kita, K.; Okino, N.; Ito, M. *Anal. Biochem.* **1997**, *247*, 52.
17. Ito, M.; Mitsutake, S.; Tani, M.; Kita, K. *Methods Enzymol.* **1999**, *311*, 682.
18. Kita, K.; Kurita, T.; Ito, M. *Eur. J. Biochem.* **2001**, *268*, 592.
19. Benjamins, J. A.; Callahan, R. E.; Montgomery, I. N.; Studzinski, D. M.; Dyer, C. A. *J. Neuroimmunol.* **1987**, *14*, 325.
20. Ishikawa, D.; Taki, T. *Methods Enzymol.* **2000**, *312*, 145.
21. Hatanaka, Y.; Hashimoto, M.; Nishihara, S.; Narimatsu, H.; Kanaoka, Y. *Carbohydr. Res.* **1996**, *294*, 95.
22. Hatanaka, Y.; Hashimoto, M.; Kanaoka, Y. *J. Am. Chem. Soc.* **1998**, *120*, 453.
23. Hashimoto, M.; Hatanaka, Y. *Chem. Pharm. Bull.* **1999**, *47*, 667.
24. Hashimoto, M.; Yang, J.; Holman, G. D. *ChemBioChem.* **2001**, *2*, 52.
25. Hashimoto, M.; Hatanaka, Y.; Yang, J.; Dhesi, J.; Holman, G. D. *Carbohydr. Res.* **2001**, *331*, 119.
26. Wenger, D. A.; Rafi, M. A.; Luzi, P.; Datto, J.; Costantino-Ceccarini, E. *Mol. Genet. Metab.* **2000**, *70*, 1.
27. Gieselmann, V.; Polten, A.; Kreysing, J.; von Figura, K. *J. Inherit. Metab. Dis.* **1994**, *17*, 500.