Synthetic Studies on Glycosphingolipids from Protostomia Phyla: Synthesis of Amphoteric Glycolipid Analogues Containing a Phosphocholine Residue from the Earthworm *Pheretima hilgendorfi*

Noriyasu HADA,[a] Koji SATO,[a] Jun-ichiro SAKUSHIMA,[b] Yukihiro GODA,[b] Mutsumi SUGITA,[c] and Tadahiro TAKEDA*,[a]

Kyoritsu College of Pharmacy,[a] 1–5–30 Shibakoen, Minato-ku, Tokyo 105–8512, Japan, National Institute of Health Sciences,[b] 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan, and Department of Chemistry, Faculty of Liberal Arts and Education, Shiga University,[c] 2–5–1 Hiratsu, Otsu, Shiga 520–0862, Japan.
Received July 26, 2001; accepted August 29, 2001

Two kinds of amphoteric glycosphingolipid analogues from the earthworm *Pheretima hilgendorfi* were synthesized as follows: The key reaction is a coupling of a phosphocholine group at the position C-6 of 1 and 6 which was attempted using 2-chloro-2-oxo-1,3,2-dioxaphospholane, followed by reaction of the resulting cyclic phosphate intermediate with anhydrous trimethylamine to give 2 and 7. Subsequent debenzylation afforded target compounds (3, 8). Their ability to inhibit the histamine release *in vitro* was examined.

Key words amphoteric glycosphingolipid; *Pheretima hilgendorfi*; chemical synthesis; phosphocholine

We have been interested in structure–activity relationships of glycosphingolipids derived from invertebrate animal species and have synthesized oligosaccharides from various protostomia phyla.[1)] Sugita *et al.*[2)] found and characterized a new neogala series of glycosphingolipids, of which core structure was a β-D-Gal*p*-(1→6)-β-D-Gal*p*- containing a mannose, glucose and phosphocholine residue from the earthworm *Pheretima* (*P.*) *hilgendorfi.* In the previous paper[1e)] we reported the synthesis of neogala series glycosphingolipids containing a mannose residue from *P. hilgendorfi.* In the present study, we attempted the synthesis of the novel neogala series of glycosphingolipids containing the phosphocholine residue. These compounds have a phosphocholine group at the C-6 position of the galactose moiety to form a zwitterionic structure A and B (Fig. 1). Furthermore Noda and his co-workers also isolated[3)] similar compounds and examined the structure–bioactivity relationships about fruiting-inducing effect.[4)] As part of our program of oligosaccharide synthesis, the preparation of neogala series containing a phosphocholine group at the C-6 position of the galactose moiety, from *P. hilgendorfi*, became of interest. Toward that end, the key reaction is a coupling of a phosphocholine group at the C-6 position of the galactose moiety. Concerning that, another novel phosphocholine-containing glycosphingolipids called GGPL-I and GGPL-III were found from the main cell membrane lipid components of *Mycoplasma fermentans* and these compounds were synthesized by Kobayashi and his coworkers.[5)] They used a phosphorodiamidite method at the C-6 position of glucose unit, *p*-nitrophenyl (*p*NP) 6-*O*-phosphocholine α-D-glucopyranoside. Up to the present, coupling incorporation of phosphocholine was performed as follows: A) phosphorylation with 2-bromoethyl phosphoryl dichloride and trimethylamine[6)]; B) a phosphorodiamidite method[5)]; C) phosphorylation with 2-chloro-2-oxo-1,3,2-dioxaphospholane[7)] in place of 2-bromoethyl phosphoryl dichloride. However, for the phosphorylation of carbohydrate hydroxy group, either A) or B)[5b,6b)] was used. In the present study, we investigated mono- and disaccharides phosphorylation by means of method C) using 2-chloro-2-oxo-1,3,2-dioxaphospholane and trimethylamine and attempted the synthesis of these novel amphoteric glycosphingolipid analogues containing fatty alkyl residue in place of ceramide. Furthermore, final compounds were examined for their ability to inhibit the histamine release *in vitro.*

Results and Discussion

Synthesis of Monosaccharide Containing Phosphocholine Octyl 2,3,4-tri-*O*-benzyl-β-D-galactopyranoside (**1**)[8)] was chosen as an acceptor prepared from octyl β-D-galactopyranoside. Introduction of a phosphocholine group at the position C-6 of **1** was attempted using 2-chloro-2-oxo-1,3,2-dioxaphospholane, followed by reaction of the resulting cyclic phosphate intermediate with anhydrous trimethylamine at 65 °C in a sealed vessel to give **2** in 68% yield for two steps. Compound **2** revealed an $[M+H]^+$ ion peak at *m*/*z* 728 in the time of flight mass spectrometer (TOF-MS) spectrum. Removal of the benzyl groups from **2** by catalytic hydrogenolysis over 10% Pd–C gave the target compound **3** (Chart 1).

Synthesis of Disaccharide Containing Phosphocholine The disaccharide derivative **5** was obtained by condensation of **1** with phenyl 1-thio-2,3,4-tri-*O*-benzyl-6-*O*-*tert*-butyldimethylsilyl-β-D-galactopyranoside (**4**) in the presence of *N*-iodosuccinimide (NIS) and trifluoromethanesulfonic acid (TfOH).[9)] Stereochemical control was achieved by using the solvent effect of nitrile[10)] to give the desired β-glycoside **5** in 91% yield and the α-glycoside was not detected. The anomeric hydrogen atom of the nonreducing-end galactose unit appeared as a signal at δ 4.22 (d, *J*=7.9 Hz). The β-D

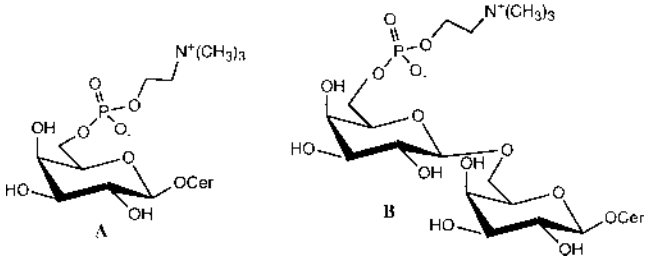
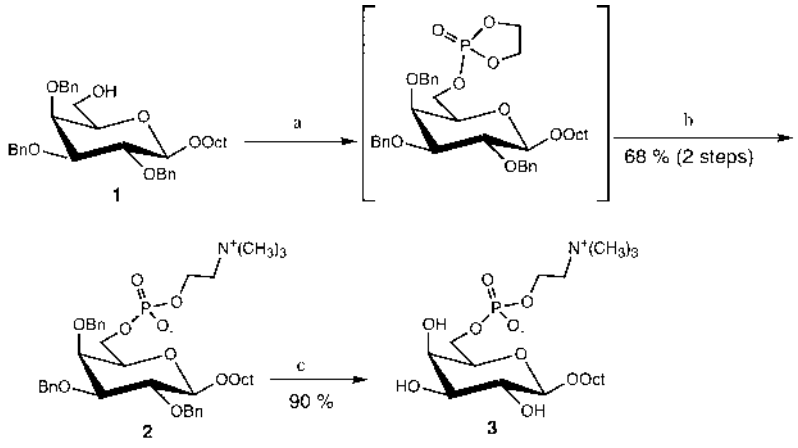


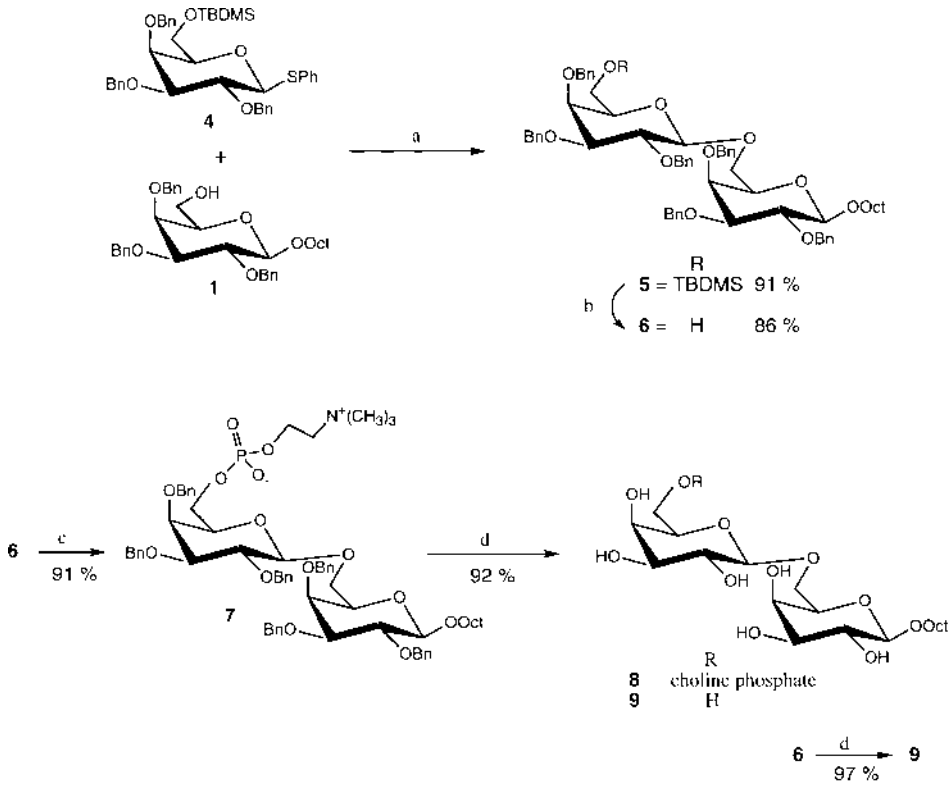
Fig. 1

* To whom correspondence should be addressed. e-mail: takeda-td@kyoritsu-ph.ac.jp



Reagents and conditions: (a) 1) 2-chloro-2-oxo-1,3,2-dioxaphospholane, Et_3N, benzene, rt, 3h. b) Me_3N, CH_3CN, 65°C, 48h. (c) Pd-C, H_2, MeOH-HCl, rt, 12h.

Chart 1



Reagents and conditions: (a) TfOH, NIS, MSAW300, C_2H_5CN. (b) Bu_4NF, THF. (c) 1) 2-chloro-2-oxo-1,3,2-dioxaphospholane, Et_3N, benzene, rt, 12h. 2) Me_3N, CH_3CN, 65°C, 48h. (d) Pd-C, H_2, MeOH-HCl, rt, 12h.

Chart 2

configuration of the newly formed glycosidic bond was also supported by the $J_{C,H}$ value of 159.7 Hz in the ^{13}C-NMR spectrum.[11] Removal of the *tert*-butyldimethylsilyl (TBDMS) group from **5** by Bu_4NF gave the disaccharide intermediate **6** in 86% yield. Coupling of a phosphocholine group with **6** as described for **2** gave the desired compound **7** (91%), and removal of the benzyl groups from **7** by catalytic hydrogenolysis over 10% Pd–C gave the target compound **8** (Chart 2).

Biological Activities The biological activity of compound **3**, **8** and phosphocholine free compound **9** were assayed *in vitro* by histamine release-inhibition test. The method using rat basophilic leukemia cells (RBL-2H3) was reported by Teshima *et al.*[12] As shown in Fig. 2, compounds **3** and **8** showed a better activity for histamine release-inhibition from RBL-2H3 cell than compound **9**. The IC_{50} values were 2.6 mM (**3**), 2.9 mM (**8**) and >10 mM (**9**). On the other hand, octyl β-D-galactopyranoside could not assay in order to show a potent cytotoxicity.

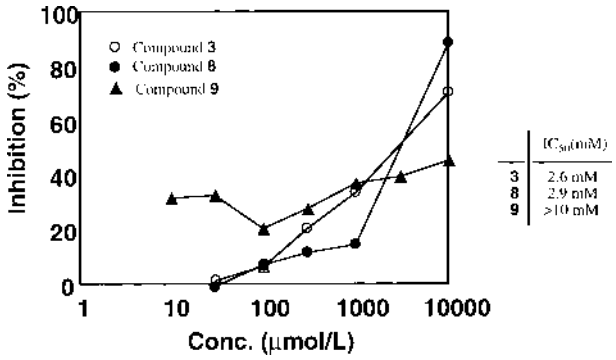


Fig. 2. Inhibitory Effect of **3**, **8** and **9** on Histamine Release from RBL-2H3

In summary, we have synthesized for the first time the oligosaccharides including phosphocholine using 2-chloro-2-oxo-1,3,2-dioxaphospholane in good yield and a phosphocholine group seemed to act as a inhibitor to a histamine release.

Experimental

Optical rotations were determined with a JASCO digital polarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded on a JNM A 500 FT NMR spectrometer in $CDCl_3$ with Me_4Si as the internal standard. Matrix-assisted laser desorption ionization (MALDI-TOF-MS) was recorded on a Perceptive Voyager RP mass spectrometer. TLC was performed on Silica gel 60 F_{254} (E. Merck) with detection by quenching of UV fluorescence and by spraying with 10% H_2SO_4. Column chromatography was carried out on Silica Gel 60 (E. Merck). Octyl 2,3,4-tri-*O*-benzyl-*β*-D-galactopyranoside (**1**) was prepared by a literature method.[8]

Octyl 2,3,4-Tri-*O*-benzyl-6-*O*-phosphocholine-*β*-D-galactopyranoside (2) To a solution of **1** (102 mg, 0.18 mmol) and triethylamine (50 μg, 0.36 mmol) in dry benzene (5 ml) was added 2-chloro-2-oxo-1,3,2-dioxaphospholane (50 μl, 0.54 mmol) at room temperature. After stirring for 3 h at room temperature, the reaction mixture was filtered to remove the precipitated salts. The filtrate and washing were combined and concentrated. This compound was transferred into a sealed vessel as a solution in 10 ml of dry acetonitrile. To the solution was added 100 μl of trimethylamine and the bottle was sealed and then heated in an oil bath at 65 °C for 48 h. Resulting mixture was concentrated and the residue was purified by Iatrobeads chromatography, eluting with $CHCl_3$–MeOH–H_2O (8 : 4 : 1) to give the desired compound (**2**) (89.1 mg, 67.5%): $[\alpha]_D^{24}$ +17.5° (c=1.2, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 4.34 (1H, d, J=7.9 Hz, H-1), 4.22 (2H, m, OC$\underline{H}_2$CH$_2$), 3.64 (2H, m, CH$_2$C$\underline{H}_2$N), 3.16 (9H, s, N(CH$_3$)$_3$). MALDI-TOF-MS: Calcd for $C_{40}H_{58}NO_9P$ m/z: 727. Found m/z: 728 [M+H]$^+$.

Octyl 6-*O*-Phosphocholine-*β*-D-galactopyranoside (3) A solution of **2** (81.5 mg, 0.11 mmol) in MeOH (4 ml) containing conc. HCl (0.1 ml) was hydrogenated over 10% Pd–C (57 mg) for 12 h at room temperature, then filtered through Celite and the filter cake was washed with methanol. Combined filtrate and washings were concentrated. Column chromatography (MeOH : H_2O=1 : 1) of the residue on Sephadex LH-20 to give **3** (52.0 mg, quant.), $[\alpha]_D^{24}$ −3.8° (c=0.4, H_2O), ^{1}H-NMR (D_2O); δ 4.23 (1H, d, J=8.6 Hz, H-1), 4.14 (2H, m, OC$\underline{H}_2$CH$_2$), 3.49 (2H, m, CH$_2$C$\underline{H}_2$N), 3.05 (9H, s, N(CH$_3$)$_3$). MALDI-TOF-MS; Calcd for $C_{19}H_{40}NO_9P$ m/z: 457. Found: 458 [M+H]$^+$.

Octyl 2,3,4-Tri-*O*-benzyl-6-*O*-*tert*-butyldimethylsilyl-*β*-D-galactopyranosyl-(1→6)-2,3,4-tri-*O*-benzyl-*β*-D-galactopyranoside (5) To a solution of **4** (342 mg, 0.52 mmol) and compound **1** (203 mg, 0.36 mmol) in dry C_2H_5CN (2 ml) was added powdered MS-AW300 (0.4 g) and the mixture was stirred for 2 h at room temperature, then cooled to −60 °C. NIS (178 mg, 0.79 mmol) and TfOH (1.5 μl, 0.36 mmol) were added to the mixture, which was stirred for 10 min. at −60 °C, then neutralized with Et_3N. The solids were filtered off and washed with $CHCl_3$. The combined filtrate and washings were successively washed with aq. $Na_2S_2O_3$ and water, dried (Na_2SO_4), and concentrated. The residue was chromatographed on silica gel using 15 : 1 benzene–acetone as an eluent to give **5** (365 mg, 91%). $[\alpha]_D^{24}$ −1.9° (c=0.9 $CHCl_3$), ^{1}H-NMR ($CDCl_3$) δ: 7.33—7.16 (30H, m, 6×Ph), 4.92—4.57 (12H, m, 6×benzyl methylene), 4.35 (1H, d, J=8.0 Hz, H-1), 4.22 (1H, d, J=7.9 Hz, H-1′), 0.83 (9H, s, C(CH$_3$)$_3$), −0.02 (6H, s, –Si(CH$_3$)$_2$). MALDI-TOF-MS: Calcd for $C_{68}H_{88}O_{11}Si$ m/z: 1109. Found: 1132 [M+Na]$^+$.

Octyl 2,3,4-Tri-*O*-benzyl-*β*-D-galactopyranosyl-(1→6)-2,3,4-tri-*O*-benzyl-*β*-D-galactopyranoside (6) A solution of compound **5** (340 mg, 0.31 mmol) in 2 ml of tetrahydrofuran (THF) was treated with 1 M Bu_4NF (1 ml) in THF solution at 0 °C. The reaction mixture was stirred at room temperature for 1.5 h, and then diluted with $CHCl_3$ and washed with water, dried (Na_2SO_4), and concentrated. The residue was chromatographed on silica gel using 4 : 1 hexane–ethyl acetate as an eluent to give compound **6** (261 mg, 86%). $[\alpha]_D^{24}$ −26.1° (c=0.5 $CHCl_3$), ^{1}H-NMR ($CDCl_3$) δ: 7.33—7.16 (30H, m, 6×Ph), 4.92—4.57 (12H, m, 6×benzyl methylene), 4.40 (1H, d, J=8.0 Hz, H-1), 4.29 (1H, d, J=7.9 Hz, H-1′). MALDI-TOF-MS: Calcd for $C_{62}H_{74}O_{11}$ m/z: 995. Found: 1018 [M+Na]$^+$.

Octyl 2,3,4-Tri-*O*-benzyl-6-*O*-phosphocholine-*β*-D-galactopyranosyl-(1→6)-2,3,4-tri-*O*-benzyl-*β*-D-galactopyranoside (7) To a solution of **6** (105 mg, 0.11 mmol) and triethylamine (21 μl, 0.15 mmol) in dry benzene (5 ml) was added 2-chloro-2-oxo-1,3,2-dioxaphospholane (46 μl, 0.50 mmol) at room temperature. After stirring for 12 h at room temperature, the reaction mixture was filtered to remove the precipitated salts. The filtrate and washings were combined and concentrated. This compound was transferred into a sealed vessel as a solution in 7 ml of dry acetonitrile. To the solution was added 95 μl of trimethylamine. The bottle was sealed and then heated in an oil bath at 65 °C for 48 h. Resulting mixture was concentrated, and the residue was purified by Iatrobeads chromatography, eluting with $CHCl_3$–MeOH (1 : 1) to give the desired compound (**7**) (116 mg, 91%): $[\alpha]_D^{24}$ +26.6° (c=1.9, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 4.44 (1H, d, J=7.9 Hz, H-1′), 4.27 (1H, d, J=7.3 Hz, H-1), 4.15 (2H, m, OC$\underline{H}_2$CH$_2$), 3.49 (2H, m, CH$_2$C$\underline{H}_2$N), 3.02 (9H, s, N(CH$_3$)$_3$). MALDI-TOF-MS: Calcd for $C_{67}H_{86}NO_{14}P$ m/z: 1160. Found m/z: 1161 [M+H]$^+$.

Octyl 6-*O*-Phosphocholine-*β*-D-galactopyranosyl-(1→6)-*β*-D-galactopyranoside (8) A solution of **7** (74.3 mg, 0.06 mmol) in MeOH (4 ml) containing conc.HCl (0.1 ml) was hydrogenated over 10% Pd–C (54 mg) for 12 h at room temperature, then filtered through Celite and the filter cake was washed with methanol. Combined filtrate and washing were concentrated. Column chromatography (MeOH : H_2O=1 : 1) of the residue on Sephadex LH-20 to give **8** (36.4 mg, 92%), $[\alpha]_D^{24}$ −9.7° (c=0.4, H_2O), ^{1}H-NMR (D_2O); δ: 4.31 (1H, d, J=7.9 Hz, H-1′), 4.22 (1H, d, J=7.9 Hz, H-1), 4.14 (2H, m, OC$\underline{H}_2$CH$_2$), 3.49 (2H, m, CH$_2$C$\underline{H}_2$N), 3.05 (9H, s, N(CH$_3$)$_3$). MALDI-TOF-MS: Calcd for $C_{25}H_{50}NO_{14}P$ m/z: 619. Found: 620 [M+H]$^+$.

Octyl *β*-D-Galactopyranosyl-(1→6)-*β*-D-galactopyranoside (9) A solution of **6** (15 mg, 15.1 μmol) in MeOH (2 ml) was hydrogenated over 10% Pd–C (20 mg) for 12 h at room temperature, then filtered through Celite and the residue was washed with methanol and concentrated. Column chromatography (1 : 1 $CHCl_3$–MeOH) of the residue on Sephadex LH-20 gave **9** (6.7 mg, 98%); $[\alpha]_D^{24}$ −24.6° (c=0.5, 1 : 1 $CHCl_3$–MeOH); ^{1}H-NMR (CD_3OD): δ: 4.30 (d, 1H, J=8.0 Hz, H-1′), 4.19 (d, 1H, J=7.5 Hz, H-1). MOLDI-TOF-MS: Calcd for $C_{20}H_{38}O_{11}$: m/z 454. Found: m/z 477 [M+Na]$^+$.

Acknowledgements This work was supported by a Grant-in-Aid for Scientific Research (No. 12672062) from the Ministry of Education, Science, Sports and Culture of Japan. We gratefully acknowledge the financial support of Uehara Memorial Foundation. The authors are grateful to Ms. J. Hada for providing NMR and MS data.

References

1) *a*) Takeda T., Hada N., Ogihara Y., *Chem. Pharm. Bull*., **40**, 1930—1933 (1992); *b*) *Idem*, *ibid*., **41**, 2058—2060 (1993); *c*) Hada N., Takeda T., Ogihara Y., *Carbohydr. Res*., **258**, 93—104 (1994); *d*) Hada N., Hayashi E., Takeda T., *ibid*., **316**, 58—70 (1999); *e*) Hada N., Matsuzaki A., Takeda T., *Chem. Pharm. Bull*., **47**, 1265—1268 (1999); *f*) Hada N., Kuroda M., Takeda T., *ibid*., **48**, 1160—1165 (2000); *g*) Hada N., Ohtsuka I., Sugita M., Takeda T., *Tetrahedron Lett*., **41**, 9065—9068 (2000).
2) *a*) Sugita M., Fujii H., Dulaney T. J., Inagaki F., Suzuki M., Suzuki A., Ohta S., *Biochim. Biophys. Acta*, **1259**, 220—226 (1995); *b*) Sugita M., Ohta S., Morikawa A., Dulaney T. J., Ichkawa S., Kushida S., Inagaki F., Suzuki M., Suzuki A., *J. Jpn. Oil. Soc*., **46**, 15—26 (1997).
3) Tanaka R., Miyahara K., Noda N., *Chem. Pharm. Bull*., **44**, 1152—1156 (1996).
4) Tanaka R., Ishizaki H., Kawano S., Okuda H., Miyahara K., Noda N., *Chem. Pharm. Bull*., **45**, 1702—1704 (1997).
5) *a*) Nishida Y., Takamori Y., Matsuda K., Ohrui H., Yamada T., Kobayashi K., *J. Carbohydr. Chem*., **18**, 985—997 (1999); *b*) Nishida Y., Takamori Y., Ohrui H., Ishizuka I., Matsuda K., Kobayashi K.,

Tetrahedron Lett., **40**, 2371—2374 (1999).
6) *a*) Ohno M., Fujita K., Nakai H., Kobayashi S., Inoue K., Nojima S., *Chem. Pharm. Bull*., **33**, 572—582 (1985); *b*) Nishida Y., Ohrui H., Meguro H., Ishizuka I., Matsuda K., Taki T., Handa S., Yamamoto N., *Tetrahedron Lett*., **35**, 5465—5468 (1994).
7) *a*) Fuji M., Watanabe F., Fujii Y., Hashizume H., Okuno T., Shirahase K., Teshirogi I., Ohtani M., *J. Org. Chem*., **62**, 6804—6809 (1997); *b*) Menger F. M., Chen X. Y., Brocchini S., Hopkins H. P., Hamilton D., *J. Am. Chem. Soc*., **115**, 6600—6608 (1993).
8) Jiao H., Hindsgaul O., *Angew. Chem. Int. Ed. Engl*., **38**, 346—348 (1999).
9) *a*) Veeneman G. H., van Leeuwen S. H., van Boom J. H., *Tetrahedron Lett*., **31**, 1331—1334 (1990); *b*) Konradsso P., Udodong U. E., Fraser-Reid B., *ibid.*, **31**, 4313—4316 (1990).
10) Schmidt R., Behrendt M., Toepfer A., *Synlett*, **1990**, 694—696.
11) Bock K., Pedersen C., *J. Chem. Soc*., *Perkin Trans*., *2*, **1974**, 293—297.
12) Teshima R., Ikebuchi H., Sekita S., Natori S., Terao T., *Int. Arch. Allergy Appl. Immunol*., **78**, 237—242 (1986).