ELSEVIER

Carbohydrate Research 315 (1999) 137–141

CARBOHYDRATE RESEARCH

Enzymatic synthesis of Kdn oligosaccharides by a bacterial α-(2→6)-sialyltransferase

Yasuhiro Kajihara [a,c,*], Shoji Akai [b], Takahiro Nakagawa [b], Reiko Sato [a], Takashi Ebata [c], Hisashi Kodama [c], Ken-ichi Sato [b,1]

[a] *Department of System Function, Faculty of Science, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan*

[b] *Laboratory of Organic Chemistry, Faculty of Engineering, Kanagawa University, Rokkakubashi, Kanagawa-ku, Yokohama 221-8686, Japan*

[c] *Life Science Research Laboratory, Japan Tobacco Inc., 6-2 Umegaoka, Midori-ku, Yokohama 227-8512, Japan*

Received 3 November 1997; accepted 1 December 1998

Abstract

Synthesis of CMP-deaminoneuraminic acid (CMP-β-D-Kdn) and its enzymatic transfer reaction using bacterial α-(2→6)-sialyltransferase were examined. CMP-β-D-Kdn was prepared from methyl 4,5,7,8,9-penta-*O*-acetyl-3-deoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosonate (**2**) in 24% overall yield. Enzymatic synthesis of Kdn oligosaccharide with CMP-β-D-Kdn (10.2 μmol), methyl β-D-lactosaminide (**7**, 8.1 μmol) and purified sialyltransferase (80 munits) afforded Kdn-α-(2→6)-Gal-β-(1→4)-GlcNAc-β-1-OMe in 77% yield.

Keywords: CMP-β-D-Kdn; Bacterial α-(2→6)-sialyltransferase; Enzymatic synthesis

1. Introduction

3-Deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (Kdn) is a unique deaminated analogue of sialic acid that has recently been isolated from the rainbow trout egg polysialoglycoprotein (PSGP) [1]. Kdn has several forms of glycosylic linkages, such as α-(2→3), α-(2→6) and α-(2→8) and has been found in a variety of tissues such as fish egg, sperm, amphibian egg, and bacteria [2–5]. To date, in order to investigate the role of the Kdn, several studies have been carried out [6], and consequently, it was deduced that the Kdn may play a role in protecting against sialidase digestion [6].

In order to investigate its further roles, enzymatic incorporation of Kdn and its analogues into intact oligosaccharides in vivo is a potential method as has been demonstrated in the research on *N*-acetylneuraminic acid (NeuAc) [7]. Although activities of Kdn-transferase have been identified [6], purification of Kdn-transferase has not been accomplished on a practical scale. On the other hand, a variety of sialyltransferases have been purified and used in enzymatic syntheses of sialoside, its analogues [8], and Kdn-α-(2→3)-Gal-β-(1→4)-Glc-β-1-OAll [9]. Recently, bacterial α-(2→6) sialyltransferase [EC 2.4.99.1], which catalyzes the transfer of NeuAc to a variety of sialyl acceptors, has been isolated [10]. Since

* Corresponding author. Tel.: +81-45-787-2210; fax: +81-45-787-2370.

E-mail address: kajihara@yokohama-cu.ac.jp (Y. Kajihara)

[1] Also corresponding author. E-mail: ksato@cc.kanagawa-u.ac.jp

0008-6215/99/$ - see front matter

PII: S0008-6215(98)00331-0

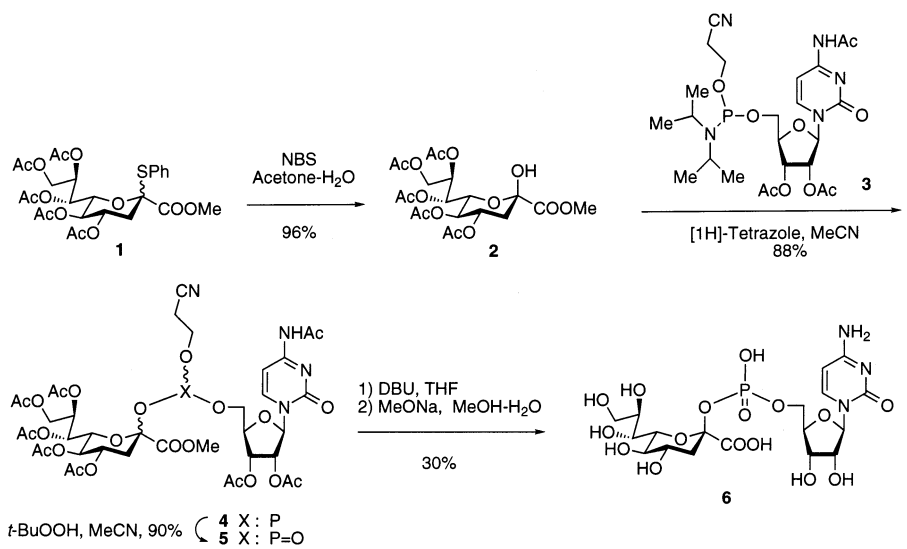
this enzyme has broad substrate specificity [11], it is expected to be useful for the syntheses of Kdn oligosaccharides or their analogues. If this enzyme can transfer Kdn from CMP-β-D-Kdn to the corresponding sialyl acceptor, modification of oligosaccharide by Kdn or its analogues in vivo will be developed.

We report here enzymatic transfer of Kdn to methyl *N*-acetyl-β-D-lactosaminide and 4-methylumbelliferyl β-D-lactoside by bacterial α-(2→6)-sialyltransferase.

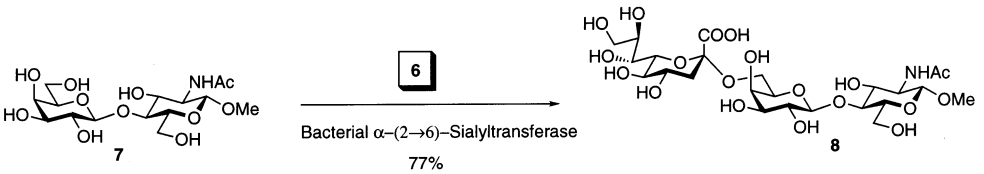
2. Results and discussion

CMP-β-D-Kdn was prepared by our previously reported method (Scheme 1) [12]. This synthetic route, however, afforded a mixture of four isomers of **4**. The derivatives were rationalized as 1:1:0.8:0.8, and this ratio is reproducible. ^{1}H NMR data suggested that the phosphite derivatives consist of an anomeric mixture of Kdn because both H-3′ and H-4′ in the Kdn unit were observed with reasonable chemical shifts (Table 2 in Ref. [13]). Since separation of each product proved to be difficult due to their lability on purification, the phosphite **4** was then used in the next step without further purification. Oxidation of **4** also afforded a mixture of four kinds of phosphate **5** in 88% yield. After deprotection, the ^{1}H NMR spectrum of the crude product showed that the desired CMP-β-D-Kdn **6** was obtained in 60% purity, and no CMP-α-D-Kdn was observed at all. These results indicated that CMP-α-D-Kdn might be more labile than **6**. Purification with a gel-permeation column (5 mM NH_4OH) afforded pure compound **6** in 30% yield, and its ^{1}H NMR data are in good agreement with reported data [4,9,14].

Enzymatic synthesis of Kdn-oligosaccharide **8** using the bacterial α-(2→6)-sialyltransferase was performed under the reported conditions [11] for the preparation of sialoside, except that the pH was adjusted to 7.5. The enzyme catalyzed the transfer of Kdn to methyl β-D-*N*-acetyllactosaminide (**7**) and afforded the desired Kdn-*N*-acetyl-β-D-lactosaminide **8** in 77% yield (Scheme 2). The structure was identified by ^{1}H and related 2D NMR spectra. Enzyme assay using 4-methylumbelliferyl β-D-lactoside indicated that the apparent K_m value of CMP-β-D-Kdn is 880 μM, which is 27 times larger than that of CMP-β-D-NeuAc (33 μM). In addition, the V_{max} value of CMP-β-D-Kdn is 15% larger than that of CMP-β-D-NeuAc. Despite the fact that substitution of the acetamido group with a hydroxyl group resulted in a decrease of both binding affinity and transfer velocity, practical enzymatic synthesis could still afford Kdn-*N*-acetyl-β-D-lactosaminide in good yield. In general, mammalian α-(2→6)-sialyltransferase cannot catalyze transfer of NeuAc to both lactose and



Scheme 1.



Scheme 2.

N-acetyl-β-D-lactosamine. However, bacterial sialyltransferase can transfer even Kdn to both lactose and *N*-acetyl-β-D-lactosamine. This finding would be useful for the synthesis of several Kdn oligosaccharides.

In summary, we found bacterial sialyltransferase catalyzes the transfer of Kdn to both methyl *N*-acetyl-β-D-lactosaminide and 4-methylumbelliferyl β-D-lactoside. A previous report [11,15] showed that this enzyme has broad substrate specificity toward disaccharides and monosaccharides. In addition, the enzyme is now available in large quantity [15]. Therefore, enzymatic synthesis using CMP-modified-Kdn and bacterial sialyltransferase would afford Kdn-oligosaccharide analogues which can be expected to clarify the role of Kdn. Since the Kdn may be resistant to sialidase digestion, the Kdn or its analogues will be expected to be used for masking of galactosides at the nonreducing end of the glycoprotein in order to change the biological response, such as blocking the hepatic clearance [16].

3. Experimental

General procedure.—^{1}H NMR spectra were recorded with either a JEOL EX 270 or Bruker DMX 500 instrument. ^{31}P NMR spectra were recorded with Bruker DMX 500 or AC 300 instrument, and chemical shifts are expressed in ppm and referenced to H_3PO_4 (0 ppm) as the external standard. Optical rotations were measured with a JASCO DIP-4. High-resolution mass spectra were recorded using a Shimazu/Kratos concept-IIH under FAB conditions. All reactions were monitored by TLC (Silica Gel 60-F_{254}, E. Merck) by charring after spraying with 5% H_2SO_4 in MeOH.

Methyl 4,5,7,8,9-penta-O-*acetyl-3-deoxy*-D-glycero-*β*-D-galacto-*2-nonulopyranosonate* (**2**). —To a solution of thiophenyl glycoside **1** [13] (682 mg, 1.17 mmol) in 10:3 acetone–H_2O (26 mL) was added NBS (609 mg), and the mixture was stirred at 0 °C. After 2 h the mixture was diluted with EtOAc and washed with a solution of $NaHCO_3$ (2 ×). The organic phase was dried with $MgSO_4$ and was concentrated in vacuo. Purification of the residue by flash column chromatography (2:1 → 1:1 *n*-hexane–EtOAc) afforded **2** (96%, 546 mg): $[\alpha]_D^{26} - 1.2°$ (*c* 0.83, $CHCl_3$); mp 106–108 °C; ^{1}H NMR (270 MHz, $CDCl_3$): *δ* 7.34–7.52 (m, 5 H, SPh), 5.20 (dd, 1 H, H-7), 5.04 (ddd, 1 H, *J* 7.4 Hz, H-8), 5.09 (ddd, 1 H, *J* 11.8 Hz, H-4), 4.79 (dd, 1 H, *J* 7.4 Hz, H-5), 4.21 (dd, 1 H, *J* 2.9 Hz, H-9), 4.19 (dd, 1 H, *J* 10.3, 2.1 Hz, H-6), 3.97 (dd, 1 H, *J* 12.4, 6.0 Hz, H-9), 3.66 (s, 3H, COOMe), 2.15 (dd, 1 H, *J* 5.1, 12.8 Hz, H-3eq), 1.83–1.93 (m, 16 H, OAc, H-3ax). Anal. Calcd for $C_{20}H_{28}O_{14}$: C, 48.78; H, 5.73. Found: C, 49.10; H, 5.50.

2-Cyanoethyl (2′,3′-di-O-*acetyl*-N^4-*acetyl-cytidine-5′-yl) (methyl 4,5,7,8,9-penta*-O-*acetyl-3-deoxy-β*-D-glycero-*β*-D-galacto-*2-nonulopyranosid-2″-yl) phosphite* (**4**).—The pentaacetyl-Kdn (**2**, 229 mg, 470 μmol) and cytidine 5′-*O*-amidite (**3**, 784 mg, 14.0 mmol) were separately dried by coevaporating twice with dry toluene. They were then combined in freshly prepared dry MeCN (2.0 mL). To this mixture was added [1*H*]-tetrazole (59 mg, 840 μmol) at −40 °C under an argon atmosphere. After 5 min, the cold bath was removed. The mixture was further stirred for 30 min at room temperature (rt), and then the mixture was diluted with EtOAc. The organic phase was washed with $NaHCO_3$ solution, dried with $MgSO_4$, and concentrated in vacuo at 40 °C. Purification of the residue by gel-permeation column chromatography twice (Sephadex LH-

20, 2 × 60 cm, MeOH,) afforded phosphite **4** (403 mg, 88%, four diastereomers, 1:1:0.8:0.8). ^{1}H NMR chemical shifts are summarized in Table 1. ^{31}P NMR (500 MHz, $CDCl_3$): δ 135.0, 135.9, 137.2, 137.3. HRFABMS: Calcd for $C_{38}H_{50}N_4O_{23}P$, 961.2604 (M + H); Found: 961.2611. Anal. Calcd for $C_{38}H_{49}N_4O_{23}P$: C, 47.50; H, 5.14; N, 5.83, Found: C, 47.09; H, 5.02; N, 6.09.

2-Cyanoethyl (2′,3′-di-O-*acetyl*-N^4-*acetyl-cytidine 5′-yl) (methyl 4,5,7,8,9-penta*-O-*acetyl-3-deoxy-β*-D-glycero-D-galacto-*2-non-ulopyranosid 2″-yl) phosphate* (**5**).—To a solution of phosphite **4** (108 mg, 110 μmol) in MeCN (1.0 mL) was added *tert*-BuOOH (2.5 M in toluene, 218 μL), and the mixture was stirred at rt. After 30 min, the mixture was diluted with EtOAc and washed with $NaHCO_3$ solution. After drying with $MgSO_4$, the organic phase was concentrated in vacuo at 40 °C. Purification of the residue by gel-permeation column chromatography (Sephadex LH-20, CH_2Cl_2) afforded phosphate **5** (99 mg, 90%, four diastereomers). ^{1}H NMR: δ 9.64 (NHAc), 8.00–8.25 (H-6), 7.39–7.50 (H-5), 6.08–6.20 (H-1′), 3.89 (COOMe), 2.78–2.88 (H-3″eq), 2.24 (NAc), 1.98–2.11 (OAc); ^{31}P NMR (500 MHz, $CDCl_3$): δ −6.33, −6.23, −6.23, −6.14. HRFABMS: Calcd for $C_{38}H_{50}N_4O_{24}P$, 977.2553 (M + H); Found: 977.2552.

CMP-β-D-*Kdn* (**6**).—To a solution of protected CMP-Kdn **5** (96 mg, 100 μmol) in THF (3.0 mL) was added 1,8-diazabicyclo-[5.4.0]-7-undecene (2.3 mg, 15.2 μmol), and the mixture was stirred at rt. After 5 min, NaOMe (174 mg, 3.24 mmol) and MeOH–H_2O (1.2–5.1 mL) were added to this mixture. After 16 h the mixture was lyophilized. Purification of the residue by gel-permeation column chromatography (Sephadex G-15, 3 × 100 cm, 5 mM NH_4OH, 4 °C) afforded CMP-β-D-Kdn **6** (19 mg 30%). ^{1}H NMR (HOD = 4.81 ppm): δ 7.96 (d, 1 H, *J* 7.5 Hz, H-6), 6.10 (d, 1 H, *J*

Table 1
^{1}H chemical shifts for four diastereomers (**4**)[a]

H	Diastereomer A	Diastereomer B	Diastereomer C	Diastereomer D
3″ax	2.10 (13.3, 13.3)	2.05 (13.4, 13.4)	2.09 (12.8, 12.8)	2.10 (12.8, 12.8)
3″eq	2.53 (5.5, 13.3)	2.58 (5.4, 13.4)	2.77 (6.4, 12.8)	2.79 (6.4, 12.8)
4″	5.24 (5.5, 10.2, 10.2)	5.25 (5.4, 10.4, 10.4)	5.06 (6.4, 10.4, 10.4)	5.02 (6.4, 10.4, 10.4)
5″	4.98 (9.9, 10.2)	5.00 (9.9, 10.4)	4.95 (9.9, 10.4)	4.93 (9.9, 10.4)
6″	4.34 (3.3, 9.9)	4.33 (3.3, 9.9)	4.33 (3.3, 9.9)	4.24 (3.3, 9.9)
7″	5.42 (3.3, 6.3)	5.41 (3.3, 6.3)	5.33	5.33
8″	5.22	5.18	5.33	5.33
9″a	4.12 (6.6, 13.2)	4.11 (6.6, 13.2)	4.05 (5.6, 12.9), 4.09 (5.6, 12.9)	4.05 (5.6, 12.9), 4.09 (5.6, 12.9)
9″b	4.49 (3.6, 13.2)	4.51 (3.6, 13.2)	4.27 (3.0, 12.9), 4.31 (3.0, 12.9)	4.27 (3.0, 12.9), 4.31 (3.0, 12.9)
1′	6.31 (5.5), 6.28 (5.3), 6.28 (5.3), 6.25 (4.3)			
2′	5.34–5.48			
3′	5.46 (3.3, 3.3), 5.42 (3.3, 3.3), 5.41, 5.39 (3.3, 3.3)			
4′	4.39 (3.3, 3.3, 6.6), 4.39 (3.3, 3.3, 6.6), 4.37 (3.3, 3.3, 6.6), 4.36 (3.3, 3.3, 6.6)			
5′	4.05–4.40			
5	7.49 (7.6), 7.45 (7.6), 7.45 (7.6), 7.42 (7.6)			
6	8.17 (7.6), 8.13 (7.6), 8.12 (7.8), 8.04 (7.6)			
NH	8.98, 8.98, 8.95, 8.95			

[a] ^{1}H chemical shifts in $CDCL_3$ (500 MHz, 298 K) are expressed relative to tetramethylsilane (0.00) ppm, and vicinal ^{1}H–^{1}H coupling constants in Hz were measured from 2D COSY spectrum (0.7 Hz/point) and are shown in parentheses.

7.5 Hz, H-5), 5.97 (d, 1 H, *J* 4.5 Hz, H-1″), 4.33 (dd, 1 H, H-4′), 4.27 (dd, 1 H, H-2′), 4.21 (d, 3 H, H-3′, 5′a, 5′b), 4.06 (d, 1 H, *J* 9.6 Hz, H-6″), 4.00 (ddd, 1 H, *J* 4.9, 9.6, 12.4 Hz, H-4″), 3.92 (d, 1 H, *J* 12.1 Hz, H-9″a), 3.88 (d, 1 H, H-7″), 3.72 (m, 1 H, H-8″), 3.65 (dd, 1 H, *J* 6.8, 12.1 Hz, H-9″b), 3.57 (dd, 1 H, *J* 9.6, 9.6 Hz, H-5″), 2.41 (dd, 1 H, *J* 4.9, 12.4 Hz), 1.57 (ddd, 1 H, *J* 5.9, 12.4, 12.4 Hz, H-3ax); ^{31}P NMR (D_2O): δ −4.43. HRFABMS: Calcd for $C_{18}H_{26}N_3O_{16}PNa_3$ ($M + Na^+$), 640.0745; Found: 640.0754.

Methyl (3-deoxy-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid)-(2→6)-(β*-D-*galactopyranosyl)-(1→4)-2-acetoamido-2-deoxy-β*-D-*glucopyranoside* (**8**).—A solution containing the CMP-β-D-Kdn (6.5 mg, 10.2 μmol, 2 sodium salt), methyl β-D-lactosaminide **7** (3.5 mg, 8.1 μmol), BSA (2 mg), alkaline phosphatase (25 u), purified sialyltransferase (80 mu) in HEPES buffer (100 mM, pH 7.5, 200 μL) was incubated at 37 °C for 12 h. The mixture was concentrated in vacuo. The residue was purified by silica gel column chromatography (Kanto Kagaku Co., Silica Gel 60, Spherical, 1 × 10 cm, 7:3:1 *n*-PrOH–H_2O–NH_4OH), and then the product was further purified on a column of Sephadex G-15 (1 × 50 cm, water) to give **8** (4.0 mg, 77%). ^{1}H NMR (500 MHz, D_2O): δ 4.47 (d, 1 H, *J* 8.1 Hz, H-1), 4.43 (d, 1 H, *J* 7.9 Hz, H-1′), 3.99 (dd, 1 H, *J* 2.1, 13.0 Hz, H-6), 3.98 (dd, 1 H, *J* 9.5, 9.5 Hz, H-6′), 3.91 (d, 1 H, *J* 3.4 Hz, H-4′), 3.82 (dd, 1 H, *J* 5.6, 13.0 Hz, H-6), 3.52 (dd, 1 H, *J* 8.0, 10.4 Hz, H-2′), 3.50 (s, 3 H, OMe), 3.47 (dd, 1 H, *J* 9.4, 9.4 Hz, H-5″), 2.61 (dd, 1 H, *J* 4.6, 12.4 Hz, H-3″eq), 2.05 (s, 3 H, Ac), 1.67 (dd, 1 H, *J* 12.4, 12.4 Hz, H-3″ax); ^{13}C NMR (D_2O, dioxane = 67.48 ppm): δ 175.39, 174.36, 174.16, 104.22, 102.51, 100.66, 81.35, 75.39, 74.61, 74.44, 73.36, 73.20, 72.86, 71.52, 70.85, 69.29, 69.00, 64.45, 63.59, 61.12, 57.89, 55.66, 40.45, 23.16. HRFABMS: Calcd for $C_{24}H_{41}NO_{19}Na$ (M + H), 670.2171; Found: 670.2205.

Enzyme assay.—Sialyltransferase assays were performed with a HEPES buffer solution (pH 7.5, 100 mM, 25 μL) containing CMP-β-D-Kdn (1.5, 3.0, 5.0 and 10.0 mM) or CMP-β-D-NeuAc (75.0, 100, 150, and 280 μM), 4-methylumbelliferyl β-D-lactoside (1.0 mM), BSA (1 μg), Triton X100 (0.02%) and sialyltransferase (for CMP-β-D-Kdn: 8.3 mU; for CMP-β-D-NeuAc: 0.1 mU). The mixture was incubated at 37 °C. The reaction was followed up to 15% consumption of donor substrate. After incubation, the mixture was allowed to cool with dry ice/acetone, and then Kdn or NeuAc transferred were quantitated with HPLC (C18-column: 1 × 25 cm, 1:4 MeCN–H_2O) equipped with fluorescence detector (excitation: 325 nm, emission: 378 nm). K_m and V_{max} values were estimated using a Lineweaver–Burk plot. The data points are the averages of at least duplicate values.

Acknowledgements

The authors would like to thank Masayosi Kusama for the mass spectroscopic analyses.

References

[1] D. Nadano, M. Iwasaki, S. Endo, K. Kitajima, S. Inoue, Y. Inoue, *J. Biol. Chem.*, 261 (1986) 11550–11557.
[2] A. Kanamori, S. Inoue, M. Iwasaki, K. Kitajima, G. Kawai, S. Yokoyama, Y. Inoue, *J. Biol. Chem.*, 265 (1990) 21811–21819.
[3] Y. Song, K. Kitajima, S. Inoue, Y. Inoue, *J. Biol. Chem.*, 266 (1991) 21929–21935.
[4] T. Terada, S. Kitazume, K. Kitajima, S. Inoue, F. Ito, F.A. Troy, Y. Inoue, *J. Biol. Chem.*, 268 (1993) 2640–2648.
[5] S. Inoue, K. Kitajima, Y. Inoue, *J. Biol. Chem.*, 271 (1996) 24341–24344.
[6] A. Rosenberg (Ed.), *Biology of the Sialic Acids*, Plenum, New York, 1995, pp 95–144.
[7] G. Herrler, H.J. Gross, A. Imhof, R. Brossmer, G. Milks, J.C. Paulson, *J. Biol. Chem.*, 267 (1992) 12501–12505.
[8] C.H. Wong, G.M. Whitesides, *Enzymes in Synthetic Organic Chemistry*, Vol. 12, Pergamon, Oxford, 1994.
[9] (a) M.D. Chappell, R.L. Halcomb, *J. Am. Chem. Soc.*, 119 (1997) 3393–3394. (b) M.D. Chappell, R.L. Halcomb, *Tetrahedron*, 53 (1997) 11109–11120.
[10] T. Yamamoto, M. Nakashizuka, H. Kodama, Y. Kajihara, I. Terada, *J. Biochem.*, 120 (1996) 104–110.
[11] Y. Kajihara, T. Yamamoto, H. Nagae, M. Nakashizuka, T. Sakakibara, I. Terada, *J. Org. Chem.*, 61 (1996) 8632–8635.
[12] Y. Kajihara, T. Ebata, K. Koseki, H. Kodama, H. Matsushita, H. Hashimoto, *J. Org. Chem.*, 60 (1995) 5732–5735.
[13] T. Terada, M. Kiso, A. Hasegawa, *J. Carbohydr. Chem.*, 12 (1993) 425–440.
[14] C. Augé, C. Gautheron, *Tetrahedron Lett.*, 29 (1988) 789–790.
[15] T. Yamamoto, H. Nagae, Y. Kajihara, I. Terada, *Biosci. Biotechnol. Biochem.*, 62 (1998) 210–214.
[16] M. Fukuda, H. Sasaki, L. Lopez, M. Fukuda, *Blood*, 73 (1989) 84–89.