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Carbohydrate Research 342 (2007) 1680–1688
Carbohydrate RESEARCH

Synthesis of CMP-9″-modified-sialic acids as donor substrate analogues for mammalian and bacterial sialyltransferases

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Received 15 February 2007; received in revised form 25 May 2007; accepted 27 May 2007
Available online 5 June 2007

Abstract—Cytidine-5′-monophospho-sialic acid (CMP-Neu5Ac) derivatives bearing a phenyl group in which the tether length between the phenyl group and the 9-position of Neu5Ac varied were synthesized and evaluated as substrates for sialyltransferases. In the synthesis of the compounds, a coupling reaction between methyl 5-acetamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate and 2-cyanoethyl 2′,3′-*O*,N^4, triacetylcytidine-5′-yl *N*,*N*-diisopropylphosphoramidite was carried out and the phosphite derivative thus obtained was oxidized and then deprotected to yield CMP-9″-azido-Neu5Ac. Modification of the 9-amino group prepared by reduction of the azido groups was performed by the use of several phenyl-substituted alkylcarboxylic acid derivatives. Using these CMP-9″-modified-Neu5Ac analogues bearing the phenyl-substituted alkyl-amide group, sialyltransferase assays were performed with both rat liver α-(2→6)-sialyltransferase and *Photobacterium* α-(2→6)-sialyltransferase. These 9-modified analogues could be transferred to disaccharide acceptors, and a practical enzymatic synthesis using CMP-9″-modified-Neu5Ac yielded sialoside analogues and sialylglycoproteins in good yield. These experiments demonstrate that the *Photobacterium* sialyltransferase can be used in the synthesis of sialoside analogues having a large substituent at the 9-position of Neu5Ac.

Keywords: CMP-Neu5Ac; Sialyltransferase; Enzymatic synthesis

1. Introduction

Sialyltransferases catalyze the transfer of sialic acid to a corresponding acceptor oligosaccharide yielding a sialyl-oligosaccharide. Mammalian sialyltransferase exhibits broad substrate specificity for CMP-9″-modified-sialic acid (CMP-9″-modified-Neu5Ac) derivatives and can catalyze the transfer of Neu5Ac analogues having a variety of substituents of different steric sizes at the 9-position. This unique substrate specificity was found by Brossmer[1–6] and several CMP-Neu5Ac analogues have been used as chemical probes. We also demonstrated that CMP-Neu5Ac immobilized on the solid support through the 9-position of Neu5Ac can be transferred to asialoglycoprotein.[7] Recently, the structures of bacterial sialyltransferase isolated from *Campyrobacter jejuni*[8] and *Pasteurella multocida*[9] have been solved. The structure of *C. jejuni* enzyme, which is bound to CMP-Neu5Ac analogue, indicates that the 9-position of Neu5Ac faces toward the water layer and this suggests that bacterial sialyltransferases can also transfer 9-modified Neu5Ac derivatives. Bacterial sialyltransferases can be isolated on a large scale compared to those obtained from a mammalian source. Chen et al. also demonstrated that the *P. multocida* enzyme exhibits broad substrate specificity toward CMP-Neu5Ac analogues and its practical enzy-

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doi:10.1016/j.carres.2007.05.029

matic synthesis yielded sialoside analogues on a large scale.[10,11] This potential advantage in yield will make it possible to modify natural products such as proteins, lipids and cells by the use of CMP-9″-modified-Neu5Ac-analogues. We report here a systematic investigation of the substrate specificity of a *Photobacterium* sialyltransferase[12] toward CMP-9″-modified-Neu5Ac.

2. Results and discussion

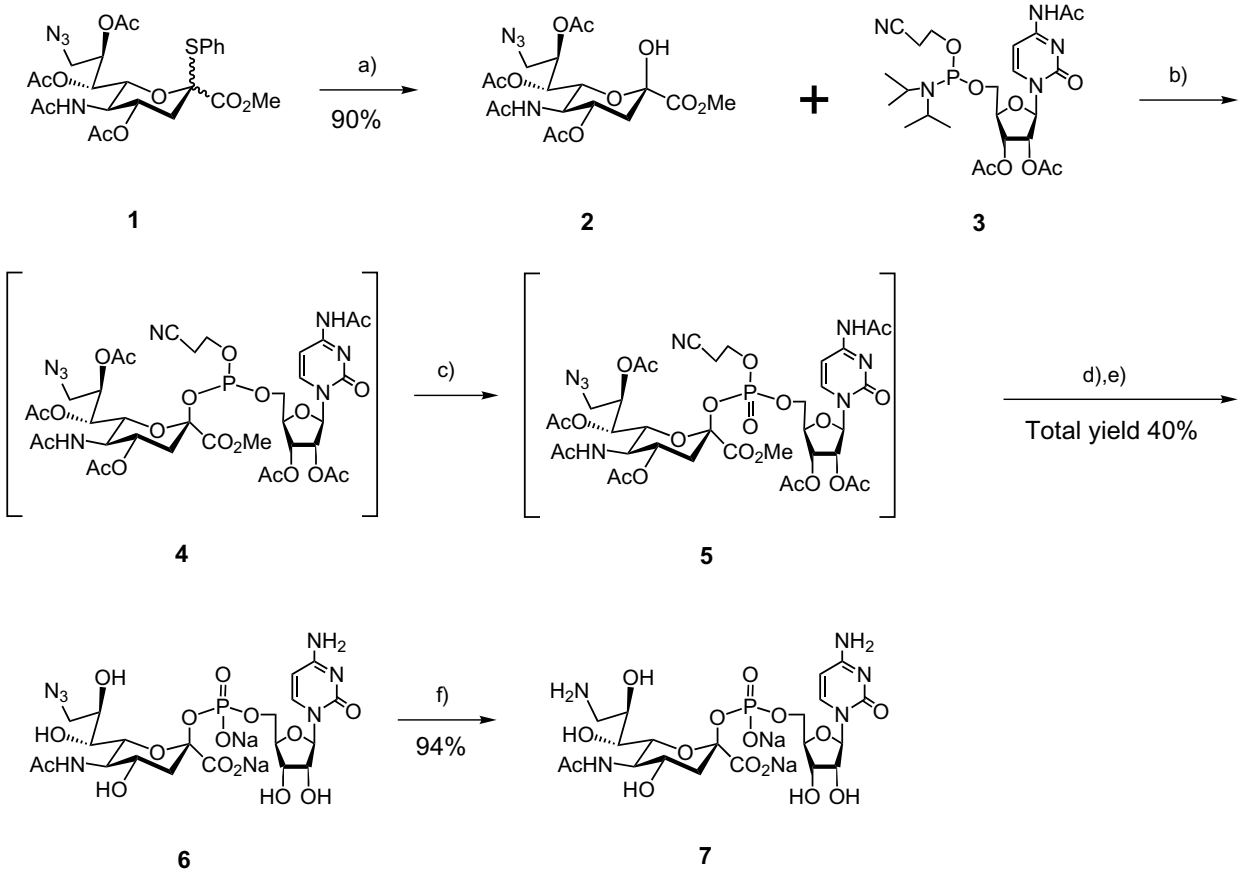
2.1. Synthesis of CMP-9″-modified-Neu5Ac

To investigate the transfer of 9-modified-Neu5Ac derivatives by the bacterial sialyltransferase, we synthesized CMP-9″-modified-Neu5Ac derivatives. We designed Neu5Ac bearing a phenyl-substituted alkyl group at the 9-position and varied the length of the tether between the phenyl group and the 9-position of Neu5Ac. These analogues can be used for the evaluation of the alkyl–tether length, which is critically important for modification with a large substituent (Scheme 1).

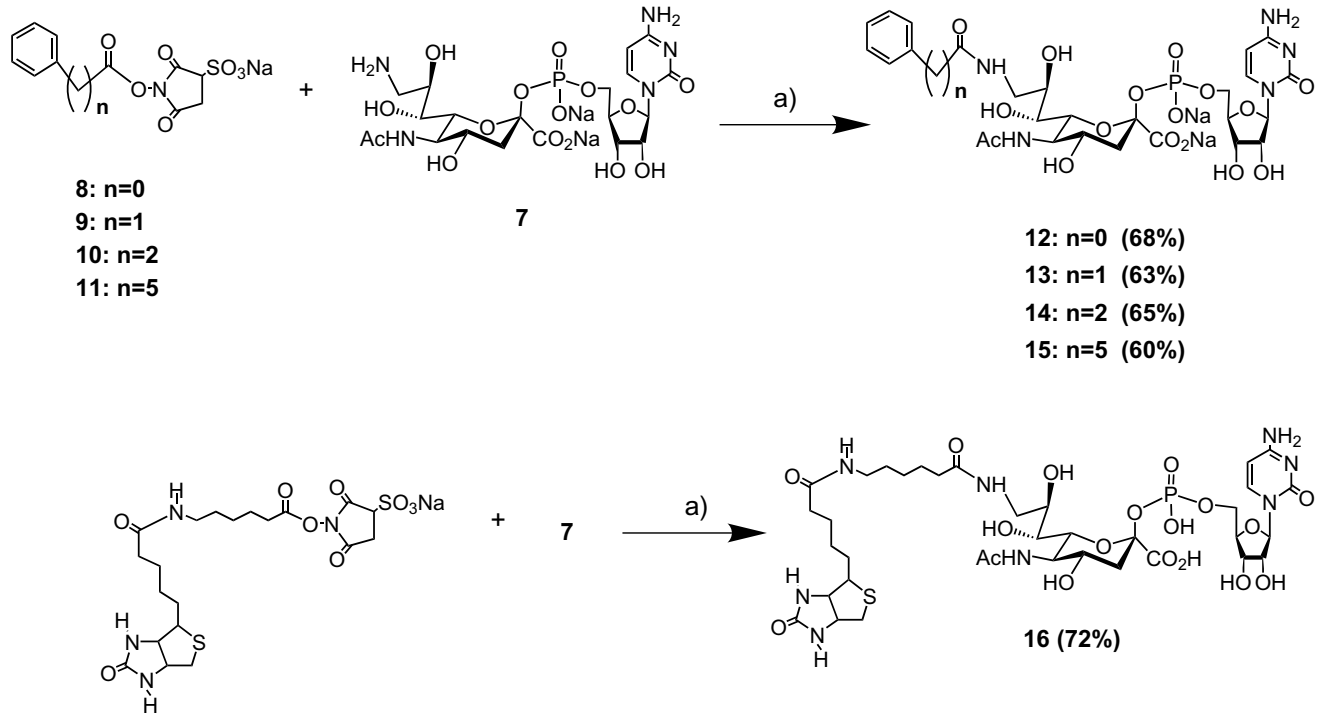
9-Azido-Neu5Ac **1**[7] was converted into alcohol derivative **2** by treatment with NBS (*N*-bromosuccinimide). This Neu5Ac-alcohol was then coupled with cytidine-5′-*O*-amidite derivative **3** to yield the corresponding phosphite derivative **4**, which was then oxidized by the use of *t*-butyl hydroperoxide.[13] After oxidation, all the protecting groups were removed to obtain CMP-9″-azido-Neu5Ac **6**. Catalytic reduction using Pd/$CaCO_3$ was then performed. Although the CMP-Neu5Ac derivative is known to be labile, this reduction afforded CMP-9″-amino-Neu5Ac **7** in good yield. To prepare CMP-9″-modified Neu5Ac **12**–**15**, CMP-9″-amino-Neu5Ac **7** was coupled with the phenyl-substituted alkylcarboxylic acid derivatives **8**–**11** under slightly basic conditions. All the coupling reactions with CMP-9″-amino-Neu5Ac smoothly afforded the desired analogues **12**–**15** (Scheme 2).

2.2. Sialyltransferase assay

To evaluate the transfer of these CMP-9″-modified Neu5Ac analogues **12**–**15**, enzymatic assays using both bacterial and mammalian α-(2→6)-sialyltransferases were performed. The enzyme assays were carried out by means of a reported fluorescence labeling method[14] and then K_m and V_{max} were estimated. The V_{max} values



Scheme 1. Reagents and conditions: (a) NBS, acetone–H_2O; (b) 1H-Tetrazole, CH_3CN/DMF; (c) *t*-BuOOH, CH_3CN; (d) DBU, THF; (e) NaOH, MeOH–H_2O; (f) Pd–$CaCO_3$, H_2, 20 mM NH_4OH.



Scheme 2. Reagents and conditions: (a) Sodium phosphate buffer (pH 7.0, 20 mM).

Table 1. Kinetic parameters of CMP-9″-modified-Neu5Ac and acceptors

	Bacterium 6STate				Rat 6STase			
	Donor		Lactose		Donor		LacNAc	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
CMP-Neu5Ac	140	100[a]	3.6	100[b]	120	100[c]	4.3	100[d]
Ph-CONH **12**	110	15	1.8	70	51	93	6.8	250
Ph–CH_2–CONH **13**	340	20	2.5	155	50	81	2.0	111
Ph–$(CH_2)_2$–CONH **14**	110	20	2.6	85	165	130	10	250
Ph–$(CH_2)_2$–CONH **15**	530	24	2.5	175	98	112	5.2	111

Donor K_m: μM; acceptor K_m: mM; V_{max} are expressed as relative values compared to that of CMP-Neu5Ac.
[a] 0.04 pmol/min.
[b] 0.01 pmol/min.
[c] 58.8 pmol/min.
[d] 20 pmol/min.

in Table 1 are expressed as relative value compared to that of native CMP-Neu5Ac. In the case of the mammalian enzyme, the parent substrate, CMP-Neu5Ac, had a K_m of 120 μM while those for analogues **12**–**15** ranged from 50 to 165 μM. The V_{max} values of these analogues were almost in the same range. For the acceptor, K_m and V_{max} values for the mammalian enzyme, K_m ranged from 2.0 mM to 10 mM and the V_{max} values of **12** and **14** exhibited a 2.5 fold larger value compared with that of the parent CMP-Neu5Ac. In the case of the bacterial enzyme, all the CMP-9″-modified-Neu5Ac analogues were substrates. Although these K_m values were in the same range, the V_{max} values of the analogues showed ~20% of the activity of the parent CMP-Neu5Ac (100%). The acceptor K_m values ranged from 1.8 mM to 3.6 mM and the relative V_{max} values ranged from 70% to 175% based on the parent CMP-Neu5Ac ($V_{max} = 100\%$). As shown in Table 1, the K_m and V_{max} values of the acceptor with both the bacterial and mammalian enzyme were not affected when the CMP-9″-modified-Neu5Ac derivatives were bound to the combining site. These data indicate that a substituent attached at the 9-position does not disturb binding of the acceptor to the combining site of enzyme.

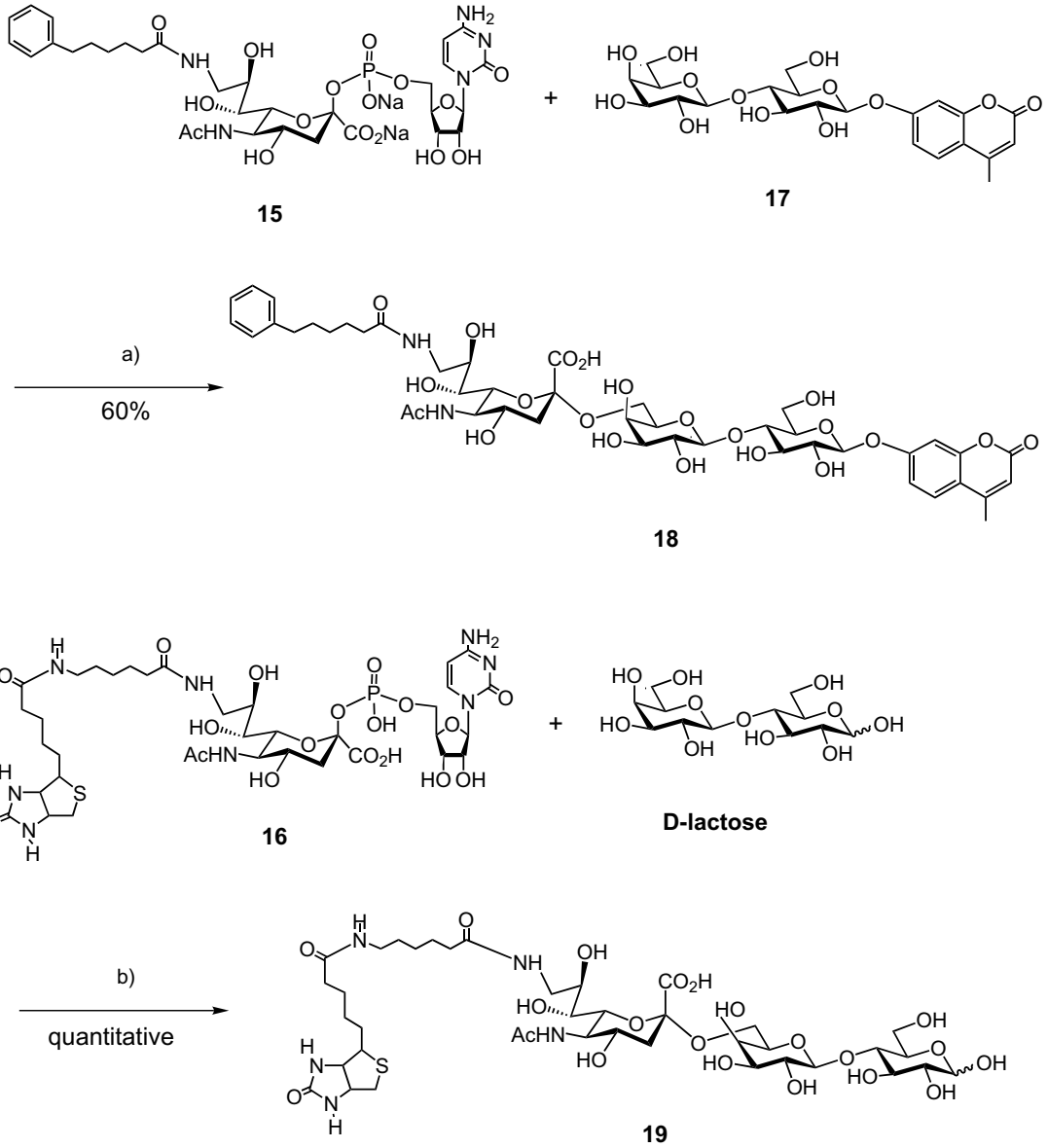
2.3. Enzymatic synthesis of sialyloligosaccharide

As shown in Table 1, all of the CMP-9″-modified-Neu5Ac derivatives were transferred by the bacterial

sialyltransferase. We examined a practical enzymatic synthesis and selected CMP-9″-(phenyl-hexanamide)-Neu5Ac, because this analogue exhibited the worst K_m value. If this analogue can afford sialosides, other CMP-9″-modified-Neu5Ac analogues could also be used for the practical enzymatic synthesis of the corresponding sialosides. A slight excess of the CMP-Neu5Ac analogue **15** was used with methyl umbelliferyl-D-lactose (**17**) and this reaction afforded the desired sialoside **18** in 60% yield. The structure was confirmed by NMR and mass spectrometric analysis. This result clearly indicates that the bacterial enzyme catalyzes the transfer of various 9-modified Neu5Ac analogues from the CMP-9″-modified Neu5Ac to the acceptor sugars.

2.4. Synthesis of CMP-9″-biotinylated Neu5Ac and its transfer to asialoglycoprotein

Because the enzyme assays showed that 9-modified Neu5Ac was transferred by both mammalian and bacterial enzymes, we were interested in using biotinylated Neu5Ac for several biological experiments. Starting from CMP-9″-amino-Neu5Ac **7**, we reacted it with sulfosuccinimidyl 6-(biotinamido)-hexanoate[15] (PIERCE, sulfo-NHS-LC-Biotin), which smoothly provided CMP-9″-biotinylated-Neu5Ac **16** (Scheme 2). Transfer of **16** to D-lactose was monitored by ESI-mass spectrometry and this reaction afforded the desired sialoside **19** in good yield (Scheme 3 and Fig. 1A: D-lactose and B:



Scheme 3. Reagents and conditions: (a) CMP-hexanamido-Neu5Ac, methyl umbelliferyl-D-lactose, BSA, alkaline phosphatase and bacterium sialyltransferase, sodium cacodylate buffer (pH 6.0, 50 mM, 0.5% triton X100) 37 °C; (b) CMP-9″-biotin-Neu5Ac, D-lactose, bacterial sialyltransferase HEPES buffer (pH 7.0, 100 mM), 37 °C.

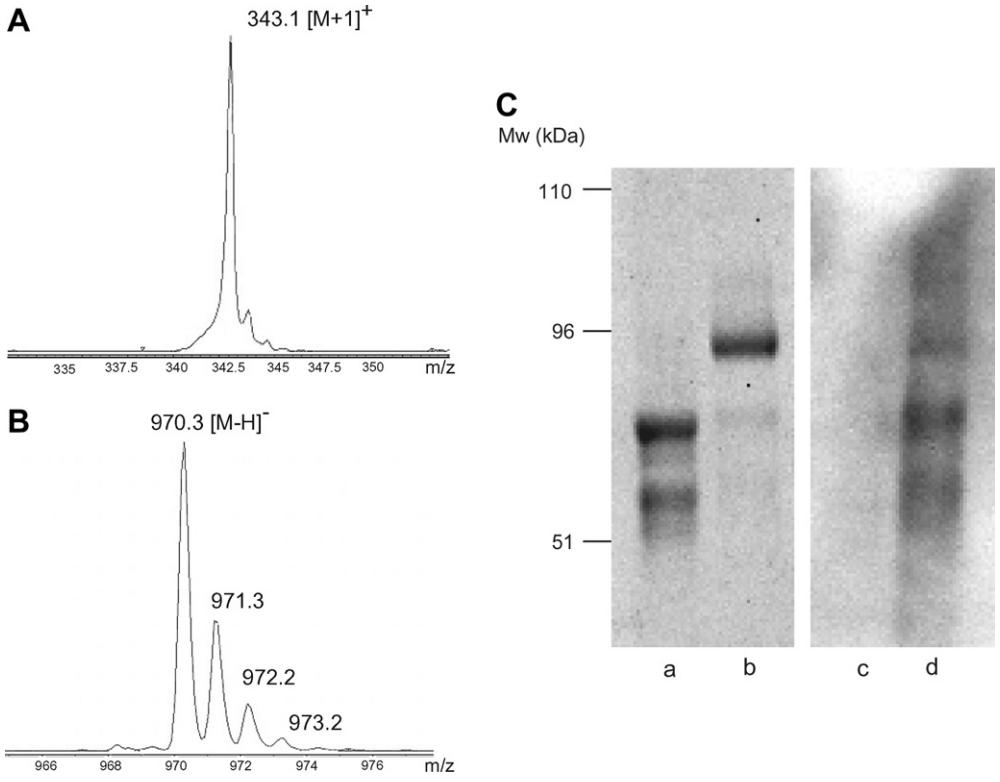


Figure 1. (A) ESI mass analysis of substrate (D-lactose); (B) ESI mass analysis of biotinylated Neu5Ac-lactose; (C) Lane a: asialofetuin stained by Sypro orange; lane b: biotinylated fetuin stained by Sypro orange; lane c: asialofetuin stained by HRP-conjugated streptavidin; lane d: biotinylated fetuin stained by HRP-conjugated streptavidin.

biotinylated-sialyl-D-lactose). In addition, this donor analogue could be transferred to a glycoprotein, asialo-Fetuin, which has complex type oligosaccharides. As shown in Figure 1C (lane a), commercially available asialofetuin (SIGMA) displays a number of glycoforms (several bands). The bands in lane b, stained by Sypro orange, correspond to biotinylated-sialylfetuin. Lanes c and d correspond with lanes a and b, respectively, but these were analyzed by Western blot. Although commercially available asialofetuin exhibits glycoforms, the sialyltransfer reaction clearly demonstrates that biotinylated glycoprotein was obtained in moderate yield. A biotinylated fucose has been known to be transferred by fucosyltransferase,[16] and our experiment demonstrates an additional biotinylation protocol by a bacterial enzyme. This finding will allow modification of glycoproteins and glycolipids on cells by use of CMP-9″-biotinylated-Neu5Ac.

3. Conclusion

We synthesized CMP-9″-modified-Neu5Ac derivatives and found that they could be transferred to oligosaccharides and asialoglycoproteins with *Photobacterium* α-(2→6)-sialyltransferase. These experiments indicate that the enzyme binds CMP-Neu5Ac, while the 9″-position of Neu5Ac does not interact with the enzyme. This finding will be useful for the modification of a number of biomolecules on a large scale.

4. Experimental

4.1. General

NMR spectra were recorded with JEOL EX-270 or Bruker AVANCE 400 instruments. The chemical shifts of ^{1}H NMR are presented in ppm and referenced to tetramethylsilane (0.00 ppm) in $CDCl_3$, HOD (4.81 ppm) in D_2O as an internal standard. The chemical shifts of ^{13}C NMR spectra are expressed in ppm and referenced to $CDCl_3$ (77.00 ppm) in $CDCl_3$. The chemical shifts of ^{31}P NMR spectra are expressed in ppm and referenced to H_3PO_4 (0.00 ppm) in D_2O. Thin-layer chromatography (TLC) was used DC-Platten Kieselgel 60 F_{254} (Merck). Column chromatography was carried out on Merck Silica gel 60 of 230–400 mesh. Methyl umbelliferyl-D-lactose was purchased from LAMBDA (Austria). ESI mass measurement was carried out on a Bruker Daltonics/Esquire3000 plus. HRMS (FAB) measurement was carried out on Shimazu-Kratos ConceptII. Bacterial sialyltransferase [EC 2.4.99.1] was isolated

from *Photobacterium damsela*[17] and rat river sialyltransferase [EC 2.4.99.1] was purchased from Novachem.

4.2. Methyl 5-acetamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate (2)

Thioglycoside **1**[7] (190 mg, 0.36 mmol, α:β = 1:2) was dissolved in acetone–H_2O (9.0 mL:1.0 mL) and then NBS (356 mg, 2.0 mmol) was added to this mixture. The mixture was stirred for 1 h at room temperature and then diluted with EtOAc and washed with 1 M $NaHCO_3$ solution. The organic phase was dried with $MgSO_4$ and then concentrated in vacuo. Purification of the residue by silica gel column chromatography (EtOAc) afforded alcohol **2** (158 mg, 90%). ^{1}H NMR ($CDCl_3$): δ 6.14 (d, 1H, J = 9.9 Hz, NH), 5.35 (dd, 1H, J = 2.0, 4.0 Hz, H-7), 5.24 (ddd, 1H, J = 5.9, 9.9, 10.6 Hz, H-4), 5.13 (bdd, 1H, J = 2.6, 8.6 Hz, H-8), 4.28 (dd, 1H, J = 10.6 Hz, H-6), 4.14 (ddd, 1H, H-5), 3.86 (s, 3H, OMe), 3.62 (dd, 1H, J = 13.2 Hz, H-9a), 3.36 (dd, 1H, J = 13.2 Hz, H-9b), 2.22 (dd, 1H, J = 10.0 Hz, H-3ax), 2.13 (dd, 1H, H-3eq), 2.16 2.13 2.02 1.90 (each s, each 3H, each Me); ^{13}C NMR ($CDCl_3$): δ 171.15, 171.03, 170.55, 170.31, 169.02, 94.97 (C-2), 73.32 (C-8), 71.32 (C-6), 69.27 (C-4), 68.64 (C-7), 53.32 (OMe) 50.64 (C-9), 36.08 (C-3); Anal. Calcd for $C_{18}H_{26}N_4O_{11}$: C, 45.57; H, 5.52; N, 11.81. Found: C, 45.62; H, 5.73; N, 11.71.

4.3. Cytidine-5′-monophospho-9″-azido-Neu5Ac

Methyl 5-acetamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate **2** (236 mg, 0.45 mmol) and 2-cyanoethyl 2′,3′-*O*,N^4, triacetylcytidine-5′-yl *N*,*N*-diisopropylphosphoramidite (632 mg, 1.10 mmol) were separately dried and then dissolved in MeCN (1.0 mL). The solution was cooled to −20 °C under an argon atmosphere. To this solution 1H tetrazole (95 mg, 1.35 mmol) was added and then stirred for 1 h. After completion of the reaction, the solution was warmed to room temperature. The solution was diluted with EtOAc (100 mL) and then poured into 1 M $NaHCO_3$. The organic phase containing the phosphite derivative was washed with $NaHCO_3$ solution and brine. The organic solution was dried with $MgSO_4$ and then concentrated in vacuo under 30 °C. Because purification of the phosphite and phosphate derivatives caused slight decomposition, isolation was done after a deprotection step. The phosphite residue was dissolved in MeCN (2.0 mL) and then *t*-BuOOH (2.5 M toluene solution, 1.76 mL, 4.40 mmol) was added to this solution at room temperature. After 1 h, the solution was diluted with EtOAc containing Me_2S (0.32 mL, 4.4 mmol) and washed with $NaHCO_3$ and brine. The organic phase was dried with $MgSO_4$ and concentrated in vacuo. To a solution of this residue in THF (1.0 mL) was added DBU (111 mg, 0.73 mmol) and then NaOMe (400 mg, 7.3 mmol) in MeOH–H_2O (4.2 mL:8.4 mL) was added to remove the acetyl group. After 12 h, the CMP-Neu5Ac derivative was extracted with H_2O and this solution was washed with CH_2Cl_2. The aqueous solution was lyophilized and then the residue was purified by column chromatography on silica gel (*n*-PrOH:20 mM NH_4OH, 4:1). Fractions containing the CMP-Neu5Ac derivative were pooled and then concentrated in vacuo. The residue was purified on a gel permeation column (Sephadex G-15, id. 1 cm × 100 cm, 5 mM NH_4OH solution). Fractions containing CMP-9-azido-Neu5Ac were pooled and then lyophilized. CMP-9-azido-Neu5Ac (111 mg) was obtained in a 40% yield. ^{1}H NMR (D_2O): δ 8.09 (d, 1H, J = 7.9 Hz, H-6), 6.24 (d, 1H, J = 7.9 Hz H-5), 6.08 (d, 1H, J = 4.0 Hz, H-1′), 3.74 (dd, 1H, J = 2.6, 13.2 Hz, H-9″a), 3.59 (dd, 1H, J = 6.0, 13.2 Hz, H-9″b), 3.56 (d, 1H, J = 9.9 Hz, H-7″), 2.58 (dd, 1H, J = 4.6, 13.2 Hz, H-3″eq), 2.15 (s, 3H, Me), 1.74 (ddd, 1H, J = 5.9, 11.2, 13.2 Hz, H-3″ax); HRMS (FAB) Calcd for $C_{20}H_{28}N_7O_{15}PNa_3$, $[M+Na]^+$: 706.1075. Found: 706.1117.

4.4. CMP-9″-amino-Neu5Ac

CMP-9-azido-Neu5Ac (50 mg, 78 μmol) was dissolved in 20 mM NH_4OH (1.0 mL) and then 5% $Pd/CaCO_3$ (5 mg) was added. This solution was stirred under a hydrogen atmosphere for 45 min. The solution was filtered through a Celite pad and the filtrate was concentrated in vacuo. Purification of the residue by gel permeation column (Sephadex G-15, id. 1 cm × 100 cm, 5 mM NH_4OH solution) afforded CMP-9″-amino-Neu5Ac 7 (45 mg, 94%). ^{1}H NMR (D_2O): δ 7.92 (d, 1H, J = 7.5 Hz, H-6), 6.14 (d, 1H, J = 7.7 Hz, H-5), 6.01 (d, 1H, J = 4.1 Hz, H-1′), 3.51 (dd, 1H, J = 2.4, 13.1 Hz, H-9a), 3.45 (br d, 1H, J = 9.1 Hz, H-7), 2.96 (dd, 1H, J = 10.3, 13.1 Hz, H-9b), 2.53 (dd, 1H, J = 4.7, 13.4 Hz, H-3eq), 2.09 (s, 3H, Me), 1.69 (ddd, 1H, J = 5.7, 11.8, 13.4 Hz, H-3ax); ^{31}P NMR (D_2O): δ −4.24; HRMS (FAB) Calcd for $C_{20}H_{30}N_5O_{15}PNa_3$, $[M+Na]^+$: 680.1170. Found: 680.1191.

4.5. CMP-9″-benzamido-Neu5Ac 12

Benzoic acid (100 mg, 0.82 mmol) and sulfohydroxy succinimide (160 mg, 0.82 mmol) were dissolved into DMF (2.0 mL). To this solution was added DCC (190 mg, 0.90 mmol) and then the solution was stirred for 24 h. This solution was cooled to 4 °C and the precipitate (urea) was filtered. To this filtrate was added EtOAc–hexane (1:1, 30 mL) and this mixture was stirred. Benzoic acid *N*-hydroxysulfosuccinimide **8** precipitate was collected through filtration and then dried in a desiccator [(170 mg, 71%), ^{1}H NMR (D_2O) δ 8.29–7.69 (m, 5H, Ph), 4.66 (dd, 1H, H-3′), 3.57 (dd, 1H,

H-4′a), 3.36 (dd, 1H, H-4′b); ^{13}C NMR ($CDCl_3$): δ 136.5 131.2 129.9 57.3 30.7]. To a solution of CMP-9″-amino-Neu5Ac (10 mg, 16 μmol) in phosphate buffer (pH 7.0, 20 mM, 1.0 mL) was added activated benzoic acid *N*-sulfosuccinimide (9.4 mg, 32 μmol) and this solution was stirred for 1 h. After completion of the reaction, the solution was lyophilized. The residue was purified by column chromatography on silica gel (*n*-PrOH:20 mM NH_4OH, 4:1). Fractions containing CMP-9″-benzamido-Neu5Ac were pooled and then concentrated in vacuo. The residue was purified on a gel permeation column (Sephadex G-15, id. 1 cm × 100 cm, 5 mM NH_4OH solution). Fractions containing CMP-9″-modified-Neu5Ac were pooled and then lyophilized. CMP-9″-benzamido-Neu5Ac **12** (8.2 mg, 68%) was obtained. ^{1}H NMR (D_2O): δ 7.99 (d, 1H, $J = 7.7$ Hz, H-6), 7.74 (d, 2H, $J = 7.6$ Hz, Ph), 7.62 (dd, 1H, $J = 7.6$, 7.6 Hz, Ph), 7.52 (dd, 2H, $J = 7.6$, 7.6 Hz, Ph), 6.10 (d, 1H, $J = 7.7$ Hz, H-5), 6.00 (d, 1H, $J = 4.7$ Hz, H-1′), 4.02 (dd, 1H, $J = 10.2$, 10.2 Hz, H-5″), 3.94 (dd, 1H, $J = 2.3$, 13.9 Hz, H-9″a), 3.47 (br d, 1H, $J = 9.0$ Hz, H-7), 3.43 (dd, 1H, $J = 8.2$, 13.8 Hz, H-9″b), 2.53 (dd, 1H, $J = 5.0$, 13.4 Hz, H-3eq), 2.07 (s, 3H, Me), 1.70 (ddd, 1H, $J = 6.2$, 11.8, 13.4 Hz, H-3ax); ^{31}P NMR (D_2O): δ −4.65; HRMS (FAB) Calcd for $C_{27}H_{34}N_5O_{16}PNa_3$, $[M+Na]^+$: 784.1432. Found: 784.1457.

4.6. CMP-9″-modified-Neu5Ac 13–15

Preparation of the phenyl-substituted alkylcarboxylic acid derivatives **9**–**11** and CMP-9″-modified-Neu5Ac **13**–**15** was performed in the same manner as in the preparation of CMP-9″-benzamide-Neu5Ac **12**. Synthetic yields of **13**–**15** were 63% (7.5 mg isolation), 65% (7.8 mg isolation) and 60% (7.5 mg isolation), respectively.

4.6.1. Data for 2-phenyl-acetic acid *N*-hydroxysulfosuccinimide. ^{1}H NMR (D_2O): δ 7.52–7.40 (m, 5H, Ph), 4.55 (dd, 1H, H-3′) 3.46 (dd, 1H, H-4′a), 3.26 (dd, 1H H-4′b), 3.16 (s, 2H, CH_2).

4.6.2. Data for 3-phenyl-propionic acid *N*-hydroxysulfosuccinimide. ^{1}H NMR (D_2O) δ 7.52–7.40 (m, 5H, Ph), 4.55 (dd, 1H, H-3′), 3.46 (dd, 1H, H-4′a), 3.26 (dd, 1H, H-4′b), 3.16 (s, CH_2); ^{13}C NMR ($CDCl_3$): δ 129.47 129.12, 127.40, 57.12, 32.63, 30.29, 30.55.

4.6.3. Data for 6-phenyl-hexanoic acid *N*-hydroxysulfosuccinimide. ^{1}H NMR (D_2O): δ 7.47–7.31 (m, 5H, Ph), 4.56 (dd, 1H, H-3′), 3.47 (dd, 1H, H-4′a), 3.27 (dd, 1H, H-4′b), 2.79 2.74 1.84 1.74 1.50 (m, 10H, CH_2); ^{13}C NMR ($CDCl_3$): δ 129.47 129.12, 127.40, 57.12, 32.63 30.29, 30.55.

4.6.4. Data for CMP-9″-phenylacetamido-Neu5Ac 13. ^{1}H NMR (D_2O): δ 7.99 (d, 1H, $J = 7.7$ Hz, H-6), 7.46–7.33 (m, 5H, Ph), 6.12 (d, 1H, $J = 7.7$ Hz, H-5), 6.04 (d, 1H, $J = 4.5$ Hz, H-1′), 4.18 (d, 1H, $J = 10.7$ Hz, H-6″), 4.10 (ddd, 1H, $J = 4.2$, 11.1, 11.1 Hz, H-4″), 4.03–3.94 (m, 2H, H-5″, 8″), 3.71 (dd, 1H, $J = 3.0$, 13.9 Hz, H-9″a), 3.37 (br d, 1H, $J = 9.6$ Hz, H-7″), 3.22 (dd, 1H, $J = 8.5$, 13.9 Hz, H-9″b), 2.52 (dd, 1H, $J = 4.6$, 13.0 Hz, H-3″eq), 2.07 (s, 3H, Me), 1.67 (ddd, 1H, $J = 6.2$, 11.1, 13.0 Hz, H3″ax); ^{31}P NMR (D_2O): δ −4.67; HRMS (Fab) Calcd for $C_{28}H_{36}N_5O_{16}PNa_3$, $[M+Na]^+$: 798.1589. Found: 798.1587.

4.6.5. Data for CMP-phenylpropionamido-Neu5Ac 14. ^{1}H NMR (D_2O): δ 7.97 (d, 1H, $J = 7.5$ Hz, H-6), 7.42–7.28 (m, 5H, Ph), 6.14 (d, 1H, $J = 7.5$ Hz, H-5), 6.02 (d, 1H, $J = 4.7$ Hz, H-1′), 4.18 (d, 1H, $J = 10.7$ Hz, H-6″), 4.10 (ddd, 1H, $J = 4.8$, 10.8, 10.8 Hz, H-4″), 3.99 (d, 1H, $J = 10.2$ Hz, H-5″), 3.94 (ddd, 1H, $J = 2.7$, 9.6, 9.6 Hz, H-8″), 3.64 (dd, 1H, $J = 2.6$, 13.9 Hz, H9″a), 3.36 (br d, 1H, $J = 9.6$ Hz, H-7″), 3.14 (dd, 1H, $J = 8.4$, 13.4 Hz, H-9″b), 2.94 (t, 2H, CH_2), 2.59 (t, 2H, CH_2), 2.52 (dd, 1H, $J = 4.6$, 13.0 Hz, H-3″eq), 2.07 (s, 3H, Me), 1.68 (ddd, 1H, $J = 6.0$, 11.5, 13.0 Hz, H-3″ax); ^{31}P NMR (D_2O): δ −4.61; HRMS (FAB) Calcd for $C_{29}H_{38}N_5O_{16}PNa_3$, $[M+Na]^+$: 812.1745. Found: 812.1782.

4.6.6. Data for CMP-phenylhexanamido-Neu5Ac 15. ^{1}H NMR (D_2O): δ 7.97 (d, 1H, $J = 7.5$ Hz, H-6), 7.42–7.28 (m, 5H, Ph), 6.12 (d, 1H, $J = 7.5$ Hz, H-5), 6.00 (d, 1H, $J = 4.7$ Hz, H-1′), 4.20 (d, 1H, $J = 10.3$ Hz, H-6″), 4.11 (ddd, 1H, $J = 4.8$, 10.4, 10.4 Hz, H-4″), 3.99 (d, 1H, $J = 10.2$ Hz, H-5″), 3.66 (dd, 1H $J = 2.6$, 13.9 Hz, H-9″a), 3.38 (br d, 1H, $J = 9.8$ Hz, H7″), 3.20 (dd, 1H, $J = 8.7$, 13.9 Hz, H-9″b), 2.66 (q, 2H, CH_2), 2.52 (dd, 1H $J = 4.6$, 13.4 Hz, H-3″eq), 2.28 (q, 2H, CH_2), 2.08 (s, 3H, Me), 1.68 (m, 3H, H-3″ax, CH2), 1.36 (m, 2H, CH_2); ^{31}P NMR (D_2O): δ −4.73; HRMS (FAB) Calcd for $C_{32}H_{44}N_5O_{16}PNa_3$, $[M+Na]^+$: 854.2215. Found: 854.2184.

4.7. Sialyltransferase assay

Enzyme assays were through the use of a fluorescence labeling method previously reported. In short, assay solutions (25 μL) containing CMP-9″-modified-Neu5Ac (0.1–1 mM), disaccharide (5 mM of lactose for the *Photobacterium* enzyme and 5 mM LacNAc for the rat liver enzyme), BSA (0.1 mg/mL) and an appropriate amount of sialyltransferase in sodium cacodylate buffer (pH 5.0, 100 mM) were incubated at 37 °C. To estimate acceptor K_m values, the concentration of disaccharide acceptor was varied (0.75–5 mM) and the concentration of

CMP-Neu5Ac was fixed (350 μM). The reaction was stopped before 15% consumption of the CMP-Neu5Ac derivative and then the transferred Neu5Ac derivative was estimated by the DMB-labeling method.[14] K_m and V_{max} values were estimated by a Lineweaver–Burk plot. The kinetic parameters are summarized in Table 1. The V_{max} values in Table 1 are expressed as relative compared to that of native CMP-Neu5Ac (*Photobacterium* enzyme: V_{max} (CMP-Neu5Ac) 0.04 pmol/min; rat liver enzyme: V_{max} (CMP-Neu5Ac) 58.8 pmol/min).

4.8. Phenyl-hexanamido-sialyllactose-UM 18

A solution containing CMP-hexanamido-Neu5Ac (2.7 mg, 3.2 μmol), methyl umbelliferyl-D-lactose (1.1 mg, 3.2 μmol), BSA (1 mg), alkaline phosphatase (5 U) and sialyltransferase (83 mU) in sodium cacodylate buffer (pH 6.0, 50 mM, 500 μL–0.5% triton × 100) was incubated for 2 h at 37 °C. The solution was directly applied to an HPLC column (ODS id. 0.5 × 15 mm, linear gradient 25 mM NH_4OAc → 25 mM NH_4OAc–MeCN 1:1 over 30 min) and then desalting of the sialoside was performed with same ODS column (linear gradient H_2O → H_2O–MeCN 1:1 over 20 min). The sialoside was obtained in 60% yield (2.0 mg). ^1H NMR (D_2O): δ 7.76 (d, 1H, Ar), 7.29–7.12 (m, Ar), 6.27 (s, 1H), 5.23 (d, 1H, $J = 7.7$ Hz, H-1), 4.45 (d, 1H, $J = 7.7$ Hz, H-1′), 3.43 (d, 1H, $J = 9.4$ Hz, H-7″), 3.30 (dd, 1H, $J = 7.6$, 13.8 Hz, H-9″a), 2.71 (dd, 1H, $J = 4.41$, 12.2 Hz, H-3″eq), 2.53 (t, 2H, $J = 7.6$ Hz, CH_2), 2.24 (t, $J = 7.6$ Hz, CH_2), 2.04 (s, 3H, Me), 1.71 (dd, 1H, $J = 12.2$, 12.2 Hz, H3″ax), 1.56 (m, 2H, CH_2); MS (FAB) Calcd for $C_{45}H_{61}N_2O_{21}$, $[M+H]^+$: 964.4. Found: 964.4.

4.9. CMP-9″-biotinylated-Neu5Ac

To a solution of CMP-9-amino-Neu5Ac (11.0 mg, 16.1 μmol) in satd $NaHCO_3$ (100 μL)–H_2O (400 μL) was added sulfosuccinimidyl 6-(biotinamido)-hexanoate[15] (PIERCE, sulfo-NHS-LC-Biotin) (19 mg, 33.5 μmol) and this solution was stirred at room temperature for 24 h. After completion, this solution was directly applied to a gel permeation column (SephadexG-15, id. 1 cm × 100 cm, H_2O) and the fractions containing CMP-9″-biotinylated-Neu5Ac **16** were pooled and then lyophilized. CMP-9″-biotinylated-Neu5Ac **16** was obtained in 72%. ^1H NMR (D_2O): δ 8.01 (d, 1H, $J = 7.8$ Hz, H-6), 6.17 (d, 1H, $J = 7.8$ Hz, H-5), 6.03 (d, 1H, $J = 4.6$ Hz, H-1′), 4.19 (d, 1H, $J = 10.3$ Hz, H-6″), 4.11 (ddd, 1H, $J = 4.8$, 10.4, 10.4 Hz, H-4″), 3.99 (d, 1H, $J = 10.2$ Hz, H-5″), 3.68 (br d, 1H $J = 13.9$ Hz, H-9″a), 3.38 (br d, 1H $J = 9.7$ Hz, H-7″), 3.34 (dd, 1H, $J = 4.5$, 13.9 Hz, H-9″b), 2.52 (dd, 1H $J = 4.6$, 13.4 Hz, H-3″eq), 2.09 (s, 3H, Me), 1.81–1.30 (m, H-3″ax, biotin); ^{31}P NMR (D_2O): δ −4.66; HRMS (FAB) Calcd for $C_{36}H_{55}N_8O_{18}PSNa_3$, $[M+Na]^+$: 1019.2787. Found: 1019.2809.

4.10. Enzymatic assay for the transfer of biotinylated Neu5Ac to D-lactose

A solution containing CMP-9″-biotin-Neu5Ac (200 μg), D-lactose (10 μg), and bacterial sialyltransferase (40 mU) in 100 mM HEPES buffer (pH 7.0, 25 μL) was incubated at 37 °C. After 30 min, an aliquot of this reaction was directly subjected to ESI mass spectrometry to analyze the product molecular weight. The desired sialoside was obtained in quantitative yield based on mass spectroscopic analysis (Fig. 1A). ESI mass Calcd for $C_{39}H_{65}N_5O_{21}S$, $[M-H]^-$: 970.4. Found: 970.3.

4.11. Enzymatic assay for the transfer of biotinylated Neu5Ac to asialofetuin

A solution containing CMP-9″-biotin-Neu5Ac (400 μg), asialofetuin (sialidase treated, SIGMA, Cat#A-1908, Lot# 93H9510, 20 μg), alkaline phosphatase (5 mU) and bacterial sialyltransferase (80 mU) in 100 mM HEPES buffer (pH 7.0, 100 μL) was incubated at 37 °C. After 48 h, an aliquot of this reaction was subjected to SDS-page analysis and Western blot. Asialofetuin and biotinylated sialylfetuin were subjected to electrophoresis on SDS-PAGE (10% acrylamide; Diichi Chemicals, Tokyo, Japan) after denaturation. Asialofetuin and biotinylated sialylfetuin (Fig. 1C: lanes a and b, respectively) were stained by Sypro orange (Molecular Probes, Invitrogen, Cat# S6650). Asialofetuin and biotinylated sialylfetuin (Fig. 1C: lanes c and d, respectively) were transferred to Immobilon-P membrane (Millipore, Billerica, MA). The blotted membranes were incubated with HRP-conjugated streptavidin (BD Pharmingen, San Diego) according to the manufacturer's instructions. After washing, biotinylated asialofetuin was visualized using ECL-plus Western Blotting Detection System (GE Healthcare, Uppsala, Sweden). The desired biotinylated-fetuin was obtained in good yield based on SDS-page and Western blot (Fig. 1C).

Acknowledgments

This work was partially supported by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science, Sports and Culture, Japan (No. 09780531) and the Takamura Foundation. The authors thank Mr. Masayoshi Kusama for mass spectroscopic analysis.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2007.05.029.

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