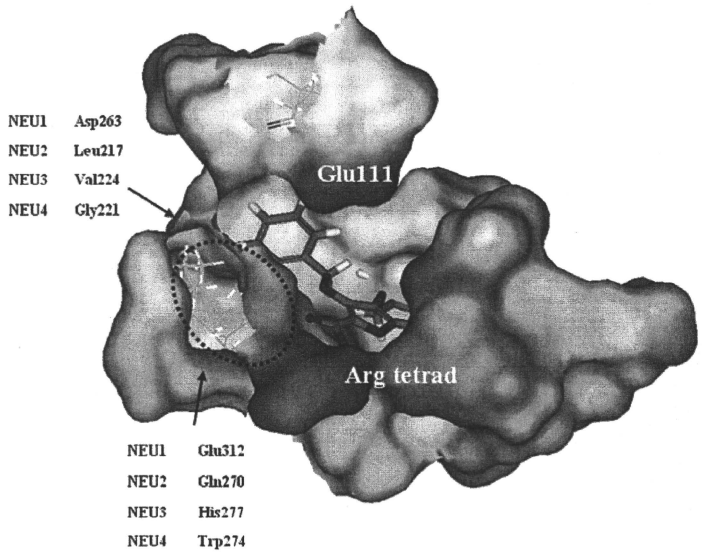

Fig. 5 Electrostatic potentials of the NEU2 surface around the ligand (compound **5**). Surface residue Glu111, the Arg tetrad, and an amino acid difference in the proposed specificity among NEUs are indicated

viruses, bacteria, and protozoa. The marginal selectivity of compound **5** to NEU2, NEU3, and NEU4 over NEU1 can be conferred to the amino acid(s) differences at the binding subsite (hydrophobic amino acids in NEU3 [Val224, His277] and NEU4 [Gly221, Trp274,], mixed hydrophobic and polar amino acids in NEU2 [Leu217, Gln270]) and completely polar amino acids in NEU1 (Asp263, Glu312), where an *O*-benzyl group of **5** is likely to form interactions (Fig. 5).

Conclusion

In summary, this study describes the discovery of a novel scaffold for human sialidase inhibition using the crystal structure of NEU2. The structure-based virtual screening results suggested several novel putative inhibitors for human sialidase inhibition. The best-hit compound, **5**, was chosen for the preliminary studies and was tested for its inhibitory effect on all four human sialidases. Although **5** showed moderate inhibitory activities, with a marginal selectivity over human lysosomal sialidase (NEU1), it seems attractive for further improvement, as it can make extra contacts with the conserved arginine triad of sialidase enzymes compared to other known inhibitors. Significant structural changes, for instance, introduction of more

flexible substituents into the core dicarboxylic furan ring system, may lead to improved affinity and selectivity not only for human sialidases, but also for sialidases of microbial origin.

Acknowledgments The first author (S.M.) is grateful to the Ministry of Education, Culture, Sports, Science & Technology, Government of Japan (MEXT), and Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, for his past and current financial assistance, respectively.

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CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.201002184

A First Total Synthesis of a Hybrid-Type Ganglioside Associated with Amyotrophic Lateral Sclerosis-Like Disorder**

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Abstract: The hybrid ganglioside X1, which was identified in the bovine brain, was synthesized for the first time. Ganglioside X1 is believed to be involved in the development of amyotrophic lateral sclerosis-like disorders in patients with neurological disorders after treatment with bovine brain gangliosides. A convergent approach using two branched glycan units, the GM2-core trisaccharide and the lacto-ganglio tetrasaccharide, efficiently provided the highly branched heptasaccharide part of ganglioside X1, which was conjugated with the ceramide part to produce the protected ganglioside X1. Global deprotection delivered homogenous ganglioside X1, with which serum from the patient was reacted.

Keywords: gangliosides · glycosides · glycosylation · immunoassays · natural products · total synthesis

Introduction

Amyotrophic lateral sclerosis (ALS) is characterized by a progressive and selective loss of motor neurons in the brain and spinal cord, and is a devastating disorder of still unknown etiology and pathogenesis. Some patients who had been misdiagnosed as having ALS carried IgM autoantibodies against the GM1 ganglioside, which is a member of a group of sialic acid containing glycosphingolipids that is enriched in nervous tissues. The patients improved after plasmapheresis and immunosuppressant treatment, suggesting that the condition is autoimmune mediated.[1] In contrast, gangliosides extracted from bovine brain, which have been used as a therapeutic agent for many neurological disorders, might have caused the patient to display ALS-like disorder.[2] The patient's IgM reacted strongly with GM2 and GalNAc-GD1a, which are minor gangliosides in bovine brain, but not with asialo-GM2, GM1, or GD1a, indicating that their terminal trisaccharides [GalNAcβ(1,4)(NeuAcα(2,3)Gal)] (GM2-core) are the epitope.[2,3] The patient who underwent plasmapheresis improved quickly, and the serum possessed killing activity to GM2-containing cells, suggesting that IgM antibodies with anti-GM2 reactivity function in the development of the ALS-like disorder.[2,4] By using the patient's IgM antibodies, further, novel GM2-core-containing gangliosides, X1 and X2 were identified in bovine brain. They were characterized as hybrid-type gangliosides, lacto-ganglio-series gangliosides, in which the core sequence of lacto-series (Galβ(1,3)GlcNAcβ(1,3)Gal) and ganglio-series (GalNAcβ(1,4)Gal) are hybridized.[3]

Their unusual structures may be immunogenic in humans to induce the pathogenic antibodies with anti-GM2 reactivity. Otherwise, X1 (**1**) and X2 (**2**) may be present in humans and may be target molecules for autoantibodies in some patients who are misjudged to have ALS. The novel GM2-core-containing gangliosides are required to identify such treatable patients with immunotherapy. Herein, we report a first total synthesis of **1** in detail.

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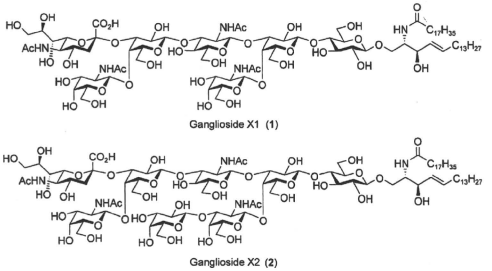
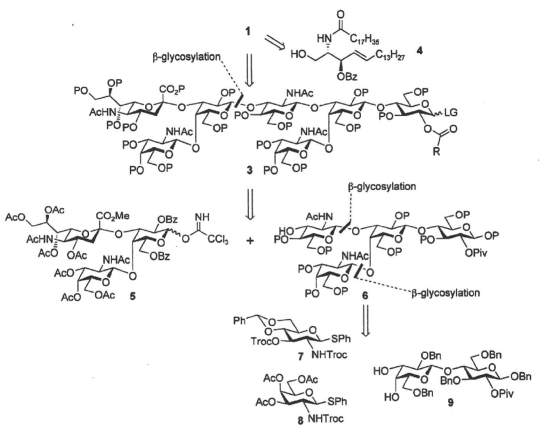
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201002184.



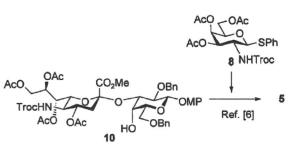
Ganglioside X1 (1)

Ganglioside X2 (2)

To build the entire ganglioside X1 structure, the glycan unit needed to be accessed through a highly efficient synthetic route and be designed to have a form suitable for straightforward introduction of the ceramide unit. The glycan structure of **1** has two galactosamine residues at the C4 hydroxyl groups of the external and internal galactose residues of sialyl lactotetraose. At first glance, direct and double galactosaminylation of the sialyl lactotetraose appeared to be a feasible approach to the assembly of the glycan part of target **1**. However, we also presumed that the glycan structure would defy this approach because the reactivity of the C4 hydroxyl group of galactose, flanked by the sialic acid residue at C-3, is markedly low. Similarly, this low reactivity could impede the selective protection–deprotection of the C4 hydroxyl group. Based on these conjectures, we devised a convergent approach to synthesize the target molecule (Scheme 1). First, the target (**1**) was divided into the glycan unit **3** and ceramide unit **4**. Then, the glycan unit was disassembled at the β(1,3)-linkage between Gal and GlcNAc, following a previous report on sialyl Lewis A,[5] thus providing two branched fragments, **5** and **6**. The left fragment, containing the GM2-core sequence, was designed as a trichloroacetimidate donor, which was developed by our research group.[6] The right fragment, containing the lacto- and ganglio-series glycan sequence, was further disassembled into glucosaminyl donor **7**, galactosaminyl donor **8**,[7] and lactose acceptor **9**.[8] For the final connection of the full-length glycan unit and ceramide, the lactose unit was designed with pivaloate at C2 to prevent orthoester formation.



Scheme 1. Retrosynthetic analysis of target compound **1**. P = protecting group, LG = leaving group, Bz = benzoyl, Piv = pivaloyl, Troc = 2,2,2-trichloroethoxycarbonyl, Bn = benzyl.

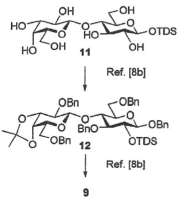


Scheme 2. Synthesis of terminal trisaccharide unit **5**. MP = *p*-methoxyphenyl.

As shown in Scheme 2, the terminal GM2-core unit **5** could be readily synthesized according to a previously reported method from *N*-Troc galactosaminyl (GalN) donor **8** and *N*-Troc sialyl galactose **10**.[6,9] which can be rapidly produced on a large scale because of its high tendency to crystallize.

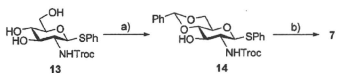
The synthesis of the tetrasaccharide counterpart commenced with the preparation of lactose acceptor **9**. The 2-*O*-pivaloyl derivative of lactoside was successfully prepared from 1-*O*-silylated lactose **11** through 2-*O*-silylated derivative **12** according to the procedure reported by Schmidt and coworkers (Scheme 3).[8b]

The glucosaminyl donor (GlcN) donor was designed in a 4,6-benzylidenated form to further incorporate the GM2-core



Scheme 3. Synthesis of lactosyl acceptor **9** bearing a pivaloate at the C2 position. TDS = thexyldimethylsilyl.

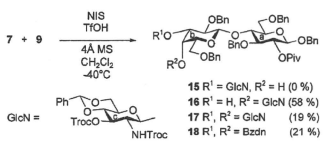
unit at the C3 hydroxyl group after assembly of the tetrasaccharide. The Troc group was chosen as the directing functionality for stereoselective β-glucosaminylation and also as a temporary protecting group for the C3 hydroxyl group, thus leading to structure **7**, which was synthesized from the reported compound **13**[10] through benzylidanation and introduction of the Troc group at the C3 hydroxyl group of **14** (Scheme 4).[11]



Scheme 4. Synthesis of glucosaminyl donor **7**. a) BDA, CSA/CH_3CN-THF, 61% (crystalline); b) TrocCl/Pyr, 94%. BDA = benzaldehyde dimethyl acetal, CSA = (±)-camphor-10-sulfonic acid, Pyr = pyridine.

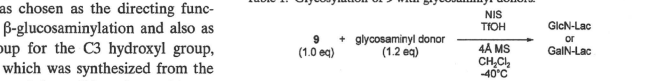
To establish the hybrid branches stemming from the galactose residue, the GlcN donor **7** was first reacted with the 3′,4′-diol lactose acceptor **9**, aiming for regioselective glycosylation at the more reactive C3 hydroxyl group rather than at the C4 hydroxyl group.

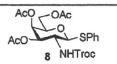
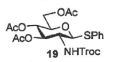
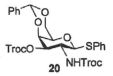
Although there are many examples of specific glycosylation of the C3 hydroxyl group of the 3,4-diol of galactose moiety by using glycosyl donors, especially sialic acid donors,[6,8,9,12] the GlcN donor **7** preferentially provided the β(1,4′)-linked product (Scheme 5). Thus, the reaction of **7** (1.2 equiv) and **9** (1.0 equiv), promoted by NIS-TfOH[13] in CH_2Cl_2 at −40 °C, yielded trisaccharide **16** (58%) with double glycosylated **17** (19%) and 3′,4′-benzylidenated lactoside **18** (21%). The position of GlcN within **16** was confirmed by comparison of the chemical shifts in ^1H NMR spectra of **16** and its acetylated derivative. Upon acetylation (Ac_2O, Pyr), the H-3 of Gal shifted downfield from $\delta = 3.8$ to 4.9 ppm, indicating that the C3 hydroxyl group was free in compound **16**. The structures of compounds **17** and **18** were confirmed by ^1H NMR spectroscopic analysis and by mass spectrometry. In contrast, the desired product **15** was not obtained from this reaction. Disappointingly, replacing GlcN **7** with GalN donor **8** in this reaction resulted in unregioselective glycosylation, generating β(1,4′)-linked (35%), β(1,3′)-linked (42%), and double glycosylated (21%) products (Table 1).



Scheme 5. Unexpected regioselectivity in the glycosylation of **9** with **7**. NIS = *N*-iodosuccinimide, TfOH = trifluoromethanesulfonic acid, MS = molecular sieves, Bzdn = benzylidene.

Table 1. Glycosylation of **9** with glycosaminyl donors.

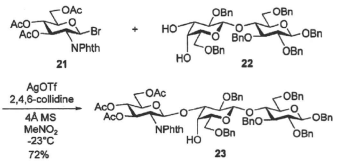


Entry	Donor	Yield of products [%] (1,3′)-linked	(1,4′)-linked	bis-linked
1	 **8**	42	35	21
2	 **19**	35	22	39
3	 **20**	63	15	20

Furthermore, triacetyl GlcN donor **19** and benzylidenated GalN donor **20**[14] were investigated. It was likely that the triacetyl GalN donor **8** and the triacetyl GlcN donor **19** were unselective (Table 1, entry 2). However, in the case of the benzylidenated GalN donor **20**, the C3 hydroxyl group was preferentially glycosylated to afford the (1,3′)-linked (63%), (1,4′)-linked (15%), and double glycosylated (20%) products as anomeric mixtures, showing a regioselectivity opposite to that observed with benzylidenated GlcN donor **7** (Table 1, entry 3). This unexpected regioselectivity also contrasts with the reaction of *N*-phthaloyl GlcN donor **21** and lactose acceptor **22** reported by Ogawa and co-workers,[15] in which the corresponding β(1,3′)glucosaminyl product **23** was obtained as the predominant product (Scheme 6).

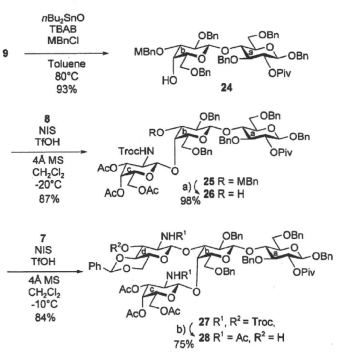
Although the unexpected results are interesting, the principle underlying these phenomena is still unclear. Because the regioselectivity is incongruous with the targeted structure, we redesigned the lactosyl acceptor as a monoalcohol derivative.

The C3 hydroxyl group of **9** was capped with the *p*-methoxybenzyl (MBn) group by stannylation and etherification (dibutyltin(IV) oxide (DBTO), MBnCl, and TBAB)[16] to



Scheme 6. The C3-selective glycosylation of 3′,4′-diol lactosyl acceptor **22** with glucosaminyl donor **21** developed by Ogawa et al.[15] Phth = phthaloyl.

provide **24** in 93% yield (Scheme 7). As expected, glycosylation of the lactose acceptor **24** (1.0 equiv) and GalN donor **8** (1.5 equiv) proceeded smoothly at −20°C to yield the gangliotriose (Gg_3) sequence **25** in 87% yield as a single



Scheme 7. Synthesis of unit **28** of the glycan part of the target compound. a) TFA/CH_2Cl_2, 98%; b) i) Zn, AcOH/MeOH; ii) Ac_2O/CH_2Cl_2-MeOH, 75%. TBAB = *n*-tetrabutylammonium bromide, MBn = *p*-methoxybenzyl, TFA = trifluoroacetic acid.

isomer. Upon treatment with trifluoroacetic acid (TFA) in CH_2Cl_2, the Gg_3 derivative was converted into C3-hydroxy acceptor **26** (98%). Subsequent glycosylation with GlcN donor **7**, mediated by NIS-TfOH in CH_2Cl_2, was also successful in converting Gg_3 into lacto-ganglio-tetraose **27** in 84% yield. Finally, successive unmasking of the three Troc groups by treatment with zinc in AcOH–MeOH and N-acetylation, delivered fragment **28** in 75% yield over two steps.

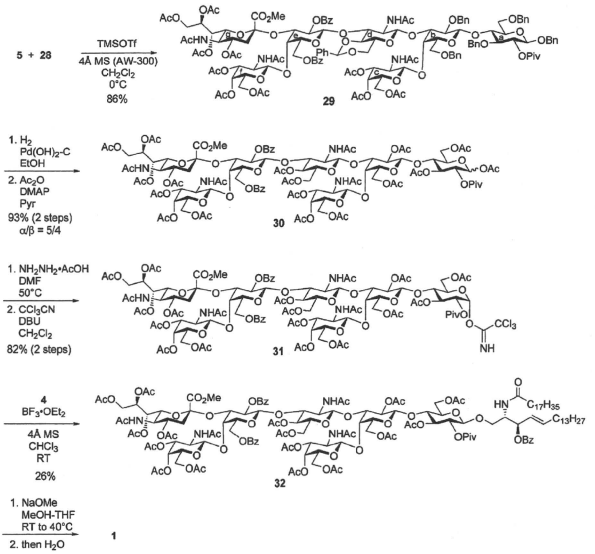
Fortunately, the convergent assembly of the heptasaccharide part proceeded in accordance with our initial expectation (Scheme 8). Thus, the left fragment, GM2-core donor **5**, was glycosidated with the right fragment **28** by using Schmidt's method[17] to provide the glycan framework of X1 **29** in 86% yield. The heptasaccharide structure of **29** was confirmed by mass spectrometry (ESI-TOF; *m/z* calcd for $C_{135}H_{160}N_4O_{52}$ [1/2*M*+Na]$^+$ 1357.4892; found 1357.4893 [1/2 *M*+Na]$^+$). However, the stereochemistry of the new glycosidic bond between Gal and GlcN could not be defined as the β form because the signals from the H-1 and H-2 protons of Gal observed by ^{1}H NMR spectroscopic analysis overlapped with other signals such as the H-1 proton from the inner GalN, H-3 from the terminal GalN, H-4 from the terminal Neu5Ac, and the CH_2 protons from the benzyl group. The protecting groups of heptasaccharide **29** were then manipulated to generate the corresponding imidate donor. This process began with sequential hydrogenolysis of the benzyl groups catalyzed by $Pd(OH)_2$/C and acetylation to provide 1-*O*-acetyl intermediate **30**, which was then advanced by hydrazinolysis of the anomer acetate and trichloroacetimidate formation by treatment with trichloroacetonitrile (CCl_3CN) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU),[18] to generate the imidate donor **31** in 76% over four steps. At this stage, the anomeric configuration of the terminal Gal was assigned as the β form from ^{1}H NMR spectroscopic analysis, whereby the H-1 proton was observed at $\delta = 4.88$ ppm as a doublet with a coupling constant of 7.5 Hz, and the H-2 proton was observed at $\delta = 5.29$ ppm as a double doublet ($J_{2,3} = 9.6$ Hz).

Finally, the obtained full-length glycan donor **31** was glycosidated with the known ceramide acceptor **4**.[19] The best result was obtained when the reaction was conducted in $CHCl_3$ at room temperature upon activation by BF_3·OEt_2 (1.2 equiv for the donor), producing the protected ganglioside X1 (**32**) in 26% yield.[8a,20] In other approaches, use of trimethylsilyl trifluoromethanesulfonate (TMSOTf) as a catalyst diminished the coupling yield to around 5 to 10%. Under the optimized reaction conditions, 61% of the glycosyl donor was recovered as the hemiacetal, which could again be converted into the donor **31** to be used for the conjugation of ceramide. Formation of the orthoester was not observed. To approach ganglioside X1, the acyl protecting groups were removed by applying Zemplén's method, followed by saponification of the methyl ester on the sialic acid residue, to successfully yield 13.5 mg of target compound **1** in pure form.

The synthetic compound **1** was immunostained with IgM antibodies and compared to natural X1 on a thin-layer chromatographic plate. Figure 1 shows that synthetic **1** had similar mobility to natural X1 from bovine brain, and that serum IgM antibodies from the patient with ALS-like disorder[2] reacted with both natural and synthetic X1.

Conclusion

We have succeeded in the total synthesis of the lacto-ganglio series ganglioside X1. The convergent approach employing the GM2-core unit and the lacto-ganglio tetraosyl unit allowed access to the target structure with high efficiency, producing homogenous X1 in sufficient quantity for biological study. Furthermore, we confirmed that the patient's serum IgM bound to the synthesized **1** on a 96-well microtiter



Scheme 8. Assembly of glycan part **29** and final conjugation with ceramide to deliver target structure **1**. TMSOTf = trimethylsilyl trifluoromethanesulfonate, DMAP = 4-dimethylaminopyridine, DMF = *N*,*N*-dimethylformamide, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene.

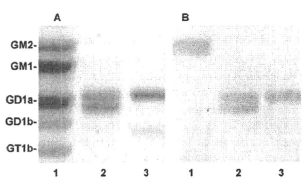


Figure 1. Reactivity of the synthesized X1 with serum that recognizes GM2 epitope [GalNAc β1-4 (NeuAc α2-3) Gal β]. A) TLC plate stained with the orcinol reagent for hexose. B) Immunostained chromatogram that had been overlaid first with serum from a patient with an ALS-like disorder[2] then with peroxidase-conjugated anti-human μ-chain specific antibodies. Lane 1: Authentic GM2, GM1, GD1a, GD1b, and GT1b. Lane 2: GM2-epitope containing gangliosides, X1 and X2, from bovine brain.[3] Lane 3: The synthesized X1 in the present study. Orcinol reagent stains GM2, GM1, GD1a, GD1b, GT1b, X1, and X2 from bovine brain and the synthesized X1. Serum IgM antibodies from the patient strongly bind to GM2, X1, and X2 from bovine brain, and the synthesized X1.

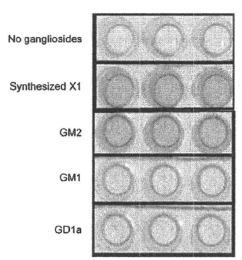


Figure 2. Reactivity of the synthesized X1 with serum that recognizes the GM2 epitope, developed on a microtiter plate. The patient's serum IgM antibodies react with X1 and GM2, but with neither GM1 nor GD1a. IgM antibodies against the X1, GM2, GM1, and GD1a (5 pmol/well) were measured in the patient's serum (1:500 dilution) according to reported procedure.[21] For structures of GM2, GM1, and GD1a, see the Supporting Information.

plate (Figure 2). Using the synthesized **1**, we will test serum samples from a number of patients who were misdiagnosed with ALS to identify those treatable with immunotherapy.

Experimental Section

General procedures: ^1H and ^{13}C NMR spectra were recorded with JEOL JNM-ECA600 spectrometers. ^1H NMR chemical shifts (δ) are expressed in ppm relative to the signal of Me_4Si as an internal standard, except when the samples were measured in [D_6]acetone, in which case the shift was referenced against the signal of acetone ($\delta = 2.09$ ppm). ^{13}C NMR chemical shifts (δ) are expressed in ppm relative to the signal of the solvent. High-resolution mass spectrometry (HRMS) was performed with a Bruker Daltonics micrOTOF (ESI-TOF) mass spectrometer. Specific rotations were measured with a Horiba SEPA-300 high-sensitivity polarimeter. Melting points were determined with an AS ONE ATM-01 apparatus. Molecular sieves were purchased from Wako Chemicals and dried at 300 °C for 2 h in a muffle furnace prior to use. Reactions were carried out under an atmosphere of argon unless otherwise specified. Solvents as reaction media were dried over molecular sieves and used without further purification. TLC analyses were performed on Merck TLC plates (silica gel $60F_{254}$ on glass), and compounds were visualized either by exposure to UV light (254 nm), by spraying with 10% H_2SO_4 solution in EtOH, or by treatment with ninhydrin reagent, followed by heating. Flash column chromatography on silica gel (Fuji Silysia Co., 80 mesh and 300 mesh) or Sephadex (Pharmacia LH-20) were performed with the solvent systems (v/v) specified. Evaporation and concentration were conducted in vacuo.

Compound 7: 2,2,2-Trichloroethyl chloroformate (200 μL, 1.45 mmol) was added to a solution of **14** (513 mg, 0.959 mmol) in pyridine (4.8 mL) at 0 °C, and the mixture was stirred for 30 min at RT. Upon completion of the reaction (confirmed by TLC analysis; EtOAc/hexane, 1:2), the reaction mixture was quenched by addition of MeOH at 0 °C and the residual solvent was removed by coevaporation with toluene. The residue was dissolved in EtOAc and the solution was washed with 2M aqueous HCl, saturated aqueous $NaHCO_3$, and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane, 1:5) to give **7** (641 mg, 94%), which was recrystallized from EtOAc/hexane. M.p. 158–160 °C; $[\alpha]_D = -26.0$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.51$–7.33 (m, 10H; 2 Ph), 5.52 (s, 1H; PhC*H*), 5.39 (t, $J_{2,3} = J_{3,4} = 9.7$ Hz, 1H; H-3), 5.29 (d, $J_{2,NH} = 8.3$ Hz, 1H; NH), 5.11 (d, $J_{1,2} = 10.3$ Hz, 1H; H-1), 4.79–4.71 (m, 4H; 2 CH_2), 4.41 (dd, $J_{5,6} = 5.2$ Hz, $J_{gem} = 10.7$ Hz, 1H; H-6), 3.82 (t, $J_{5,6'} = 10.3$ Hz, 1H; H-6′), 3.74 (t, $J_{4,5} = 9.7$ Hz, 1H; H-4), 3.68–3.60 ppm (m, 2H; H-2, H-5); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 153.9$, 153.8, 136.6, 133.1, 131.5, 129.2, 129.2, 128.6, 128.2, 126.1, 101.4, 95.2, 94.2, 86.8, 78.4, 76.9, 74.6, 70.4, 68.4, 55.5 ppm; HRMS: *m/z* calcd for $C_{25}H_{23}Cl_6NO_8SNa^+$: 729.9168 [*M*+Na]$^+$; found: 729.9168.

Compounds 16, 17, and 18: Molecular sieves (4 Å, 200 mg) were added to a solution of compounds **7** (98 mg, 137 μmol) and **9** (101 mg, 115 μmol) in CH_2Cl_2 (2.50 mL). The suspension was stirred for 30 min at −40 °C, whereupon NIS (51 mg, 227 μmol) and TfOH (2.0 μL, 22.6 μmol) were added. Stirring was continued for 40 min at −40 °C, at which point completion of the reaction was indicated by TLC (EtOAc/hexane 2:5, developed twice). The reaction mixture was filtered through Celite and the removed molecular sieves were washed with $CHCl_3$. The combined filtrate and washings were extracted with $CHCl_3$, and organic layer was washed with saturated aqueous $NaHCO_3$, saturated aqueous $Na_2S_2O_3$, and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane 1:4→1:3→2:5) to give **16** (97 mg, 58%), **17** (57 mg, 19%), and **18** (30 mg, 21%) as diastereoisomers (a/b = 13:17).

Compound 16: $[\alpha]_D = -45.5$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR (600 MHz, [D_6]acetone): $\delta = 7.50$–7.21 (m, 30H; 6 Ph), 7.18 (d, $J_{2,NH} = 8.9$ Hz, 1H; NH), 5.72 (s, 1H; PhC*H*), 5.38 (t, $J_{2,3} = J_{3,4} = 9.7$ Hz, 1H; H-3c), 5.36 (d, $J_{1,2} = 8.2$ Hz, 1H; H-1c), 5.21 (d, $J_{gem} = 10.3$ Hz, 1H; CH_2), 5.01 (t, $J_{1,2} = J_{2,3} = 8.6$ Hz, 1H; H-2a), 4.99 (d, $J_{gem} = 11.0$ Hz, 1H; CH_2), 4.89 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.87–4.73 (m, 6H; OH, 4 CH_2), 4.66 (d, $J_{1,2} = 8.6$ Hz, 1H; H-1a), 4.62 (d, $J_{gem} = 10.3$ Hz, 1H; CH_2), 4.57 (d, $J_{1,2} = 7.6$ Hz, 1H; H-1b), 4.54 (d, $J_{gem} = 11.0$ Hz, 1H; CH_2), 4.52 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.42 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.39 (d, $J_{gem} = 12.3$ Hz, 1H; CH_2), 4.21–4.18 (m, 2H; H-6c, CH_2), 4.13 (d, $J_{3,4} = 1.4$ Hz, 1H; H-4b), 4.06 (t, $J_{3,4} = J_{4,5} = 9.3$ Hz, 1H; H-4a), 3.95 (t, $J_{4,5} = 9.7$ Hz, 1H; H-4c), 3.93–3.75 (m, 5H; H-6a, H-6′a, H-3b, H-2c, H-6′c), 3.72 (t, $J_{2,3} = J_{3,4} = 8.6$ Hz, 1H; H-3a), 3.68–3.57 (m, 4H; H-5a, H-2b, H-6b, H-5c), 3.55 (t, $J_{5,6} = J_{5,6'} = 5.5$ Hz, 1H; H-5b), 3.32 (dd, $J_{5,6'} = 5.5$ Hz, $J_{gem} = 9.6$ Hz, 1H; H-6′b), 1.16 ppm (s, 9H; *t*Bu); ^{13}C NMR (150 MHz, [D_6]acetone): $\delta = 176.9$, 155.4, 154.5, 140.3, 140.2, 139.5, 138.6, 138.5, 129.6, 129.0, 129.0, 128.9, 128.9, 128.8, 128.8, 128.6, 128.5, 128.3, 128.3, 128.2, 128.1, 128.0, 127.8, 127.7, 127.1, 103.5, 102.7, 101.9, 100.8, 96.9, 95.7, 81.9, 81.8, 79.6, 78.3, 77.4, 77.3, 77.1, 76.0, 75.1, 74.9, 74.6, 74.5, 73.6, 73.5, 73.1, 70.9, 70.0, 69.0, 68.9, 66.7, 58.0, 39.2, 27.4 ppm; HRMS: *m/z* calcd for $C_{71}H_{77}Cl_6NO_{20}Na^+$: 1496.3062 [*M*+Na]$^+$; found: 1496.3063.

Compound 17: $[\alpha]_D = -37.0$ ($c = 0.5$, $CHCl_3$); ^{1}H NMR (600 MHz, [D_6]DMSO): $\delta = 8.24$ (d, $J_{2,NH} = 8.9$ Hz, 1H; NHd), 7.41–7.19 (m, 35H; 7 Ph), 5.77 and 5.75 (2 s, 2H; 2 PhC*H*), 5.37 (t, $J_{2,3} = J_{3,4} = 9.6$ Hz, 1H; H-3c), 5.30–5.27 (m, 2H; H-1c, H-3d), 5.04 (d, $J_{gem} = 10.3$ Hz, 1H; CH_2), 4.99 (d, $J_{1,2} = 8.2$ Hz, 1H; H-1d), 4.98–4.94 (m, 2H; 2 CH_2), 4.90 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.85–4.82 (m, 3H; 3 CH_2), 4.76–4.68 (m, 4H; H-1a, H-2a, 2 CH_2), 4.63 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.58 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.53 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.37–4.32 (m, 4H; H-1b, H-6d, 2 CH_2), 4.26 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.23 (d, $J_{gem} = 12.3$ Hz, 1H; CH_2), 4.20 (t, $J_{3,4} = J_{4,5} = 9.6$ Hz, 1H; H-4d), 4.15 (brs, 1H; H-4b), 4.14–4.07 (m, 3H; H-2d, H-6c, CH_2), 4.05 (d, $J_{gem} = 10.3$ Hz, 1H; CH_2), 3.91 (t, $J_{3,4} = J_{4,5} = 9.6$ Hz, 1H; H-4c), 3.84–3.71 (m, 6H; H-4a, H-3b, H-2c, H-5c, H-6′c, H-6′d), 3.61 (dd, $J_{5,6} = 4.5$ Hz, $J_{gem} = 12.0$ Hz, 1H; H-6a), 3.58–3.42 (m, 5H; H-3a, H-5a, H-6′a, H-2b, H-5d), 3.41 (t, $J_{5,6} = J_{5,6'} = 5.5$ Hz, 1H; H-5b), 3.16 (m, 1H; H-6′b), 1.11 ppm (s, 9H; *t*Bu); ^{13}C NMR (150 MHz, [D_6]DMSO): $\delta = 176.2$, 154.6, 153.6, 153.4, 139.1, 139.1, 139.0, 138.4, 137.6, 137.4, 137.4, 129.1, 128.4, 128.2, 128.2, 128.2, 127.8, 127.8, 127.0, 127.6, 127.6, 127.5, 127.3, 127.3, 127.2, 126.4, 126.3, 102.6, 101.7, 100.5, 100.3, 99.6, 96.4, 95.8, 95.0, 95.0, 81.9, 80.5, 78.8, 77.9, 77.0, 76.6, 76.5, 76.2, 76.1, 74.7, 74.5, 74.4, 74.2, 73.6, 73.6, 72.5, 72.4, 72.3, 72.1, 70.2, 68.9, 67.9, 67.5, 65.3, 64.7, 56.6, 55.8, 38.4, 27.0 ppm; HRMS: *m/z* calcd for $C_{90}H_{94}Cl_{12}N_2O_{28}Na^+$: 2093.2148 [*M*+Na]$^+$; found: 2093.2139.

Compound 18a: $[\alpha]_D = -8.4$ ($c = 1.6$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.41$–7.16 (m, 30H; 6 Ph), 5.95 (s, 1H; PhC*H*), 5.15 (t, $J_{1,2} = J_{2,3} = 8.6$ Hz, 1H; H-2a), 4.92 (d, $J_{gem} = 11.0$ Hz, 1H; CH_2), 4.88 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.82 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.74 (d, $J_{gem} = 11.0$ Hz, 1H; CH_2), 4.62–4.59 (m, 3H; 3 CH_2), 4.48 (d, $J_{1,2} = 8.6$ Hz, 1H; H-1a), 4.48 (d, $J_{1,2} = 8.3$ Hz, 1H; H-1b), 4.45 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.39 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.34 (t, $J_{2,3} = J_{3,4} = 6.2$ Hz, 1H; H-3b), 4.23 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.13 (d, $J_{4,5} = 1.4$, 9.6 Hz, 1H; H-4b), 4.09 (t, $J_{3,4} = J_{4,5} = 9.3$ Hz, 1H; H-4a), 3.90 (dd, $J_{5,6} = 4.1$ Hz, $J_{gem} = 11.0$ Hz, 1H; H-6a), 3.77 (dd, $J_{5,6'} = 1.4$ Hz, $J_{gem} = 11.0$ Hz, 1H; H-6′a), 3.67 (t, $J_{1,2} = J_{2,3} = 8.6$ Hz, 1H; H-3a), 3.65–3.61 (m, 2H; H-5b, H-6b), 3.49–3.44 (m, 3H; H-5a, H-2b, H-6′b), 1.13 ppm (s, 9H; *t*Bu); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 176.7$, 138.7, 138.2, 138.2, 137.2, 129.2, 128.4, 128.4, 128.3, 128.0, 127.9, 127.8, 127.8, 127.7, 127.7, 127.6, 127.6, 127.6, 127.5, 127.2, 126.3, 103.3, 102.0, 99.8, 81.1, 80.3, 77.9, 76.5, 75.5, 73.9, 73.7, 73.5, 73.4, 73.3, 72.3, 72.2, 70.1, 69.0, 68.0, 38.7, 27.1 ppm; HRMS: *m/z* calcd for $C_{59}H_{64}O_{12}Na^+$: 987.4290 [*M*+Na]$^+$; found: 987.4290.

Compound 18b: $[\alpha]_D = -15.4$ ($c = 1.3$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.43$–7.16 (m, 30H; 6 Ph), 5.91 (s, 1H; PhC*H*), 5.13 (t, $J_{1,2} = J_{2,3} = 8.6$ Hz, 1H; H-2a), 4.89 (d, $J_{gem} = 10.3$ Hz, 1H; CH_2), 4.87 (d, $J_{gem} = 11.0$ Hz, 1H; CH_2), 4.68 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.60–4.55 (m, 4H; 4 CH_2), 4.46–4.41 (m, 3H; H-1a, H-1b, CH_2), 4.39 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.25 (d, $J_{gem} = 11.0$ Hz, 1H; CH_2), 4.19–4.16 (m, 2H; H-3b, H-4b), 4.06 (t, $J_{3,4} = J_{4,5} = 9.3$ Hz, 1H; H-4a), 3.85 (dd, $J_{5,6} = 6.9$ Hz, $J_{gem} = 9.7$ Hz, 1H; H-6a), 3.76–3.72 (m, 2H; H-6′a, H-5b), 3.65 (dd, $J_{5,6} = 6.9$ Hz, $J_{gem} = 9.7$ Hz, 1H; H-6b), 3.61 (dd, $J_{2,3} = 8.6$ Hz, $J_{3,4} = 9.3$ Hz, 1H; H-3a), 3.45 (dd, $J_{5,6'} = 6.2$ Hz, $J_{gem} = 9.7$ Hz, 1H; H-6′b), 3.42 (m, 1H; H-5a), 3.37 (m, 1H; H-2b), 1.13 ppm (s, 9H; *t*Bu); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 176.7$, 138.7, 138.3, 138.2, 138.2, 137.8, 137.2, 129.2, 128.4, 128.3, 128.3, 128.3, 128.2, 128.0, 127.8, 127.8, 127.8, 127.7, 127.6, 127.5, 127.5, 127.1, 126.6, 104.3, 102.1, 99.8, 81.1, 80.9, 79.0, 76.3, 76.1, 75.4, 73.6, 73.5, 73.3, 73.3, 72.2, 71.8, 70.1, 68.8, 68.0, 38.7, 27.1 ppm; HRMS: *m/z* calcd for $C_{59}H_{64}O_{12}Na^+$: 987.4290 [*M*+Na]$^+$; found: 987.4290.

Compound 24: Dibutyltin(IV) oxide (37 mg, 148 μmol), 4-methoxybenzyl chloride (18.6 μL, 137 μmol), and tetrabutylammonium bromide (45 mg, 138 μmol) were added to a solution of **9** (101 mg, 115 μmol) in toluene (1.1 mL). The mixture was stirred for 9 h at 80 °C (completion of the reaction was confirmed by TLC analysis; EtOAc/hexane, 1:1), then triethylamine was added and the mixture was concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane 1:3) to give **24** (106 mg, 93%). $[\alpha]_D = -7.0$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.34$–7.17 (m, 27H; 6 ArH), 6.84 (d, $J = 8.2$ Hz, 2H; ArH), 5.13 (t, $J_{1,2} = J_{2,3} = 8.3$ Hz, 1H; H-2a), 4.97 (d, $J_{gem} = 11.0$ Hz, 1H; CH_2), 4.87 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.76 (s, 2H; CH_2), 4.63 (d, $J_{gem} = 11.0$ Hz, 1H; CH_2), 4.62–4.57 (m, 3H; 3 CH_2), 4.56 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.46 (d, $J_{1,2} = 8.3$ Hz, 1H; H-1a), 4.42–4.40 (m, 2H; H-1b, CH_2), 4.33 (d, $J_{gem} = 12.0$ Hz, 1H; CH_2), 4.28 (d, $J_{gem} = 11.0$ Hz, 1H; CH_2), 4.05 (t, $J_{3,4} = J_{4,5} = 9.3$ Hz, 1H; H-4a), 3.96 (brs, 1H; H-4b), 3.82 (dd, $J_{5,6} = 4.5$ Hz, $J_{gem} = 10.3$ Hz, 1H; H-6a), 3.79 (s, 3H; OCH_3), 3.73 (d, $J_{gem} = 10.3$ Hz, 1H; H-6′a), 3.63 (dd, $J_{2,3} = 8.3$ Hz, $J_{3,4} = 9.3$ Hz, 1H; H-3a), 3.57 (t, $J_{1,2} = J_{2,3} = 8.6$ Hz, 1H; H-2b), 3.52 (dd, $J_{5,6} = 7.2$ Hz, $J_{gem} = 8.6$ Hz, 1H; H-6b), 3.43 (dd, $J_{4,5} = 9.3$ Hz, $J_{5,6} = 4.5$ Hz, 1H; H-5a), 3.35 (dd, $J_{2,3} = 8.6$ Hz, $J_{3,4} = 4.1$ Hz, 1H; H-3b), 3.35 (dd, $J_{5,6'} = 4.1$ Hz, $J_{gem} = 8.6$ Hz, 1H; H-6′b), 3.31 (dd, $J_{5,6} = 7.2$ Hz, $J_{5,6'} = 4.1$ Hz, 1H; H-5b), 2.39 (s, 1H; OH), 1.13 ppm (s, 9H; *t*Bu); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 176.7$, 159.3, 138.8, 138.6, 138.2, 138.0, 137.2, 129.9, 129.4, 128.3, 128.2, 128.2, 128.0, 127.8, 127.7, 127.6, 127.6, 127.6, 127.5, 127.4, 127.0, 113.8, 102.7, 99.7, 81.0, 80.7, 79.4, 76.4, 75.5, 75.3, 73.8, 73.4, 73.1, 72.8, 72.2, 71.7, 70.0, 68.5,

68.1, 66.2, 55.2, 38.7, 27.1 ppm; HRMS: *m/z* calcd for $C_{60}H_{68}O_{13}Na^+$: 1019.4552 $[M+Na]^+$; found: 1019.4552.

Compound 25: Molecular sieves (4 Å, 415 mg) were added to a solution of **8** (125 mg, 218 μmol) and **24** (145 mg, 145 μmol) in CH_2Cl_2 (3.65 mL). The suspension was stirred for 30 min at −20 °C, whereupon NIS (74 mg, 327 μmol) and TfOH (2.9 μL, 32.7 μmol) were added. Stirring was continued for 15 min at −20 °C, when completion of the reaction was indicated by TLC (EtOAc/hexane 1:2, developed twice). The reaction mixture was filtered through Celite and the removed molecular sieves were washed with $CHCl_3$. The combined filtrate and washings were extracted with $CHCl_3$, and organic layer was washed with saturated aqueous $NaHCO_3$, saturated aqueous $Na_2S_2O_3$, and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane, 1:2→1:1→3:2, then EtOAc/Toluene, 1:5) to give **25** (184 mg, 87 %). $[\alpha]_D = -12.0$ ($c = 1.0$, $CHCl_3$); ^1H NMR (600 MHz, $[D_6]$DMSO): $\delta = 7.57$ (d, $J_{2,NH} = 9.0$ Hz, 1H; NH), 7.43–7.16 (m, 27H; 6 ArH), 6.84 (d, $J = 8.2$ Hz, 2H; ArH), 5.30 (d, $J_{3,4} = 2.7$ Hz, 1H; H-4c), 5.18 (dd, $J_{2,3} = 11.4$ Hz, $J_{3,4} = 2.7$ Hz, 1H; H-3c), 5.03 (d, $J_{gem} = 10.3$ Hz, 1H; CH_2), 4.84 (d, $J_{1,2} = 8.3$ Hz, 1H; H-1c), 4.78 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.76–4.68 (m, 5H; H-1a, H-2a, 3 CH_2), 4.66 (d, $J_{gem} = 10.3$ Hz, 1H; CH_2), 4.55 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.54 (d, $J_{gem} = 12.3$ Hz, 1H; CH_2), 4.44–4.41 (m, 3H; 3 CH_2), 4.38 (d, $J_{1,2} = 7.5$ Hz, 1H; H-1b), 4.31 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.24 (d, $J_{gem} = 12.3$ Hz, 1H; CH_2), 4.11 (dd, $J_{5,6} = 6.2$ Hz, $J_{gem} = 11.0$ Hz, 1H; H-6c), 4.05 (dd, $J_{5,6'} = 6.2$ Hz, $J_{gem} = 11.0$ Hz, 1H; H-6′c), 4.02 (s, 1H; H-4b), 3.99 (t, $J_{5,6} = J_{5,6'} = 6.2$ Hz, 1H; H-5c), 3.96 (d, $J_{gem} = 12.3$ Hz, 1H; CH_2), 3.81 (t, $J_{3,4} = J_{4,5} = 9.3$ Hz, 1H; H-4a), 3.77–3.65 (m, 8H; H-3a, H-5a, H-6a, H-6′a, H-2c, OCH_3), 3.56 (dd, $J_{1,2} = 7.5$ Hz, $J_{2,3} = 8.6$ Hz, 1H; H-2b), 3.53 (dd, $J_{5,6} = 4.2$ Hz, $J_{gem} = 11.0$ Hz, 1H; H-6b), 3.47 (d, $J_{2,3} = 8.6$ Hz, 1H; H-3b), 3.40 (dd, $J_{5,6} = 4.2$ Hz, $J_{5,6'} = 5.2$ Hz, 1H; H-5b), 3.28 (dd, $J_{5,6'} = 5.2$ Hz, $J_{gem} = 11.0$ Hz, 1H; H-6′b), 2.12, 1.97 and 1.87 (3×s, 9H; 3 Ac), 1.14 ppm (s, 9H; *t*Bu); ^{13}C NMR (150 MHz, $[D_6]$DMSO): $\delta = 176.2$, 170.2, 170.1, 169.6, 158.9, 154.3, 139.0, 138.9, 138.8, 138.3, 137.5, 130.8, 129.6, 128.8, 128.4, 128.3, 128.3, 128.2, 127.8, 127.8, 127.7, 127.5, 127.5, 127.4, 127.2, 113.7, 102.1, 101.5, 99.6, 96.4, 80.8, 80.3, 79.5, 79.3, 76.4, 74.7, 74.4, 74.3, 73.5, 73.5, 73.4, 72.5, 72.3, 72.3, 71.0, 70.3, 70.2, 69.8, 69.7, 67.8, 66.7, 61.6, 55.1, 52.5, 40.2, 38.4, 27.0, 20.6, 20.6, 20.5 ppm; HRMS: *m/z* calcd for $C_{75}H_{86}Cl_3NO_{22}Na^+$: 1480.4599 $[M+Na]^+$; found: 1480.4599.

Compound 26: TFA (41.0 μL, 552 μmol) was added to a solution of **25** (101 mg, 69.0 μmol) in CH_2Cl_2 (1.4 mL) at 0 °C, and the mixture was stirred for 7.5 h at RT (completion of the reaction was confirmed by TLC; EtOAc/hexane, 1:1), then the reaction mixture was quenched by the addition of triethylamine at 0 °C. The residue was extracted with $CHCl_3$ and the solution was washed with saturated aqueous $NaHCO_3$ and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane 2:3) to give **26** (90 mg, 98 %). $[\alpha]_D = -23.5$ ($c = 1.0$, $CHCl_3$); ^1H NMR (600 MHz, $[D_6]$DMSO): $\delta = 7.65$ (d, $J_{2,NH} = 8.9$ Hz, 1H; NH), 7.44–7.18 (m, 25H; 5 Ph), 5.30 (d, $J_{3,OH} = 5.5$ Hz, 1H; OH), 5.29 (d, $J_{3,4} = 3.5$ Hz, 1H; H-4c), 5.12 (dd, $J_{2,3} = 11.0$ Hz, $J_{3,4} = 3.5$ Hz, 1H; H-3c), 5.03 (d, $J_{gem} = 10.3$ Hz, 1H; CH_2), 4.99 (d, $J_{1,2} = 8.3$ Hz, 1H; H-1c), 4.85 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.83 (d, $J_{gem} = 10.3$ Hz, 1H; CH_2), 4.78–4.73 (m, 3H; H-2a, 2 CH_2), 4.70 (d, $J_{1,2} = 8.3$ Hz, 1H; H-1a), 4.60 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.55 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.44–4.40 (m, 3H; H-1b, 2 CH_2), 4.35 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.28 (d, $J_{gem} = 12.3$ Hz, 1H; CH_2), 4.10 (dd, $J_{5,6} = 5.5$ Hz, $J_{gem} = 11.0$ Hz, 1H; H-6c), 4.05 (dd, $J_{5,6'} = 6.2$ Hz, $J_{gem} = 11.0$ Hz, 1H; H-6′c), 4.00 (brs, 1H; H-5c), 3.96 (d, $J_{gem} = 12.3$ Hz, 1H; CH_2), 3.90 (s, 1H; H-4b), 3.84 (m, 1H; H-2c), 3.83 (t, $J_{3,4} = J_{4,5} = 9.6$ Hz, 1H; H-4a), 3.73 (dd, $J_{5,6} = 4.2$ Hz, $J_{gem} = 11.0$ Hz, 1H; H-6a), 3.70–3.61 (m, 4H; H-3a, H-5a, H-6′a, H-3b), 3.53 (dd, $J_{5,6} = 4.2$ Hz, $J_{gem} = 10.5$ Hz, 1H; H-6b), 3.50–3.46 (m, 2H; H-2b, H-5b), 3.25 (dd, $J_{5,6'} = 6.6$ Hz, $J_{gem} = 10.5$ Hz, 1H; H-6′b), 2.16, 1.97 and 1.87 (3×s, 9H; 3 Ac), 1.14 ppm (s, 9H; *t*Bu); ^{13}C NMR (150 MHz, $[D_6]$DMSO): $\delta = 176.2$, 170.1, 170.1, 169.6, 153.9, 139.3, 139.0, 138.8, 138.4, 137.6, 128.8, 128.4, 128.2, 128.2, 127.8, 127.7, 127.5, 127.5, 127.4, 127.3, 127.2, 102.3, 101.5, 99.7, 96.5, 80.7, 80.3, 76.4, 76.3, 74.8, 74.4, 73.6, 73.5, 73.0, 72.5, 72.3, 70.9, 70.2, 69.9, 69.6, 67.9, 66.9, 61.6, 52.6, 38.4, 27.0, 20.6, 20.6 ppm; HRMS: *m/z* calcd for $C_{67}H_{78}Cl_3NO_{21}Na^+$: 1360.4024 $[M+Na]^+$; found: 1360.4026.

Compound 27: Molecular sieves (4 Å, 450 mg) were added to a solution of **7** (130 mg, 183 μmol) and **26** (163 mg, 122 μmol) in CH_2Cl_2 (3.0 mL). The suspension was stirred for 30 min at −10 °C, whereupon NIS (83 mg, 366 μmol) and TfOH (3.2 μL, 36.6 μmol) were added. Stirring was continued for 40 min at −10 °C (completion of the reaction was indicated by TLC, EtOAc/hexane 1:1). The reaction mixture was filtered through Celite and the removed molecular sieves were washed with $CHCl_3$. The combined filtrate and washings were extracted with $CHCl_3$, and organic layer was washed with saturated aqueous $NaHCO_3$, saturated aqueous $Na_2S_2O_3$, and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/hexane 1:2→1:1) to give **27** (198 mg, 84 %). $[\alpha]_D = -35.0$ ($c = 1.0$, $CHCl_3$); ^1H NMR (600 MHz, $[D_6]$ DMSO): $\delta = 8.23$ (d, $J_{2,NH} = 8.9$ Hz, 1H; NHd), 7.42–7.17 (m, 30H; 6 Ph), 7.08 (d, $J_{2,NH} = 9.6$ Hz, 1H; NHc), 5.68 (s, 1H; PhC*H*), 5.32 (d, $J_{3,4} = 3.4$ Hz, 1H; H-4c), 5.29 (t, $J_{2,3} = J_{3,4} = 10.0$ Hz, 1H; H-3d), 5.25 (dd, $J_{2,3} = 11.7$ Hz, $J_{3,4} = 3.4$ Hz, 1H; H-3c), 5.16 (d, $J_{1,2} = 8.2$ Hz, 1H; H-1c), 5.00 (d, $J_{1,2} = 8.9$ Hz, 1H; H-1d), 5.00 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.98 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.87 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.85 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.78 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.74–4.69 (m, 4H; H-1a, H-2a, 2 CH_2), 4.58 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.58 (d, $J_{gem} = 11.0$ Hz, 1H; CH_2), 4.53 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.36 (d, $J_{1,2} = 7.6$ Hz, 1H; H-1b), 4.36 (d, $J_{gem} = 10.3$ Hz, 1H; CH_2), 4.32 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.30–4.21 (m, 5H; H-6c, H-4d, H-6d, 2 CH_2), 4.15 (d, $J_{3,4} = 2.1$ Hz, 1H; H-4b), 4.13–4.02 (m, 4H; H-5c, H-6′c, H-2d, CH_2), 3.95 (m, 1H; H-2c), 3.93 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 3.83 (t, $J_{5,6'} = J_{gem} = 10.0$ Hz, 1H; H-6′d), 3.77–3.73 (m, 2H; H-4a, H-3b), 3.62 (dd, $J_{5,6} = 3.9$ Hz, $J_{gem} = 10.7$ Hz, 1H; H-6a), 3.60–3.43 (m, 7H; H-3a, H-5a, H-6′a, H-2b, H-5b, H-6b, H-5d), 3.18 (dd, $J_{5,6'} = 7.9$ Hz, $J_{gem} = 12.3$ Hz, 1H; H-6′b), 2.19, 1.98 and 1.91 (3×s, 9H; 3×Ac), 1.13 ppm (s, 9H; *t*Bu); ^{13}C NMR (150 MHz, $[D_6]$DMSO): $\delta = 176.2$, 170.2, 170.0, 169.8, 154.6, 154.5, 153.4, 139.1, 139.0, 138.8, 138.4, 137.6, 137.3, 129.1, 128.7, 128.3, 128.2, 128.1, 127.7, 127.7, 127.7, 127.6, 127.5, 127.3, 127.2, 126.3, 102.5, 101.6, 100.6, 100.2, 99.6, 96.6, 95.7, 95.0, 82.3, 80.4, 78.7, 77.9, 76.5, 76.3, 76.1, 74.7, 74.6, 74.2, 73.7, 73.5, 73.0, 72.5, 72.3, 72.1, 71.0, 70.2, 69.8, 69.5, 67.9, 67.5, 67.2, 65.2, 61.7, 55.9, 52.5, 38.4, 27.0, 20.7, 20.6 ppm; HRMS: *m/z* calcd for $C_{86}H_{95}Cl_9N_2O_{29}Na^+$: 1957.3109 $[M+Na]^+$; found: 1957.3103.

Compound 28: Zinc powder (3.00 g) was added to a solution of **27** (204 mg, 105 μmol) in a mixture of MeOH (5.2 mL) and AcOH (5.2 mL), and the mixture was stirred for 30 min at RT (completion of the reaction was confirmed by TLC analysis; $CHCl_3$/MeOH, 20:1). The reaction mixture was filtered through Celite and the removed zinc powder was washed with $CHCl_3$. The combined filtrate and washings were concentrated and the residue was extracted with $CHCl_3$. The organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried (Na_2SO_4), concentrated, and exposed to high vacuum for 4 h. The residue was treated with a solution of Ac_2O (24.0 μL, 254 μmol) in MeOH (600 μL) and CH_2Cl_2 (600 μL) for 40 min (completion of the reaction was confirmed by TLC analysis; $CHCl_3$/MeOH, 15:1). The reaction mixture was extracted with EtOAc and this solution was washed with saturated aqueous $NaHCO_3$ and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 50:1→40:1→30:1) to give **28** (118 mg, 75 %). $[\alpha]_D = -41.5$ ($c = 1.0$, $CHCl_3$); ^1H NMR (600 MHz, CD_3CN): $\delta = 7.52$–7.20 (m, 30H; 6 Ph), 7.16 (d, $J_{2,NH} = 10.3$ Hz, 1H; NHc), 6.75 (d, $J_{2,NH} = 9.7$ Hz, 1H; NHd), 5.63 (s, 1H; PhC*H*), 5.32 (d, $J_{3,4} = 3.4$ Hz, 1H; H-4c), 5.10 (d, $J_{1,2} = 8.9$ Hz, 1H; H-1c), 5.04 (dd, $J_{2,3} = 11.7$ Hz, $J_{3,4} = 3.4$ Hz, 1H; H-3c), 5.01 (d, $J_{gem} = 10.3$ Hz, 1H; CH_2), 4.78 (t, $J_{1,2} = J_{2,3} = 8.9$ Hz, 1H; H-2a), 4.77 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.69 (d, $J_{gem} = 11.0$ Hz, 1H; CH_2), 4.65 (d, $J_{1,2} = 8.9$ Hz, 1H; H-1d), 4.61 (d, $J_{gem} = 12.4$ Hz, 1H; CH_2), 4.56 (d, $J_{gem} = 11.0$ Hz, 1H; CH_2), 4.53 (d, $J_{1,2} = 8.9$ Hz, 1H; H-1a), 4.47 (d, $J_{gem} = 10.3$ Hz, 1H; CH_2), 4.42 (d, $J_{gem} = 11.7$ Hz, 1H; CH_2), 4.37 (m, 1H; H-2c), 4.35 (d, $J_{1,2} = 8.2$ Hz, 1H; H-1b), 4.30–4.26 (m, 3H; H-6d, 2 CH_2), 4.17 (dd, $J_{5,6} = 5.5$ Hz, $J_{gem} = 9.6$ Hz, 1H; H-6c), 4.08 (d, $J_{3,4} = 2.8$ Hz, 1H; H-4b), 4.07–3.98 (m, 4H; H-5c, H-6′c, H-2d, CH_2), 3.86 (t, $J_{3,4} = J_{4,5} = 9.3$ Hz, 1H; H-4a), 3.81 (t, $J_{5,6'} = J_{gem} = 10.0$ Hz, 1H; H-6′d), 3.77 (t, $J_{2,3} = J_{3,4} = 9.7$ Hz, 1H; H-3d), 3.72–3.66 (m, 3H; H-6a, H-4d, OH), 3.62–3.59 (m, 2H; H-3a, H-3b), 3.55 (d, $J_{gem} = 9.6$ Hz, 1H; H-6′a), 3.51–3.43 (m, 3H; H-5a, H-6b, H-5d), 3.40–3.37 (m, 2H; H-2b, H-5b), 3.18 (dd, $J_{5,6'} = 6.2$ Hz, $J_{gem} = 10.3$ Hz, 1H; H-6′b), 2.18, 1.96, 1.95, 1.92 and 1.75 (5×s, 15H; 5×Ac), 1.16 ppm (s, 9H; *t*Bu);

^{13}C NMR (150 MHz, CD_3CN): δ = 176.2, 170.3, 170.1, 169.9, 169.1, 139.0, 139.0, 138.8, 138.4, 137.9, 137.6, 129.1, 128.8, 128.3, 128.2, 128.1, 127.8, 127.6, 127.5, 127.5, 127.2, 127.2, 127.0, 126.6, 103.5, 101.5, 101.0, 101.0, 99.7, 81.5, 81.2, 80.2, 79.3, 76.3, 74.9, 74.8, 74.4, 74.2, 72.9, 72.4, 72.4, 72.2, 71.6, 70.2, 70.1, 69.7, 69.4, 68.1, 67.6, 67.1, 66.0, 61.9, 56.4, 49.1, 38.4, 27.0, 23.1, 23.1, 20.7, 20.6 ppm; HRMS: *m/z* calcd for $C_{81}H_{96}N_2O_{23}Na^+$: 1519.6194 [*M*+Na]$^+$; found: 1519.6194.

Compound 29: AW-300 molecular sieves (4 Å, 500 mg) were added to a solution of **5** (153 mg, 115 μmol) and **28** (113 mg, 75.5 μmol) in CH_2Cl_2 (1.90 mL). The suspension was stirred for 30 min at 0 °C, whereupon TMSOTf (1.0 μL, 5.54 μmol) was added. Stirring was continued for 30 min at 0 °C (completion of the reaction was indicated by TLC analysis; $CHCl_3$/MeOH, 12:1; developed twice). The reaction mixture was filtered through Celite and the removed molecular sieves were washed with $CHCl_3$. The combined filtrate and washings were extracted with $CHCl_3$, and organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 40:1→35:1→30:1→25:1) to give **29** (174 mg, 86%). $[\alpha]_D = -16.5$ (c=1.0, $CHCl_3$); ^{1}H NMR (600 MHz, CD_3CN): δ = 8.30 (d, J = 7.5 Hz, 2H; Ph), 7.83 (d, J = 7.6 Hz, 2H; Ph), 7.65 (t, J = 7.3 Hz, 1H; Ph), 7.58 (t, J = 7.2 Hz, 1H; Ph), 7.50 (t, J = 7.3 Hz, 2H; Ph), 7.46–7.11 (m, 32H; 7 Ph), 7.03 (d, $J_{2,NH}$ = 9.6 Hz, 1H; NHe), 6.41 (d, $J_{2,NH}$ = 9.7 Hz, 1H; NHf), 6.30 (d, $J_{2,NH}$ = 9.6 Hz, 1H; NHd), 6.12 (d, $J_{2,NH}$ = 10.3 Hz, 1H; NHg), 5.62 (s, 1H; PhC*H*), 5.34 (d, $J_{3,4}$ = 3.5 Hz, 1H; H-4c), 5.22 (d, $J_{1,4}$ = 2.8 Hz, 1H; H-4f), 5.18–5.12 (m, 3H; H-3c, H-7g, H-8g), 5.08–4.99 (m, 5H; H-1c, H-1e, H-2e, H-3f, H-4g), 4.98 (d, J_{gem} = 10.3 Hz, 1H; CH_2), 4.86 (d, $J_{1,2}$ = 8.3 Hz, 1H; H-1f), 4.76 (t, $J_{1,2}=J_{2,3}$ = 9.0 Hz, 1H; H-2a), 4.75 (d, J_{gem} = 12.4 Hz, 1H; CH_2), 4.62–4.48 (m, 7H; H-1a, H-1d, H-3e, H-9g, 3 CH_2), 4.46 (d, J_{gem} = 10.3 Hz, 1H; CH_2), 4.40 (d, J_{gem} = 12.4 Hz, 1H; CH_2), 4.37 (q, $J_{1,2}=J_{2,3}=J_{2,NH}$ = 9.6 Hz, 1H; H-2c), 4.29 (d, $J_{1,2}$ = 8.2 Hz, 1H; H-1b), 4.26–4.22 (m, 3H; H-6d, 2 CH_2), 4.18–4.02 (m, 12H; H-4b, H-5c, H-6c, H-6′c, H-2d, H-3d, H-6e, H-2f, H-5f, H-5g, H-9g, CH_2), 3.99 (d, $J_{3,4}$ = 2.1 Hz, 1H; H-4e), 3.95–3.92 (m, 3H; H-5e, H-6′e, H-6f), 3.84–3.81 (m, 3H; H-4a, H-6′d, H-6g), 3.75 (s, 3H; $COOCH_3$), 3.71 (dd, $J_{5,6}$ = 6.2 Hz, J_{gem} = 11.0 Hz, 1H; H-6′f), 3.63–3.56 (m, 2H; H-6a, H-4d), 3.54 (dd, $J_{2,3}$ = 9.6 Hz, $J_{3,4}$ = 2.7 Hz, 1H; H-3b), 3.50 (d, J_{gem} = 10.3 Hz, 1H; H-6′a), 3.45–3.40 (m, 3H; H-5a, H-6b, H-5d), 3.36 (t, $J_{5,6}=J_{5,6′}$ = 6.2 Hz, 1H; H-5b), 3.33 (dd, $J_{1,2}$ = 8.2 Hz, $J_{2,3}$ = 9.6 Hz, 1H; H-2b), 3.16 (dd, $J_{5,6′}$ = 6.2 Hz, J_{gem} = 11.0 Hz, 1H; H-6′b), 2.22–2.15 (m, 7H; H-3g_{ax}, 2 Ac), 2.11, 2.05 and 1.98 (3×s, 9H; 3 Ac), 1.96–1.89 (m, 19H; H-3g_{eq}, 6 Ac), 1.82, 1.78 and 1.75 (3×s, 9H; 3 Ac), 1.16 ppm (s, 9H; *t*Bu); ^{13}C NMR (150 MHz, CD_3CN): δ = 177.6, 171.5, 171.3, 171.2, 171.1, 171.0, 171.0, 170.8, 170.6, 170.5, 170.4, 169.2, 166.4, 165.3, 140.0, 139.9, 139.7, 139.3, 138.4, 138.4, 134.3, 134.1, 130.9, 130.7, 130.7, 130.1, 129.8, 129.6, 129.4, 129.4, 129.2, 129.2, 129.1, 129.0, 128.9, 128.9, 128.7, 128.6, 128.4, 128.3, 128.2, 128.1, 127.9, 127.0, 103.9, 103.1, 102.9, 102.2, 101.9, 100.9, 100.8, 100.2, 82.2, 81.4, 81.3, 80.3, 79.0, 78.7, 77.0, 75.9, 75.8, 75.5, 75.3, 74.3, 74.0, 73.7, 73.5, 73.2, 72.6, 72.4, 72.2, 72.1, 71.7, 71.3, 71.2, 70.8, 70.6, 70.3, 69.1, 68.6, 68.5, 68.3, 67.8, 67.6, 67.0, 63.8, 63.0, 62.4, 62.2, 55.4, 53.9, 50.8, 50.4, 48.7, 39.3, 35.7, 27.4, 23.4, 23.2, 23.1, 23.0, 21.4, 21.0, 21.0, 20.8, 20.8, 20.7, 20.5 ppm; HRMS: *m/z* calcd for 1/2 ($C_{135}H_{160}N_4O_{52}$) + Na$^+$: 1357.4892 [1/2*M*+Na]$^+$; found: 1357.4893.

Compound 30: Pd(OH)$_2$/C (20%, 234 mg) was added to a solution of **29** (153 mg, 115 μmol) in EtOH (1.90 mL) and the suspension was stirred under a hydrogen stream for 62 h at RT (completion of the reaction was confirmed by TLC analysis; $CHCl_3$/MeOH/H_2O, 14:3:0.1). The reaction mixture was filtered through Celite and washed thoroughly with $CHCl_3$. The combined filtrate and washings were concentrated and exposed to high vacuum for 4 h. The residue was then treated with a solution of Ac_2O (500 μL, 5.29 mmol) and DMAP (1 mg, 8.15 μmol) in pyridine (500 μL) for 21 h (completion of the reaction was confirmed by TLC analysis; $CHCl_3$/MeOH, 18:1; developed twice), then the reaction mixture was coevaporated with toluene. The residue was extracted with EtOAc and the solution was washed with 2 M aqueous HCl, saturated aqueous $NaHCO_3$, and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 30:1→25:1) to give **30** (97 mg, 93%, α/β = 5/4). ^{1}H NMR (600 MHz, CD_3CN): δ = 6.16 (d, $J_{1,2}$ = 3.4 Hz, 1H; H-1aα), 5.75 ppm (d, $J_{1,2}$ = 8.3 Hz, 0.8H; H-1aβ); HRMS: *m/z* calcd for 1/2 ($C_{107}H_{146}N_4O_{59}$) + Na$^+$: 1235.3931 [1/2*M*+Na]$^+$; found: 1235.3931.

Compound 31: Hydrazine acetate (10 mg, 111 μmol) was added to a solution of **30** (97 mg, 40.0 μmol) in DMF (800 μL) and the mixture was stirred for 10 min at 50 °C (completion of the reaction was confirmed by TLC analysis; $CHCl_3$/MeOH, 15:1; developed twice). The reaction mixture was diluted with EtOAc and the solution was washed with water and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 20:1→15:1) to give the 1-OH derivative, which was exposed to high vacuum for 7 h. The residue was then treated with a solution of trichloroacetonitrile (80.0 μL, 79.8 μmol) and DBU (7.5 μL, 50.1 μmol) in CH_2Cl_2 for 50 min at RT (completion of the reaction was confirmed by TLC analysis; $CHCl_3$/MeOH, 10:1). The mixture was concentrated and the residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 25:1) to give **31** (83 mg, 82%). $[\alpha]_D = -2.0$ (c=1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.65 (s, 1H; NH), 8.14 (d, J = 6.9 Hz, 2H; Ph), 8.05 (d, J = 7.6 Hz, 2H; Ph), 7.59 (t, J = 7.2 Hz, 1H; Ph), 7.55 (t, J = 7.2 Hz, 1H; Ph), 7.48–7.43 (m, 4H; Ph), 6.49 (d, $J_{1,2}$ = 3.5 Hz, 1H; H-1a), 6.43–6.35 (m, 2H; NHc, NHf), 5.85 (d, $J_{2,NH}$ = 11.0 Hz, 1H; NHd), 5.53 (m, 1H; H-8g), 5.52 (t, $J_{2,3}=J_{3,4}$ = 10.0 Hz, 1H; H-3a), 5.37 (d, $J_{3,4}$ = 2.7 Hz, 1H; H-4c), 5.36 (d, $J_{3,4}$ = 3.4 Hz, 1H; H-4f), 5.29 (dd, $J_{1,2}$ = 7.5 Hz, $J_{2,3}$ = 9.6 Hz, 1H; H-2e), 5.23–5.20 (m, 3H; H-3c, H-3f, H-7g), 5.04 (dd,$J_{1,2}$ = 3.5 Hz, $J_{2,3}$ = 10.0 Hz, 1H; H-2a), 5.02–4.98 (m, 3H; H-1c, H-1f, NHg), 4.95 (dd, $J_{1,2}$ = 8.3 Hz, $J_{2,3}$ = 10.3 Hz, 1H; H-2b), 4.88 (d, $J_{1,2}$ = 7.5 Hz, 1H; H-1e), 4.83 (td, $J_{3ax,4}=J_{4,5}$ = 11.2 Hz, $J_{3eq,4}$ = 4.2 Hz, 1H; H-4g), 4.76 (brs, 1H; H-4d), 4.62 (dd, $J_{5,6}$ = 7.2 Hz, J_{gem} = 11.0 Hz, 1H; H-6e), 4.40–4.38 (m, 2H; H-6c, H-3e), 4.32 (dd, $J_{5,6′}$ = 4.8 Hz, J_{gem} = 11.0 Hz, 1H; H-6′e), 4.26 (d, $J_{1,2}$ = 8.3 Hz, 1H; H-1b), 4.26–4.23 (m, 3H; H-6b, H-1d, H-6f), 4.20 (dd, $J_{8,9}$ = 1.4 Hz, J_{gem} = 12.3 Hz, 1H; H-9g), 4.16–4.11 (m, 2H; H-6′b, H-6′c), 4.08–4.05 (m, 3H; H-5a, H-6a, H-6d), 4.03 (d, $J_{3,4}$ = 2.0 Hz, 1H; H-4b), 3.99 (dd, $J_{8,9}$ = 5.5 Hz, J_{gem} = 12.3 Hz, 1H; H-9g), 3.96–3.78 (m, 10H; H-4a, H-6′a, H-3d, H-5d, H-6′d, H-4e, H-5e, H-5f, H-6′f, H-5g), 3.76 (dd, $J_{5,6}$ = 11.0 Hz, $J_{6,7}$ = 2.0 Hz, 1H; H-6g), 3.73 (s, 3H; $COOCH_3$), 3.62–3.57 (m, 5H; H-3b, H-5b, H-2c, H-2d, H-2f), 2.67 (dd, $J_{3eq,4}$ = 4.2 Hz, J_{gem} = 12.3 Hz, 1H; H-3g_{eq}), 2.15, 2.14, 2.14, 2.09, 2.08, 2.06, 2.05, 2.05, 2.05, 2.04, 2.02, 2.01, 2.01 and 1.96 (14×s, 42H; 14 Ac), 1.84–1.79 (m, 10H; H-3g_{ax}, 3 Ac), 1.77 (s, 3H; Ac), 1.13 ppm (s, 9H; *t*Bu); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 178.0, 171.5, 171.3, 171.3, 171.2, 171.1, 171.0, 171.0, 170.7, 170.4, 170.3, 170.0, 169.1, 166.9, 165.3, 160.7, 134.4, 134.3, 131.0, 130.6, 130.6, 130.3, 129.6, 129.4, 103.6, 103.0, 102.0, 101.6, 100.8, 100.3, 93.4, 91.3, 80.0, 79.0, 78.7, 76.6, 75.9, 75.2, 74.4, 72.9, 72.8, 72.6, 72.3, 72.2, 72.1, 71.6, 71.3, 70.9, 70.5, 70.4, 70.3, 70.0, 68.5, 68.0, 67.9, 67.4, 64.4, 64.1, 63.2, 62.9, 62.5, 62.4, 62.1, 53.9, 50.9, 50.0, 48.7, 39.3, 35.8, 27.0, 23.4, 23.2, 23.0, 22.9, 21.4, 21.2, 21.0, 21.0, 20.9, 20.8, 20.8, 20.7, 20.7 ppm; HRMS: *m/z* calcd for $C_{107}H_{138}Cl_3N_3O_{58}Na^+$: 2548.6961 [*M*+Na]$^+$; found: 2548.6960.

Compound 32: Molecular sieves (4 Å, 250 mg) were added to a solution of **31** (88 mg, 34.8 μmol) and **4** (35 mg, 52.2 μmol) in $CHCl_3$ (880 μL). The suspension was stirred for 30 min at RT, whereupon $BF_3{\cdot}OEt_2$ (5.2 μL, 41.0 μmol) was added. Stirring was continued for 6.5 h at RT (completion of the reaction was indicated by TLC analysis; $CHCl_3$/MeOH, 20:1; developed twice). The reaction mixture was filtered through Celite and the removed molecular sieves were washed with $CHCl_3$. The combined filtrate and washings were extracted with $CHCl_3$, and the organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 40:1→30:1→20:1, then acetone/hexane, 3:2) to give **32** (27 mg, 26%). $[\alpha]_D = -14.6$ (c=2.3, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.12 (d, J = 6.9 Hz, 2H; Ph), 8.05 (d, J = 6.8 Hz, 2H; Ph), 8.00 (d, J = 6.9 Hz, 2H; Ph), 7.60–7.53 (m, 3H; 3 Ph), 6.44–6.31 (m, 2H; NHc, NHf), 5.86 (dt, $J_{4,5}$ = 15.2 Hz, $J_{5,6}$ = $J_{5,6′}$ = 7.2 Hz, 1H, H-5^{Cer}), 5.82 (d, $J_{2,NH}$ = 10.3 Hz, 1H; NHd), 5.72 (d, $J_{2,NH}$ = 9.6 Hz, 1H; NH^{Cer}), 5.54–5.51 (m, 2H; H-8g, H-3^{Cer}), 5.45 (dd, $J_{3,4}$ = 7.9 Hz, $J_{4,5}$ = 15.2 Hz,1H, H-4^{Cer}), 5.37 (d, $J_{3,4}$ = 2.7 Hz, 1H; H-4c), 5.36 (d, $J_{3,4}$ = 3.4 Hz, 1H; H-4f), 5.28 (t, $J_{1,2}=J_{2,3}$ = 9.0 Hz, 1H; H-2e), 5.22–5.20 (m, 3H; H-3c, H-3f, H-7g), 5.15 (t, $J_{2,3}=J_{3,4}$ = 8.6 Hz, 1H; H-3a), 5.02–4.97 (m, 2H; H-1c, NHg), 4.91–4.87 (m, 4H; H-2a, H-2b, H-1e, H-1f), 4.83 (td, $J_{3ax,4}=J_{4,5}$ = 11.0 Hz, $J_{3eq,4}$ = 4.4 Hz, 1H; H-4g), 4.72 (brs, 1H; H-4d), 4.62 (dd, $J_{5,6}$ = 7.6 Hz, J_{gem} = 10.3 Hz, 1H; H-6e), 4.46 (m, 1H;

H-2^{Cer}), 4.38 (d, $J_{1,2}$=7.6 Hz, 1H; H-1a), 4.36 (m, 1H; H-3e), 4.32 (dd, $J_{5,6'}$=4.5 Hz, J_{gem}=10.3 Hz, 1H; H-6'e), 4.25–4.17 (m, 6H; H-1b, H-6c, H-1d, H-6d, H-6f, H-9g), 4.12 (dd, $J_{5,6}$=7.2 Hz, J_{gem}=12.0 Hz, 1H; H-6'c), 4.08–3.79 (m, 14H; H-6a, H-6'a, H-4b, H-6b, H-6'b, H-3d, H-5d, H-6'd, H-4e, H-5e, H-6'f, H-5g, H-9g, H-1^{Cer}), 3.75 (dd, $J_{5,6}$=11.0 Hz, $J_{6,7}$= 2.1 Hz, 1H; H-6 g), 3.73 (s, 3H; $COOCH_3$), 3.69 (dd, $J_{3,4}$=8.6 Hz, $J_{4,5}$= 9.6 Hz, 1H; H-4a), 3.66–3.55 (m, 8H; H-3b, H-5b, H-2c, H-5c, H-2d, H-2f, H-5f, H-1'^{Cer}), 3.49 (m, 1H; H-5a), 2.64 (dd, $J_{9eq,4}$=4.4 Hz, J_{gem}= 10.3 Hz, 1H; H3 g_{eq}), 2.15, 2.14, 2.13, and 2.10 (4×s, 12H; 4 Ac), 2.06–2.00 (m, 30H; H-3g_{ax}, H-6^{Cer}, H-6'^{Cer}, 9 Ac), 1.96, 1.92, 1.88, 1.83, 1.83, 1.81, and 1.75 (7×s, 21H; 7 Ac), 1.33–1.22 (m, 54H; 27 CH_2), 1.14 (s, 9H; *t*Bu), 0.88 ppm (2×t, J=6.6 Hz, 6H; 2 CH_2CH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ=177.0, 172.6, 171.8, 171.1, 170.8, 170.8, 170.7, 170.7, 170.5, 170.3, 170.3, 170.2, 170.1, 170.1, 170.0, 169.9, 169.9, 169.6, 168.9, 168.3, 165.7, 165.0, 164.8, 164.7, 137.7, 133.3, 133.2, 132.9, 130.3, 130.3, 129.8, 129.7, 129.6, 128.5, 128.5, 128.3, 124.7, 100.4, 100.2, 100.1, 99.6, 99.6, 97.9, 74.7, 73.8, 73.6, 73.5, 73.4, 73.0, 72.7, 72.1, 71.9, 71.9, 71.4, 71.2, 70.7, 70.5, 70.1, 69.9, 68.7, 68.6, 67.4, 67.1, 66.9, 66.9, 66.5, 63.2, 62.6, 62.4, 62.3, 62.1, 61.4, 61.2, 52.9, 52.8, 50.5, 49.2, 38.8, 36.9, 36.5, 32.3, 31.9, 30.0, 29.9, 29.8, 29.8, 29.7, 29.6, 29.5, 29.5, 29.4, 29.3, 29.2, 28.9, 27.1, 26.9, 25.7, 23.6, 23.3, 23.2, 23.1, 22.7, 21.2, 20.9, 20.8, 20.7, 20.7, 20.6, 20.6, 20.5, 20.4, 20.3, 14.1 ppm; HRMS: *m/z* calcd for 1/2 ($C_{148}H_{211}N_5O_{61}$)+Na^+: 1540.1674[1/2*M*+Na]$^+$; found: 1540.1673.

Ganglioside X1 (1): A solution of sodium methoxide (28% in MeOH, 0.4 mg) was added to a solution of **32** (22 mg, 7.25 μmol) in MeOH (500 μL) and THF (500 μL). The mixture was stirred for 45 h at RT, whilst monitoring the reaction by TLC ($CHCl_3$/MeOH/12 mM $MgCl_2$ aq, 5:4:1). The mixture was then heated at 40°C and stirring was continued for 32 h at 40°C. Water (200 μL) was added and stirring was continued for 35 h at 40°C. After neutralization with Dowex-50 (H^+), the mixture was filtered through cotton wool, and the removed resin was washed with mixed solvent ($CHCl_3$/MeOH, 1:1). The combined filtrate and washings were concentrated and the residue was purified by column chromatography on Sephadex LH-20 ($CHCl_3$/MeOH, 1:1) and column chromatography on silica gel ($CHCl_3$/MeOH/H_2O, 5:4:0.6→5:4:0.7) to give **1** (13.5 mg, 95%). $[\alpha]_D$=+1.4 (c=0.7, MeOH); ^{1}H NMR (600 MHz, CD_3OD): δ=5.67 (dt, $J_{4,5}$=15.1 Hz, $J_{5,6}$=$J_{5,6'}$=7.2 Hz, 1H; H-5^{Cer}), 5.44 (dd,$J_{4,5}$=15.1 Hz, $J_{3,4}$=7.8 Hz, 1H; H-4^{Cer}), 4.88–4.85 (m, 2H; 2 anomeric H), 4.60 (d, $J_{1,2}$=8.9 Hz, 1H; anomeric H), 4.40 (d, $J_{1,2}$=8.3 Hz, 1H; anomeric H), 4.31 (d, $J_{1,2}$=7.6 Hz, 1H; anomeric H), 4.28 (d, $J_{1,2}$= 7.5 Hz, 1H; anomeric H), 4.26 (d, $J_{3,4}$=2.0 Hz, 1H; H-4), 4.19 (dd, J= 9.9 Hz, J=4.5 Hz, 1H), 4.12 (d, $J_{3,4}$=2.8 Hz, 1H; H-4), 2.75 (dd, J_{gem}= 12.8 Hz, $J_{9eq,4}$=5.2 Hz, 1H; H-3g_{eq}), 2.03–2.00 (m, 14H; H-6^{Cer}, H-6'^{Cer}, 4 Ac), 2.16 (t, J=7.6 Hz, 2H; $NHCOCH_2$), 1.89 (t, $J_{3ax,4}$=12.0 Hz, 1H; H-3g_{ax}), 1.57 (m, 2H; $NHCOCH_2CH_2$),1.39–1.28 (m, 50H; 25 CH_2), 0.90 ppm (2×t, J=7.3 Hz, 6H; 2 CH_2CH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ=175.9, 175.6, 175.0, 174.7, 174.7, 174.5, 135.1, 131.4, 105.2, 105.0, 104.6, 104.4, 104.3, 103.4, 103.4, 101.3, 84.7, 83.5, 78.9, 77.7, 76.9, 76.5, 76.5, 76.3, 76.1, 75.8, 75.7, 75.1, 74.9, 74.2, 73.8, 73.4, 73.0, 71.3, 70.7, 70.4, 70.3, 70.0, 69.9, 69.7, 65.4, 63.0, 62.9, 61.8, 61.8, 56.2, 54.7, 54.3, 54.1, 53.8, 38.8, 37.4, 33.5, 33.1, 30.9, 30.8, 30.8, 30.8, 30.7, 30.6, 30.5, 30.5, 30.4, 27.2, 23.7, 23.6, 23.6, 23.5, 22.6, 14.5 ppm; HRMS: *m/z* calcd for $C_{99}H_{157}N_3O_{41}$–H: 1951.0281 [*M*–H]$^-$; found: 1951.0280.

TLC with immunostaining: The novel GM2-epitope-containing gangliosides, X1 and X2, and authentic gangliosides GM2, GM1, GD1a, GD1b, and GT1b were prepared from bovine brain gangliosides as described elsewhere.[3,22] These gangliosides and the synthesized X1 were layered on precoated Silica Gel 60 plates (Merck, Darmstadt, Germany). The plates were developed with a solvent system of chloroform/methanol/12 mM magnesium chloride in water (5:4:1, by volume), dipped in *n*-hexane containing 0.4% > polyisobutylmethacrylate for 1 min, then dried under an air stream. The TLC plate was overlaid with serum from the patient with ALS-like disorder (1:50 dilution with phosphate-buffered saline/0.5% casein) and kept at 4°C overnight. The plates were washed and overlaid with peroxidase-conjugated anti-human *m*-chain-specific antibodies (Dako, Glostrup, Denmark; 1:250 dilution with phosphate-buffered saline/0.5% casein), kept at 20°C for 2 h, then washed. Binding activities were made visible with 4-chloro-1-naphtol.

Acknowledgements

This work was financially supported by MEXT of Japan (WPI program and a grant-in-aid for Scientific Research to M.K., nos. 1701007 and 22380067). We thank Kiyoko Ito for technical assistance.

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Received: July 30, 2010
Published online: November 5, 2010

Journal of Medical Virology 83:501–509 (2011)

Target Epitopes of HTLV-1 Recognized by Class I MHC-Restricted Cytotoxic T Lymphocytes in Patients With Myelopathy and Spastic Paraparesis and Infected Patients With Autoimmune Disorders

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Human T-cell lymphotropic virus type I (HTLV-1) causes adult T-cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). The different patterns of clinical diseases are thought to be linked to immunogenetic host factors. A variety of autoimmune diseases, such as Sjögren's syndrome, have been reported in persons infected with HTLV-1, although the precise relationship between these disorders and HTLV-1 infection remains unknown. There is no report on the repertoire of HTLV-1-specific $CD8^+$ T-cells in HAM/TSP patients or carriers with autoimmune diseases, both characterized by an abnormal immune state. In this study, to characterize HTLV-1-specific $CD8^+$ T-cells in asymptomatic HTLV-1 carriers, HAM/TSP patients and carriers with autoimmune diseases, we examined the frequency and diversity of HTLV-1-specific $CD8^+$ T-cells using HTLV-1 tetramers. HTLV-1 Env-specific $CD8^+$ T-cells were significantly more frequent in HAM/TSP and carriers with autoimmune diseases compared with asymptomatic HTLV-1 carriers, while the frequency of HTLV-1 Tax-specific $CD8^+$ T-cells was not significantly different among them. $CD8^+$ cells binding to HTLV-1 Tax tetramers in carriers with autoimmune diseases were significantly reduced compared with HAM/TSP patients. This study demonstrates the importance of $CD8^+$ T-cells recognizing HTLV-1 Env-tetramers in HAM/TSP patients and carriers with autoimmune diseases, thereby suggesting that the diversity, frequency and repertoire of HTLV-1 Env-specific $CD8^+$ T-cell clones may be related to the hyperimmune response in HAM/TSP and carriers with autoimmune diseases, although different immunological mechanisms may mediate the hyperimmunity in these conditions. ***J. Med. Virol. 83:501–509, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: HTLV-1; HAM/TSP; autoimmune diseases; MHC; CD8+ T cells

INTRODUCTION

Adult T-cell leukemia/lymphoma (ATL) [Poiesz et al., 1980; Hinuma et al., 1981; Tsukasaki et al., 2009] and human T-cell lymphotropic virus type I (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [Gessain et al., 1985; Osame et al., 1986; Casseb, 2009] are two of the most important diseases associated with long-term infection with HTLV-1, which has infected approximately 10–20 million people world-

The authors declare no financial or commercial conflict of interest.

Grant sponsor: Japanese Ministry of Health, Labour, and Welfare (to T.K. and N.A.); Grant sponsor: Kagoshima University for Frontier Science Research Center Program (to N.A.).

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Accepted 30 September 2010

DOI 10.1002/jmv.21985

Published online in Wiley Online Library (wileyonlinelibrary.com).

wide, particularly in Equatorial Africa, the Caribbean basin, South America, Melanesia, and southern Japan [Proietti et al., 2005]. The different patterns of clinical disease are thought to be linked to host immunogenetic factors. HTLV-1 is also associated with a variety of autoimmune disorders including T cell alveolitis, myopathy, uveitis, arthritis, and Sjögren's syndrome [Sugimoto et al., 1987; Vernant et al., 1988; Nishioka et al., 1989; Terada et al., 1994], although the precise relationship between these disorders and HTLV-1 infection remains unclear. Patients with systemic lupus erythematosus (SLE) and concomitant HTLV-1 infection, for example, seem to have a more indolent clinical course compared with patients with SLE who are not infected with HTLV-1 [Akimoto et al., 2007b].

HTLV-1 Tax-specific cytotoxic T lymphocytes (CTLs) play an important role in suppressing proliferation of HTLV-1-infected or transformed T cells in vitro [Jacobson et al., 1990; Bangham, 2008, 2009]. HTLV-1 Tax and envelope (Env) epitopes recognized by HLA class I molecules [Yashiki et al., 2001], and the association between HTLV-1 Tax-specific CTL frequency, as well as anti-HTLV-1 Tax antibody titers, with reduced HTLV-1 proviral load in asymptomatic HTLV-1 carriers have been described previously [Kozako et al., 2006, 2009c; Akimoto et al., 2007a]. In HAM/TSP, HTLV-1 specific $CD8^+$ CTLs target infected $CD4^+$ cells that have entered the central nervous system, resulting in direct and/or bystander injury [Ijichi et al., 1993]. These CTLs primarily target p40 Tax epitopes, although the less well-characterized *env* and *pol* encoded epitope targets have also been detected [Jacobson et al., 1991; Kannagi et al., 1991; Parker et al., 1992; Furukawa et al., 1994]. Like HAM/TSP, it has been demonstrated using vaccinia virus-HTLV-1 recombinants that $CD8^+$ CTLs from patients with Sjögren's syndrome show major histocompatibility (MHC) class I restricted cytotoxicity to target cells expressing various HTLV-1 proteins, primarily HTLV-1 Tax [Kannagi et al., 1991].

There appears to be no difference in the frequency of amino acid residues favoring MHC class I restricted binding when Env and Tax epitopes are compared, but among non-binding peptides, Env peptides seem more likely to have detrimental amino acid residues at the anchor sites [Pique et al., 1996]. Furthermore, because there seems to be no difference in the functional capacity of HTLV-1 virus-encoded envelope proteins originating from HAM/TSP patients compared with virus from ATL patients, host factors seem to play an important role in mediating immunopathogenesis [Pique et al., 1994]. Our current understanding of the immunological response to HTLV-1 envelope epitopes includes the broad immunogenicity of the surface protein Env175–199 that elicits helper T cell, cytotoxic T cell, as well as humoral responses [Baba et al., 1993]. Despite the apparent association between some autoimmune diseases and HTLV-1 infection, CTL responses targeting HTVL-1 Env epitopes have been less well characterized in individuals infected with HTLV-1 with autoimmune diseases.

MATERIALS AND METHODS

Subjects and PBMCs

The study sample consisted of 26 asymptomatic HTLV-1 carriers (range, 22- to 82-year-old; median, 60-year-old), 18 HAM/TSP patients (range, 34- to 73-year-old; median, 54-year-old), and 25 individuals with autoimmune disorders and HTLV-1 infection (range, 32- to 80-year-old; median, 60-year-old) from the Kagoshima University Hospital. The individuals with autoimmune disorders included eight with Sjögren's syndrome, seven with SLE, five with rheumatoid arthritis (RA), three with systemic sclerosis (SSc), and two with polymyositis (PM). Diagnoses of Sjögren's syndrome, SLE, and RA were made according to the revised Japan criteria for Sjögren's syndrome [Miyawaki, 2000], the revised criteria of the American College of Rheumatology (ACR) [Tan et al., 1982], and the 1987 ACR criteria [Silman, 1988], respectively. Anti-HTLV-1 antibody was measured by electrochemiluminescence immunoassay (ECLIA) (Picolumi®HTLV-I; Eisai, Tokyo, Japan), using beads coated with purified HTLV-1 antigen and synthetic Env peptides. This study protocol was in compliance with the Helsinki Declaration, and approved by the Medical Ethical Committee of Kagoshima University, and participants provided informed consent. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood by centrifugation on Ficoll Hypaque (Amersham Biosciences, Uppsala, Sweden). For some experiments, cells were cryopreserved in liquid nitrogen until assayed [Kozako et al., 2006, 2009b,c].

HLA Typing of PBMCs

Based on prior reports, HLA type analysis revealed that 88% of people infected with HTLV-1 were HLA-A*02 or HLA-A*24 [Kozako et al., 2006], consistent with other studies of HLA allele types in the population of Southern Kyushu, Japan [Sonoda et al., 2000]. PBMC samples were screened initially by serological staining with monoclonal antibodies (mAbs) for HLA-A*02 supertype (clone BB7.2) and HLA-A*24 supertype (clone 17A10; Medical and Biological Laboratories, Nagoya, Japan), followed by secondary staining with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Immunotech, Praka, Czech) and subjected to flow cytometry on a FACScan (BD Biosciences, San Jose, CA). Individuals with neither HLA-A*02 nor HLA-A*24 were excluded from this study. HLA allele types of asymptomatic HTLV-1 carriers were also confirmed by the Luminex method using DNA isolated from cryopreserved PBMCs, as has been previously described (G&G Science, Fukushima, Japan) [Itoh et al., 2005].

Tetramer Assay for HTLV-1 Tax/Env-Specific CD8+ T Cells

Sixteen distinct phycoerythrin (PE)-conjugated HLA-A*0201 and HLA-A*2402 tetramers for HTLV-1 Tax and Env peptides (Medical and Biological Laboratories)

based on known HTLV-1 Tax and Env CTL epitope mapping data were used in this study [Yashiki et al., 2001] (Table II). HLA tetramers were produced as previously described [Baenziger et al., 1986; Altman et al., 1996; Kozako et al., 2006, 2009c]. Aliquots of 1×10^6 PBMCs were incubated with each of 16 distinct HTLV-1 Tax or Env peptides, followed by staining with FITC-conjugated mouse anti-human CD8 mAbs (Beckman Coulter, Fullerton, CA) and peridin chlorophyll a protein (PerCP)-conjugated anti-CD45 (BD Biosciences) according to the manufacturer's instructions. $CD45^+$ lymphocytes were applied to a FACScan [Kuzushima et al., 2001] and 1×10^5 events analyzed with FlowJo software (Tree Star, San Carlos, CA) [Betts et al., 2004]. Human immunodeficiency virus (HIV)/HLA tetramers (Medical and Biological Laboratories) were also stained as negative controls. Based on the negative controls, a cut-off point of 0.1% for HTLV-1/HLA tetramer positivity in $CD8^+CD45^+$ T lymphocytes was used, as previously described [Akimoto et al., 2007a; Kozako et al., 2006].

Statistical Analysis

The proportion of people with HTLV-1-specific $CD8^+$ T cell positivity for respective subject groups was compared with a χ^2 test or Fisher's exact test as appropriate. The percentage of tetramer positive cells was compared using the Mann–Whitney *U*-test. SPSS for Windows (version 14.0J; SPSS, Inc., Chicago, IL) was used for statistical analyses, and $P < 0.05$ was considered statistically significant.

RESULTS

Adopting HLA-A*0201 Tetramers for Carriers Possessing HLA-A*0201 and HLA-A*0206

Consistent with prior studies in the Southern Kyushu population [Sonoda et al., 2000], most of the asymptomatic HTLV-1 carriers with the HLA-A*02 haplotype were either HLA-A*0201 or HLA-A*0206, and nearly all HLA-A*24 subjects were HLA-A*2402. Of 26 asymptomatic HTLV-1 carriers, six were HLA-A*0201, five were HLA-A*0206, 18 were HLA-A*2402, and three were heterozygous for HLA-A*0201 and HLA-A*2402. Because existing HTLV-1/HLA tetramers were developed for carriers possessing HLA-A*0201 or HLA-A*2402, but not for HLA-A*0206, HLA-A*0201 tetramers were adopted for subjects of the HLA-A*0206 haplotype. In comparing asymptomatic HTLV-1 carriers with HLA-A*0201 and HLA-A*0206 haplotypes, there was no significant difference in frequency of Tax-specific $CD8^+$ T cells (27% and 16%, respectively; $P = 0.52$), or the proportion of individuals with detectable $CD8^+$ T cells for all HTLV-1 tetramers tested (Table I). Furthermore, the CMV p65/HLA-A*0201 tetramer was recognized by $CD8^+$ T cells from individuals with HLA-A*0201 ($n = 6$) and HLA-A*0206 ($n = 4$) haplotypes. These normally HLA-A*0201-restricted tetramers were thus also considered to be reliable in selecting $CD8^+$ T cells in HLA-A*0206 individuals. The lower limit of this assay for detecting tetramer specific $CD8^+$ T cells was 0.1%, defined using HIV-1 tetramers as negative control, and consistent with previous reports [Kozako et al., 2006, 2009a,b,c]. The percentage of HTLV-1 tetramer positive cells in the $CD8^+CD45^+$ T cell subset ranged from 0% to 1.28% (Fig. 1).

Env-Derived Epitopes are Recognized More Frequently by Patients With Autoimmune Diseases

The proportion of individuals with detectable $CD8^+$ T cells binding the various envelope epitope tetramers tested was significantly higher in HAM/TSP patients and carriers with autoimmune diseases (15% and 25%, respectively), compared with asymptomatic HTLV-1 carriers (1%, $P < 0.001$, Table II). There was no statistically significant difference in the proportion of individuals with detectable $CD8^+$ T cells specific for the different HTLV-1 Tax epitope tetramers. Individuals with autoimmune diseases had detectable $CD8^+$ T cells that primarily recognized envelope-derived epitopes to a greater extent than Tax-derived epitope tetramers. A

TABLE I. Frequency of HTLV-1-Specific CTL Positivity in Asymptomatic HTLV-1 Carriers Possessing HLA-A*0201 and HLA-A*0206

Tetramers	HLA-A*0201	HLA-A*0206
T11	100% (6/6)[a]	60% (3/5)
T123	17% (1/6)	0% (0/5)
T155	0% (0/6)	0% (0/5)
T178	0% (0/6)	20% (1/5)
T307	17% (1/6)	0% (0/5)
E175	0% (0/6)	0% (0/5)
E239	0% (0/6)	0% (0/5)
E442	0% (0/6)	0% (0/5)
HTLV-1 Tax CTL positives	27% (8/30)	16% (4/25)*
HTLV-1 Env CTL positives	0% (0/18)	0% (0/15)
Total HTLV-1-specific CTL positives	17% (8/48)	10% (4/40)**
CMV pp65-specific CTL positives	100% (6/6)	100% (4/4)

[a]Epitopes detected by HTLV-1/HLA tetramers/number of tetramers tested.
*$P = 0.52$.
**$P = 0.55$.

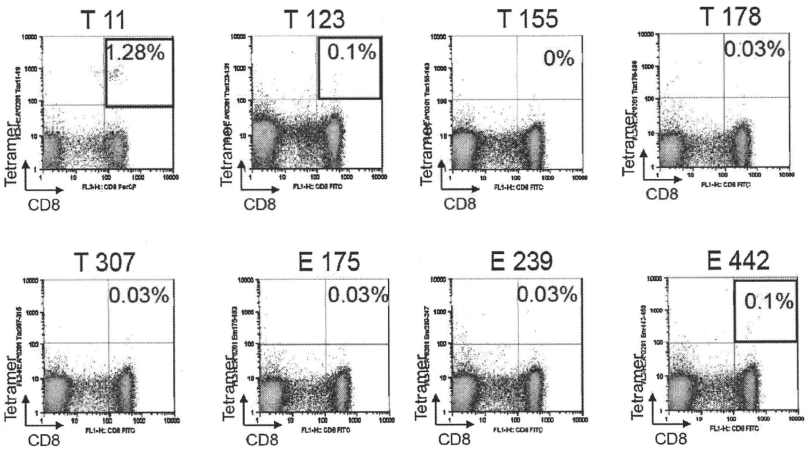

Fig. 1. Variety of HTLV-1-specific $CD8^+$ T cells in fresh PBMCs. Tetramer$^+$$CD8^+$ T cells were estimated among the $CD45^+$ T lymphocyte population. Fresh PBMCs isolated from carriers with autoimmune diseases were stained with eight distinct HTLV-1/HLA-A*0201-tetramers (T11, T123, T155, T178, T307, E175, E239, and E442). Numbers in the upper right quadrants represent the percentages of tetramer$^+$$CD8^+$ T cells among $CD8^+CD45^+$ T lymphocytes.

higher proportion of HTLV-1 infected individuals with Sjögren's syndrome, SLE, or SSc had detectable levels of envelope tetramer specific $CD8^+$ T cells (30%, 29%, and 33%, respectively), compared with asymptomatic carriers (1.1%, $P < 0.001$). In contrast, the proportion of individuals with detectable Tax-specific $CD8^+$ T cells was not significantly different among the subject groups, except for individuals with Sjörgren's syndrome, who were more likely than asymptomatic carriers to have detectable Tax-specific $CD8^+$ T cells (34% vs. 22%, respectively). In fact, individuals with Sjörgren's syndrome were more likely to have detectable overall tetramer (Tax- and Env-) specific $CD8^+$ T cells compared with asymptomatic carriers (34% vs. 14%, respectively; $P < 0.001$). There were no significant differences observed in the proportion of individuals with detectable tetramer specific $CD8^+$ T cells when individuals with rheumatoid arthritis or polymyositis were compared with asymptomatic carriers for Tax-specific (24%, 40%, and 22%, respectively) or Env-specific $CD8^+$ T cells (6.7%, 17%, and 1.1%, respectively). Table III summarizes the proportion of individuals with detectable $CD8^+$ T cells binding the various epitope tetramers tested. There was no significant difference in the proportion of individuals with $CD8^+$ T cells binding Tax-derived epitope tetramers, but a higher proportion of HAM/TSP patients (50%) and carriers with autoimmune diseases (64%) had Env-tetramer specific $CD8^+$ T cells overall, when compared with asymptomatic HTLV-1 carriers (3.8%, $P < 0.001$).

$CD8^+$ T Cells From Asymptomatic HTLV-1 Carriers Exhibit a Narrower HTLV-1 Epitope Repertoire Than HAM/TSP Patients and Carriers With Autoimmune Diseases

Fifty-nine individuals were assessed using eight distinct tetramers corresponding to the immunodominant HLA-A*02 or HLA-A*24 restricted epitopes. In the case of individuals with both HLA alleles (three asymptomatic HTLV-1 carriers, seven HAM/TSP patients, and three HTLV-1 carriers with autoimmune diseases), epitope-specific $CD8^+$ T-cells were analyzed using eight tetramers for each HLA type. The array of HTLV-1 epitopes recognized by $CD8^+$ T cells varied, not only by HLA/HTLV-1 peptide, but also according to clinical diagnosis (Table II). Among asymptomatic HTLV-1 carriers, $CD8^+$ T cells predominantly recognized the HLA-A*0201-restricted Tax11–19 and the HLA-A*2402-restricted Tax301–309 tetramers. None of the asymptomatic HTLV-1 carriers in this study had detectable $CD8^+$ T cells binding T155, T289, or T311 tetramers (Table II), whereas these tetramers were recognized by HAM/TSP patients and HTLV-1 infected carriers with autoimmune diseases. Envelope epitopes were rarely recognized by $CD8^+$ T cells from asymptomatic carriers, while HAM/TSP patients and carriers with autoimmune diseases consistently showed detectable levels of Env-specific $CD8^+$ T cells binding HLA-A*0201-restricted Env175–183, Env239–246, Env442–450; and HLA-A*2402-restricted Env11–19,

TABLE II. Diversity of HTLV-1 Epitopes Recognized by CTL m Asympiomatic HTLV-1 Carriers, HAM/TSP Patients, and Earners With Autoimmune Diseases

Tetramers	HLA allele	HTLV-l peptide	Asymptomatic HTLV-1 carriers	HAM TSP	Carriers with autoimmune diseases
T11	A*0201	Taxll–19	73% (8/11)[a]	90% (9/10)	55% (6/11)
T123	A*0201	Taxl23–131	9% (1/11)	20% (2/10)	45% (5/11)
T155	A*0201	Taxl55–163	0% (0/11)	10% (1/10)	0% (0/11)
T178	A*0201	Taxl78–186	9% (1/11)	20% (2/10)	18% (2/11)
T3O7	A*0201	Tax307–315	9% (1/11)	0% (0/10)	0% (0/11)
E175	A*0201	Envl75–183	0% (0/11)	0% (0/10)	9% (1/11)
E239	A*0201	Env239–247	0% (0/11)	10% (1/11)	18% (2/11)
E442	A*0201	Env442–450	0% (0/12)	0% (0/12)	18% (2/12)
T12	A*2402	Taxl2–20	11% (2/13)	13% (2/13)	24% (4/13)
T187	A*2402	Taxl87–195	11% (2/18)	7% (1/15)	18% (3/17)
T289	A*2402	Tax289–297	0% (0/18)	13% (2/15)	18% (3/17)
T301	A*2402	Tax301–309	94% (17/18)	87% (13/15)	76% (13/17)
T311	A*2402	Tax311–319	0% (0/16)	7% (1/16)	6% (1/16)
E11	A*2402	Euvll–19	6% (1/17)	40% (6/17)***	47% (8/17)***
E21	A*2402	Env21–29	0% (0/18)	13% (2/18)	35% (6/18)***
B153	A*2402	Envl 53–161	0% (0/18)	13% (2/I?)	24% (4/17)
Tax CTL positives			22% (32/145)	26% (33/125)	26% (37/140)
Env CTL positives			1% (1/87)	15% (11/75)*	27% (23/84)*
Total CTL positives			14% (33/232)	22% (44/200)***	27% (60/224)**

Tetramers	Sioaren's syndrome	SLE	RA	Sarcoidosis	PM	SSc
T11	100% (3/3)	25% (1/4)	33% (1/3)	100% (1/1)	100% (1/1)	NT
T123	67% (2/3)	50% (2/4)	33% (1/3)	0% (0/1)	0% (0/1)	NT
T155	0% (0/3)	0% (0/4)	0% (0/3)	0% (0/1)	0% (0/1)	NT
T178	0% (0/3)	25% (1/4)	33% (1/3)	0% (0/1)	0% (0/1)	NT
T307	0% (0/3)	0% (0/4)	0% (0/3)	0% (0/1)	0% (0/1)	NT
E175	33% (1/3)	0% (0/4)	0% (0/3)	0% (0/1)	0% (0/1)	NT
E239	33% (1/3)	25% (1/4)	0% (0/3)	0% (0/1)	0% (0/1)	NT
E442	33% (1/3)	25% (1/4)	0% (0/3)	0% (0/1)	0% (0/)	NT
TI2	29% (2/7)	0% (0/4)	0% (0/2)	50% (1/2)	[00% (1/)	33% (1/3)
T187	43% (3/7)	0% (0/4)	0% (0/2)	0% (0/2)	0% (0/)	0% (0/3)
T2S9	0% (0/7)	0% (0/4)	50% (1/2)	0% (0/2)	100% (1/)	33% (1/3)
T301	86% (6/7)	50% (2/4)	100% (2/2)	100% (2/)	100% (1/)	67% (2/3)
T311	14% (1/7)	0% (0/4)	0% (0/2)	0% (0/)	0% (0/)	0% (0/3)
Ell	43% (3/7)	50% (2/4)	0% (0/2)	100% (2/2)***	0% (0/1)	33% (1/3)
E21	14% (1/7)	50% (2/4)	50% (1/2)	0% (0/2)	100% (1/1)	33% (1/3)
El 53	29% (2/7)	25% (1/4)	0% (0/2)	0% (0/2)	0% (0/1)	33% (1/3)
Tax CTT, positives	34% (17/50)	15% (6/40)	24% (6/25)	27% (4/15)	40% (4/10)	27% (4/15)
Env CTL positives	30% (9/40)*	29% (7/24)*	7% (1/15)	22% (2/9)***	17% (1/6)	33% (3/9)**
Total CTL positives	33% (26/80)*	20% (13/64)	18% (7/40)	25% (6/24)	31% (5/16)	29% (7/24)

NT, not tested.
[a]Epitopes detected by HTLV-1/HLA tetramers/number of tetramers tested.
*$P < 0.001$.
**$P < 0.01$.
***$P < 0.05$, versus ACs.

Env21–29, and Env153–161 epitope tetramers. The epitope repertoire of HTLV-1 Env-specific $CD8^+$ cells in asymptomatic carriers showed considerably less breadth than that of HAM/TSP patients and carriers with autoimmune diseases.

Differences in Frequency of HTLV-1-Specific Tetramer Binding $CD8^+$ T Cells Among Asymptomatic HTLV-1 Carriers, HAM/TSP Patients, and Carriers With Autoimmune Diseases

There were significant differences related to clinical status with respect to the percentages of Tax-specific $CD8^+$ T cells among individuals. Among HLA-A*0201 subjects, the percentage of $CD8^+$ T cells binding Tax11–19/HLA-A*0201 tetramer in $CD8^+/CD45^+$ T lymphocytes ranged from 0.03% to 3.77% in asymptomatic HTLV-1 carriers, 0–17.1% in HAM/TSP patients, and 0–1.21% in carriers with autoimmune diseases. A similar trend was observed among HLA-A*2402 subjects, for whom the percentages of $CD8^+$ T cells binding Tax301–309/HLA-A*2402 tetramer in $CD8^+/CD45^+$ T lymphocytes ranged from 0.09% to 15.6% in asymptomatic HTLV-1 carriers, 0–26.0% in HAM/TSP patients, and 0–3.83% in carriers with autoimmune diseases. For both immunodominant HTLV-1 Tax epitopes, the mean percentage of tetramer-specific T cells within the $CD8^+$ T cell subset in HAM/TSP patients was significantly greater than in asymptomatic HTLV-1 carriers

TABLE III. The Number of Subjects Positive for HTLV-1-Specific CD8+ T Cells in Asymptomatic HTLV-1 Carriers, HAM/TSP Patients, and Carriers With Autoimmune Diseases

HLA allele	Tetramers	Asymptomatic HTLV-1 carriers	HAM/TSP	Carriers with autoimmune diseases
A*02	Tax	82% (9/11)	90% (9/10)	64% (7/11)
A*02	Env	0% (0/11)	10% (1/10)	27% (3/11)
A*24	Tax	94% (17/18)	100% (15/15)	88% (15/17)
A*24	Env	6% (1/18)	40% (6/15)*	82% (14/17)*
Tax CTL positivities		96% (25/26)	100% (18/18)	80% (20/25)
Env CTL positivities		4% (1/26)	50% (9/10)*	64% (16/25)*

*$P < 0.001$, versus ACs.

($P < 0.05$%; Fig. 2), and carriers with autoimmune diseases ($P < 0.01$; Fig. 2), who in turn consistently had the lowest percentages of tetramer-specific $CD8^+$ T cells. HTLV-1 proviral load in HTLV-1-infected persons with autoimmune diseases was significantly lower than in asymptomatic HTLV-I carriers (28.8 and 62.2 copies/1,000 PBMCs, respectively; $P < 0.05$). With respect to Env11-19/HLA-A*2402 tetramer-binding, asymptomatic carriers consistently had the lowest percentages of Env-tetramer specific $CD8^+$ T cells (mean ± SD, 0.036% ± 0.026), which was significantly lower than in carriers with autoimmune diseases (0.067% ± 0.043, $P < 0.05$), and marginally lower than in HAM/TSP patients (0.066% ± 0.042, $P = 0.064$).

DISCUSSION

Myriad autoimmune diseases, including T cell alveolitis, myopathy, uveitis, certain types of arthritis, Sjögren's syndrome, and SLE, are seen in persons infected with HTLV-1 [Sugimoto et al., 1987; Vernant et al., 1988; Nishioka et al., 1989; Terada et al., 1994; Akimoto et al., 2007b]. The precise nature of the association between HTLV-1 and these diseases, however, remains unclear. Previous studies have focused on the rather robust cytotoxic T cell response to the HTLV-1 Tax protein in patients with HAM/TSP [Jacobson et al., 1990; Kubota et al., 2003; Bangham, 2008], but there has been little attention given to other HTLV-1

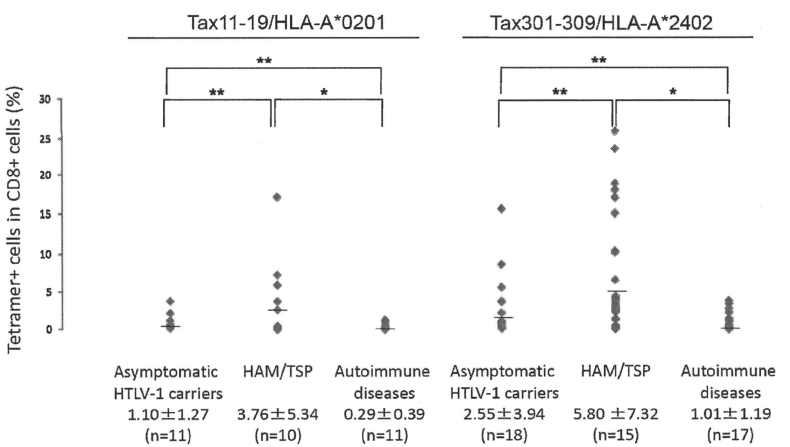

Fig. 2. Frequency of Tax11-19/HLA-A*0201 or Tax301-309/HLA-A*0201 tetramer binding $CD8^+$ T cells in asymptomatic HTLV-1 carriers, HAM/TSP patients, and carriers with autoimmune diseases. The percentage of $tetramer^+$ cells in $CD8^+$ lymphocytes in asymptomatic HTLV-1 carriers, HAM/TSP patients, and carriers with autoimmune diseases. Horizontal bars indicate the mean percentages of $tetramer^+$. The numbers below each subject group are the means ± SD. *$P < 0.01$; **$P < 0.05$ (significantly different as determined by Mann–Whitney U-test).

preferential CTL targets, particularly the HTLV-1 envelope [Pique et al., 1994, 1996]. In this study, HLA class I tetramers were used to measure the CTL response to HTLV-1 Tax and Env in a group of asymptomatic people infected with HTLV-1, patients with HAM/TSP, and persons with autoimmune diseases and concomitant HTLV-1 infection. The proportion of individuals with detectable Env tetramer-specific CTL responses was significantly higher in patients with HAM/TSP or autoimmune diseases than in asymptomatic carriers (Table II). Furthermore, patients with HAM/TSP or autoimmune diseases recognized a significantly broader repertoire of Env epitopes (four and six out of a total of six Env epitope tetramers tested, respectively), compared with asymptomatic carriers (one out of six Env epitope tetramers tested). Consistently, patients with HAM/TSP or autoimmune disease had a higher percentage of Env11-19/HLA-A*2402 specific CTLs. These findings were more pronounced for people with HLA-A*2402 than HLA-A*0201 alleles.

Antigen-mediated activation of $CD4^+$ helper T cells (Th) is essential for $CD8^+$ CTL activation. Antigen specific Th cells can be activated by Env-specific B cells, and $CD4^+$ T cells specific for intracellular viral antigens, giving cognate assistance to B cells [Scherle and Gerhard, 1986]. HTLV-1 Env epitopes eliciting both Th and B cell responses have been previously described [Jacobson et al., 1991]. Kitze et al. [1998] have also demonstrated a $CD4^+$ Th response to HTLV-1 Env glycoprotein gp21, an important target antigen in patients with HAM/TSP. The enhanced $CD4^+$ T cell responsiveness known to be characteristic of autoimmune conditions, and similarly described in HAM/TSP [Yamano et al., 1997], may therefore explain the broader Env epitope repertoire and increased Env specific CTL frequency in people with HAM/TSP or autoimmune diseases observed in this study. Previous studies examining the breadth of the T cell receptor repertoire in $CD8^+$ T cells in people infected with HTLV-1 have reported conflicting results. One study found no significant difference in the number of expanded $CD8^+$ T cell clones when asymptomatic carriers were compared with HAM/TSP patients [Eiraku et al., 1998]. Another study, using Immunoscope methods to examine the breadth of T cell clonal expansion, reported significantly greater breadth of the CTL repertoire in HAM/TSP patients compared with asymptomatic carriers [Ureta-Vidal et al., 2001]. Peripheral blood derived T lymphocytes, examined using reverse transcription-polymerase chain reaction/single-stranded conformational polymorphism methods, were also found to have a wider variety of HTLV-1 specific T-cell clonotypes in HAM/TSP patients compared with carriers [Hoger et al., 1997]. This study also demonstrates clearly, that a greater proportion of HAM/TSP patients had detectable Env specific CTLs, and that HAM/TSP patients had a broader repertoire of CTLs recognizing Env epitopes, as well as higher mean percentage of Env-specific CTLs within the $CD8^+CD45^+$ T cell subset, compared with asymptomatic carriers. This study goes further to demonstrate that like HAM/TSP patients, people with autoimmune diseases and HTLV-I infection, also have a broader CTL repertoire and higher frequency of Env-specific CTLs compared with asymptomatic carriers.

Increased proviral load in asymptomatic carriers is associated with an increased risk for progression to HAM/TSP. The Tax-specific CTL response is critical in controlling proviral load. Although increased in HAM/TSP compared with asymptomatic carriers [Nagai et al., 1998], the CTLs in HAM/TSP have been demonstrated to have lower cytolytic efficiency [Bangham, 2008; Kattan et al., 2009]. The higher Tax-specific CTL frequency, but with higher proviral load, observed in HAM/TSP patients compared with asymptomatic persons infected with HTLV-1 is consistent with low CTL efficiency. Conversely, higher CTL efficiency could explain the lower $CD8^+$ Tax-specific CTL frequency seen in people with autoimmune diseases, who have a significantly lower proviral load than asymptomatic carriers in this study. This difference in CTL efficiency remains to be confirmed.

In this study, it was demonstrated that the difference in frequency of HTLV-1 Tax-specific $CD8^+$ T cells was not statistically significant between asymptomatic HTLV-1 carriers, HAM/TSP or carriers with autoimmune diseases, while HTLV-1 Env-specific $CD8^+$ T cells were significantly more frequent in HAM/TSP and carriers with autoimmune diseases than those in asymptomatic HTLV-1 carriers. These results suggest that the diversity, frequency, and repertoire of HTLV-1-specific $CD8^+$ T cell clones, especially HTLV-1 Env $CD8^+$ T cells may be related to the hyperimmune response in HAM/TSP and carriers with autoimmune diseases, although different immunological mechanisms may mediate the hyperimmune responses in HAM/TSP and autoimmune diseases.

ACKNOWLEDGMENTS

We thank Mrs. Hokkoku and Mrs. Higashi for technical assistance.

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