excess amount of $SO_3 \cdot NMe_3$ in anhydrous DMF at 50 °C and the reaction progress was monitored by TLC (disappearance of the starting material and if possible for product formation). However, in this case, we expected high efficiency of a complete O-sulfation, and it was quite impossible to observe the fully *O*-sulfated compound in TLC. Instead, we stopped the reaction by cooling down and separated the product from the reagents with a Sephadex G-15 column eluted with water. Fractions stained with Azure reagent[38] were combined and concentrated to dryness. Then, we lyophilized the fractions and took an NMR. If the reaction was not completed, we observed several peaks corresponding to the methyl ester of **18** or **33**. After stirred at 50 °C for 5 days, we were able to see the single peak for the methyl ester of sulfated compounds. The sulfur content of the sulfated compounds was calculated by elemental analysis.

The β(1→2)- (**1**) and β(1→6)-tetramers (**4**) showed interesting and potent biological activities as mentioned. We anticipated that those activities might relate to their conformation. In our previous study, CD spectroscopy data of a β(1→2)-linked hexamer suggested the possibility of a 14-helical structure.[21] In addition, a β(1→2)-linked oligomer can be particularly assumed as a unique β-peptide with functionality on molecules such as hydroxyl groups and ring oxygen. Therefore, we focused on a β(1→2)-linked oligomer for conformational study among our carbopeptoid oligomers **1**–**4**. Conformational search calculations were performed with the package of MacroModel ver. 8.1 (Schrödinger Inc.) on an SGI workstation. The Monte Carlo Multiple Minimum (MCMM) method and AMBER* force field were used to find the global and local minimum energy conformation. As the initial structure, an extended structure was used. More than 10,000 conformers were optimized. Figure 5 shows the result of conformational analysis of a β(1→2)-linked decamer with a molecular modeling calculation. Interestingly, it revealed that a typical right-handed 16-helix was the most stable conformation. The 16-helix structure was stabilized with a hydrogen bond between oxygen of the C-1 carbonyl group and nitrogen of the C-2 amino group of another unit as shown in Figure 5. Although we examined various calculations, β(1→2)-linked oligomers from dimer to decamer have never had left-handed conformation except tetramers. This finding corresponds to the result of β-amino acid oligomer reported by Gellman et al.[23,24] In the case of the tetramer, one reason why it possessed sinistral structure might be steric hinderance caused by amino acids.

This unique conformation of a β(1→2)-linked oligomer may give various functions such as protein–protein interaction. In an earlier work in this field, Seebach demonstrated that β-peptide hairpins could bind somatostatin receptors with high affinity and specificity.[39,40] Seebach and co-workers,[41] DeGrado and co-workers,[42,43] and Gellman and co-workers[44–46] demonstrated that amphiphathic β-peptides could perform a variety of functions including the inhibition of cholesterol and fat uptake,[41] potent antibacterial activity,[42–45] and RNA binding.[46] Recently, some groups reported the synthesis and investigation of the helical structure of α, β, and γ-peptide including molecular dynamics simulation.[47–51] Some evaluated biological activity, for example, Kritzer et al. studied protein–protein interaction inhibitors, which were made from 14-helical β-peptide.[47] Meanwhile Porter et al. reported structure–activity trends among

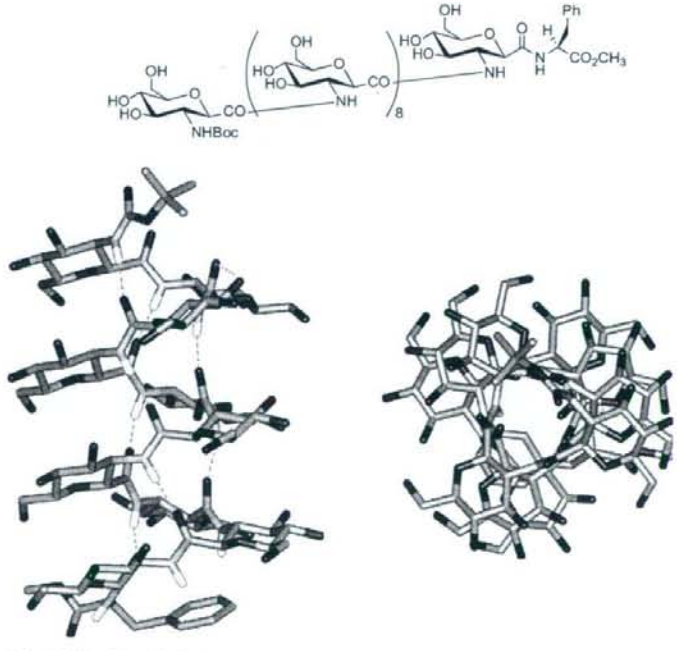

Figure 5. Conformational analysis of β(1→2)-amide-linked analogue.

helix-forming β-amino acid oligomers that are intended to mimic α-helical host-defense peptides.[48] Their β-peptide displayed antimicrobial activity. These findings indicate that a β-peptide field is now poised to make significant contributions to chemical biology. Protein–protein interactions are extremely difficult to target; however, the foldamer scaffold may provide a general platform for controlling crucial interactions with high potency and specificity. Unlike the case of reported peptides, our carbopeptoid oligomers have peptide as well as carbohydrate properties, therefore, modification of their hydroxyl groups can be a new methodology for therapeutic agents.

3. Conclusion

We have shown the synthetis strategy for a new class of oligomers composed of carbopeptoids, which possess a carboxylate at the anomeric position and an amino group replacing one of the hydroxyl groups of monosaccharide, and this synthesis can be effectively accomplished in a stereo- and regioselective manner. We also examined conformational analysis with molecular modeling and found that the β(1→2)-linked oligomer formed a rigid secondary structure, therefore it had the property of a folding β-peptide. Some *O*-sulfated oligomers effectively inhibited HIV infection, sialyl Lewis x-mediated cell adhesion, and heparanase activity in a linkage-specific manner, as reported in the previous paper. Although further investigation is required to clarify the structure–activity relationships, these findings may lead to the design of a new class of biologically active analogues of peptides or oligosaccharides. Thus, our analogues are especially interesting as tools for mimicking native peptide and protein structures, and may stimulate synthesis work in this field.

4. Experimental

4.1. General

Unless otherwise indicated, all starting materials were obtained from commercial suppliers and without further purification. All reactions were conducted under Ar atmosphere. When reactions were worked up by extraction with CH_2Cl_2, $CHCl_3$, or EtOAc, organic solutions were dried with molecular sieves 4 Å and concentrated with a rotary evaporator. Silica gel column chromatography was performed using Fisher Scientific S704-25 60–200-mesh silica gel. Reactions and chromatography fractions were analyzed using Merck silica gel 60 F-254 TLC plates. ^{1}H NMR and ^{13}C NMR spectra were measured in $CDCl_3$ at 300 and 75 MHz, respectively. Chemical shifts are expressed in parts per million relative to internal $CHCl_3$. *J* values are in Hertz. Melting points were determined in Pyrex capillaries. IR spectra were measured as solutions in the solvent indicated.

4.1.1. 4,5,7-Tetra-*O*-acetyl-3-phthalimido-D-*glycero*-D-*gluco*-hepturonamide (6). A suspension of 3-phthalimido-4,5,7-tetra-*O*-acetyl-2,6-anhydro-D-*gulo*-heptononitrile (**5**, 2-phthalimido-3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl cyanide; 7.71 g, 17.3 mmol) in 30% (w/w) hydrogen bromide–glacial acetic acid (17 mL) was stirred in a closed vessel for 3 h at room temperature. The resulting solution was then poured into stirred ice and water (200 mL), which was immediately extracted with chloroform (2×200 mL). The extracts were combined, successively washed with saturated aqueous sodium hydrogen carbonate solution (2×100 mL) and water (100 mL), and then processed as described under Section 4.1. Crystallization from chloroform (30 mL)–diethyl ether (120 mL) gave **6** (6.81 g, 14.7 mmol; 85% yield) as heteromorphic crystals, mp 223–226 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.86–7.83 (m, 2H, Phthalimide), 7.75–7.70 (m, 2H, Ph), 6.47 (s, 1H, NH), 5.94 (t, 1H, *J*=9.8 Hz, H-3), 5.69 (s, 1H, NH), 5.16 (t, 1H, *J*=9.7 Hz, H-4), 4.84 (d, 1H, *J*=10.8 Hz, H-1), 4.44 (t, 1H, *J*=10.6 Hz, H-2), 4.33 (dd, 1H, *J*=4.8, 12.4 Hz, H-6a), 4.23 (dd, 1H, *J*=2.1, 12.5 Hz, H-6b), 3.96–3.91 (m, 1H, H-5), 2.13, 2.05, 1.88 (3s, 3H each, 3×CH_3CO); ^{13}C NMR (72.5 Hz, $CDCl_3$): δ 170.7, 169.9, 169.4 (3×CH_3CO), 167.7, 153.9 (CO), 134.1, 123.6 (Ph), 75.7, 72.3, 71.0, 68.7, 61.9, 51.9 (C-1, 2, 3, 4, 5, 6), 13.8, 13.4, 13.1 (3×CH_3CO); Anal. Calcd for $C_{21}H_{22}O_{10}N_2 \cdot 1/2H_2O$: C, 53.52; H, 4.92; N, 5.94. Found: C, 53.32; H, 4.94; N, 5.33.

4.1.2. (2*R*,3*R*,4*R*,5*S*,6*R*)-Methyl tetrahydro-4,5-dihydroxy-6-(hydroxymethyl)-3-(1,3-dioxoisoindolin-2-yl)-2*H*-pyran-2-carboxylate (7). The amide (**6**) (12.0 g, 30.0 mmol) and the resin of Dowex 50W-X8 [H$^+$] were combined with sufficient solvent (MeOH) to cover the resin (~50 mL). For slightly soluble amides, the volume of solvent was doubled. Gentle agitation was provided by a magnetic spin bar small enough to prevent powdering of the resin. The flask was tightly stoppered and warmed to the reflux temperature of the solvent for 16 h. The progress of the reaction was monitored by TLC. Removal of the resin by filtration and evaporation of the solvent provided the crude product, which was purified by silica gel column chromatography (1:1–0:1 hexane/EtOAc) to afford **7** (8.80 g, 25.0 mmol; 97%) as a white powder, mp 94–97 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.90–7.82 (m, 4H, Ph), 4.74 (d, 1H, *J*=8.6 Hz, H-1), 4.41 (dd, 1H, *J*=8.3, 10.3 Hz, H-2), 4.14 (t, 1H, *J*=10.6 Hz, H-3), 3.92 (dd, 1H, *J*=1.3, 12.1 Hz, H-6a), 3.74 (dd, 1H, *J*=4.7, 12.0 Hz, H-6b), 3.45–3.43 (m, 2H, H-4, 5); ^{13}C NMR (72.5 Hz, CD_3OD): δ 170.4 (CO_2CH_3), 169.43, 169.37 (2×PhCON), 135.7, 132.8, 124.3 (Ph), 82.0, 74.8, 73.0, 72.1, 62.5, 55.7, 53.0 (C-1,2,3,4,5,6, CO_2CH_3); Anal. Calcd for $C_{16}H_{17}O_8N \cdot 1/2H_2O$: C, 53.33; H, 5.04; N, 3.89. Found: C, 53.51; H, 4.99; N, 3.66.

4.1.3. (2*R*,3*R*,4*R*,5*S*,6*R*)-3-Amino-tetrahydro-4,5-dihydroxy-6-(hydroxymethyl)-2*H*-pyran-2-carboxylic acid hydrochloride (8). Compound **7** (11.3 g, 35.2 mmol) was dissolved in 100 mL of MeOH/H_2O 3:1, cooled in an ice bath for 10 min, and then added to a suspension of 8.85 g of LiOH·H_2O (211 mmol) in 20 mL of MeOH/H_2O 3:1 at 0 °C, then the mixture was stirred at 60 °C for 16 h. After cooling to room temperature, the pH of the solution was adjusted to neutral by the addition of 1 N hydrochloric acid, and then the solvent was evaporated. Furthermore, 300 mL of 3 N hydrochloric acid was added to the residue and the acidic mixture was brought to reflux for 3 h. After the solution was concentrated to remove HCl, the resin was dissolved in 2 L of water and the pH adjusted to 7.0–7.5 using 1 N aqueous NH_4OH. The crude reaction mixture was then eluted onto a Dowex 50W-X8 [H$^+$] column, and washed with water, MeOH, water (each 500 mL), and then

eluted with 1 L of 1 N aqueous NH_4OH. Ninhydrin-active fractions were collected and concentrated, then the resin was suspended in 50 mL of 1 N hydrochloric acid and evaporated. Lyophilization with a small amount of water gave **8** (8.10 g, 33.2 mmol; 95%) as a colorless amorphous powder: ^{1}H NMR (300 MHz, D_2O): δ 4.10 (d, 1H, J=10.5 Hz, H-1), 3.63 (1H, H-6a), 3.51–3.42 (m, 2H, H-4, 6b), 3.31–3.16 (m, 2H, H-2, 5), 3.10 (t, 1H, J=10.4 Hz, H-3); ^{13}C NMR (72.5 Hz, D_2O): δ 171.8 (CO_2H), 80.5, 74.02, 73.93, 70.2, 61.4, 53.7 (C-1,2,3,4,5,6).

4.1.4. (2*R*,3*R*,4*R*,5*S*,6*R*)-2-Oxo-2-phenylethyl 4,5-diacetoxy-6-(acetoxymethyl)-3-(*tert*-butoxycarbonylamino)-tetrahydro-2*H*-pyran-2-carboxylate (9). To a stirred solution of **8** (4.2 g, 12.0 mmol) and Et_3N (7.21 mL, 51.7 mmol) in water (30 mL) was added a solution of BOC-ON (6.43 g, 25.9 mmol) in 1,4-dioxane (30 mL). After 3 h at room temperature, the solution was concentrated, and the residue was dissolved in 50 mL of water. The water solution was then adjusted to neutral using Dowex 50W-X8 [H^+] and washed with 50 mL of CH_2Cl_2, followed by evaporation of the aqueous layer. To a DMF solution (50 mL) of the residue was added 2-bromoacetophenone (phenacylbromide) (5.25 g, 25.8 mmol) and Et_3N (3.6 mL, 25.8 mmol). The mixture was stirred at room temperature for 3 h, then pyridine (60 mL) and acetic anhydride (30 mL) were added to the solution and stirred at room temperature for 12 h. The mixture was poured into ice water and extracted three times with EtOAc (200 mL each), and organic layers were washed with 0.5 N hydrochloric acid, water, saturated aqueous $NaHCO_3$, and brine. After drying ($MgSO_4$), the solution was filtered and concentrated. The residue was purified by silica gel column chromatography (hexane/EtOAc 5:1) to afford **9** (4.81 g, 8.70 mmol; 73% overall) as a white powder, mp 194–197 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.90 (2H, Ph), 7.62 (1H, Ph), 7.49 (2H, Ph), 5.47 (t, 1H, J=9.5 Hz, H-3), 5.40 (t, 2H, J=16.3 Hz, CH_2COPh), 5.10 (t, 1H, J=9.6 Hz, H-4), 4.45 (d, 1H, J=10.4 Hz, H-1), 4.27 (dd, 1H, J=5.2, 12.5 Hz, H-6a), 4.15 (dd, 1H, J=2.2, 12.4 Hz, H-6b), 3.88 (1H, H-2), 3.80–3.75 (m, 1H, H-5), 2.08, 2.06, 2.03 (3s, 3H each, 3×CH_3CO), 1.40 (s, 9H, NHCO$_2t$*Bu*); ^{13}C NMR (72.5 Hz, $CDCl_3$): δ 170.5, 170.4, 169.4, 167.1 (CO), 133.9, 128.8, 127.7 (Ph), 76.8, 75.9, 72.7, 68.7, 66.7, 62.3 (C-1,2,3,4,5,6), 28.1 (*t*-Bu), 20.6, 20.51, 20.45 (3×CH_3CO); mass spectrum (EI) *m/e* 569.2349 (M^+, calcd 569.2347).

4.1.5. (2*R*,3*R*,4*R*,5*S*,6*R*)-4,5-Diacetoxy-6-(acetoxymethyl)-3-(*tert*-butoxycarbonylamino)-tetrahydro-2*H*-pyran-2-carboxylic acid (β(1→3)-linked monomer) (10). Compound **9** (2.70 g, 4.90 mmol) was dissolved in EtOAc/EtOH 2:1 (60 mL), and 10% Pd/C (400 mg) was added to the solution. The resulting mixture was stirred at room temperature for 16 h under H_2. Analysis of the reaction mixture by TLC ($CHCl_3$/MeOH, 2:1) indicated the disappearance of the starting material. The catalyst was filtered off and washed with MeOH. The combined solution was concentrated in vacuo to provide a crude product. Column chromatography ($CHCl_3$/MeOH, 20:1) of the residue gave compound **10** (1.91 g, 4.38 mmol; 90%) as a white powder, mp 193–195 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 5.17 (t, 1H, J=9.4 Hz, H-3), 4.99 (t, 1H, J=9.6 Hz, H-4), 4.29 (dd, 1H, J=5.0, 12.4 Hz, H-6a), 4.10 (dd, 1H, J=2.0, 12.3 Hz, H-6b), 3.97–3.86 (m, 2H, H-1, 2), 3.79–3.75 (m, 1H, H-5), 2.04, 2.00, 1.99 (3s, 3H each, 3×CH_3CO), 1.40 (s, 9H, NHCO$_2t$*Bu*); ^{13}C NMR (72.5 Hz, CD_3OD): δ 172.5 (CO_2H), 171.9, 171.4 (3×CH_3CO), 157.6 (NHCO_2tBu), 80.9, 76.9, 75.9, 70.3, 63.8, 54.4 (C-1,2,3,4,5,6), 28.7 (*t*-Bu), 20.7, 20.68, 20.61 (3×CH_3CO); mass spectrum (EI) *m/e* 451.1928 ($(M+NH_4)^+$, calcd 451.1928).

4.1.6. (2*R*,3*S*,4*R*,5*R*,6*R*)-2-(Acetoxymethyl)-5-(*tert*-butoxycarbonylamino)-6-(1-methoxy-1-oxo-3-phenylpropan-2-ylcarbamoyl)-tetrahydro-2*H*-pyran-3,4-diyl diacetate (11). DEPC (524 μL, 3.21 mmol) was added to a stirred solution of compound **10** (1.16 g, 2.68 mmol), L-phenylalanine methyl ester (692 mg, 3.21 mmol), and Et_3N (821 μL, 5.89 mmol) in DMF (30 mL) at 0 °C under argon. The mixture was stirred at 0 °C for 1 h, and then at room temperature for 12 h. The reaction mixture was diluted with EtOAc (200 mL), and then washed successively with water (200 mL), and saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over anhydrous $MgSO_4$, and concentrated to dryness in vacuo. The residue was chromatographed on silica gel using toluene/EtOAc 5:1 to give **11** (1.23 g, 2.30 mmol; 86%) as a white powder, mp 231–234 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.27–7.12 (m, 5H, Ph), 6.87 (d, 1H, J=8.1 Hz, NH), 5.27 (m, 2H, H-1, 3), 5.03 (t, 1H, J=9.7 Hz, H-4), 4.80 (m, 1H, CHCO$_2$CH$_3$), 4.21 (dd, 1H, J=5.0, 12.4 Hz, H-6a), 4.14 (dd, 1H, J=1.2, 11.9 Hz, H-6′), 4.03 (d, 1H, J=9.9 Hz, H-6b), 4.03 (d, 1H, J=9.9 Hz, NH), 3.81–3.70 (m, 5H, H-2, 5, CO$_2$CH_3), 3.18–3.02 (m, 2H, CH_2Ph of L-Phe), 2.07, 2.06, 2.02 (3s, 3H each, 3×CH_3CO), 1.39 (s, 9H, NHCO$_2t$*Bu*); ^{13}C NMR (72.5 MHz, $CDCl_3$): δ 171.1, 170.4, 169.2 (3×CH_3CO), 170.2 (CO_2CH_3), 166.8 (NHCO), 154.9 (NHO*C*O*t*Bu), 135.6, 129.0, 128.2, 126.9 (Ph), 79.5, 76.8, 75.2, 73.1, 68.4, 62.0 (C-1,2,3,4,5,6), 52.6 (*C*HCO$_2$CH$_3$), 52.0 (CO_2CH_3), 37.6 (*C*H$_2$Ph of L-Phe), 27.9 (*t*-Bu), 20.44, 20.38, 20.33 (3×CH_3CO); mass spectrum (EI) *m/e* 595.2513 ($(M+H)^+$, calcd 595.2503).

4.1.7. β(1→2)-Linked dimer (13). A solution of 4 N HCl in EtOAc (10 mL) was added to a stirred solution of compound **11** (887 mg, 1.49 mmol) in EtOAc (10 mL) at 0 °C. The mixture was stirred at room temperature for 1 h. After evaporation of the solvent, the residue was washed with ether to give **12** as a white powder. This compound was reacted without further purification. Compound **12**, BOP reagent (792 mg, 1.79 mmol), and DIEA (572 μL, 3.94 mmol) were added to a solution of compound **10** (776 mg, 1.79 mmol) in DMF (20 mL) at room temperature under argon. The reaction mixture was stirred at room temperature for 16 h, then diluted with EtOAc (200 mL), and successively washed with water, saturated aqueous $NaHCO_3$, and brine (150 mL each). The organic layer was dried over anhydrous $MgSO_4$ and filtered. The filtrate was concentrated and the residue was chromatographed on silica gel using toluene/EtOAc 5:1–1:1 to afford **13** (800 mg, 879 μmol; 59%) as a white powder, mp 198–203 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.29–7.11 (m, 5H, Ph), 7.01 (d, 1H, J=7.9 Hz, NH), 6.70 (d, 1H, J=7.1 Hz, NH), 5.38 (t, 1H, J=9.7 Hz, 1H-3), 5.21 (t, 1H, J=9.5 Hz, 2H-3), 5.07–4.98 (m, 3H, 1H-4, 2H-1, 4), 4.72 (dd, 1H, J=6.3, 14.0 Hz, CHCO$_2$CH$_3$), 4.29–4.08 (m, 5H, 1H-6a, 6b, 2H-1, 6a, 6b), 3.97–3.87 (m, 2H, 1H-2, 5), 3.77–3.55 (m, 5H, 2H-2, 5, CO$_2$CH_3), 3.18–3.06 (m, 2H, CH_2Ph of L-Phe), 2.08, 2.06, 2.05, 2.02

(s, 3H each, 6×CH_3CO), 1.39 (s, 9H, NHCO$_2$*tBu*); ^{13}C NMR (72.5 MHz, $CDCl_3$): δ 171.1, 170.9, 170.6, 170.5, 169.30, 169.25 (6×CH_3CO), 170.1 (CO_2CH_3), 167.7, 166.8 (NH*C*O), 155.2 (NHO*C*O*t*Bu), 135.8, 129.0, 128.4, 126.9 (Ph), 79.8, 76.3, 76.2, 75.5, 73.3, 72.3, 68.4, 68.3, 62.1, 61.9, 52.9 (*C*HCO$_2$CH$_3$), 52.2 (CO_2CH_3), 37.4 (*C*H$_2$Ph of L-Phe), 28.0 (*t*-Bu), 20.6, 20.5, 20.4 (6×*C*H$_3$CO); mass spectrum (FAB) *m/e* 932.2 ((M+Na)$^+$, calcd 932.3), 810.1 ((M−Boc+2H)$^+$ calcd 810.3).

4.1.8. β(1→2)-Linked trimer (15). As described for **13**, 4 N HCl in EtOAc (10 mL) was added to a stirred solution of compound **13** (712 mg, 783 μmol) in EtOAc (10 mL), followed by treatment with BOP reagent (415 mg, 938 μmol), DIEA (300 μL, 1.72 mmol), and compound **10** (407 mg, 938 μmol) in DMF (20 mL) at room temperature under argon to give **15** (478 mg, 390 μmol; 50%) as a white powder, mp 118–121 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.28–7.11 (m, 5H, Ph), 6.94 (d, 1H, *J*=6.9 Hz, NH), 5.25 (t, *J*=8.7 Hz, 2H), 5.14–4.98 (m, 4H), 4.77 (dd, 1H, *J*=5.5, 12.1 Hz, *C*HCO$_2$CH$_3$), 4.35–4.00 (m, 10H), 3.78–3.72 (m, 6H), 3.55 (dd, 1H, *J*=8.8, 15.5 Hz), 3.22–3.08 (2H, *C*H$_2$Ph of L-Phe), 2.07–1.99 (s, 3H each, 9×*C*H$_3$CO), 1.45 (s, 9H, NHCO$_2$*tBu*); ^{13}C NMR (72.5 MHz, $CDCl_3$): δ 170.9, 170.4, 170.3, 170.2, 170.1, 169.9, 169.3, 169.2, 169.1 (9×CH_3CO, CO_2CH_3), 168.2, 167.9, 166.8 (NH*C*O), 156.2 (NHO*C*O*t*Bu), 135.5, 129.0, 128.7, 126.9 (Ph), 80.4, 77.2, 77.1, 75.3, 75.2, 74.9, 73.4, 73.3, 72.7, 68.9, 68.4, 62.4, 62.1, 62.0, 52.7 (*C*HCO$_2$CH$_3$), 52.0 (CO_2CH_3), 37.3 (*C*H$_2$Ph of L-Phe), 27.9 (*t*-Bu), 20.3, 20.26, 20.18 (9×*C*H$_3$CO); mass spectrum (FAB) *m/e* 1247.5 ((M+Na)$^+$, calcd 1247.4), 1125.3 ((M−Boc+2H)$^+$ calcd 1125.4).

4.1.9. β(1→2)-Linked tetramer (17). As described for **13**, 4 N HCl in EtOAc (10 mL) was added to a stirred solution of compound **15** (478 mg, 390 μmol) in EtOAc (10 mL), followed by treatment with BOP reagent (207 mg, 468 μmol), DIEA (150 μL, 859 μmol), and compound **10** (203 mg, 468 μmol) in DMF (10 mL) at room temperature under argon to give **17** (269 mg, 175 μmol; 45%) as a white powder, mp 134–138 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.29–7.14 (m, 5H, Ph), 7.09 (d, 1H, *J*=7.5 Hz, NH), 7.00 (d, 1H, *J*=7.7 Hz, NH), 5.46 (m, 1H), 5.37–5.28 (m, 3H), 5.10–4.99 (m, 4H), 4.73 (dd, 1H, *J*=6.0, 13.6 Hz, *C*HCO$_2$CH$_3$), 4.30–4.10 (m, 13H), 3.94–3.83 (m, 2H), 3.73–3.61 (m, 8H), 3.21–3.07 (m, 2H, *C*H$_2$Ph of L-Phe), 2.11–2.00 (s, 3H each, 12×*C*H$_3$CO), 1.41 (s, 9H, NHCO$_2$*tBu*); ^{13}C NMR (72.5 MHz, $CDCl_3$): δ 171.0, 170.7, 170.6, 170.5, 170.4, 170.2, 169.5, 169.4, 169.3, 169.1 (12×CH_3CO, CO_2CH_3), 168.7, 168.0, 167.7, 167.1 (NH*C*O), 156.1 (NHO*C*O*t*Bu), 135.6, 129.2, 128.9, 127.1 (Ph), 79.8, 76.7, 75.4, 75.2, 75.0, 74.7, 72.3, 68.6, 68.4, 68.2, 62.2, 62.1, 62.0, 53.0 (*C*HCO$_2$CH$_3$), 52.3 (CO_2CH_3), 37.5 (*C*H$_2$Ph of L-Phe), 28.3 (*t*-Bu), 20.8, 20.6, 20.5, 20.4, 20.3 (12×*C*H$_3$CO); mass spectrum (FAB) *m/e* 1562.7 ((M+Na)$^+$, calcd 1562.5), 1540.6 ((M+Na)$^+$, calcd 1540.5), 1440.5 ((M−Boc+2H)$^+$, 1440.5).

4.1.10. β(1→2)-Linked tetramer (1). 30% MeONa in MeOH was added to a solution of compound **17** (269 mg, 175 μmol) in MeOH (10 mL) at room temperature and the pH was adjusted to 10–11. The mixture was stirred at room temperature for 3 h. Dowex 50W [H$^+$] was added and the pH was adjusted to neutral. After the removal of Dowex 50W [H$^+$], the filtrate was concentrated. The residue was purified by Sephadex G-10 to afford **1** (123 mg, 120 μmol; 68%) as a white powder, mp 125–128 °C; ^{1}H NMR (300 MHz, D_2O): δ 7.32–7.17 (m, 5H, Ph), 4.57 (dd, 1H, *J*=6.5, 8.2 Hz, *C*HCO$_2$CH$_3$), 3.87–3.58 (m, 21H), 3.50–3.32 (m, 10H), 3.10 (dd, 1H, *J*=8.2, 14.0 Hz, *C*H$_2$Ph of L-Phe), 2.95 (dd, 1H, *J*=6.5, 14.0 Hz, *C*H$_2$Ph of L-Phe), 1.35 (s, 9H, NHCO$_2$*tBu*); ^{13}C NMR (72.5 MHz, D_2O): δ 173.5 (CO_2CH_3), 171.8, 171.5, 171.1, 170.5 (NH*C*O), 158.3 (NHO*C*O*t*Bu), 136.7, 129.6, 129.2, 127.7 (Ph), 81.8, 79.6, 79.5, 79.4, 78.1, 77.1, 77.0, 75.1, 74.9, 74.7, 74.4, 70.0, 69.7, 69.6, 61.0, 60.9, 60.7, 55.8, 54.3, 54.2, 54.1, 53.5, 53.3 (CO_2CH_3), 37.0 (*C*H$_2$Ph of L-Phe), 28.1 (*t*-Bu); mass spectrum (FAB) *m/e* 1036.3 ((M+H)$^+$, calcd 1036.4).

4.1.11. (2*R*,3*R*,4*S*,5*S*,6*R*)-Methyl tetrahydro-3,4,5-trihydroxy-6-(hydroxymethyl)-2*H*-pyran-2-carboxylate (19). Compound **19** was prepared from D-glucose pentaacetate by a known sequence in large quantities as a colorless oil: ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.31–3.74 (br, 3H, OH), 3.66–3.61 (m, 5H, H-1, 6, 6′), 3.37 (dd, 1H, *J*=6.0, 11.9 Hz, H-5), 3.29 (t, 1H, *J*=9.2 Hz, H-3), 3.19–3.11 (m, 4H, H-4 and CO_2CH_3), 3.04 (dd, 1H, *J*=9.1, 17.8 Hz, H-2); ^{13}C NMR (72.5 MHz, CD_3OD): δ 171.5 (CO_2CH_3), 81.3, 79.4, 78.3, 72.5, 70.4, 62.1, 52.9 (C-1,2,3,4,5,6, CO_2CH_3); mass spectrum (FAB) *m/e* 223.1 ((M+H)$^+$, calcd 223.1).

4.1.12. (2*R*,3*R*,4*S*,5*S*,6*R*)-Methyl tetrahydro-3,4,5-trihydroxy-6-((*tert*-butyldimethylsilyloxy)methyl)-2*H*-pyran-2-carboxylate (20). To a solution of **19** (9.60 g, 43.2 mmol) in DMF (50 mL) was added *tert*-butyldimethylsilyl chloride (7.81 g, 51.8 mmol) and imidazole (7.35 g, 10.8 mmol) at 0 °C, and the suspension was stirred for 2 h at room temperature. The reaction mixture was diluted with EtOAc (300 mL), and successively washed with water (2×200 mL) and brine (200 mL). The organic layer was dried ($MgSO_4$), and concentrated in vacuo. The residue was purified by silica gel column chromatography (1:1 toluene/EtOAc to EtOAC only) to afford **20** (12.1 g, 40.0 mmol; 83%) as a white heteromorphic crystals: ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.64–3.58 (m, 2H, H-6, 6′), 3.34 (s, 3H, CO_2CH_3), 3.32–3.24 (m, 2H, H-1, 5), 3.21–3.13 (m, 2H, H-3, 4), 3.09–3.05 (m, 1H, H-2), 0.83 (s, 9H, $(CH_3)_3CSi(CH_3)_2$), 0.006, −0.013 (s, 6H, $(CH_3)_3CSi(CH_3)_2$); ^{13}C NMR (72.5 MHz, CD_3OD): δ 171.3 (CO_2CH_3), 82.3, 80.2, 79.0, 72.9, 71.0, 64.2, 52.6 (C-1,2,3,4,5,6, CO_2CH_3), 26.4 ($(CH_3)CSi(CH_3)_2$), 19.2 ($(CH_3)CSi(CH_3)_2$), −4.99, −5.04 ($(CH_3)CSi(CH_3)_2$); mass spectrum (FAB) *m/e* 337.2 ((M+H)$^+$, calcd 337.2).

4.1.13. (2*R*,3*R*,4*S*,5*R*,6*R*)-Methyl 3,4,5-tris(benzyloxy)-6-((*tert*-butyldimethylsilyloxy)methyl)-tetrahydro-2*H*-pyran-2-carboxylate (21a) and (2*R*,3*R*,4*S*,5*R*,6*R*)-benzyl 3,4,5-tris(benzyloxy)-6-((*tert*-butyldimethylsilyloxy)-methyl)-tetrahydro-2*H*-pyran-2-carboxylate (21b). To a solution of **20** (10.0 g, 29.7 mmol) in DMF (100 mL), were added BnBr (31.8 mL, 267 mmol) and Ag_2O (41.4 g, 178 mmol) at room temperature, and the suspension stirred for 20 h in a reaction vessel covered to exclude light. The reaction mixture was diluted with EtOAc (1 L), and then the precipitate was filtered off. The filtrate was washed with saturated aqueous $NaHCO_3$ (2×1 L), brine (1 L), dried

($MgSO_4$) and concentrated in vacuo. The residue was chromatographed on silica gel (heptane only to 5:1 heptane/EtOAc) to give mixture of **21a** and **21b** (1:1) (12.2 g, 75%) as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.17–7.00 (m, 30H, Ph), 5.03–4.34 (m, 12H, CH_2Ph), 3.89–3.28 (m, 14H), 3.39 (3H, CO_2CH_3), 0.83 (s, 18H, $(CH_3)_3CSi(CH_3)_2$), 0.006, −0.013 (s, 12H, $(CH_3)_3CSi(CH_3)_2$).

4.1.14. (2*R*,3*R*,4*S*,5*R*,6*R*)-Methyl 3,4,5-tris(benzyloxy)-tetrahydro-6-(hydroxymethyl)-2*H*-pyran-2-carboxylate (22). Compounds **21a** and **21b** (10.0 g) were dissolved in AcOH/THF/H_2O (3:1:1) (500 mL) at room temperature, and the solution was stirred for 15 h at room temperature. The mixture was concentrated in vacuo and the residue was azeotropied with toluene twice. The resin was then dissolved in MeOH (200 mL), and 30% NaOMe in MeOH (2.0 mL) was added to the solution. The reaction mixture was stirred at room temperature for 15 min, and then concentrated. Purification by silica gel chromatography (20:1 toluene/EtOAc to 5:1) afforded **22** (7.60 g, 15.4 mmol, 78% overall) as a white powder, mp 76–78 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.31–7.21 (m, 15H, Ph), 4.88–4.52 (m, 6H, CH_2Ph), 3.97–3.66 (m, 7H), 3.62 (s, 3H, CO_2CH_3); ^{13}C NMR (72.5 MHz, CD_3OD): δ 169.8 (CO_2CH_3), 138.3, 137.8, 137.7 (*C*Ph), 128.52, 128.47, 128.44, 128.04, 127.96, 127.90, 127.7 (Ph), 86.1, 80.0, 79.9, 78.0, 77.4 (C-1,2,3,4,5), 75.6, 75.2, 75.1 (CH_2Ph), 61.8 (C-6), 52.5 (CO_2CH_3); mass spectrum (FAB) *m/e* 493.2 ((M+H)$^+$, calcd 493.2).

4.1.15. (2*R*,3*R*,4*S*,5*R*,6*R*)-Methyl 6-(azidomethyl)-3,4,5-tris(benzyloxy)-tetrahydro-2*H*-pyran-2-carboxylate (23). To a solution of **22** (7.60 g, 15.4 mmol) in CH_2Cl_2 (100 mL) was added TsCl (5.94 g, 31.7 mmol) and pyridine (3.74 mL, 46.2 mmol) at 0 °C under argon, and the reaction mixture was stirred for 12 h at room temperature. The solution was poured into stirred ice and water (200 mL), and immediately extracted with chloroform (2×200 mL). The combined extracts were successively washed with 0.5 N hydrochloric acid (100 mL), water (100 mL), saturated aqueous $NaHCO_3$ (100 mL) and brine (100 mL), dried ($MgSO_4$), and concentrated in vacuo. The residue was used for the next coupling reaction without further purification. Sodium azido (2.03 g, 31.7 mmol) was added to the residue (prepared from 15.4 mmol of **22**) in DMF (50 mL) at room temperature under Ar, and the reaction mixture was stirred for 12 h at 60 °C. The reaction mixture was diluted with EtOAc (300 mL) and washed successively with water (300 mL), saturated aqueous $NaHCO_3$ (300 mL), and brine (300 mL), dried ($MgSO_4$), and concentrated in vacuo. The residue was chromatographed on silica gel (5:1 to 3:1 hexane/EtOAc) to give **23** (6.50 g, 12.6 mmol; 81% overall) as white needles, mp 76–77 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.36–7.19 (m, 15H, Ph), 4.95–4.77 (m, 4H, CH_2Ph), 4.62 (d, 1H, *J*=9.3 Hz, CH_2Ph), 3.95 (d, 1H, *J*=9.5 Hz, H-1), 3.84 (t, 1H, *J*=8.6 Hz, H-3), 3.77–3.71 (t, 1H, *J*=8.1 Hz, H-4), 3.61–3.48 (m, 2H, H-6a, 6b), 3.33 (dd, 1H, *J*=5.5, 13.6 Hz, H-2); ^{13}C NMR (72.5 MHz, $CDCl_3$): δ 169.2 (CO_2CH_3), 138.3, 137.80, 137.75 (*C*Ph), 128.54, 128.50, 128.44, 128.04, 128.00, 127.9, 127.8, 127.5 (Ph), 86.1, 79.9, 78.7, 78.1 (C-1,2,3,4,5), 75.6, 75.2, 75.1 (CH_2Ph), 52.4 (CO_2CH_3), 51.1 (C-6); mass spectrum (FAB) *m/e* 490.2 ((M−N_2+H)$^+$, calcd 490.2).

4.1.16. (2*R*,3*R*,4*S*,5*R*,6*R*)-6-(Azidomethyl)-3,4,5-tris(benzyloxy)-tetrahydro-2*H*-pyran-2-carboxylic acid (24). To the solution of **23** (4.40 g, 8.50 mmol) in MeOH/THF/H_2O (3:3:1) was added LiOH·H_2O (713 mg, 17.0 mmol), and the reaction mixture was stirred at room temperature for 12 h. The solution was evaporated and the residue was chromatographed on silica gel (10:1 EtOAc/MeOH) to afford **24** (3.51 g, 6.97 mmol; 82%) as a white powder, mp 68–70 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.35–7.07 (m, 15H, Ph), 4.8–4.53 (m, 12H, CH_2Ph), 3.85–3.64 (m, 3H), 3.58–3.31 (m, 4H); ^{13}C NMR (72.5 MHz, CD_3OD): δ 138.9, 137.8, 138.4 (*C*Ph), 129.0, 128.9, 128.7, 128.5, 128.4, 128.3, 128.2 (Ph), 86.6, 82.2, 81.1, 78.4, 76.0, 75.5, 75.3; mass spectrum (FAB) *m/e* 526.2 ((M+Na)$^+$, calcd 526.2).

4.1.17. (2*R*,3*R*,4*S*,5*R*,6*R*)-6-(*tert*-Butoxycarbonylaminomethyl)-3,4,5-tris(benzyloxy)-tetrahydro-2*H*-pyran-2-carboxylic acid (25). A mixture of **24** (3.51 g, 6.97 mmol) and Lindlar catalyst (2.0 g) in MeOH (100 mL) was stirred in an H_2 atmosphere at room temperature for 3 h, and then filtered through Celite. The filtrate was evaporated, then the resin was dissolved in MeOH/H_2O (3:1). To the solution were added LiOH·H_2O (585 mg, 13.9 mmol) and BOC_2O (2.35 g, 10.5 mmol), and the reaction mixture was stirred at room temperature for 12 h. The solution was evaporated and the residue was chromatographed on silica gel (10:1 EtOAc/MeOH) to give **25** (2.17 g, 3.76 mmol; 54% overall) as white needles, mp 110–112 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.34–7.22 (m, 15H, Ph), 4.87 (d, 1H, *J*=11.3 Hz, CH_2Ph), 4.83 (d, 1H, *J*=13.2 Hz, CH_2Ph), 4.78 (d, 1H, *J*=3.9 Hz, CH_2Ph), 4.75 (d, 1H, *J*=2.5 Hz, CH_2Ph), 4.66 (d, 1H, *J*=9.9 Hz, CH_2Ph), 4.62 (d, 1H, *J*=10.1 Hz, CH_2Ph), 3.78 (t, 1H, *J*=4.8 Hz, H-3), 3.71–3.62 (m, 2H, H-1, 4), 3.54 (dd, 1H, *J*=3.8, 13.9 Hz, H-6a), 3.47–3.29 (m, 3H, H-2, 5, 6b), 1.45 (s, 9H, $NHCO_2tBu$); ^{13}C NMR (72.5 MHz, CD_3OD): δ 172.5 (C-1), 166.5 (*NHCO*), 154.7 (NHO*CO*tBu), 138.3, 137.78, 137.66, 135.6 (*C*Ph), 129.2, 128.7, 128.5, 128.4, 128.3, 128.0, 127.8, 127.6, 127.4 (Ph); mass spectrum (FAB) *m/e* 600.3 ((M+Na)$^+$, calcd 600.3), 478.2 ((M−Boc+2H)$^+$, calcd 478.2).

4.1.18. (*S*)-Methyl 2-((2*R*,3*R*,4*S*,5*R*,6*R*)-3,4,5-tris(benzyloxy)-6-(*tert*-butoxycarbonylaminomethyl)-tetrahydro-2*H*-pyran-2-carboxamido)-3-phenylpropanoate (26). DEPC (339 μL, 1.93 mmol) was added to a cooled solution of **25** (800 mg, 1.38 mmol), L-phenylalanine methyl ester (358 mg, 1.67 mmol), and Et_3N (579 μL, 4.15 mmol) in DMF (10 mL) at 0 °C under Ar, and the mixture was stirred for 1 h at 0 °C, and then for an additional 12 h at room temperature. The reaction mixture was diluted with EtOAc (200 mL), and successively washed with water (200 mL), saturated aqueous $NaHCO_3$, and brine, dried ($MgSO_4$), and concentrated in vacuo. The residue was chromatographed on silica gel (toluene/EtOAc 5:1) to give **26** (941 mg, 1.27 mmol; 92% yield) as a white powder, mp 111–115 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.31–7.23 (m, 18H, Ph), 7.10 (2H, Ph), 6.57 (d, 1H, *J*=7.5 Hz, NH), 4.92–4.77 (m, 4H, CH_2Ph), 4.67–4.56 (m, 3H, CH_2Ph, $CHCO_2CH_3$), 3.80 (d, 1H, *J*=9.0 Hz, H-1), 3.73–3.53 (m, 7H, H-3, 4, 6a, 6b, CO_2CH_3), 3.41–3.31 (m, 2H, H-2, 5), 3.25 (1H, NH), 3.15 (dd, 1H, *J*=5.8, 14.0 Hz, CH_2Ph of L-Phe), 3.10 (dd, 1H, *J*=6.2, 14.0 Hz, CH_2Ph of L-Phe), 1.47 (s, 9H, $NHCO_2tBu$); ^{13}C NMR (72.5 MHz, $CDCl_3$): δ 171.6, 168.3, 155.7 (C-1,

CO), 138.3, 137.78, 137.66, 135.6 (*C*Ph), 129.2, 128.7, 128.5, 128.4, 128.3, 128.0, 127.8, 127.6, 127.4 (Ph), 85.7, 80.4, 79.5, 78.5, 77.9, 75.5 (C-2,3,4,5,6,7), 75.0 (CH_2Ph), 52.6 ($CHCO_2CH_3$ of L-Phe), 52.3 (CO_2CH_3 of L-Phe), 37.4 (CH_2Ph of L-Phe), 28.4 (*t*-Bu); mass spectrum (FAB) *m/e* 761.3 ((M+Na)$^+$, calcd 761.4), 739.3 ((M+H)$^+$, calcd 739.4), 639.2 ((M−Boc+2H)$^+$, calcd 639.3).

4.1.19. β(1→6)-Linked dimer (28). A solution of 4 N HCl in EtOAc (30 mL) was added to a stirred solution of compound **26** (864 mg, 1.17 mmol) in EtOAc (30 mL) at 0 °C. The mixture was stirred at room temperature for 1 h. After evaporation of the solvent, the residue was washed with ether to give **27** as a white powder. This compound was used without further purification. Compound **27**, DEPC (292 μL, 1.75 mmol), and Et_3N (498 μL, 3.51 mmol) were added to a solution of compound **25** (826 mg, 1.43 mmol) in DMF (20 mL) at room temperature under argon. The reaction mixture was stirred at room temperature for 16 h, then diluted with EtOAc (200 mL), and successively washed with water, saturated aqueous $NaHCO_3$, and brine (150 mL each). The organic layer was dried over anhydrous $MgSO_4$ and filtered. The filtrate was concentrated and the residue was chromatographed on silica gel using toluene/EtOAc 5:1–1:1 to afford **28** (1.24 g, 1.05 mmol; 90%) as a white powder, mp 128–131 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32–7.16 (m, 33H, Ph), 7.08 (m, 2H, Ph), 6.88 (d, 1H, *J*=6.9 Hz, NH), 6.44 (1H, NH), 4.93–4.61 (m, 13H, CH_2Ph, $CHCO_2CH_3$), 3.74–3.63 (m, 10H), 3.48–3.33 (m, 7H), 3.07 (2H, CH_2Ph of L-Phe), 1.43 (s, 9H, $NHCO_2tBu$); ^{13}C NMR (72.5 MHz, $CDCl_3$): δ 171.8 (C-1), 168.8, 168.2 (NH*C*O), 156.0 (NH*C*O*t*Bu), 138.3, 138.2, 137.91, 137.86, 137.79, 137.6, 135.8 (*C*Ph), 129.2, 128.6, 128.5, 128.4, 128.33, 128.27, 128.13, 128.08, 128.0, 127.9, 127.8, 127.7, 127.2 (Ph), 85.7, 85.5, 80.3, 79.7, 79.4, 78.7, 78.2, 77.2, 75.4, 75.0, 74.8, 53.0 ($CHCO_2CH_3$ of L-Phe), 52.3 (CO_2CH_3 of L-Phe), 37.6 (CH_2Ph of L-Phe), 28.2 (*t*-Bu); mass spectrum (FAB) *m/e* 1198.7 ((M+Na)$^+$, calcd 1198.6), 1098.6 ((M−Boc+2H)$^+$, calcd 1098.6).

4.1.20. β(1→6)-Linked trimer (30). As described for **28**, deprotection of the Boc group of compound **28** (1.2 g, 1.00 mmol) with 4 N HCl in EtOAc (30 mL) followed by treatment with DEPC (248 μL, 1.50 mmol), Et_3N (424 μL, 3.00 mmol) and compound **25** (702 mg, 1.22 mmol) in DMF (20 mL) at room temperature under argon gave **30** (1.40 g, 8.52 mmol; 85%) as a white powder, mp 156–158 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32–7.17 (m, 48H, Ph), 7.04 (m, 2H, Ph), 6.95 (d, 1H, *J*=6.9 Hz, NH), 6.75 (2H, NH), 4.96–4.60 (m, 19H, CH_2Ph, $CHCO_2CH_3$), 3.74–3.54 (m, 15H), 3.45–3.30 (m, 9H), 3.02 (2H, CH_2Ph of L-Phe), 1.41 (s, 9H, $NHCO_2tBu$); ^{13}C NMR (72.5 MHz, $CDCl_3$): δ 171.6 (C-1), 169.0, 168.8, 168.2 (NH*C*O), 156.0 (NH*C*O*t*Bu), 138.3, 138.2, 137.9, 137.8, 137.7, 135.8 (*C*Ph), 129.2, 129.0, 128.6, 128.5, 128.4, 128.33, 128.29, 128.2, 128.0, 127.79, 127.75, 127.69, 127.2 (Ph), 85.7, 85.6, 85.5, 80.1, 79.7, 79.3, 78.6, 78.4, 78.2, 78.1, 77.8, 77.3, 75.5, 75.3, 74.9, 74.8, 53.1 ($CHCO_2CH_3$ of L-Phe), 52.3 (CO_2CH_3 of L-Phe), 37.6 (CH_2Ph of L-Phe), 28.4 (*t*-Bu); mass spectrum (FAB) *m/e* 1681.0 ((M+H+Na)$^+$, calcd 1681), 1558.8 ((M−Boc+3H)$^+$, calcd 1558.7).

4.1.21. β(1→6)-Linked tetramer (32). As described for **28**, 4 N HCl in EtOAc (30 mL) was added to a stirred solution of compound **30** (581 mg, 350 μmol) in EtOAc (10 mL), followed by treatment with DEPC (86.5 μL, 526 μmol), Et_3N (148 μL, 1.05 mmol), and compound **25** (245 mg, 424 μmol) in DMF (10 mL) at room temperature under argon to give **32** (600 mg, 283 μmol; 81%) as a white powder, mp 165–167 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.29–7.17 (m, 60H, Ph), 7.08–7.00 (m, 5H, Ph), 6.77, 6.71, 6.42 (br s, 3H, NH), 5.02–4.57 (m, 25H, CH_2Ph, $CHCO_2CH_3$), 3.86–3.52 (m, 19H), 3.47–3.27 (m, 12H), 3.07 (2H, CH_2Ph of L-Phe), 1.39 (s, 9H, $NHCO_2tBu$); ^{13}C NMR (72.5 MHz, $CDCl_3$): δ 171.8 (C-1), 169.22, 169.16, 168.9, 168.2 (NH*C*O), 156.1 (NH*C*O*t*Bu), 138.4, 138.2, 138.1, 138.0, 137.9, 137.83, 137.78, 137.5, 135.8 (*C*Ph), 129.2, 128.51, 128.46, 128.4, 128.3, 128.2, 128.11, 128.08, 128.00, 127.9, 127.8, 127.74, 127.69, 127.62, 127.1 (Ph), 86.1, 85.8, 85.7, 85.4, 80.6, 80.1, 79.8, 79.4, 79.2, 78.9, 78.6, 78.3, 78.1, 78.0, 77.5, 77.2, 77.0, 76.8, 76.6, 75.6, 75.4, 75.21, 75.16, 75.0, 74.8, 74.7, 53.3 ($CHCO_2CH_3$ of L-Phe), 52.2 (CO_2CH_3 of L-Phe), 37.8 (CH_2Ph of L-Phe), 28.4 (*t*-Bu); mass spectrum (FAB) *m/e* 2117.8 ((M+2H)$^+$, calcd 2118.0), 2017.7 ((M−Boc+3H)$^+$, calcd 2017.9).

4.1.22. β(1→6)-Linked tetramer (4). A mixture of **32** (300 mg, 142 μL) and 20% $Pd(OH)_2$/C (400 mg) in MeOH (20 mL), THF (20 mL), and H_2O (4 mL) was stirred in an atmosphere of H_2 at 20 °C for 12 h, and then filtered through Celite, and concentrated in vacuo. The residue was chromatographed on Sephadex G-25 (H_2O) followed by lyophilization to give **4** (126 mg, 122 μmol; 86%) as a white powder: ^{1}H NMR (300 MHz, D_2O): δ 7.23–7.11 (m, 5H), 4.60 (m, 1H, $CHCO_2CH_3$), 3.74–3.07 (m, 31H), 2.95 (CH_2Ph of L-Phe), 1.29 (s, 9H, $NHCO_2tBu$); ^{13}C NMR (72.5 MHz, $CDCl_3$): δ 172.5 (CO), 129.7, 129.0, 127.5 (Ph), 28.6 (*t*-Bu); mass spectrum (FAB) *m/e* 1036.4 ((M−H)$^+$ calcd 1036.4), 936.4 ((M−Boc+H)$^+$, calcd 936.4).

4.1.23. Sulfated compounds 18 and 33. O-Sulfation of the oligomers was performed based on the published procedure.[21] A mixture of the tetramer **1** or **4** and a large excess of SO_3·NMe_3 (10 equiv per free OH groups) in DMF was stirred at 50 °C for 5 days, and cooled. Saturated aqueous $Ba(OH)_2$ solution was added to the reaction mixture until it became slightly basic (pH 8.0 judged by a pH paper). The formed white precipitate was removed by centrifugation with microcentrifuge (Eppendorf Centrifuge 5415C) at 13×1000 cpm for 10 min. The clear supernatant was collected and was passed through a column of Dowex 50W-X8 [Na]$^+$(1×5 cm), and then eluted with water. The eluate was collected and concentrated in vacuo. The residue was chromatographed, for desalting, on a column of Sephadex G-15 (1×65 cm), and then eluted with water. Each fraction was examined with an Azure assay and the positive fractions were pooled and concentrated in vacuo. The residue was lyophilized from water (10 mL) and NMR was taken. If the reaction was completed, only single peak corresponding to the methyl ester was observed. (If not, several peaks for the methyl ester were observed.)

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References and notes

1. Varki, A. *Glycobiology* **1993**, *3*, 97–130.
2. Ogawa, S.; Sugawa, I.; Shibata, Y. *Carbohydr. Res.* **1991**, *211*, 147–155.
3. Wei, A.; Haudrechy, A.; Audin, C.; Jun, H.-S.; Haudrechy-Bretel, N.; Kishi, Y. *J. Org. Chem.* **1995**, *60*, 2160–2169.
4. Hasegawa, A.; Ogawa, H.; Ishida, H.; Kiso, M. *Carbohydr. Res.* **1992**, *224*, 175–184.
5. Joseph, B.; Rollin, P. *Carbohydr. Res.* **1995**, *266*, 321–325.
6. Grindly, T. B.; Namazi, H. *Tetrahedron Lett.* **1996**, *37*, 991–994.
7. Eyrisch, O.; Fessner, W.-D. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1639–1641.
8. Haneda, T.; Goekjan, P. G.; Kim, S. H.; Kishi, Y. *J. Org. Chem.* **1992**, *57*, 490–498.
9. Yoshimura, J.; Ando, H.; Sato, T.; Tsuchida, S.; Hashimoto, H. *Bull. Chem. Soc. Jpn.* **1976**, *49*, 2511–2514.
10. Fuchs, E.-F.; Lehmann, J. *Chem. Ber.* **1975**, *108*, 2254–2260.
11. Nicolaou, K. C.; Flörke, H.; Egan, M. G.; Barth, T.; Estevez, V. A. *Tetrahedron Lett.* **1995**, *36*, 1775–1778.
12. Müller, C.; Kitas, E.; Wessel, H. P. *J. Chem. Soc., Chem. Commun.* **1995**, 2425–2426.
13. Wessel, H. P.; Mitchell, C. M.; Lobato, C. M.; Schmid, G. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2712–2713.
14. Von Roedern, E. G.; Kessler, H. *Angrew. Chem., Int. Ed. Engl.* **1994**, *33*, 687–689.
15. Hirschmann, R.; Nicolaou, K. C.; Pietranico, S.; Salvino, J.; Leahy, E. M.; Sprengeler, P. A.; Furst, G.; Smith, A. B., III; Strader, C. D.; Cascier, M. A.; Candelore, M. R.; Donaldson, C.; Vale, W.; Maechler, L. *J. Am. Chem. Soc.* **1992**, *114*, 9217–9218.
16. Smith, M. D.; Long, D. D.; Martin, A.; Marquess, D. G.; Claridge, T. D. W.; Fleet, G. W. J. *Tetrahedron Lett.* **1999**, *40*, 2191–2194.
17. Long, D. D.; Hungerford, N. L.; Smith, M. D.; Brittain, D. E. A.; Marquess, D. G.; Claridge, T. D. W.; Fleet, G. W. J. *Tetrahedron Lett.* **1999**, *40*, 2195–2198.
18. Clarigde, T. D. W.; Long, D. D.; Hungerford, N. L.; Aplin, R. T.; Smith, M. D.; Marquess, D. G.; Fleet, G. W. J. *Tetrahedron Lett.* **1999**, *40*, 2199–2202.
19. Long, D. D.; Smith, M. D.; Martin, A.; Wheatley, J. R.; Watkin, D. G.; Müller, M.; Fleet, G. W. J. *J. Chem. Soc., Perkin Trans. 1* **2002**, 1982–1998.
20. Suhara, Y.; Hildreth, J. E. K.; Ichikawa, Y. *Tetrahedron Lett.* **1996**, *37*, 1575–1578.
21. Suhara, Y.; Yamaguchi, Y.; Collins, B.; Schnaar, R. L.; Yanagishita, M.; Hildreth, J. E. K.; Shimada, I.; Ichikawa, Y. *Bioorg. Med. Chem.* **2002**, *10*, 1999–2013.
22. Suhara, Y.; Ichikawa, M.; Hildreth, J. E. K.; Ichikawa, Y. *Tetrahedron Lett.* **1996**, *37*, 2549–2552.
23. Appella, D. H.; Christianson, L. A.; Karle, I. L.; Powell, D. R.; Gellman, S. H. *J. Am. Chem. Soc.* **1996**, *118*, 13071–13072.
24. Appella, D. H.; Christianson, L. A.; Klein, D. A.; Powell, D. R.; Huang, X.; Barchi, J. J., Jr.; Gellman, S. H. *Nature* **1997**, *387*, 381–384.
25. Snow, C. D.; Nguyen, H.; Pande, V. S.; Gruebele, M. *Nature* **2002**, *420*, 102–106.
26. Glättli, A.; Daura, X.; Seebach, D.; van Gunsteren, W. F. *J. Am. Chem. Soc.* **2002**, *124*, 12972–12978.
27. Simmerling, C.; Strockbine, B.; Roitberg, A. E. *J. Am. Chem. Soc.* **2002**, *124*, 11258–11259.
28. Wu, H. W.; Wang, S. M.; Brooks, B. R. *J. Am. Chem. Soc.* **2002**, *124*, 5282–5283.
29. Baron, R.; Bakowies, D.; van Gunsteren, W. F. *Angrew. Chem., Int. Ed.* **2004**, *43*, 4055–4059.
30. Mayes, B. A.; Simon, L.; Watkin, D. J.; Ansell, C. W. G.; Fleet, G. W. J. *Tetrahedron Lett.* **2004**, *45*, 157–162.
31. Baron, R.; Bakowies, D.; van Gunsteren, W. F. *J. Pept. Sci.* **2005**, *11*, 74–84.
32. Grynkiewicz, G.; Bemiller, J. N. *Carbohydr. Res.* **1983**, *112*, 324–327.
33. Myers, R. W.; Lee, L. C. *Carbohydr. Res.* **1984**, *132*, 61–82.
34. Itoh, M.; Hagiwara, D.; Kamiya, T. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 718–721.
35. Hayashi, K.; Hamada, Y.; Shioiri, T. *Tetrahedron Lett.* **1992**, *33*, 5075–5076.
36. Anisfeld, S. T.; Lansbury, P., Jr. *J. Org. Chem.* **1990**, *55*, 5560–5562.
37. Corey, E. J.; Nicolaou, K. C.; Balanson, R. D.; Machida, Y. *Synthesis* **1975**, 590–591.
38. Schnaar, R. L.; Needham, L. K. *Methods Enzymol.* **1994**, *230*, 371–389.
39. Gademann, K.; Ernst, M.; Hoyer, D.; Seebach, D. *Angew. Chem., Int. Ed.* **1999**, *38*, 1223–1226.
40. Gademann, K.; Kimmerlin, T.; Hoyer, D.; Seebach, D. *J. Med. Chem.* **2001**, *44*, 2460–2468.
41. Werder, M.; Hauser, H.; Abele, S.; Seebach, D. *Helv. Chim. Acta* **1999**, *82*, 1774–1783.
42. Hamuro, Y.; Schneider, J. P.; DeGrado, W. F. *J. Am. Chem. Soc.* **1999**, *121*, 12200–12201.
43. Liu, D. H.; DeGrado, W. F. *J. Am. Chem. Soc.* **2001**, *123*, 7553–7559.
44. Porter, E. A.; Wang, X. F.; Lee, H. S.; Weisblum, B.; Gellman, S. H. *Nature* **2000**, *404*, 565.
45. Arvidsson, P. I.; Ryder, N. S.; Weiss, H. M.; Gross, G.; Kretz, O.; Woessner, R.; Seebach, D. *ChemBiochem* **2003**, *4*, 1345–1347.
46. Gellman, M. A.; Richter, S.; Cao, H.; Umezawa, N.; Gellman, S. H.; Rana, T. M. *Org. Lett.* **2003**, *5*, 3563–3565.
47. Kritzer, J. A.; Stephens, O. M.; Guarracino, D. A.; Reznik, S. K.; Schepartz, A. *Bioorg. Med. Chem.* **2005**, *13*, 11–16.
48. Porter, E. A.; Weisblum, B.; Gellman, S. H. *J. Am. Chem. Soc.* **2005**, *127*, 11516–11529.
49. Trzesniak, D.; Glättli, A.; Jaun, B.; van Gunsteren, W. F. *J. Am. Chem. Soc.* **2005**, *127*, 14320–14329.
50. Salaün, A.; Farve, A.; Le Grel, B.; Potel, M.; Le Grel, P. *J. Org. Chem.* **2006**, *71*, 150–158.
51. Bldauf, C.; Günther, R.; Hofmann, H. J. *J. Org. Chem.* **2006**, *71*, 1200–1208.

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Methyl-introduced A-ring Analogues of 1α,25-Dihydroxyvitamin D_3: Synthesis and Biological Evaluation

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Abstract. *The hormonally-active metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3 (1), has a wide variety of biological activities, which makes it a promising therapeutic agent for the treatment of cancer, psoriasis and osteoporosis. Insights into the structure-activity relationships of the A-ring of 1 are needed to assist the development of more potent and selective analogues, as well as to define the molecular mode of action. All possible A-ring stereoisomers of 2-methyl-1,25-dihydroxyvitamin D_3 and 2,2-dimethyl-1,25-dihydroxyvitamin D_3, which differ in stereochemistry at the C1-, C2- and C3-positions, were designed and efficiently synthesized by employing the convergent method. Biological evaluation of the analogues, in terms of the vitamin D receptor-binding affinity and HL-60 cell differentiation-inducing activity, as well as the transcriptional potency in ROS 17/2.8 cells, revealed the importance of substituents at the C2-position in certain orientations.*

Cholecalciferol, known as vitamin D_3, is metabolized *via* 25-hydroxyvitamin D_3 to produce the hormonally-active form, 1α,25-dihydroxyvitamin D_3 (**1**; Figure 1), the formation of which is strictly regulated (1). In addition to its classic role in calcium and phosphorus homeostasis, 1α,25-dihydroxyvitamin D_3 dominates the cell cycle in many malignant cells, regulating proliferation, differentiation and apoptosis. Most of the biological activities of **1** are considered to be mediated by a ligand-inducible transcriptional factor, the vitamin D receptor (VDR), which belongs to the nuclear receptor superfamily. The specific interaction of the ligands with the ligand-binding domain of VDR has been a major focus of attention, since it triggers the whole sequence of biological responses: conformational change of the VDR, particularly of the AF-2 domain, heterodimerization with retinoid X receptors (RXRs), recruitment of co-activators and binding to the DNAs. Insights into the structure-function relationships of a variety of ligands are essential to understand how the subtype-free, singular VDR can deliver the diverse biological activites of **1**, as well as allowing the development of potent therapeutic agents with selective activity profiles for the treatment of cancers or ostereoporosis.

Structural modification of **1** in the A-ring, which possesses two critical hydroxyl groups at the C1- and C3-positions, has become of interest in recent years, because the other three stereoisomers have proven to exhibit unique activity profiles, being different from the natural hormones (2, 3). Our study of all eight possible A-ring stereoisomers of 2-methyl-1,25-dihydroxyvitamin D_3 and their 20-epimers showed that introduction of a simple methyl group into the parent **1** yielded analogues with distinct activity profiles (4-6). These methyl-introduced analogues, which differ in stereochemistry at the C1-, C2- and C3-positions, exhibited cell differentiation- or apoptosis-inducing activity towards HL-60 cells, depending on their A-ring structures (7). Some of the synthesized 2α-substituted analogues of **1** showed remarkably high affinity for VDR (8-10). 2β-Methyl introduction into the A-ring, on the other hand, in combination with the 1β-hydroxy or 3α-hydroxy groups, resulted in antagonists of the nongenomic, but not genomic, actions in NB-4 cells (11).

The X-ray crystal structure of VDR complexed with **1** (12) indicated the presence of an extra space in the vicinity of the A-ring, suggesting that the substituents of synthetic A-ring analogues could occupy this additional space. Our study of the 2-methyl analogues of **1** revealed that 2α-methyl-1α,25-dihydroxyvitamin D_3 (**2a**) was a four-fold

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Key Words: Vitamins, hormones, receptors, chemical synthesis.

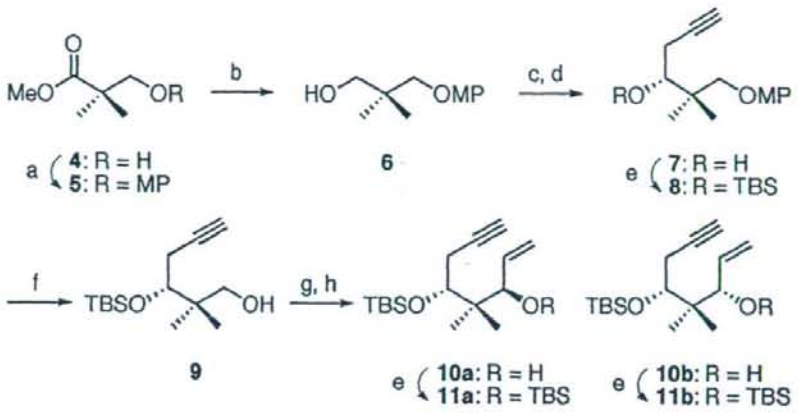

Figure 2. *Reagents and conditions: (a) 4-methoxyphenol, DEAD, Ph_3P/ THF, 98%; (b) $LiAlH_4$/ THF, 97%; (c) PDC, 4A MS/ CH_2Cl_2, 89%; (d) allenylmagnesium bromide/ ether, 68%; (e) TBSOTf, 2,6-lutidine/ CH_2Cl_2, 81% for 8, quant. for 11a,b; (f) CAN/ CH_3CN-H_2O, 77%; (g) TPAP, NMO, 4A MS/ CH_2Cl_2, 69%; (h) vinylmagnesium bromide/ toluene, 60%. (MP: 4-methoxyphenyl, TBS: t-butyldimethylsilyl).*

induce distinctive conformational changes in the VDR to modulate the stability of the transcriptional machinery in certain cells. The differential stability of the protein complexes caused by ligands would be an interesting approach for separating the activities of **1**. The results will be reported elsewhere in detail.

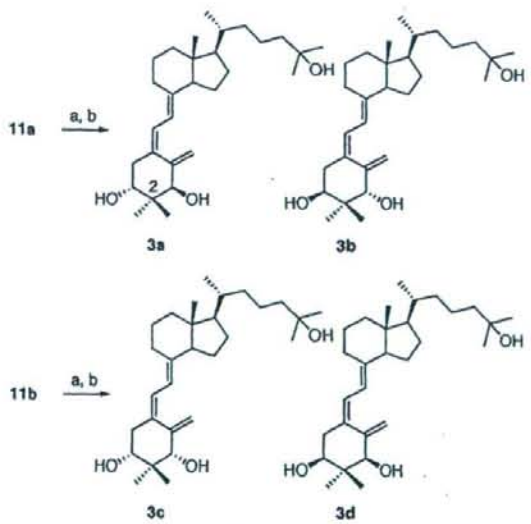

Figure 3. *Reagents and conditions: (a) CD-ring portion, $(Ph_3P)_4Pd$/ Et_3N-toluene, 63-66%; (b) TBAF/ THF, 29-63%.*

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References

1 Feldman D, Pike JW and Glorieux FH (eds.). Vitamin D. Burlington, Elsevier Academic Press, 2005.

2 Norman AW, Bouillon R, Farach-Carson MC, Bishop JE, Zhou LX, Nemere L, Zhao J, Muralidoharan KR and Okamura WH: Demonstration that 1α,25-dihydroxyvitamin D_3 is the nongenomic but not genomic biologic responses and biologic profiles of the three A-ring diastereomers of 1α,25-dihydroxyvitamin D_3. J Biol Chem *268*: 20022-20030, 1993.

3 Bischof MG, Siu-Caldera ML, Weiskop Af, Vouros P, Cross HS, Peterlik M and Reddy GS: Differentiation related pathways of 1α,25-dihydroxycholecalciferol metabolism in human colon adenocarcinoma-derived Caco-2 cells. Production of 1α,25-dihydroxy-3-epi-cholecalciferol. Exp Cell Res *241*: 194-201, 1998.

4 Konno K, Maki S, Fujishima T, Liu ZP, Miura D, Chokki M and Takayama H: A novel and practical route to A-ring enyne synthon for 1α,25-dihydroxyvitamin D_3 and 2-methyl-1,25-dihydroxyvitamin D_3. Bioorg Med Chem Lett *8*: 151-156, 1998.

5 Konno K, Fujishima T, Maki S, Liu ZP, Miura D, Chokki M, Ishizuka S, Yamaguchi K, Kan Y, Kurihara M, Miyata N, Smith C, DeLuca HF and Takayama H: Synthesis, biological evaluation, and conformational analysis of A-ring diastereomers of 2-methyl-1,25-dihydroxyvitamin D_3 and their 20-epimers: unique activity profiles depending on the stereochemistry of the A-ring and at C-20. J Med Chem *43*: 4247-4265, 2000.

6 Fujishima T, Konno K, Nakagawa K, Tanaka M, Okano T, Kurihara M, Miyata N and Takayama H: Synthesis and biological evaluation of all A-ring stereoisomers of 5,6-*trans*-1,25-dihydroxyvitamin D_3 and their 20-epimers: possible binding modes of potent A-ring analogues to vitamin D receptor. Chem Biol *8*: 1011-1024, 2001.

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Synthesis of Various Chiral Cyclic α,α-Disubstituted Amino Acids and Conformational Analysis of Their Peptides

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A chiral cyclic α,α-disubstituted α-amino acid; {(3R,4R)-1-amino-3,4-diazidocyclopentanecarboxylic acid; (R,R)-Ac_5c^{dN3}} was synthesized starting from dimethyl L-(+)-tartrate. The amino acid (R,R)-Ac_5c^{dN3} could be converted into several cyclic α,α-disubstituted α-amino acids having various functional groups.

Keywords: α,α-disubstituted α-amino acid, side-chain chiral center, peptide conformation, cyclic amino acid

Introduction

Helices shown in peptides and proteins, as secondary structures, almost always form the right-handed (*P*) helical screw direction. Scientists undoubtedly believe that the right-handedness is a result of the asymmetric center on the α-carbon atom of terrestrial L-α-amino acids. Among proteinogenic L-α-amino acids, isoleucine and threonine possess an additional chiral center, besides the α-carbon atom, on the side chain of amino acid. However, so far no attention has been paid as to how the chiral centers on the side-chain of amino acids affect the secondary structure of their peptides. Recently, we have reported that side-chain chiral centers of amino acids affect the secondary structures of their peptides [1]. That is, we designed and synthesized chiral cyclic α,α-disubstituted α-amino acids (dAAs); (*S*,*S*)-Ac_5c^{dOM} and (*R*,*R*)-$Ab_{5,6=}c$, in which the α-carbon atoms are not chiral centers but the asymmetric centers exist at the side-chain cycloalkane skeleton. The conformational analysis revealed that the (*S*,*S*)-Ac_5c^{dOM} homopeptides form left-handed (*M*) 3_{10}- and α-helices, and the (*R*,*R*)-$Ab_{5,6=}c$ homopeptides form both right-handed (*P*) and left-handed (*M*) 3_{10}-helices. These results indicate that the side-chain chiral environment (bulkiness or flexibility) may be important for control of the helical-screw sense of peptides. Thus herein, we report the synthesis of a chiral cyclic dAA; (3*R*,4*R*)-1-amino-3,4-diazidocyclopentanecarboxylic acid {(*R*,*R*)-Ac_5c^{dN3}}, in which the azido group can be converted into several functional groups, such as amino, triazole, and amide groups. Furthermore, we describe the peptides containing (*R*,*R*)-Ac_5c^{dN3} in Aib residues.

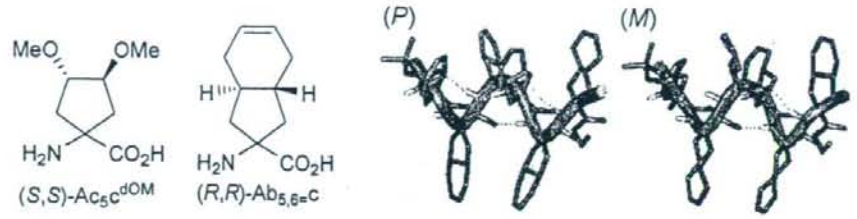

Fig. 1. Structures of (S,S)-Ac_5c^{dOM} and (R,R)-$Ab_{5,6=}c$, and X-ray structure of (R,R)-$Ab_{5,6=}c$ hexapeptide.

Results and Discussion

We synthesized an optically active (*R*,*R*)-Ac_5c^{dN3} starting from dimethyl L-(+)-tartrate. After conversion of dimethyl L-(+)-tartrate to diiodide (**1**), dimethyl malonate was bisalkylated with diiodide **1** to give a chiral cyclic diester (**2**). Deprotection of the MOM ether, conversion of diol into diazido function, and subsequent Curtius rearrangement generated the chiral cyclic (*R*,*R*)-Ac_5c^{dN3}. As expected, the amino acid (*R*,*R*)-Ac_5c^{dN3} could be efficiently converted into several dAAs having various functional groups, such as amino, triazole, amide functional groups.

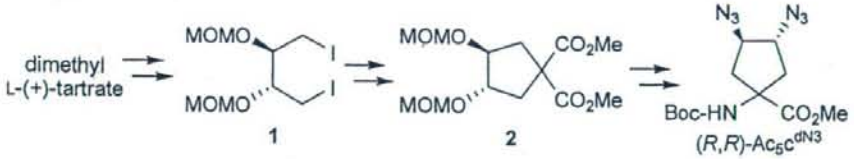

Fig. 2. Synthesis of (R,R)-Ac_5c^{dN3} from dimethyl L-(+)-tartrate.

We prepared peptides containing (*R*,*R*)-Ac_5c^{dN3} in Aib sequences. These peptides seemed to form 3_{10}-helical structures both in solution and in the solid state. The conformational analysis in detail will be published elsewhere.

Acknowledgments

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References

1. a) Tanaka, M., Demizu, Y., Doi, M., Kurihara, M., Suemune, H. (2004) *Angew. Chem. Int. Ed.*, **43**, 5360-5363; b) Tanaka, M., Anan, K., Demizu, Y., Kurihara, M., Doi, M., Suemune, H. (2005) *J. Am. Chem. Soc.*, **127**, 11570-11571.

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The Japanese Peptide Society (2006)

Chiral Cyclic α,α-Disubstituted α-Amino Acids Bearing Two Chiral Centers and Conformation of Their Peptides

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Two diastereomeric cyclic α,α-disubstituted α-amino acids {(1S,3S)-Ac_5c^{OM} and (1R,3S)-Ac_5c^{OM}} having two chiral centers at the side-chain cyclopentane ring and on the backbone α-carbon have been synthesized. The conformational analysis of their peptides seemed to form helical secondary structures.

Keywords: α,α-disubstituted α-amino acids, side-chain chiral center, helical-screw sense, secondary structure, peptide conformation

Introduction

Helical structures in proteins almost always form a right-handed (*P*) helical-screw sense, which is believed to result from the asymmetric center at the α-position of L-α-amino acids. Besides an asymmetric center at the α-position, L-Ile and L-Thr possess an additional chiral center at the side-chain β-position. However, no attention has been paid as to how the asymmetric center on the side chain affects the secondary structure of peptides. Recently, we have reported that the chiral centers on the side chain of cyclic α,α-disubstituted α-amino acids (dAAs) affect the helical secondary structures of their peptides [1]. That is to say, we synthesized chiral cyclic dAAs; (*S,S*)-Ac_5c^{dOM} and (*R,R*)-$Ab_{5,6=}c$, in which the α-carbon atoms are not chiral centers but the asymmetric centers exist at the side-chain cycloalkane skeleton, and studied the conformation of their peptides. The X-ray crystallographic analysis revealed that the (*S,S*)-Ac_5c^{dOM} homopeptides form left-handed (*M*) 3_{10}- and α-helices, while the (*R,R*)-$Ab_{5,6=}c$ homopeptides form both right-handed (*P*) and left-handed (*M*) 3_{10}-helices. Herein, we wish to report the synthesis of chiral cyclic dAAs; (1*S*,3*S*)-1-amino-3-methoxycyclopentanecarboxylic acid {(1*S*,3*S*)-Ac_5c^{OM}} and (1*R*,3*S*)-1-amino-3-methoxycyclopenatnecarboxylic acid {(1*R*,3*S*)-Ac_5c^{OM}}, which have two chiral centers at the side-chain cyclopentane ring and on the backbone α-carbon, and the conformational study of their homopeptides.

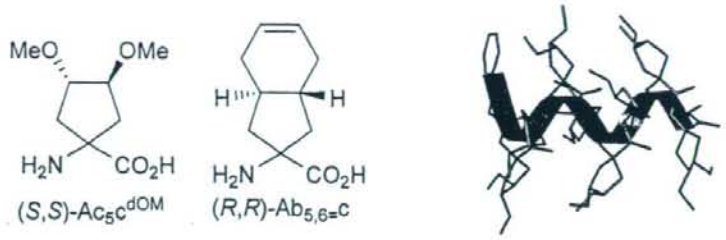

Figure 1. Structure of (S,S)-Ac_5c^{dOM} and (R,R)-$Ac_{5,6=}c$, and the X-ray structure of (S,S)-Ac_5c^{dOM} octapeptide.

Results and Discussion

Chiral cyclic dAAs (1*S*,3*S*)-Ac_5c^{OM} and (1*R*,3*S*)-Ac_5c^{OM} were synthesized starting from L-(-)-malic acid. That is to say, at first, the malic acid was converted to diiodide (**1**) by esterification, methylation of alcohol, reduction of esters, and substitution with iodide. Then, bisalkylation of dimethyl malonate with the diiodide **1** gave diester (**2**). Hydrolysis of monoester in **2**, and subsequent Curtius rearrangement afforded the chiral cyclic dAAs (1*S*,3*S*)-Ac_5c^{OM} and (1*R*,3*S*)-Ac_5c^{OM} in the ratio of 3 : 1. Recrystallization from hexane/ether afforded the pure chiral cyclic (1*S*,3*S*)-Ac_5c^{OM}.

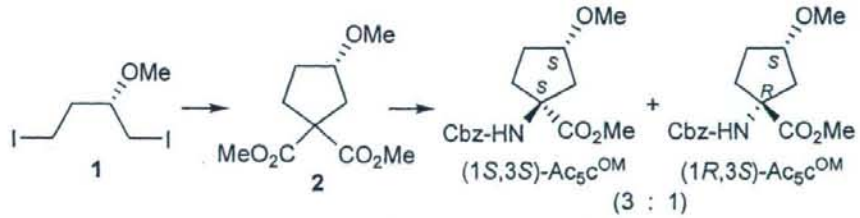

Figure 2. Synthesis of (1S,3S)-Ac_5c^{OM} and (1R,3S)-Ac_5c^{OM} from L-(-)-malic acid

We prepared (1*S*,3*S*)-Ac_5c^{OM} homopeptides by solution-methods, and studied the conformation by using the ^{1}H NMR, FT-IR, and X-ray crystallographic analysis. The peptides seemed to preferentially form helical structures. The detail of conformation analysis will be published elsewhere.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science, and also by the Sasakawa Scientific Research Grant from the Japan Science Society.

References

1 a) Tanaka, M., Demizu, Y., Doi, M., Kurihara, M., Suemune, H. (2004) *Angew. Chem. Int. Ed.*, **43**, 5360-5363; b) Tanaka, M., Anan, K., Demizu, Y., Kurihara, M., Doi, M., Suemune, H. (2005) *J. Am. Chem. Soc.*, **127**, 11570-11571.

COMPUTATIONAL STUDY ON CONFORMATION OF OLIGOPEPTIDES CONTAINING CHIRAL CYCLIC α,α-DISUBSTITUTED

Masaaki Kurihara[1], Yukiko Sato[1], Wataru Hakamata[1], Haruhiro Okuda[1], Yosuke Demizu[2], Masanobu Nagano[2], Naomi Kawabe[2], Mitsunobu Doi[3], Masakazu Tanaka[2] and Hiroshi Suemune[2]

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Introduction

Prediction of the conformation of peptides using computational simulation is an interesting challenge for the design of functionalized and bioactive peptides. We have shown the Monte Carlo conformational search using MacroModel is useful for conformational study of oligopeptides prepared from α,α-disubstituted α-amino acids. Moreover, we have studied conformational analysis of oligopeptides containing chiral α,α-disubstituted α-amino acids to predict the helical screw sense of helical structures (α-helix, 3_{10}-helix). Here we report computational study on conformation of oligopeptides containing cyclic α,α-disubstituted α-amino acids with side-chain chiral centers.

Fig. 1. Helical structures of oligopeptides

Results and Discussion

Conformational search calculations of oligopeptides 1, 2, containing chiral cyclic α,α-disubstituted amino acids, have performed using the Monte Carlo method of MacroModel (ver. 8.1, Schrödinger, Inc.). When AMBER* force field was used, the global minimum energy conformation of peptide 1 was a left-handed α-helix, which was more stable than a left-handed 3_{10}-helix by 4.2 kcal/mol. The results were in agreement with its X-ray structure, which showed a left-handed α-helix [1]

The global minimum energy conformation of peptide 2 was a right-ha helix, which was more stable than a left-handed 3_{10}-helix by 1.6 kcal/m results were obtained by STO-3G level molecular orbital calculat difference of energies was small. There were both right- and left-handed 3 in the solid state [2, 3]. These results indicated computational simulati conformational search calculations could predict the helical screw oligopeptides containing chiral cyclic α,α-disubstituted amino acids.

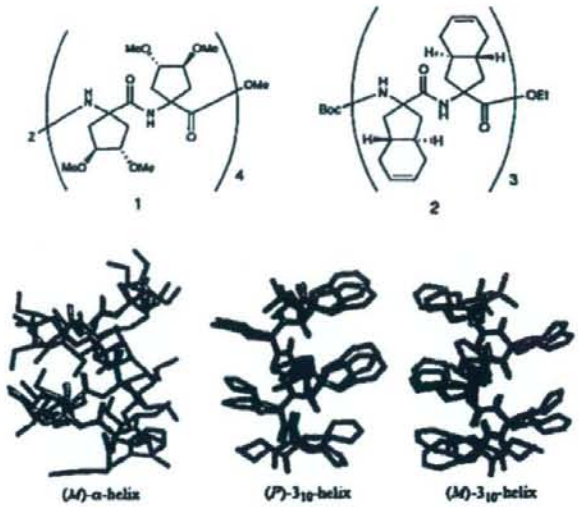

Fig. 2. Modeled structures (light) and X-ray structures (dark) of oligopeptides 1, 2.

Acknowledgements

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References

1. Tanaka M., Demizu Y., Doi M., Kurihara M. and H. Suemune, Angew. Chem. Int. Ed., 43 (2004) 5360.
2. Tanaka M., Anan K., Demizu Y., Kurihara M., Doi M. and H. Suemune, J. Am. Chem. Soc., 127 (2005) 11570.
3. Kurihara, M., Sato, Y., Hakamata, W., Okuda, H., Demizu, Y., Nagano, M., Kawabe, N., Doi, M., Tanaka, M. and Suemune, H., In: Wakamiya, T. (Ed.) Peptide Science 2005 (Proceedings of the 42nd Japanese Peptide Society), JPS, 2006, p. 371.

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Purification and characterization of *Vibrio parahaemolyticus* extracellular chitinase and chitin oligosaccharide deacetylase involved in the production of heterodisaccharide from chitin

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Abstract A chitin-degrading bacterial strain, KN1699, isolated from Yatsu dry beach (Narashino, Chiba Prefecture, Japan), was identified as *Vibrio parahaemolyticus*. Treatment of powdered chitin with crude enzyme solution prepared from the supernatant of KN1699 cultures yielded a disaccharide, β-D-*N*-acetylglucosaminyl-(1,4)-D-glucosamine (GlcNAc-GlcN), as the primary chitin degradation product. The extracellular enzymes involved in the production of this heterodisaccharide, chitinase (*Pa*-Chi; molecular mass, 92 kDa) and chitin oligosaccharide deacetylase (*Pa*-COD; molecular mass, 46 kDa), were isolated from the crude enzyme solution, and their hydrolysis specificities were elucidated. These studies confirmed that (1) *Pa*-Chi hydrolyzes chitin to produce $(GlcNAc)_2$ and (2) *Pa*-COD hydrolyzes the acetamide group of reducing end GlcNAc residue of $(GlcNAc)_2$. These findings indicate that GlcNAc-GlcN is produced from chitin by the cooperative hydrolytic reactions of both *Pa*-Chi and *Pa*-COD.

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Introduction

Chitin, a β-(1,4) polymer of *N*-acetylglucosamine (GlcNAc), is one of the most abundant biomass polysaccharides, composing the shells of crustaceans such as crab and shrimp, the exoskeletons of insects, and the cell walls of fungi. Oligosaccharides obtained by the hydrolysis of chitin have shown physiological activities. For example, the antitumor (Suzuki et al. 1986; Tokoro et al. 1988; Tsukada et al. 1990) and antimicrobial actions (Tokoro et al. 1989; Kobayashi et al. 1990) of hexa-*N*-acetylchitohexaose [$(GlcNAc)_6$] in mice are known to be caused by enhancement of the immunological defense system. Various enzymes involved in chitin hydrolysis [i.e., chitinase (EC 3.2.1.14), β-*N*-acetylhexosaminidase (EC 3.2.1.52), chitin deacetylase (EC 3.5.1.41), and chitin oligosaccharide deacetylase (EC 3.5.1)] are known. Chitinase catalyzes the degradation of water-insoluble chitin into water-soluble chitin oligosaccharides by its hydrolytic reaction. A number of chitinases have been isolated from bacteria, and their properties have been investigated (Dahiya et al. 2006). In addition, the genes encoding a variety of chitinases have been cloned. Based on their amino acid sequences, these chitinases are classified in either glycoside hydrolase (GH) family 18 or 19 (http://afmb.cnrs-mrs.fr/CAZY/). Most bacterial chitinases belong to GH family 18. β-*N*-Acetylhexosaminidase catalyzes the hydrolysis of chitin oligosaccharides to release the monosaccharide GlcNAc. The enzymes from various sources have been classified in GH families 3, 20 (the main family), and 84.

An excellent review of microbial β-*N*-acetylhexosaminidases has been published (Scigelova and Crout 1999). Chitin deacetylase and chitin oligosaccharide deacetylase are involved in the hydrolysis of the acetamide group of the GlcNAc residue of chitin and chitin oligosaccharides (Tsigos et al. 2000). Both enzymes, isolated from various sources, are classified in carbohydrate esterase (CE) family 4 (http://afmb.cnrs-mrs.fr/CAZY/). Several microbial chitin deacetylases and chitin oligosaccharide deacetylases have been isolated and their properties elucidated (Kafetzopoulos et al. 1993; Tsigos and Bouriotis 1995; Gao et al. 1995; Tokuyasu et al. 1996; Ohishi et al. 1997).

To obtain the enzymes that produce specific oligosaccharide from chitin effectively, we screened chitin-degrading bacteria isolated from dry beach soil and from the contents of marine fish intestines. The screen used was the formation of clear zones around colonies on colloidal chitin-agar plates. A number of bacterial strains having chitin-degrading activity were isolated from Yatsu dry beach (Narashino, Chiba Prefecture, Japan). We chose one kind of bacterium (strain KN1699), from these bacteria isolated, through the tests with both chitin decomposition and oligosaccharide production. In this paper, we report the identification of the strain KN1699, oligosaccharide production by extracellular enzyme of this strain, and purification and characterization of the enzyme involved in the oligosaccharide production.

Materials and methods

Microorganism

Gram-stain and physiological characterization of strain KN1699 was accomplished using a Color Gram 2 kit (bioMerieux) and an API 20 E kit (bioMerieux), respectively, and the strain was classified according to Bergey's Manual of Systematic Bacteriology (Farmer et al. 2005). The genotype of strain KN1699 was investigated by comparing the nucleotide sequence of its 16S rDNA to the sequence database Basic Local Alignment Search Tool for Nucleotide (BLASTN; http://www.ncbi.nlm.nih.gov/blast/). Chromosomal DNA from KN1699 was isolated using a High Pure Polymerase Chain Reaction (PCR) Template Preparation Kit (Roche). The 16S rDNA was amplified from the isolated DNA by PCR using a MicroSeq 16S rDNA Full Gene PCR kit (Applied Biosystems), and the resulting PCR product was purified using Quantum Prep PCR Kleen Spin Columns (Bio-Rad). DNA sequence analysis was performed by the dideoxynucleotide method (Sanger et al. 1977). The nucleotide sequence of the PCR product was determined using an automated DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems).

Chemicals

Glucosamine (GlcN), GlcNAc, chitin oligosaccharides [$(GlcNAc)_{2-6}$], and β-chitin flake were purchased from Seikagaku Kogyo. Powdered α-chitin was obtained from Wako Pure Chemical Industries. β-Chitin flake was ground to a powder using a blender (Wonder Blender WB-1, Osaka Chemical). Colloidal chitin was prepared using powdered α-chitin according to the method of Shimahara and Takiguchi (1988). Artificial seawater was prepared using the Sealife (Marine Tech) salt mixture. All other chemicals were of analytical grade.

Preparation of the crude enzyme solution

Strain KN1699 was grown at 28°C for 16 h with shaking (135 rpm) in 10 ml of half-strength artificial seawater containing 1% (*w*/*v*) peptone, 0.1% (*w*/*v*) yeast extract, and 0.5% (*w*/*v*) powdered α-chitin. To produce sufficient chitinase for purification and characterization, 5 ml of the above culture was added to 1 l of fresh medium and cultivated for 60 h as described above. After centrifuging the cells (6,000×*g*, 15 min at 4°C), proteins in the supernatant were precipitated by adding $(NH_4)_2SO_4$ (80% saturation) and collected by centrifugation (6,000×*g*, 20 min at 4°C). The resulting precipitate was dissolved in 50 ml of 20 mM sodium phosphate buffer (pH 7.0), and the solution was dialyzed against the same buffer to afford crude enzyme solution.

Purification of enzyme

Purification of chitinase (*Pa*-Chi): The crude enzyme solution was loaded on a DEAE-Toyopearl 650M resin (Tosoh) column (φ2.5×20 cm), pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0), and the enzyme was eluted with a linear gradient of 0–0.4 M NaCl in the same buffer (total volume, 800 ml). The active fractions were collected, dialyzed against 20 mM sodium phosphate buffer (pH 7.0), then the enzyme solution was again loaded on the DEAE-Toyopearl 650M resin column and eluted with a linear gradient of 0–0.3 M NaCl in the same buffer (total volume, 800 ml). After the active fractions were collected and concentrated by diaflow filtration using an Amicon PM-10 membrane, *Pa*-Chi was further purified by gel filtration chromatography using a Toyopearl HW-55F resin (Tosoh) column (φ1.6×100 cm) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 0.2 M NaCl.

Purification of chitin oligosaccharide deacetylase (*Pa*-COD): Ten milliliters of the KN1699 starter culture was diluted into 2 l of half-strength artificial seawater containing 1% (*w*/*v*) peptone and 0.1% (*w*/*v*) yeast extract, and the

culture was incubated at 28°C for 48 h with shaking (135 rpm). The cells were removed by centrifugation (6,000×*g*, 15 min at 4°C), and proteins in the supernatant were precipitated with $(NH_4)_2SO_4$ (80% saturation) and collected by centrifugation (6,000×*g*, 20 min at 4°C). The precipitate was dissolved in 20 mM sodium phosphate buffer (pH 7.0) and dialyzed against the same buffer. The resulting enzyme solution was loaded on a DEAE Sepharose Fast Flow resin (GE Healthcare Bio-science) column (ϕ3.2×11 cm) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0) and eluted with a linear gradient of 0–0.4 M NaCl in the same buffer (total volume, 800 ml). The active enzyme fractions were collected, dialyzed against 20 mM sodium phosphate buffer (pH 7.0), and loaded on a DEAE-Toyopearl 650M resin column (ϕ1.5×10 cm) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0). Enzyme was eluted with a linear gradient of 0–0.4 M NaCl in the same buffer (total volume, 200 ml). The active fractions were collected, dialyzed as above, and loaded on a Phenyl Sepharose HP resin (GE Healthcare Bio-science) column (ϕ1.0×26 cm) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 0.7 M $(NH_4)_2SO_4$. *Pa*-COD was eluted from the column with a linear gradient of 0.7–0 M $(NH_4)_2SO_4$ in the same buffer (total volume, 200 ml).

Protein analysis

The homogeneities of the enzyme preparations were confirmed by native- and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In the SDS-PAGE, 2-mercaptoethanol was adopted as a reducing agent and was added to the sample buffer. Proteins in the polyacrylamide gels were stained using Coomassie Brilliant Blue R250 (Tokyo Kasei). Protein concentrations of the enzyme solutions were determined by the Lowry's method using bovine serum albumin (Sigma) as a standard. The *N*-terminal amino acid sequences of the enzymes were determined using a Perkin Elmer Biosystems model Procise 49X HT protein sequencer.

Assay of enzyme

Assay of chitinase activity: The assay mixture, consisting of 50 µl enzyme solution and 950 µl 0.5% (*w*/*v*) powdered β-chitin in 20 mM sodium phosphate buffer (pH 7.0), was incubated at 37°C for 30 min. The enzymatic reaction was terminated by heating at 100°C for 5 min in a hot dry bath, and then the reaction mixture was centrifuged at 10,000×*g* for 4 min. The amount of reducing sugar released was determined using a modification of the Schales methodology (Imoto and Yagashita 1971), using GlcNAc as a standard. One unit of chitinase activity was defined as the amount of enzyme required to liberate reducing sugar equivalent to 1 µmol of GlcNAc per minute under the assay conditions.

Assay of chitin oligosaccharide deacetylase activity: The assay mixture consisted of 75 µl enzyme solution and 425 µl 1-mM $(GlcNAc)_2$ in 20 mM sodium phosphate buffer (pH 7.0). The reaction mixture was incubated at 37°C for 30 min, then the reaction was stopped by heating at 100°C for 5 min in a hot dry bath. The amount of GlcN residues produced by the enzymatic reaction was determined according to the method of Dische and Borenfreund (1950) using GlcN as a standard. One unit of chitin oligosaccharide deacetylase activity was defined as the amount of enzyme required to produce 1 µmol of GlcN residues per minute under the assay conditions.

To investigate the effects of pH on the activities and stabilities of the enzymes, they were incubated in following buffers: pH 3.0–6.0, sodium citrate buffer; pH 6.0–8.0, sodium phosphate buffer; pH 8.0–11.0, sodium borate buffer. Temperature studies on the activity and the stability of the enzymes were conducted in sodium phosphate buffer (pH 7.0).

Thin layer chromatography analysis

Oligosaccharides produced from β-chitin, $(GlcNAc)_2$, and $(GlcNAc)_3$ by the enzymatic reactions were analyzed by Silica Gel thin layer chromatography (TLC) using 5:4:3 (*v*/*v*/*v*) *n*-butanol/methanol/16% aqueous ammonia as the mobile phase. Silica Gel 60 TLC plates (0.25 mm) were obtained from E. Merck. After developing the TLC plates twice, compounds were visualized by spraying with an aqueous solution of 2.4% (*w*/*v*) phosphomolybdic acid, 5% (*v*/*v*) H_2SO_4, and 1.5% (*v*/*v*) H_3PO_4 (phosphomolybdic acid reagent) or ethanol containing 0.5% (*w*/*v*) ninhydrin (ninhidrin reagent), followed by heating.

Purification and identification of oligosaccharide

Aqueous solutions containing the oligosaccharide produced by incubation of powdered β-chitin with crude enzyme were filtered through no. 4 filter paper (Kiriyama), then the filtrate was loaded on a charcoal (Wako Pure Chemical) column (φ2.0×15 cm) pre-equilibrated with water. The oligosaccharide was eluted from the column with water and lyophilized. After the resulting white powder was dissolved in a small amount of water, the solution was loaded on a Toyopearl HW-40F resin (Tosoh) column (ϕ2.5×90 cm) pre-equilibrated with water. The oligosaccharide was eluted from the column with 20% (*v*/*v*) ethanol. The oligosaccharide-containing fractions were collected and dried then re-dissolved in a small amount of water and lyophilized to afford the oligosaccharide as a white powder.

The structure of the oligosaccharide was characterized by ^1H nuclear magnetic resonance (NMR) spectrometry using D_2O as a solvent and mass spectrometry.

The ^{1}H NMR spectra were recorded with a Varian Mercury 400 spectrometer at 20°C. Chemical shifts were expressed in ppm downfield shift from $(CH_3)_4Si$. Mass spectra were obtained with a Waters MicroMass ZQ instrument under positive or negative ion electron-spray ionization conditions.

Results

Identification of the strain

The bacterial isolate obtained from soil of Yatsu dry beach, strain KN1699, was identified from its morphological and physiological characteristics (Table 1) and from the nucleotide sequence encoding its 16S rDNA. According to Bergey's Manual of Systematic Bacteriology, the isolate was classified as a bacterium belonging to the genus *Vibrio*. A search for similarity with 16S rDNA in the BLASTN database confirmed that the isolate was most closely related to *Vibrio parahaemolyticus* O3:K6 (Table 2). These taxonomic studies allowed us to conclude that strain KN1699 was *V. parahaemolyticus*.

Analysis of the oligosaccharide produced by enzymes in the culture fluid

The oligosaccharide produced by the action of crude enzyme (prepared from the supernatant of *V. parahaemolyticus* KN1699 cultures) on β-chitin was analyzed using TLC. When phosphomolybdic acid reagent was used to visualize the oligosaccharide, TLC analysis of the reaction mixture showed a single product (Fig. 1a), which was also visualized by ninhydrin reagent (Fig. 1b). These results suggest that the product is an oligosaccharide possessing a free amino group. Although the mobility of this compound corresponded to that of chitobiose [$(GlcN)_2$], it is necessary to confirm its structure. Therefore, the compound was purified and its structure analyzed by electron-spray ionization mass spectrometry (ESIMS) and ^{1}H NMR. The ESIMS spectra of the product corresponded to $[M-H]^-$ and $[M+H]^+$ species at *m/z* of 381 and 383, respectively, indicating that this compound is a disaccharide consisting of GlcN and GlcNAc. The ^{1}H NMR signals obtained (400 MHz, D_2O) were assigned as follows; α-anomer (non-reducing end sugar moiety) δ 1.93 (s, 3H, $COCH_3$), 3.31–3.53 (m, 3H, H-3, H-4, and H-5), 3.61–3.64 (m, 2H, H-2, and H-6a), 3.80 (dd, 1H, $J_{5,6b}$ 1.2 Hz, $J_{6a,6b}$ 12.4 Hz, H-6b), 4.43 (d, 1H, $J_{1,2}$ 8.4 Hz, H-1), (reducing end sugar moiety) δ 2.61 (dd, 1H, $J_{1,2}$ 3.6 Hz, $J_{2,3}$ 10.6 Hz, H-2), 3.31–3.53 (m, 1H, H-4), 3.55–3.59 (m, 1H, H-6a), 3.68 (dd, 1H, $J_{5,6b}$ 2.4 Hz, $J_{6a,6b}$ 12.4 Hz, H-6b), 3.68 (dd, 1H, H-3), 3.73 (ddd, 1H, $J_{4,5}$ 10.0 Hz, H-5), 5.06 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1); β-anomer (non-reducing end sugar moiety) δ 1.93 (s, 3H, $COCH_3$), 3.31–3.53 (m, 3H, H-3, H-4, and H-5), 3.61–3.64 (m, 2H, H-2, and H-6a), 3.80 (dd, 1H, $J_{5,6b}$ 1.2 Hz, $J_{6a,6b}$ 12.4 Hz, H-6b), 4.44 (d, 1H, $J_{1,2}$ 8.4 Hz, H-1), (reducing end sugar moiety) δ 2.48 (t, 1H,

Table 1 Morphological and physiological characteristics of strain KN1699

Characteristics	Results
Form	Rods
Gram stain	Negative
Motility	Positive
Oxidase	Positive
Nitrate reduction	Positive
Indole production	Positive
Gas from D-glucose	Negative
Acetoin production	Negative
Hydrogen sulfide production	Positive
Gelatin hydrolysis	Negative
Urea hydrolysis	Negative
Utilization of citrate	Negative
Utilization of sucrose	Negative
Arginine dihydrolase	Negative
Lysine decarboxylase	Positive
Ornithine decarboxylase	Positive
β-Galactosidase	Negative
Growth in NaCl	0.5–7%
Growth on TCBS	Green

Table 2 BLASTN results of comparison of the 16S rDNA of strain KN1699 to the sequence database

Accesion number	Species	Strain number	Similarity (%)
BA000031	*Vibrio parahaemolyticus*	03:K6	100
BA000032	*Vibrio parahaemolyticus*	03:K6	99.9
AY345403	*Bacterium*	K2-74	99.9
AF319769	*Vibrio* sp.	Ex25	99.9
AY911391	*Vibrio parahaemolyticus*	MP-2	99.9
AF500207	*Vibrio* sp.	CJ11052	99.8
AJ874352	*Vibrio natriegens*	01/097	99.8
AY542526	*Vibrio* sp.	KYJ962	99.8
AY738129	*Vibrio campbelli*	90-69B3	99.7
AF388387	*Vibrio parahaemolyticus*	ATCC17802	99.5
AF246980	*Vibrio* sp.	98CJ11027	99.4
AY911396	*Vibrio harveyi*	SW-3	99.3
AF319770	*Vibrio* sp.	Ex97	99.3
AF064637	*Vibrio* sp.	NAP-4	99.3
AF388389	*Vibrio parahaemolyticus*	ATCC17802	99.3
AJ874353	*Vibrio natrieggens*	01/252	99.2

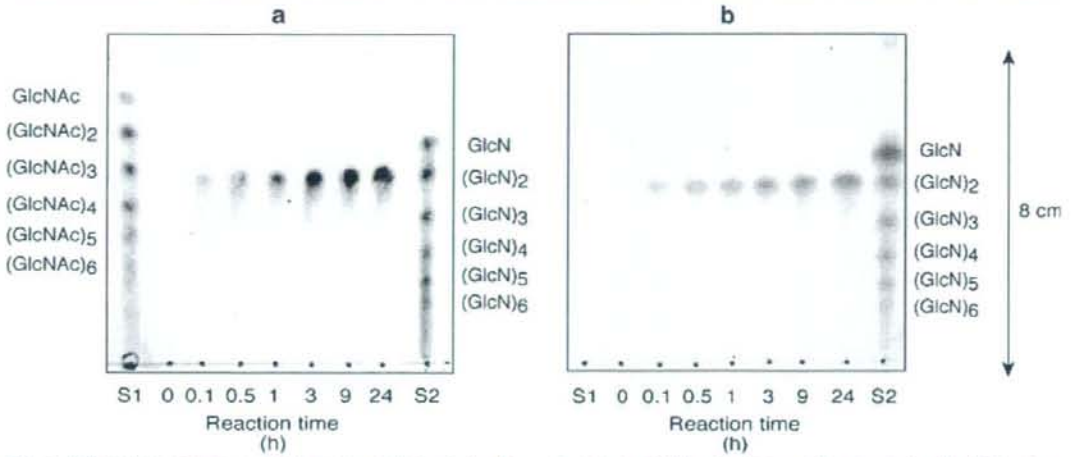

Fig. 1 TLC analysis of oligosaccharide produced by incubating β-chitin with crude enzyme solution. Crude enzyme solution (200 μl) prepared from the supernatant of *V. parahaemolyticus* KN1699 cultures was added to 3.8 ml 20-mM sodium phosphate buffer (pH 7.0) containing 20 mg powdered β-chitin, then the mixture was incubated at 37°C with stirring. After developing the TLC plates, reaction products produced after various incubation times were visualized using the following reagents: **a** phosphomolybdic acid reagent and **b** ninhydrin reagent. *Lane S1* *N*-acetylchitooligosaccharide standards, *lane S2* chitooligosaccharide standards

$J_{1,2}$=$J_{2,3}$ 8.8 Hz, H-2), 3.31–3.53 (m, 3H, H-3, H-4, and H-5), 3.55–3.59 (m, 1H, H-6a), 3.68 (dd, 1H, $J_{6a,6b}$ 12.4 Hz, H-6b), 4.45 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1). The NMR signals obtained corresponded well to the those of β-D-*N*-acetylglucosaminyl-(1,4)-D-glucosamine (GlcNAc-GlcN) (Ohishi et al. 1997; Tokuyasu et al. 1999). From these results, it became clear that the compound produced from β-chitin by the action of crude enzyme, which was prepared from the supernatant of *V. parahaemolyticus* KN1699 cultures, is GlcNAc-GlcN.

This heterodisaccharide seemed to be produced by the reactions of both chitinase (*Pa*-Chi) and chitin oligosaccharide deacetylase (*Pa*-COD), which were secreted into the culture medium by *V. parahaemolyticus* KN1699. Then, to confirm this supposition, we purified these enzymes and analyzed their reactions.

Purification of the enzymes

Purification of *Pa*-Chi: The *Pa*-Chi was purified in four steps from 1 l of culture fluid (Table 3). Each column chromatographic step produced a single peak showing chitinase activity. The purified enzyme gave a single band on both SDS-PAGE (Fig. 2a) and native-PAGE (Fig. 2b), indicating that the enzyme protein is in a high state of purity. *Pa*-Chi was purified 9.67-fold with 31.2% recovery of initial total activity. The specific activity of the purified enzyme towards powdered β-chitin was 2.32 U/mg of protein.

Table 3 Purification of *Pa*-Chi and *Pa*-COD from culture fluid of strain KN1699

Purification step	Total activity (U)	Specific activity (U/mg of protein)	Yield (%)	Fold
Pa-Chi[a]				
$(NH_4)_2SO_4$ precipitation	34.4	0.24	100	1
DEAE-Toyopearl 650M (first)	18.2	1.61	52.9	6.71
DEAE-Toyopearl 650M (second)	14.9	2.13	43.2	8.88
Toyopearl HW-55F	10.7	2.32	31.2	9.67
Pa-COD[b]				
$(NH_4)_2SO_4$ precipitation	1.95	0.01	100	1
DEAE Sepharose FF	1.04	3.05	53.3	305
DEAE-Toyopearl 650M	0.76	9.50	39.0	950
Phenyl Sepharose HP	0.62	31.0	31.8	3,100

[a] *Pa*-Chi was purified from 1 l of culture fluid.
[b] *Pa*-COD was purified from 2 l of culture fluid.