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Pentamer is the minimum structure for oligomannosylpeptoids to bind to concanavalin A

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Abstract—Enzyme-linked lectin assay (ELLA) was performed for oligomannosylpeptoids, which were immobilized on microtiter plates through a streptavidin–biotin interaction. The other immobilization methods, a hydrophobic adsorption and a covalent attachment, were found inapplicable to the oligomannosylpeptoids. Penta- and hexamannosylpeptoids with a shorter or longer spacer were found to be significantly recognized by concanavalinA (ConA), while the smaller peptoids showed no bindings. A proportional relationship between the amount of bound ConA and the peptoid density on the microtiter plate was observed, indicating the absence of both cluster and overdense effects that would assist or inhibit the binding increasingly with the ligand density.

Oligopeptoids are N-substituted glycine oligomers used as the mimics of oligopeptides or the scaffold of functional groups for combinatorial libraries.[1] When sugars are arrayed on the peptoid backbones, these molecules can be regarded as the mimics of glycopeptides[2] or oligosaccharides.[3] Thus, the library of the oligoglycosylpeptoids is a potential substitute for a glycopeptide or oligosaccharide library. The use of mimics in the drug discovery based on combinatorial approach is especially important for oligosaccharides since oligosaccharide synthesis is generally too tedious to cover the extensive structural variation in a library and to be profitable in a mass drug production.[4] In our previous study, we synthesized oligomannosylpeptoids $\mathbf{1_n}$ labeled with a dansyl group at the N-terminals (Fig. 1) and investigated the binding abilities of these peptoids to concanavalin A (ConA) by a fluorescence anisotropy assay in solution.[3] The combination of oligomannosylpeptoids and ConA was selected as a starting point of the library development because the synthesis of the mannosylpeptoid unit is easy, ConA is commercially available, and its properties are well known. As a result, the binding abilities of di- and trimannosylpeptoids ($\mathbf{1_2}$, $\mathbf{1_3}$) were comparable to those of the natural counterparts, di- and trimannosides, respectively. The relatively strong binding abilities were partially attributed to the specific binding of the mannose residues as suggested from the increment of binding free energy (ca 0.5 kcal/mol) with the increasing number of mannose residue from monomer to trimer. However, a non-specific hydrophobic contact between the dansyl group and ConA[5] was suggested to be the main attractive interaction from the fact that the monomannosylpeptoid $\mathbf{1_1}$ showed a better binding ability to ConA than methyl mannoside. Such an assistance by non-specific binding should be avoided in library assays, since a general purpose of the assay is the selection of lead ligands that specifically and strongly bind to a protein. As our aim was to realize the library assay for oligoglycosylpeptoids, we decided to take a step closer to this goal by examining enzyme-linked lectin assay (ELLA) on the microtiter plate carrying the oligomannosylpeptoids devoid of using dansyl group.

As discussed in a review article,[6] there are mainly three methods to immobilize carbohydrates on a microtiter plate: (1) physical adsorption, (2) covalent immobilization, and (3) streptavidin–biotin immobilization. At first,

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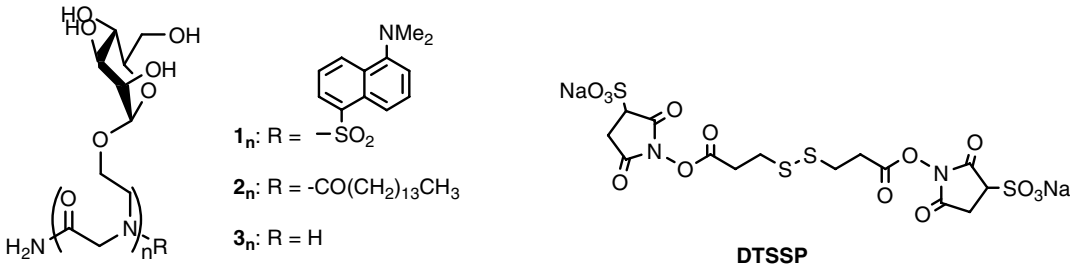
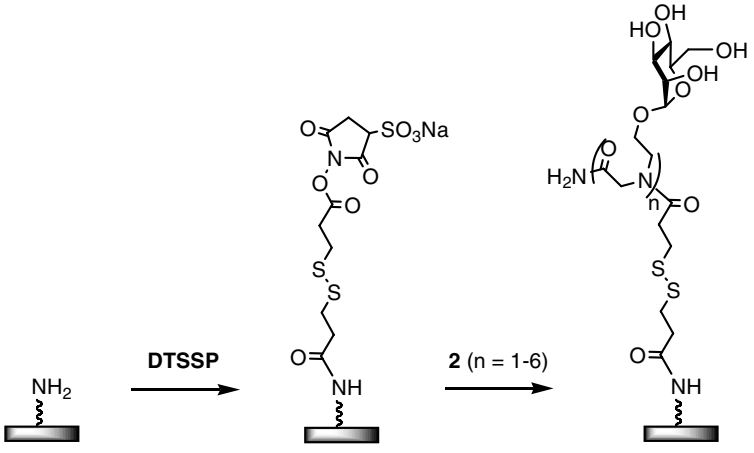


Figure 1. Structures of oligomannosylpeptoids $\mathbf{1_n}$, $\mathbf{2_n}$, $\mathbf{3_n}$, and DTSSP.

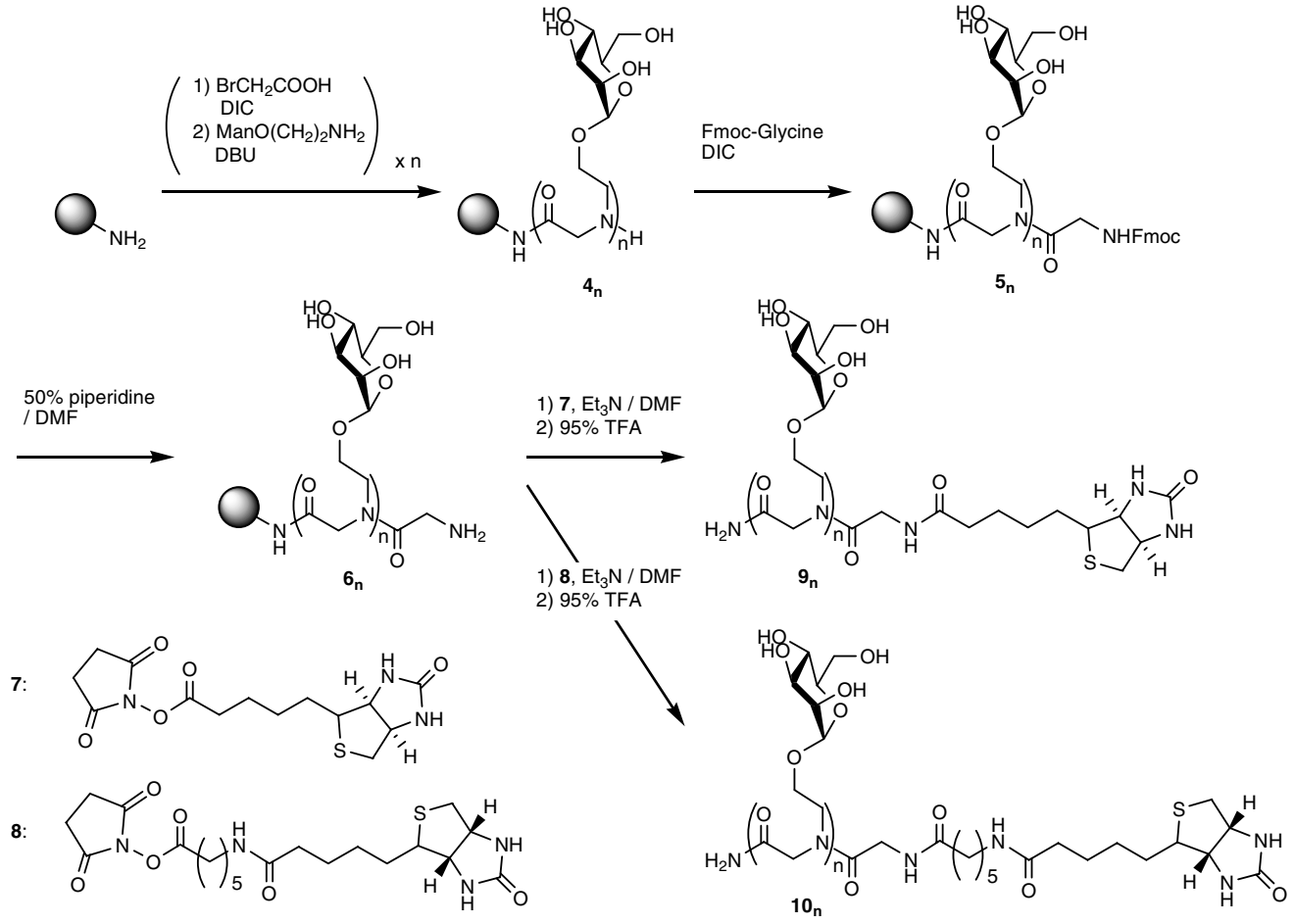
we attempted adsorption of the oligomannosylpeptoids attached with a C14 chain ($\mathbf{2_n}$) onto a polystyrene 96-well microtiter plate (Fig. 1). The chain length of C14 was selected because it was reported that oligosaccharyl lipids with C14 aglycon were almost completely retained on a polystyrene microtiter plate after washing steps.[7] However, compounds $\mathbf{2_n}$ ($n = 1$–3) were completely desorbed from the well surface after three washings with water, as quantified by sulfuric acid–phenol assay (SPA). The hydrophilic property of the peptoid chain seems incompatible with the physical adsorption method. We thus turned to the use of a covalent bond to immobilize the oligomannosylpeptoids.

The use of 3,3′-dithiobis(propionate) (DTS) as a covalent linker has an advantage of being cleavable at the disulfide bond by reduction after the immobilization of carbohydrates, thereby permitting SPA quantification of the loaded carbohydrates.[8,9] In a test run of immobilization, a water soluble active ester, 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP) (Fig. 1), was reacted with the amino groups of a commercially available amino-coated microtiter plate (Scheme 1).[10] Then oligomannosylpeptoids $\mathbf{3_n}$ ($n = 1$–6), which were synthesized on solid supports,[3] were added to the wells (2.0×10^{-7} mol/well) to react with the unreacted succinimide moieties of DTSSP.[11] The amounts of the loaded oligomannosylpeptoids per well were determined by SPA after reduction with dithiothreitol: $\mathbf{3_1}$, 2.4×10^{-8}; $\mathbf{3_2}$, 9.8×10^{-9}; $\mathbf{3_3}$, 5.1×10^{-9}; $\mathbf{3_4}$, 3.8×10^{-9}; $\mathbf{3_5}$, 3.0×10^{-9}; $\mathbf{3_6}$, 3.7×10^{-9} mol/well. The loading efficiencies tended to decrease with the increasing number of the mannose moieties. For ELLA trials, the loading amounts were adjusted to 2.0×10^{-9}, 2.0×10^{-10}, 2.0×10^{-11}, or 2.0×10^{-12} mol/well by adequately diluting the DTTSP and $\mathbf{3_n}$ solutions. In quadruplicate ELLA experiments,[12] however, there were no indications of significant bindings between HRP-ConA and immobilized $\mathbf{3_n}$ regardless of the number of mannose residues and the density of the ligands. We were uncertain at this point whether the binding abilities of oligomannosylpeptoids were too weak to be detected by ELLA. The length of DTS might be too short to surmount the BSA block, expose the mannose residues to the top surfaces, and deliver them to ConA pockets. We thus decided to use the third immobilization method, the streptavidin-biotin protocol, rather than going into detail to find a good covalent linker, since a number of precedent studies suggested that this method seemed most promising for the weak ligands.[6]

Biotinylated oligomannosylpeptoids with shorter ($\mathbf{9_n}$) and longer spacers ($\mathbf{10_n}$) were synthesized in solid phase as shown in Scheme 2. Bromoacetylation and coupling with 1-mannosyl-2-aminoethanol on Rink amide resin were repeated for n times to give on-polystyrene oligomannosylpeptoids $\mathbf{4_n}$, which were the same products as those previously reported.[3] The secondary amino groups of $\mathbf{4_n}$ were reacted with Fmoc-glycine for 3 hrs in the presence of N,N'-diisopropylcarbodiimide (DIC) to give $\mathbf{5_n}$, which were subjected to removal of Fmoc group to give $\mathbf{6_n}$. Though the yields of $\mathbf{6_n}$ decreased with the



Scheme 1.



Scheme 2.

increasing number of mannose residues as determined by quantification of liberated Fmoc by UV absorption, they were improved by raising temperature from room temperature (RT) to 50 °C at the Fmoc–Glycine coupling step: $\mathbf{6_1}$, 83% (RT); $\mathbf{6_2}$, 76% (RT); $\mathbf{6_3}$, 43% (RT); $\mathbf{6_4}$, 7% (RT), 61% (50 °C); $\mathbf{6_5}$, 5% (RT), 39% (50 °C); $\mathbf{6_6}$, 5% (RT), 10% (50 °C). The primary amino groups of $\mathbf{6_n}$ were reactive enough toward NHS-activated carboxylates **7** and **8** to afford the biotinylated products with a shorter or longer spacer ($\mathbf{9_n}$, $\mathbf{10_n}$) after liberation from the solid support.[13,14] The biotinylated oligomannosylpeptoids $\mathbf{9_n}$ and $\mathbf{10_n}$ were used in ELLA without further purification, because the strong streptavidin–biotin binding would permit a thorough washing of non-biotinylated impurities.

The ELLAs for streptavidin-biotin immobilized ligands were carried out basically with the reported method.[15] The results are shown in Figures 2a and b, as presented by the absorbance at 490 nm indicating the relative amounts of the bound HRP-ConA. As shown in Figure 2a, the amounts of the bound ConA for $\mathbf{9_1}$, $\mathbf{9_2}$, $\mathbf{9_3}$, $\mathbf{9_4}$, and **11** were less than or comparable to that of the blank experiment. On the other hand, $\mathbf{9_5}$ and $\mathbf{9_6}$ indicated significantly higher binding abilities to ConA. The same trend was observed for $\mathbf{10_n}$ and only $\mathbf{10_5}$ and $\mathbf{10_6}$ showed the binding to ConA. Since the replacement of spacer of tetramannosylpeptoid from the shorter ($\mathbf{9_4}$) to longer ones ($\mathbf{10_4}$) did not improve the binding ability to ConA, the low binding abilities of the tetramer are unlikely due to the shortage of spacer length. We, therefore, can suggest that pentamannosylpeptoid, which has five mannosyl residues, is the minimum structure for oligomannosylpeptoids that is specifically recognized by ConA. This binding profile is different from that obtained by the fluorescent anisotropy assay in solution for the dansyl oligomannosylpeptoids $\mathbf{1_n}$ ($n = 1$–6),[3] in which the trimer $\mathbf{1_3}$ showed the maximum binding ability to ConA. The solution assay is generally more sensitive to a weak interaction than ELLA and the hydrophobic effect of the dansyl group would mainly act on the relatively strong binding of $\mathbf{1_3}$ to ConA. Thus, the binding profile for oligomannosylpeptoids $\mathbf{9_n}$ and $\mathbf{10_n}$ obtained by ELLA is more likely to serve the information about useful ligands and this study showed that the pentamannosylpeptoid and hexamannosylpeptoid are good ligands of ConA.

The opposite responses of ConA against the pentamer and hexamer for shorter ($\mathbf{9_5 < 9_6}$) and longer ($\mathbf{10_5 > 10_6}$) spacers could be explained as follows. The peptoid backbone has a considerable freedom of mo-

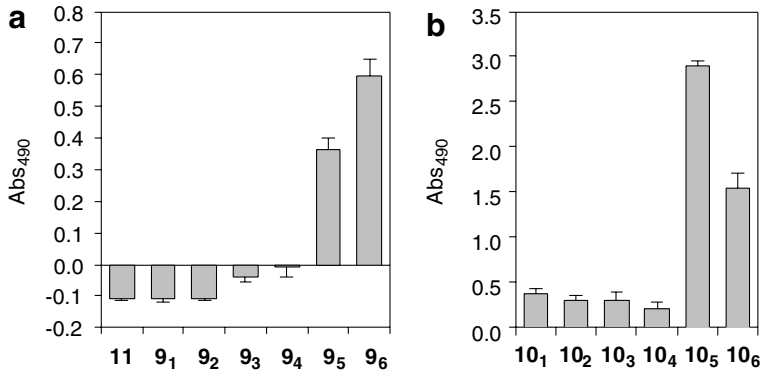
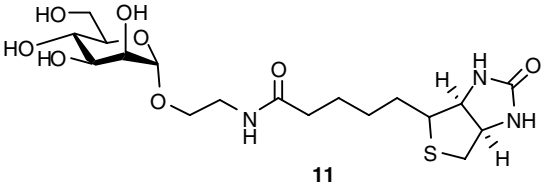


Figure 2. The ELLA results for oligomannosylpeptoids $\mathbf{9_n}$ with a shorter spacer (**a**) and $\mathbf{10_n}$ with a longer spacer (**b**). Streptavidin (4 μg/well) in 100 μL TBS buffer (10 mM, pH 7.4) was added to each well and kept for 24 h at 4 °C and the wells were washed carefully to afford a streptavidin-coated microtiter plate. Excess oligomannosylpeptoids $\mathbf{9_n}$, $\mathbf{10_n}$ (50–100 eq.) in 50 μL PBS buffer were added to each well of the above microtiter plate and they were kept for 2 h at 37 °C. After removal of the peptoid solutions, the wells were washed three times with 250 μL TBS buffer. Blocking of the wells with BSA and quantification with HRP-ConA were performed as those described above for covalently linked ligands.[12] The experiment with the same conditions was repeated four times. The same assay was performed for biotin as a blank and for a non-peptoid mannose derivative, *N*-biotinyl-*O*-α-mannosylethanolamine **11**, for comparison. Error bars stand for standard error.

tions and the longer oligomers would be entropically unfavorable in the binding to ConA, while the binding enthalpy would be enhanced as the number of the mannose residue increases as far as there are unoccupied recognition sites in the lectin pocket. The oligomannosylpeptoids with the shorter spacer are more restricted in motions, so that the enthalpic contribution could surpass the entropic effects in the binding where ConA favors the hexamer $\mathbf{9_6}$ over the pentamer $\mathbf{9_5}$. On the other hand, the longer spacer would somewhat liberalize the restriction, emphasizing the entropic effects. Thus we suggest that while the longer spacer would allow for the easier access of the peptoid ligands into ConA pocket, the smaller affinity of the hexamer $\mathbf{10_6}$ relative to the pentamer $\mathbf{10_5}$ would be due to the enhanced entropical disadvantage.



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We observed a proportional increase of ConA binding to $\mathbf{9_6}$ with the increasing amount of immobilized streptavidine from 0.2 to 4 μg/well (Fig. 3a). This proportional relationship indicates that in the tested density range there was neither a cluster effect, which would give an exponential increase of the binding ability with the increasing density, nor an overdense effect, which would hinder the binding of ConA to give a saturation curve. Therefore, each output observed in this ELLA study reflects the binding ability of a single peptoid ligand to ConA, not that of the multivalent ligand array on the well surface. The number of washing step after the addition of ConA to the $\mathbf{9_6}$-immobilized wells significantly influenced ConA retainment on a well as shown in Figure 3b, indicating that the binding ability of $\mathbf{9_6}$ is not extraordinarily strong.

In conclusion, we demonstrated by ELLA that pentamer was the minimum structural requirement for oligomannosylpeptoids to be efficiently recognized by ConA without any additional assistance. The largest separation of two mannose residues in the pentamer $\mathbf{9_5}$ was calculated to be ca 25 Å from a molecular model,

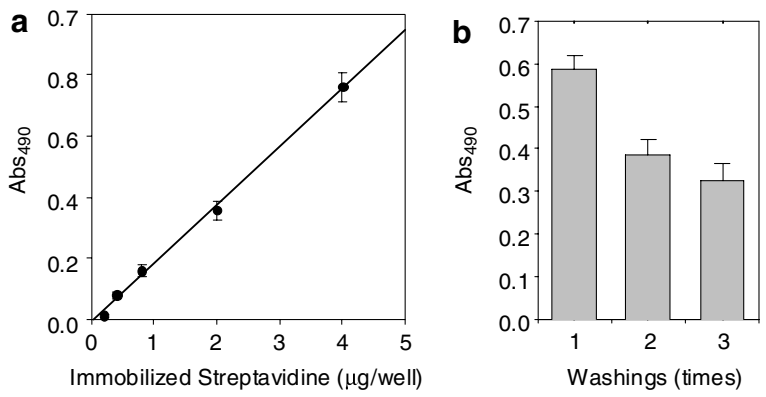

Figure 3. The relationship between the binding amount of ConA on microtiter plate and the density of $\mathbf{9_6}$ in a well (**a**). The effect of washings on the retainment of ConA (**b**). Error bars stand for standard error.

being much smaller than 65 Å,[16] the separation between two binding sites of ConA tetramer. Therefore, the ligand cannot bridge two binding sites of ConA. Thus, the results that the penta- and hexamannosyl peptoids were active ligands in ELLA are likely to mean that these lignads fit in a single binding site of ConA. While even a monomannosylpeptoid was considered sufficient for the recognition by ConA in our previous study, this study demonstrated that pentamer is the minimum structure for the practical use of oligomannosylpeptoids as the ligands of ConA.

Acknowledgments

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9. We carried out SPA in glass test tubes to avoid a possible damage on the polystyrene microtiter plate by the SPA reagents.
10. MS-8696F, Sumitomo Bakelite, Co., Ltd., Tokyo, Japan.
11. Immobilization: A solution of DTSSP in 50 μL PBS (100 mM, pH 9.0) was added into each well of the amino-coated microtiter plate. After 30 min, the solution was removed and each well was washed three times with 300 μL of water. A mannopeptoid $\mathbf{3_n}$ in 50 μL PBS (100 mM, pH 9.0) was added to each well and kept for 30 min. The sugar solution was removed and each well was washed three times with 300 μL of water. When it was necessary to quantify the sugar contents by SPA method, the sugars were released by the reduction of the disulfide bond with 3 equiv aqueous dithiothreitol (50 μL).
12. ELLA: To avoid non-specific adsorption of ConA, the wells were blocked with bovine serum albumin (BSA): 3% BSA in 200 μL TBS buffer was kept in each well for 30 min. After removal of BSA solution, each well was soaked for 1 h with the solution of ConA–horseradish peroxidase fusion protein (HRP-ConA, 2.5 μg/mL) in 200 μL TBS buffer and then washed three times with 250 μL TBS buffer. Peroxidase activity was measured by adding the solution of 0.6 mg *O*-phenylenediamine and 0.6 μL of 30% H_2O_2 in 150 μL citrate buffer (100 mM, pH 5.0) and then adding 50 μL of 4 M H_2SO_4 after 7 min to stop the chromogenic reaction. Absorption at 490 nm was read with a microplate reader.
13. Compound $\mathbf{9_1}$ was also synthesized in liquid phase to confirm the structure, in which 2,4-dimethoxy-benzylamine was used as the soluble substitute for Rink amide resin: $[\alpha]_D^{25}$ -12.6 (c 0.92, methanol); mp 133–138 °C; ^{1}H NMR (270 MHz, D_2O) δ 4.69 (s, 1H), 4.50–4.42 (m, 1H), 4.32–4.25 (m, 1H), 4.12 (s, 2H), 3.98 (s, 1.4H), 3.90 (s, 0.6H), 3.85–3.33 (m, 10H), 3.25–3.15 (m, 1H), 2.85 (dd, 1H, J 4.7, 12.9 Hz), 2.63 (d, 1H, J 12.9 Hz), 2.22 (t, 2H, J 7.1 Hz), 1.68-1.23 (m, 6H); ^{13}C NMR (67.8 MHz, DMSO-d_6) d 172.5, 172.4, 170.4, 170.0, 169.4, 169.3, 162.9, 162.8, 74.1, 71.0, 70.1, 67.0, 64.6, 64.3, 61.2, 61.0, 59.3, 55.4, 50.3, 48.8, 47.3, 46.9, 34.9, 28.2, 28.0, 25.3; ESI-HMS *m/z* calcd for $C_{22}H_{37}N_5O_{10}SNa$ (M+Na) 586.2159. Found: 586.2159.
14. Compound $\mathbf{9_2}$: ESI-HMS *m/z* calcd for $C_{32}H_{55}N_6O_{17}S$ (M+H) 827.3344. Found: 827.3344. Compound $\mathbf{9_3}$: ESI-HMS *m/z* calcd for $C_{42}H_{72}N_7O_{24}S$ (M+H) 1090.4349. Found: 1090.4327. Compound $\mathbf{9_4}$: MALDI-TOFMass *m/z* calcd for $C_{52}H_{88}N_8O_{31}SNa$ (M+Na) 1375.52. Found: 1375.48. Compound $\mathbf{9_5}$: MALDI-TOFMass *m/z* calcd for $C_{62}H_{105}N_9O_{38}SNa$ (M+Na) 1638.62. Found: 1638.56. Compound $\mathbf{9_6}$: MALDI-TOFMass *m/z* calcd for $C_{72}H_{122}N_{10}O_{45}SNa$ (M+Na) 1901.72. Found: 1901.61. Compound $\mathbf{10_1}$: ESI-HMS *m/z* calcd for $C_{28}H_{48}N_6O_{11}SNa$ (M+Na) 699.2999. Found: 699.3063. Compound $\mathbf{10_2}$: ESI-HMS *m/z* calcd for $C_{38}H_{65}N_7O_{18}SNa$ (M+Na) 962.4405. Found: 962.4387. Compound $\mathbf{10_3}$: ESI-HMS *m/z* calcd for $C_{48}H_{83}N_8O_{25}S$ (M+H) 1203.5190. Found: 1203.5116. Compound $\mathbf{10_4}$: MALDI-TOFMass *m/z* calcd for $C_{58}H_{99}N_9O_{32}SNa$ (M+Na) 1488.60. Found: 1488.61. Compound $\mathbf{10_5}$: MALDI-TOFMass *m/z* calcd for $C_{68}H_{116}N_{10}O_{39}SNa$ (M+Na) 1751.70. Found: 1751.74. Compound $\mathbf{10_6}$: MALDI-TOFMass *m/z* calcd for $C_{78}H_{133}N_{11}O_{46}SNa$ (M+Na) 2014.80. Found: 2014.69.
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