Facile Synthesis of a Glycopeptide Building Block of Antifreeze Glycoprotein

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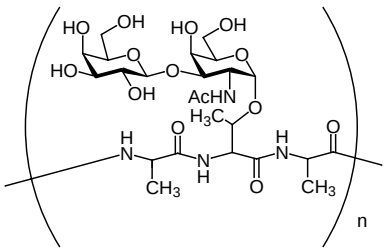
Abstract: A new procedure for synthesizing a building block of antifreeze glycoprotein (AFGP) **1**, Moz-Ala-(Galβ1→3GalNAcα1)Thr-Ala-OH **7** has been developed. The onium salt is an important intermediate to generate α-glycopeptide **5** of excellent yield with high selectivity as a key step. One can successfully remove the Moz protective group of glycopeptide selectively with formic acid without cleaving the glycosidic bond.

Key words: antifreeze, glycopeptides, solid phase synthesis, tetramethylurea, protecting groups, Moz

Four distinct macromolecular antifreezes [antifreeze peptide type I, type II, type III and AFGP (antifreeze glycoprotein)] have been isolated and characterized from different marine fish.[1] The AFGP has a simple, repeating unit structure, and may be expressed as Ala-[Ala-(Galβ1→3GalNAcα1)Thr-Ala]-Ala. Due to the heterogenity of the AFGPs, which is usually isolated as a mixture, a good characterization of AFGP is difficult.[2] The antifreeze peptides can be obtained by molecular cloning, but not the carbohydrate containing AFGP with the presently available techniques. The synthesis of mixtures of AFGP with high molecular weight has been reported recently.[3] A computer modeling structure of AFGP was generated to explain the interaction of AFGP with planes of ice, whereas, homogeneous AFGP for biological study is still not available.[4,5] The synthesis of these antifreezes has generated great research interest and thus, a facile and efficient synthesis of AFGP is urgently required.[6] In this report, we describe a new procedure for synthesizing, Ala-(Galβ1→3GalNAcα1)Thr-Ala, a building block of AFGP **1**.



1 n=4-55

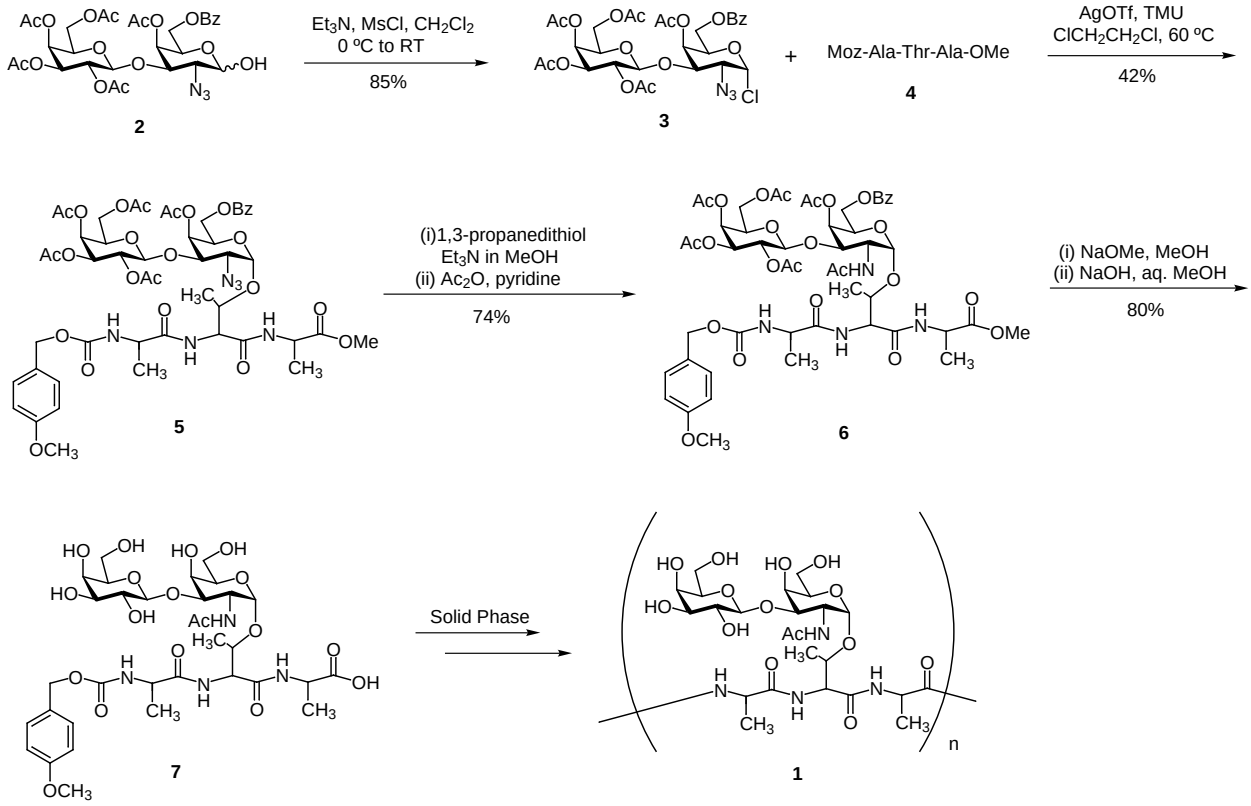
Antifreeze glycoprotein (AFGP)

In our study, we found that the choice of *N*-terminal protective group in peptide is very important in preparing an efficient glycopeptide building block for future synthesis of AFGP by solid phase method. Especially, we were interested in using unprotected disaccharide moiety to synthesize antifreeze glycoprotein analogs by solid phase method. The Fmoc protective group has been widely used in a solid phase synthesis of biologically active glycopeptide.[7] The final cleavage of elongated glycopeptide from resin was carried out with trifluoroacetic acid. The acidic condition can cause the cleavage of the *O*-glycosidic linkage, especially, β-anomer linkage or unprotected glycosidic bond.[8] For this reason, we chose the acid labile *p*-methoxybenzyloxycarbonyl (Moz)[9,10] as an *N*-terminal protective group of the building block to suppress the possibility of its removal from the resin. The cleavage from the resin in Moz protocol can be processed under basic or photolytic condition.[11] Furthermore, the condition for deprotection of the extremely acid-labile Moz protective group will keep the *O*-glycosidic bond intact. On this basis, the Moz-protected glycopeptide **7** will be a suitable building block to synthesize antifreeze glycoprotein analogs by facile solid phase synthesis.

Our facile and efficient synthesis of an AFGP building block is the first attempt to synthesize AFGPs by solid phase. We designed the sequence of tripeptide (Ala-Thr-Ala), in which the threonyl residue in the middle of tripeptide will give the C-terminal and *N*-terminal alanine more free space to be coupled on solid phase synthesis. The synthetic approach for the building block glycopeptide **7** is shown in Scheme 1.

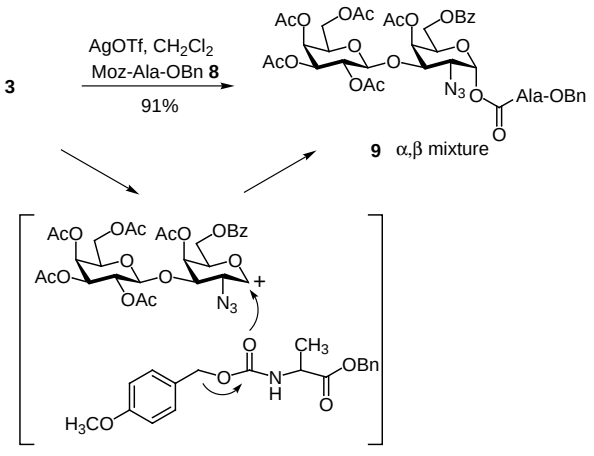
Disaccharide **2** was prepared by established method,[7,12] and was converted to α-glycosyl chloride **3** by treatment with methanesulfonyl chloride and triethylamine at 0 °C to room temperature for 36 hours (85%). The glycosyl donor **3** was directly coupled with the available Moz-Ala-Thr-Ala-OMe tripeptide **4** at the threonyl residue to give **5**. Due to acid-labile protective groups such as Boc and Moz groups, side reactions occurred in the glycosylation step when an active catalyst such as AgOTf, $AgClO_4$, $AgClO_4/Ag_2CO_3$ was used in dichloromethane or dichloromethane/toluene. A satisfactory yield could not be obtained even at lower temperature (<15% at –20 °C) and the imidate method[13] was also found to be unsuitable (<10%).

According to the analysis of the side products, they were generated from the deprotection of Moz-group triggering a sequence of side reactions under these conditions. In order to investigate the side reaction (Scheme 2), the highly nucleophilic carbamate group of Moz-Ala-OBn **8** was re-



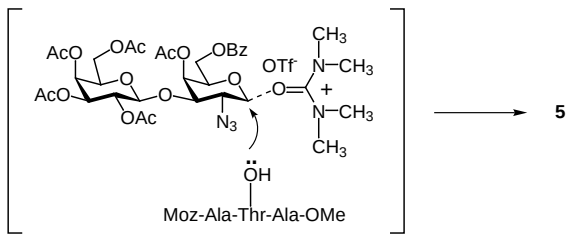
Scheme 1

acted with α-glycosyl chloride **3** in the presence of AgOTf to produce α,β carbamate-linked glycopeptide **9** in 91% yield. This result showed that the carbamate could successfully compete with the OH group of threonyl residue in tripeptide **4** to initiate the nucleophilic substitution (Scheme 2).



Scheme 2

In order to circumvent this problem, we modified Sasaki's work[14] so that the glycosyl donor **3** was catalyzed by AgOTf in the presence of hindered tetramethylurea (TMU) in 1,2-dichloroethane/toluene to form the onium salt (Scheme 3).[15] Then the available tripeptide **4** was added and stirred at 60 °C overnight. This efficient procedure afforded α-glycoside **5** in 42% yield with high selectivity. We believe that the formation of onium salt (by adding TMU) may decrease the positive charge at the anomeric center and suppress the degradation of the acid sensitive Moz-group, thus elevating the coupling yield of glycosylated tripeptide. This result can explain the reason why acid-labile *tert*-butyloxycarbonyl (Boc) is not a suitable protecting group in glycosylation reaction.[16] By using the same procedure as described above one can overcome the problem of degradation of the Boc substituent.



Scheme 3

The azide group of intermediate **5** was converted into an acetamide with triethylamine and propane-1,3-dithiol in methanol followed by acetylation to provide the glyco-

peptide **6**[17] in 74% yield. The fully protected glycoside **6** was de-*O*-acetylated with a catalytic amount of sodium methoxide in methanol followed by saponification to obtain the desired building block **7** (80%).[18] The removal of Moz-group with trifluoroacetic acid/dichloromethane or hydrochloric acid/methanol in peptide synthesis is well documented.[9,10] This acidic condition may be hazardous to the acid-labile glycosidic linkage of the disaccharide.[8] The Moz-group of **7** was removed by treatment with formic acid at room temperature for 30 minutes. This condition was mild enough to leave the glycosidic linkage intact (as judged by ^{1}H NMR spectrum in the region of the glycoside bond) and can be applied to a solid phase glycopeptide synthesis using disaccharide **7** as a building block.

In conclusion, we have described a complete procedure to obtain Moz-Ala-(Galβ1→3GalNAcα1)Thr-Ala as a building block of AFGP. This method can overcome the previous limitation while using monosaccharide for AFGP synthesis. Further work on solid phase synthesis is in progress.

Column chromatography was performed on silica gel 60 (70–230 and 230–400 mesh, Merck) and preparative HPLC on nucleosil 5 C18. 1,2-Dichloroethane and toluene were dried by CaH_2 and distilled. The ^{1}H NMR spectra were recorded on a Bruker ASPECT 3000 (400 MHz) spectrometer. The value of δ are expressed in ppm relative to the solvent signal as internal standard ($CHCl_3$, 7.24 ppm; HOD, 4.7 ppm). Mass spectra were measured with Autospec (micromass, UK). All reagents are commerically available.

2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-4-*O*-acetyl-2-azido-6-*O*-benzoyl-2-deoxyl-α-D-galactopyranosyl Chloride (3)

Compound **2** (0.68 g, 1.00 mmol) and $MeSO_2Cl$ (0.16 mL, 2.00 mmol) were dissolved in CH_2Cl_2 (5 mL) at 0 °C. Et_3N (0.42 mL, 3.00 mmol) was added dropwise under N_2. After 36 h, the mixture was diluted with EtOAc (60 mL), the solution was washed with aq satd $NaHCO_3$ solution (50 mL), dried ($MgSO_4$), and concentrated in vacuo to give a yellow liquid. The crude product was purified by column chromatography over silica gel (eluent:7:3 hexane/EtOAc) to give **3** (0.60 g, 85%) as a colorless foam.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.96, 2.03, 2.07, 2.13 (4 s, 15 H, 5 $COCH_3$), 3.91 (t, *J* = 6.6 Hz, 1 H, H-5), 4.04–4.18 (m, 4 H, H-3, 6), 4.33 (d, 2 H, *J* = 6.1 Hz, H-6'), 4.53 (t, 1 H, *J* = 6.1 Hz, H-5'), 4.73 (d, 1 H, *J* = 7.8 Hz, H-1), 4.98 (dd, 1 H, *J* = 10.4, 3.3 Hz, H-3'), 5.17 (dd, 1 H, *J* = 10.4, 7.8 Hz, H-2'), 5.34 (d, 1 H, *J* = 3.3, H-4'), 5.62 (d, 1 H, *J* = 3.1 Hz, H-4), 6.17 (d, 1 H, *J* = 3.7 Hz, H-1), 7.42 (br t, 2 H_m, C_6H_5), 7.55 (br t, 1 H_p, C_6H_5), 7.99 (br d, 2 H_o, C_6H_5).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 20.5, 30.6, 20.8, 60.7, 61.0, 62.3, 66.7, 68.7, 70.7, 71.0, 74.8, 93.1, 101.3, 128.4, 129.4, 129.7, 133.3, 166.0, 169.3, 169.5, 170.1, 170.3, 170.4.

MS: m/z (%) = 700 (M^++H, 22), 698 (40), 674 (65), 664 (58), 656 (42), 640 (52), 331 (100).

***N*-(4-Methoxylbenzyloxycarbonyl)-L-alanyl-*O*-[2,3,4,6-tetra-*O*-acetyl-β-D-galacto-pyranosyl-(1→3)-4-*O*-acetyl-2-azido-6-*O*-benzoyl-2-deoxyl-α-D-galactopyranosyl]-L-threonyl-L-alanine Methyl Ester (5)**

Tripeptide **4** (1.32g, 3.00 mmol), tetramethylurea (0.48 mL, 4.00 mmol), AgOTf (0.77 g, 3.00 mmol) were dissolved in anhyd $ClCH_2CH_2Cl$/toluene (1:2, 15 mL) in the presence of Drierite (4 g). The mixture was stirred at r.t. After 1 h, a solution of compound **3** (0.70 g, 1.00 mmol) in $ClCH_2CH_2Cl$ (5 mL) was added at r.t., and stirred at 60 °C overnight. The resulting solution was diluted with EtOAc and filtered. The filtrate was washed with aq satd $NaHCO_3$ solution (60 mL), dried ($MgSO_4$), and concentrated in vacuo to give a brown liquid. The crude product was purified by column chromatography over silica gel (eluent: 8:2 CH_2Cl_2/EtOAc) to give **5** (0.47 g, 42%) as a foam.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.02, 1.34, 1.41 (3 d, *J* = 6.1 Hz, *J* = 7.1 Hz, *J* = 7.2 Hz, 9 H, 2 × Ala-β-CH_3, Thr-γ-CH_3), 1.80, 1.99, 2.00, 2.14 (4 s, 15 H, 5 × $COCH_3$), 3.74 (s, 3 H, CO_2CH_3), 3.77 (s, 3 H, PhOCH_3), 3.88–3.95 (m, 2 H), 4.05–4.38 (m, 9 H), 4.50 (br s, 1 H), 4.61 (m, 1 H), 4.80 (d, 1 H, *J* = 7.8 Hz, H-1), 4.90–5.10 (m, 3 H, PhCH_2, H-3'), 5.17 (dd, 1 H, *J* = 10.4, 7.8 Hz, H-2'), 5.26 (d, 1 H, *J* = 3.7 Hz, H-1, overlapped with NH), 5.32 (d, 1 H, *J* = 3.3, H-4'), 5.57 (d, 1 H, *J* = 3.1 Hz, H-4), 6.84 (br d, 1 H, *o*-$MeOC_6H_4$), 7.24 (br d, 1 H, *m*-$MeOC_6H_4$), 7.38 (br t, 2 H_m, C_6H_5), 7.50 (br t, 1 H_p, C_6H_5), 7.97 (br d, 2 H_o, C_6H_5).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 15.8, 18.4, 18.7, 20.5, 20.6, 20.7, 48.0, 50.7, 52.4, 52.2, 60.5, 60.9, 63.2, 66.7, 66.9, 68.0, 68.8, 69.9, 70.7, 70.9, 74.0, 75.5, 97.8, 101.5, 113.9, 128.1, 128.4, 129.6, 130.0, 133.2, 156.0, 160.6, 166.1,167.5, 169.4, 169.6, 170.2, 170.3, 170.4, 172.1, 173.2.

MS: *m/z* (%) = 1103 (M^++H, 62), 1059 (81), 664 (51), 391 (100), 331 (55).

***N*-(4-Methoxylbenzyloxycarbonyl)-L-alanyl-*O*-[2,3,4,6-tetra-*O*-acetyl-β-D-galacto-pyranosyl-(1→3)-2-acetamido-4-*O*-acetyl-6-*O*-benzoyl-2-deoxyl-α-D-galactopyranosyl]-L-threonyl-L-alanine Methyl Ester (6)**

To a solution of azide **5** (1.10g, 1.00 mmol) in absolute MeOH (50 mL) under argon were added propane-1,3-dithiol (1.0 mL, 10 mmol) and Et_3N (1.4 mL, 10 mmol) at r.t. The mixture was stirred at r.t. for 24 h. The solvent and excess reagents were removed under vacumn, and the residue was treated with pyridine (20 mL) and Ac_2O (5 mL). After 1 h, the solution was concentrated in vacuo and the crude product was purified by column chromatography over silica gel (eluent:2:8 CH_2Cl_2/EtOAc) to yield **6** (0.83 g, 74%) as a colorless foam.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.11, 1.35, 1.40 (3 d, *J* = 6.2 Hz, *J* = 7.0 Hz, *J* = 7.1 Hz, 9 H, 2 × Ala-β-CH_3, Thr-γ-CH_3), 1.94, 1.97, 1.99, 2.09, 2.12, 2.13 (6 s, 18 H, 5 × $COCH_3$), 3.70 (s, 3 H, CO_2CH_3), 3.76 (s, 3 H, $PhOCH_3$), 3.95–4.15 (m, 3 H), 4.15–4.40 (m, 5 H), 4.40–4.58 (m, 3 H), 4.60 (d, 1 H, *J* = 7.8 Hz, H-1'), 4.88–5.10 (m, 5 H, PhCH_2, H-1, 2', 3'), 5.27 (d, 1 H, *J* = 3.0 Hz, H-4'), 5.44 (d, 1 H, *J* = 2.5 Hz, H-4), 5.62 (br d, 1 H, *J* = 6.5 Hz, NH), 6.68 (br d, 1 H, *J* = 8.5 Hz, NH), 6.82 (br d, 2 H, *o*-$MeOC_6H_4$), 7.11 (br d, 1 H, *J* = 5.5 Hz, NH), 7.21 (d, 2 H, *m*-$MeOC_6H_4$), 7.36 (br t, 2 H_m, C_6H_5), 7.49 (br t, 1 H_p, C_6H_5), 7.94 (br d, 2 H_o, C_6H_5).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 17.6, 17.7, 17.8, 20.7, 20.7, 23.0, 48.4, 49.1,50.8, 52.8, 55.2, 56.2, 60.6, 63.4, 66.7, 67.0, 67.8, 68.6, 69.3, 70.3, 70.7, 73.4, 75.6, 99.1, 101.2, 113.9, 114.0, 127.7, 128.4, 129.5, 129.6, 129.7, 129.9, 133.2, 156.5, 159.7, 166.4, 169.4, 170.2, 170.3, 170.4, 170.5, 172.8, 173.4.

MS: *m/z* (%) = 1119 (M^++H, 67), 1141 (M^++Na, 88), 1077 (43), 955 (25), 789 (41), 680 (72), 331 (100).

***N*-(4-Methoxylbenzyloxycarbonyl)-L-alanyl-*O*-[β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxyl-α-D-galactopyranosyl]-L-threonyl-L-alanine (7)**

Compound **6** (0.56 g, 0.50 mmol) was dissolved in MeOH (15 mL). A 0.5 M solution of NaOMe in MeOH was added dropwise until pH = 10.5 (pH paper). The reaction was complete after 6 h. The mixture was neutralized with AcOH and the solvents were removed in vacuo. The crude product in MeOH (3 mL) was cooled in an ice-

water bath and 0.5 N NaOH (3 mL) was added, and stirring was continued for 2 h (0 °C → r.t.). The mixture was acidified to pH ≈ 3 with citric acid. The resulting solution was concentrated in vacuo. The crude product was purified by column chromatography over silica gel (eluent: 70:30:1 CH_2Cl_2/EtOH/AcOH) to yield **7** (0.32 g, 80%) as a colorless foam; $[\alpha]_D^{23}$+57.7 (*c* = 0.1, EtOH).

^{1}H NMR (400 MHz, D_2O): δ = 1.32, 1.41, 1.63 (1 m, 9 H, 2 × Ala-β-CH_3, Thr-γ-CH_3), 2.03 (s, 3 H, NCOCH_3), 3.85 (s, 3 H, PhOCH_3), 5.07 (s, 2 H, PhCH_2), 7.02 (d, *J* = 7.6 Hz, 2 H, *o*-MeOC$_6H_4$), 7.36 (m, 3 H, *m*-MeOC$_6H_4$ and NH).

^{13}C NMR (100 MHz, D_2O containing 4 drops of DMS*O*-d_6): δ = 15.9, 18.0, 19.3, 22.7, 48.4, 50.1, 50.6, 54.7, 55.1, 60.5, 60.7, 65.4, 68.1, 71.0, 72.1, 72.3, 73.4, 75.6, 75.9, 97.5, 103.3, 113.8, 128.8, 129.7, 155.8, 159.0, 166.7, 169.9, 172.3, 175.6.

MS: *m/z* (%): = 791 (M^++H, 100), 813 (M^++Na, 80), 695 (12), 671 (8), 629 (5).

***N*-[2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4-*O*-acetyl-2-azido-6-*O*-benzoyl-2-deoxyl-α,β-D-galactopyranosyloxycarbonyl]-L-alanine Benzyl Ester (9)**

Moz-Ala-OBn **8** (686 mg, 2.00 mmol) and AgOTf (771 mg, 3.00 mmol) were dissolved in anhyd CH_2Cl_2 (5 mL) in the presence of Drierite (4 g) and the mixture stirred at r.t. After 1 h, a solution of **3** (700 mg, 1.00mmol) in CH_2Cl_2 (3 mL) was added at r.t., and stirring was continued for 1 h. The resulting solution was diluted with EtOAc and filtered. The filtrate was washed with aq satd $NaHCO_3$ solution (60 mL), dried ($MgSO_4$) and concentrated in vacuo to give a yellow liquid. The crude product was purified by column chromatography over silica gel (eluent: 6:4 hexane/EtOAc) to give **9** (806 mg, 91%) as a foam.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.37–1.50 (d, 3 H, α, β form Ala-β-CH_3), 4.70–4.75 (d, 1 H, α, β form H-1'), 6.21 (d, *J* = 3.6 Hz, α form H-1), 7.24–7.45 (m, 7 H, OCH_2Ph and H_m, C_6H_5), 7.45–7.55 (m, 1 H_p, C_6H_5), 7.95–8.05 (br d, 2 H_o, C_6H_5).

MS: *m/z* (%) = 909 (M^++Na, 9), 861 (41), 827 (15), 664 (80), 331 (100).

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(15) Glycosyl donor **3** was added to a mixture of AgOTf and TMU, then the spot of donor **3** (R_f 0.9) disappeared and a new spot of a highly polar compound (onium salt) was generated. Glycopeptide **5** (R_f 0.55) appeared gradually when tripeptide **4** (R_f 0.2) was added (detected by TLC, CH_2Cl_2/EtOAc, 6:4).
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