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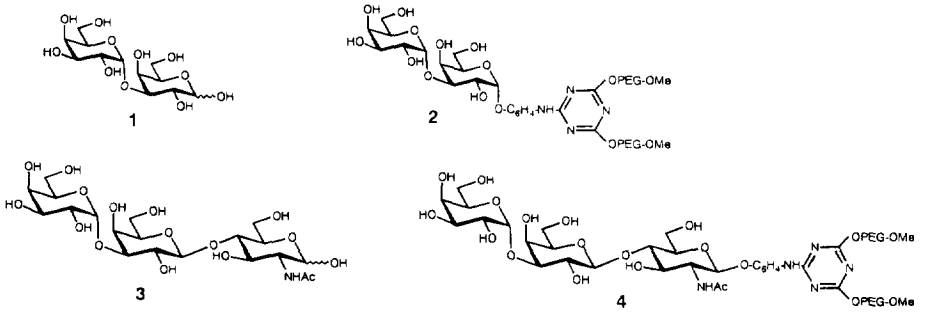
CHEMOENZYMATIC SYNTHESIS OF GALα1-3GAL, GALα1-3GALβ1-4GLCNAC, AND THEIR PEG-CONJUGATES.

Ichiro Matsuo, Hiroshi Fujimoto, Megumi Isomura, and Katsumi Ajisaka*

Meiji Institute of Health Science, Meiji Milk Products Co., Ltd., 540 Naruda, Odawara 250, Japan

Abstract: Galα1-3Gal-pNP was prepared enzymatically from Gal-pNP using α-galactosidase from coffee beans. PEG was attached after the reduction of nitro group into amino group to give Galα1-3Gal-PEG conjugate. After removing the pNP group in Galα1-3Gal-pNP, the obtained disaccharide was used for the synthesis of Galα1-3Galβ1-4GlcNAc and corresponding trisaccharide-PEG conjugate.

Galα1-3Gal **1** as well as Galα1-3Galβ1-4GlcNAc **3** are well known epitopes found on the surface of pig endothelial cells.[1,2] These oligosaccharides have been shown to be effective in removing the cytotoxic activity of human serum to pig cells and pig-to-primate xeno graft rejection during xeno-transplantation.[3] However, as these oligosaccharides have a low molecular weight, they are excreted soon after introduction into the blood stream. Therefore a modification of these oligosaccharides is necessary in order to increase the residence time in the circulation. In this study, we were able to attach polyethylene glycol (PEG) to di- and trisaccharide derivatives in order to increase their molecular size.

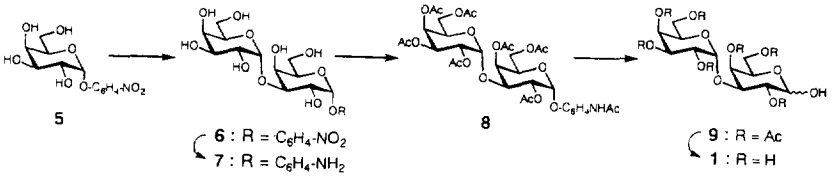


In order to reduce the number of protection and deprotection steps during the chemical synthesis of **1**, we utilized the transglycosylation activity of α-galactosidase from coffee beans.[4] Galα1-3Gal-pNP was synthesized in one step from Gal-pNP which was used as both the donor and the acceptor. The nitro group of pNP in Galα1-3Gal-pNP was then reduced to the corresponding amino group, and PEG was attached using 2,4-bis(O-

methoxypolyethylene glycol)-6-chloro-s-triazine (activated PEG[5], the average molecular weight of the methoxypolyethylene glycol was 5,000 dalton). The aminophenyl group was converted into acetamidophenyl group, so that it could be easily removed using cerium ammonium nitrate (CAN) in high yield. The resulting disaccharide was used as the glycosyl donor for coupling with GlcNAc derivatives of benzyl glycoside or pNP glycoside. The benzyl glycoside derivative of Galα1-3Galβ1-4GlcNAc was deprotected to provide trisaccharide **3** and the corresponding pNP glycoside was converted into the Galα1-3Galβ1-4GlcNAc-PEG conjugate **4**.

Synthesis of Galα1-3Gal **1**

Galα1-3Gal-pNP, **6**, was synthesized[6] enzymatically using *para*-nitrophenyl-α-D-galactopyranoside (Gal-pNP), **5**, as both the donor and the acceptor. The disaccharide derivative **6** was stirred with zinc-dust in a 2% cupric sulfate solution for 1 hr. Concentration after filtration of the catalyst afforded **7** in quant. yield. After acetylation of compound **7**, the disaccharide derivative **8** was dissolved in toluene-acetonitrile-water (3 : 4 : 3). The solution was cooled in an ice bath, and after the addition of CAN (10 eq.) it was stirred for 30 min at room temperature. Following purification by silica gel column chromatography, the disaccharide derivative **9** was obtained in 89% yield. The deacetylation of **9** with sodium methoxide afforded disaccharide **1** in 80% yield. Fukase *et al* have also reported a similar procedure to remove a pNP group using CAN.[7]



Scheme 1

Synthesis of Galα1-3Gal-PEG **2**

The *para*-aminophenyl glycoside derivative **7** was dissolved in 0.1M sodium borate buffer (pH 9.9) and activated PEG (0.9 eq.) was added. After stirring for 3 hrs, the reaction mixture was concentrated using a membrane filter of YM-3 (Amicon). Freeze-drying of the solution afforded a white powder of the disaccharide-PEG conjugate **2**[8] in approximately 90% yield.[9] The structure was confirmed by measuring the ratio of the peak areas of the aromatic proton signal of the pNP group and the methyl proton signal of methoxy PEG.

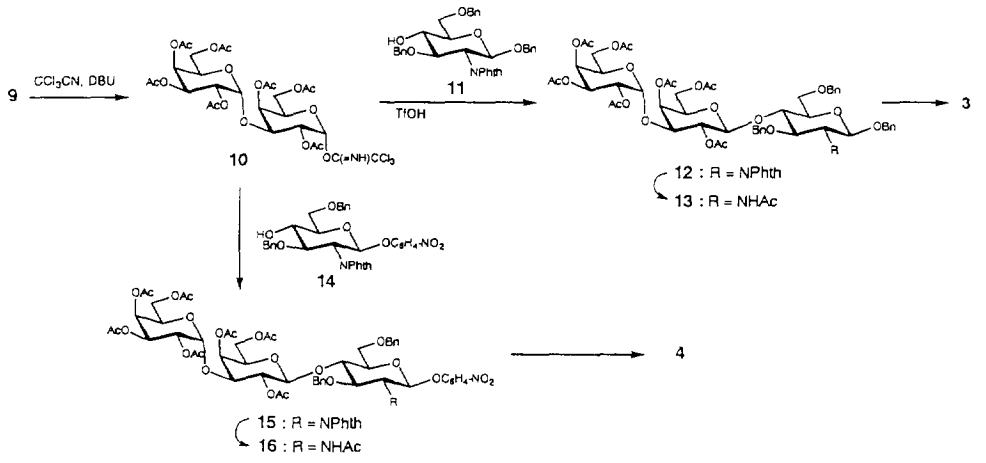
Synthesis of Galα1-3Galβ1-4GlcNAc **3**

The disaccharide hemiacetal **9** was converted into the trisaccharide **10** using trichloroacetonitrile and DBU. The trichloroacetimidate **10** was coupled with 0.9 eq. of benzyl 2-deoxy-2-phthalimido-3,6-di-O-benzyl-β-D-glucopyranoside, **11**,[10] in the presence of AW300 molecular sieves and trifluoromethanesulfonic acid at -78°C for 15 min. After work-up of the reaction in a standard manner, **12**[11] was obtained in 70% yield. The trisaccharide derivative **12** was dissolved in butanol containing ethylenediamine and the mixture stirred at 90°C for 12 hrs.[12] After removal of the volatiles, the residue was acetylated to provide trisaccharide derivative **13** in 91% yield. Removal of the acetyl group by sodium methoxide and subsequent debenzylation with H_2/palladium

black gave trisaccharide **3** quantitatively.

Synthesis of Galα1-3Galβ1-4GlcNAc-PEG **4**

The trisaccharide **15**[13] was obtained in 81% yield by the coupling of trichloroacetimidate **10** with *para*-nitrophenyl 2-deoxy-2-phthalimido-3,6-di-O-benzyl-β-D-glucopyranoside (1.1 eq.), **14**, in the presence of AW300 molecular sieves and trifluoromethanesulfonic acid at -78°C for 15 min. Resulting *para*-nitrophenyl glycoside **15** was treated in a similar manner as described above and subsequent coupling with activated PEG afforded Galα1-3Galβ1-4GlcNAc-PEG-conjugate **4**[14] in approximately 90% yield.



Scheme 2

By combining enzymatic and chemical methods, we were able to decrease the number of protection and deprotection steps necessary for this synthesis. In particular, the process for the synthesis of Galα1-3Gal and its PEG conjugate is straightforward and practical for large scale preparation.

To the best of our knowledge, this is the first example of a preparation of oligosaccharide-PEG conjugate in order to extend the circulation time of the oligosaccharide *in vivo*. In preliminary experiments, the PEG-conjugates of the di- and trisaccharides have shown residence times in the circulation of mice which have been extended 3 ~ 4 fold in comparison with the corresponding unmodified oligosaccharides.

Acknowledgment

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References and Notes

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3. D. K. C. Cooper, E. Coren, R. Oriol, *Xeno*, **1994**, 2, 22-25.
4. K. G. I. Nilsson, *Carbohydr. Res.*, **1987**, 167, 95-103.
5. Activated PEG, commercial name of Activated PEG-2, was purchased from Seikagaku-Kogyo Co. (Osaka, Japan).
6. Galα1-3Gal-pNP was synthesized according to the method of Nilsson. We succeeded to increase the yield to about 1.7 times of Nilsson's report by the improvement of the reaction condition.

   Gal-pNP (1.0g) was dissolved in 4ml of 0.1M potassium phosphate buffer (pH 6.0) containing 30% DMF, the solution was incubated for 4hrs at 37°C after the addition of 26.7 units of galactosidase from coffee beans. After the deactivation of enzyme by heating the solution in boiling water bath for 5 minutes, the solution was applied to Sephadex G-15 column (2.5cmϕ x 100cm). By the elution with water, 169.2mg of Galα1-3Gal-pNP was isolated and 28.6mg of Galα1-2Gal-pNP was also obtained as a by-product.
7. K. Fukase, T. Yasukochi, Y. Nakai, and S. Kusumoto, *Tetrahedron Letters*, **1996**, 37, 3343-3344.
8. Spectral data for **2**: ^{1}H NMR (500 MHz, D_2O) ; δ7.55(2H, d, J=8.95Hz), 7.22(2H, d, J=8.95Hz), 5.70(1H, d, J=2.53Hz, Gal, H-1), 5.22(1H, d, J=2.50Hz, Gal', H-1), 3.40(6H, s, OCH_3). Non-reducing end galactose residue was named as Gal'. The ratio of the integral intensity of the signals at δ7.55 and δ3.40 was 1 : 3.
9. The calculation of the yield was based on that the average molecular weight of the activated PEG as 10,000 dalton.
10. T. Ogawa and S. Nakabayashi, *Carbohydr. Res.*, **1981**, 97, 81-86.
11. Spectral data for **12**: ^{1}H NMR (500 MHz, $CDCl_3$); δ5.21(1H, d, J=3.0Hz, Gal', H-1), 5.11(1H, d, J=8.5Hz, GlcNAc, H-1), 4.64(1H, d, J= 8.0Hz, Gal, H-1); ^{13}C NMR (125MHz, $CDCl_3$); δ100.438(Gal, C-1), 97.472(GlcNAc, C-1), 93.964(Gal', C-1).
12. O. Kanie, S. C. Crawley, M. M. Palcic, and O. Hindsgaul, *Carbohydr. Res.*, **1993**, 243, 139-164.
13. Spectral data for **15**: ^{1}H NMR (500 MHz, $CDCl_3$); δ5.86(1H, δ, ϑ=8.47H, GlcNAc, H-1), 5.23(1H, d, J=3.67Hz, Gal', H-1), 4.61(1H, d, J=8.02Hz, Gal, H-1), 2.17, 2.15, 2.07, 2.05, 2.02, 1.96, 1.63(3H x 7, s, OAc x 7) , ^{13}C NMR (125MHz, $CDCl_3$); δ100.586(Gal, C-1), 95.511(GlcNAc, C-1), 93.933(Gal', C-1).
14. Spectral data for **4**: ^{1}H NMR (500 MHz, D_2O); δ7.54(2H, d, J=8.95Hz), 7.12(2H, d, J=8.95Hz), 5.16(1H, d, J=2.53Hz, Gal', H-1), 5.17(1H, d, J=7.87Hz, GlcNAc, H-1), 5.18(1H, d, Gal, H-1), 3.39(6H, s, OCH_3).

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