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Development and Applications of a Chemical Method for Sequential Analysis of Reducing Oligosaccharides

Seon-Pyo Hong[1], Yong-Moon Lee[2] and Hiroshi Nakamura[1]

[1]Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo, Ichigaya-funagawara-machi, Shinjuku, Tokyo 162, Japan; [2]College of Pharmacy, Chungbuk National University, Chongju 361-763, Korea

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A new method based on the chemical reaction has been devised for the sequential analysis of reducing oligosaccharides using 8-amino-2-naphthalenesulfonic acid (ANS), a fluorescent precolumn derivatization reagent for reducing saccharides. The procedure established includes 1) the derivatization of a reducing oligosaccharide to produce a Schiff base, 2) the reduction of the base with sodium cyanoborohydride ($NaBH_3CN$), 3) the methoxycarbonylation of the resultant secondary amino group, 4) the cleavage of the glycoside bond next to the reducing end, based on the intramolecular acid hydrolysis by the action of a sulfonic acid group of the ANS derivative, 5) the identification of the liberated reducing end by high-performance liquid chromatography (HPLC), and finally 6) the recovery of the resultant oligosaccharide fragment from the cleavage reaction mixture. The extensive examination of the conditions for the sequential analysis of reducing oligosaccharides resulted in the procedure of simplicity , high selectivity and high recovery . This procedure was found to be useful for the sequential analysis of di-, tri- and tetrasaccharides.

Key words : Reducing oligosaccharide, 8-Amino-2-naphthalenesulfonic acid, HPLC, Cleavage reaction, Sequential analysis, Methoxycarbonylation

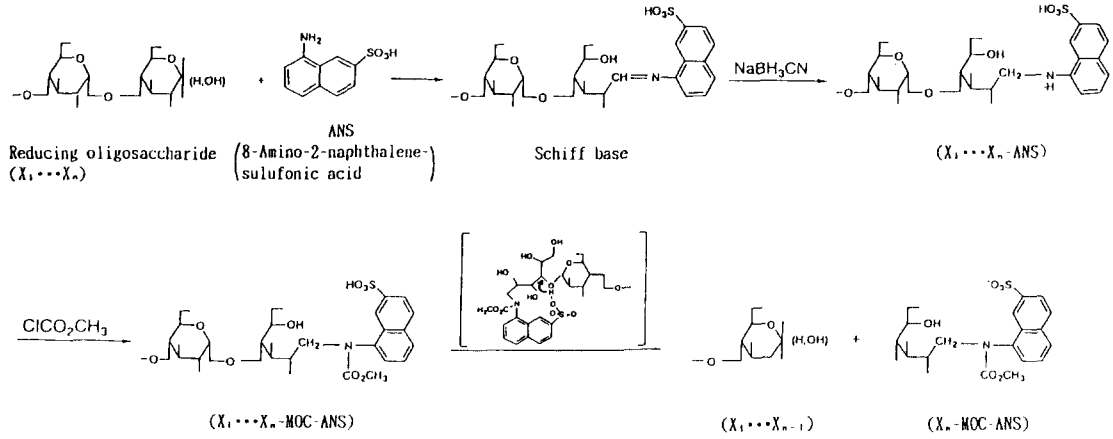
INTRODUCTION

Although saccharides play vital roles in maintenance of life, the unknown but important saccharides are naturally expected to exist in organism and various kinds of biological resources. Therefore, the development of a simple sequential analysis method for oligosaccharides is highly required for their structural elucidation and successive utilization.

Until now, enzymatic (Furukawa *et al.*, 1990; Mizouchi *et al.*, 1984; Tarentino *et al.*, 1985; Fukuda *et al.*, 1984), MS (Fukuda *et al.*, 1985; Fukuda *et al.*, 1986), NMR (Korrel *et al.*, 1984) and chemical methods (Fife *et al.*, 1981; Fife *et al.*, 1991) have been reported as tools for sequential analysis of oligosaccharides. Among them, the chemical methods focused on the chemical hydrolysis of O-glycosidic (1→4) linkage have been studied based on the ground that the carboxyl groups from glutamic acid-35 and aspartic acid-52 located in the active site of lysozyme are involved in the cleavage reaction of O-glycosidic (1→4) linkage. In addition to the fundamental studies related to the cleavage reaction of O-glycosidic (1→4) linkage by the carboxyl groups, acid hydrolysis (Roy *et al.*, 1968; De Bruyne *et al.*, 1974), partial acetolysis (Kocourek *et al.*, 1969), periodate oxidation methods (Hase *et al.*, 1985; Irimura *et al.*, 1980) have been reported. However, these methods are applicable only to a limited number of oligosaccharides for their partial sequential analysis.

Ideally, such a chemical method for the sequential analysis of oligosaccharides, as the Edman degradation in protein chemistry, is highly desirable owing to its simplicity and generality, though the general method has not been developed as yet. We have attempted to develop a new method for the sequential analysis of reducing oligosaccharides using 8-amino-2-naphthalenesulfonic acid (ANS) developed as a fluorescent precolumn derivatization reagent of reducing saccharides (Scheme) (Hong *et al.*, 1996). In the preliminary report (Hong *et al.*, 1994), we established a procedure for the cleavage reaction and the recovery of the resultant oligosaccharide fragment from the cleavage reaction mixture. In this paper, we went into the full details about the conditions of the cleavage reaction and the recovery of the resultant oligosaccharide fragment from the cleavage reaction

Correspondence to: Yong-Moon Lee, College of Pharmacy, Chungbuk National University, Chongju 361-763, Korea



Scheme 1. Sequential analysis of reducing oligosaccharides using ANS, $NaBH_3CN$ and methyl chloroformate as derivatization reagents.

mixture. In addition, we tried to apply this method to other oligosaccharides.

MATERIALS AND METHODS

Reagents and Apparatus

N,N-Dimethylformamide (DMF), dimethylacetamide (DMA), 1,2-dimethoxyethane (DME), mercury (II) chloride, kryptofix 111 (4, 10, 15-trioxa-1,7-diazabicyclo-[5.5.5]heptadecane and trifluoromethanesulfonic acid (CF_3SO_3H) were purchased from Kanto Chemical (Tokyo, Japan). Lactose, 4-O-α-D-galactopyranosyl-D-galactopyranose and 4'-galactosyllactose were obtained from Wako Pure Chemical (Osaka, Japan). Other reagents and HPLC conditions used are the same as those reported in a previous paper (Hong *et al.*, 1994; Hong *et al.*, 1996), unless stated otherwise. Evaporation of solvents was carried out by using a Model ES-57CS centrifugal evaporator (Sakuma, Tokyo, Japan). The mobile phase was a 10 mM sodium phosphate buffer (pH 3.8) containing 0.2% (v/v) 1-butanol.

Conditions of the Cleavage Reaction and the Recovery

A reducing oligosaccharide [schematically represented as $X_1 \cdots X_n$ (reducing end)] was derivatized with ANS followed by the reduction of the resultant Schiff base with $NaBH_3CN$ as described previously ($X_1 \cdots X_n$-ANS was formed)(Hong *et al.*, 1994). After derivatization reaction, ANS remained was removed by extraction with 1-butanol, and then the solution was passed through an anion-exchange column packed with 0.1 ml of QAE Sephadex A-25 (OH- form) (Pharmacia), by which non-derivatized sugars were removed while ANS-derivatized sugars were adsorbed. The ANS-sugars adsorbed were eluted by 1% CF_3SO_3H. To remove an excess of CF_3SO_3H, the effluent was passed through a Sep-Pak C18 cartridge (Waters Assoc., Milford, MA, USA). Acetonitrile-water (4:1) (5 ml) was then passed through the cartridge to elute the derivative. After an aliquot of the effluent was evaporated to dryness, the residue was dissolved in an appropriate solvent and submitted to the cleavage reaction. The reducing end (Xn-ANS) released was identified by HPLC with fluorescence detection as reported previously (Hong *et al.*, 1994). After the reaction mixture was evaporated to dryness, the residue was dissolved in 0.2 ml of water and the solution was passed through a column of QAE Sephadex A-25 (OH- form)(Pharmacia), by which ANS-sugars were trapped and free saccharides (X1···Xn-1) were effused. After the effluent was evaporated to dryness, residue was derivatized with ANS, and then submitted to the HPLC analysis with fluorescence detection. Following reducing oligosaccharides were used : glucose (M1), maltose (M2), maltotriose (M3) and maltotetraose (M 4). Those derivatized with ANS and NaBH3CN were designated as M1-ANS, M2-ANS, M3-ANS and M4-ANS, respectively.

Stability of Oligosaccharides to the Reaction Solvents

DMF, DMA, DME and 2-methoxyethanol were separately added to M4 and heated at appropriate temperature for a given period. After evaporation to dryness, the residue was derivatized with ANS/$NaBH_3CN$ and submitted to the HPLC analysis with fluorescence detection.

Sequential Analysis through Methoxycarbonylation (MOC) of ANS-aminated Oligosaccharides

To suppress the formation of an intramolecular zwitter ion between the sulfonic acid and the secondary amino groups of the reduction products of ANS-reducing sugars and to increase the reactivity of sulfonic acid group, introduction of a methoxycarbonyl group into the secondary amino group with methyl chloroformate was attempted using M3 derivatized with ANS (M3-

ANS) as a model (Hong *et al.*, 1996; Sano *et al.*, 1976). Experimental procedure was the same as those reported in a previous paper (Hong *et al.*, 1996).

RESULTS AND DISCUSSION

Cleavage Reaction and the Recovery

All the cleavage reaction described below was examined using 40 mM M4-ANS in the reaction mixture. When the cleavage reaction of M4-ANS was carried out in water at low temperature, it didn't progress; even at high temperature, M1-ANS was not obtained, but M4-ANS itself was decomposed. When the reaction mixture was heated in 80% dioxane at 50°C for 2 h, M1-ANS was almost not obtained, and M4-ANS itself was decreased as well (Fig. 1). From this result, it was thought that the breakdown reaction of derivative itself occurred prior to the cleavage reaction of O-glycoside linkage.

When M4-ANS was heated in 1-butanol-DMF (6:4) at 104°C for 90 min, the cleavage reaction progressed a little with small formation of M1-, M2- and M3-ANS almost without change of M4-ANS (Fig. 2). The condition was not practical owing to no selectivity and low yield of the desired product (M1-ANS).

In contrast to the preceding methods, the method to use kryptofix 111, which converts the $-SO_3H$ group of M4-ANS into $-SO_3^-$ group, was carried out to examine to what extent the cleavage reaction was accelerated by sulfonate ion. When M4-ANS was heated in 1-butanol-DMSO (6:4) containing 40 mM kryptofix 111, the reaction was hardly proceeded. Therefore it was proved that the $-SO_3H$ form was necessary for the cleavage reaction.

When M4-ANS was heated in 1-butanol-DMSO (6:4) containing 8 mM mercury (II) chloride as Lewis acid catalyst at 104°C for 4 h, M1-ANS was detected about 4 times as much as M2- and M3-ANS (Fig. 3). Although the procedure showed some reaction selectivity, it had to use the harmful heavy metal and the recovery of the resultant oligosaccharide fragment from the cleavage reaction mixture was very poor.

When M4-ANS was heated in 1-butanol-2-methox-

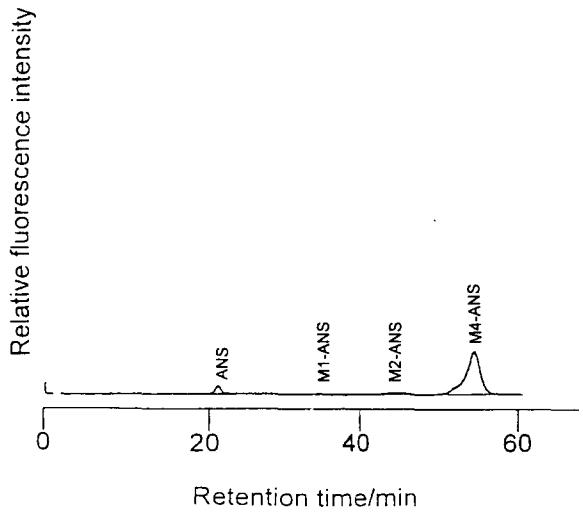


Fig. 1. Chromatogram of M4-ANS after a cleavage reaction. Cleavage reaction: M4-ANS (40 mM) in 80% dioxane was heated on a dry block heater at 50°C for 2 h.

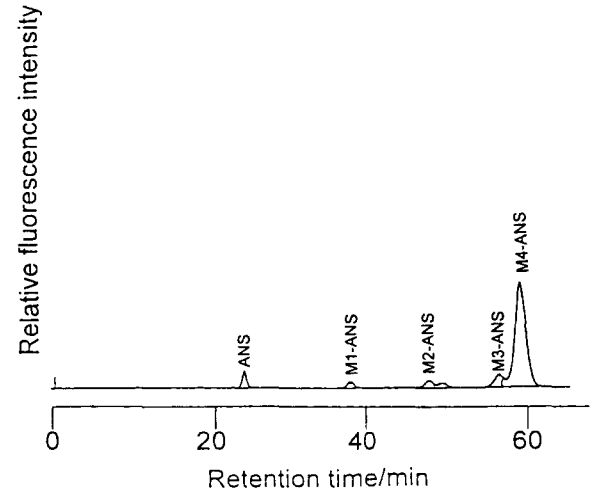


Fig. 2. Chromatogram of M4-ANS after a cleavage reaction. Cleavage reaction: M4-ANS (40 mM) in 1-butanol-DMF (6:4) was heated on a dry block heater at 104°C for 1.5 h.

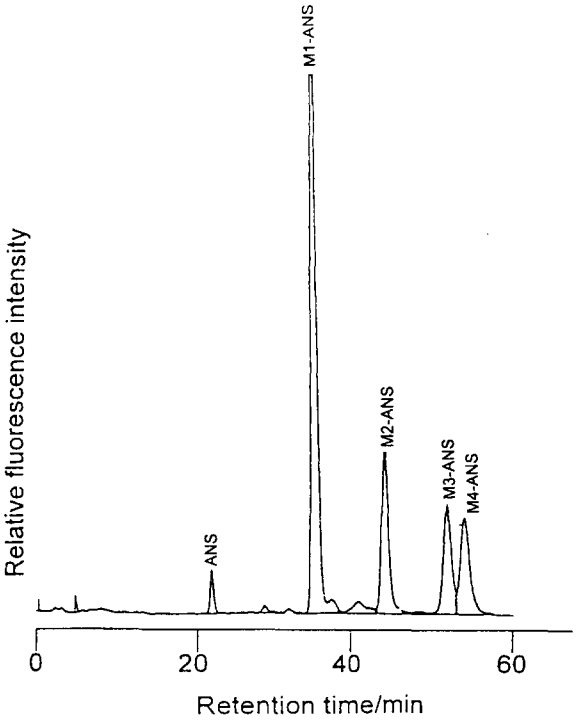


Fig. 3. Chromatogram of M4-ANS after a cleavage reaction. Cleavage reaction: M4-ANS (40 mM) in 1-butanol-DMSO (6:4) containing 8 mM $HgCl_2$ was heated on a dry block heater at 104°C for 4 h.

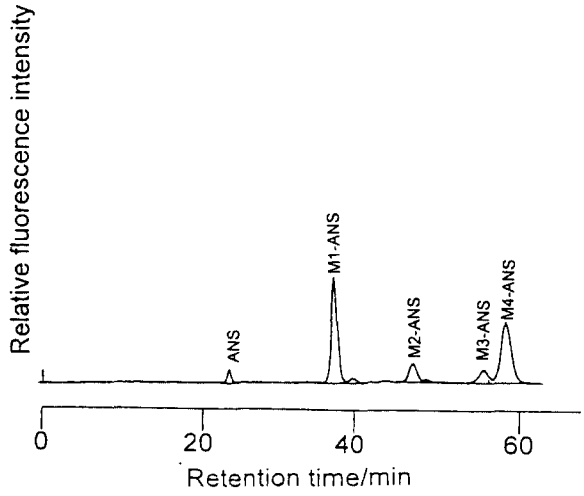


Fig. 4. Chromatogram of M4-ANS after a cleavage reaction. Cleavage reaction: M4-ANS (40 mM) in 1-butanol-2-methoxyethanol (6:4) was heated on a dry block heater at 80°C for 1.5 h.

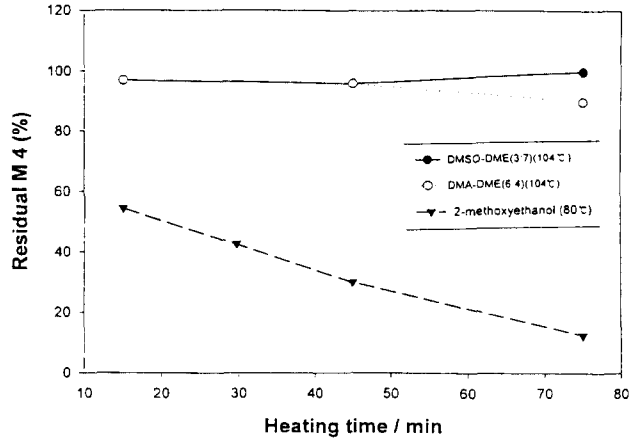


Fig. 5. Chemical stability of M4 in various solvents. M4 (40 mM) in various organic solvents was heated, and the residual amount of M4 was determined by HPLC after derivatization with ANS/$NaBH_3CN$. Solvent: ●, DMSO-DME (3:7) at 104°C: ○, DME-DMA (6:4) at 104°C: ▼, 2-methoxyethanol at 80°C.

yethanol (6:4) at 80°C for 1.5 h, M1-ANS was detected about 6 times as much as M2-and M3-ANS (Fig. 4). The cleavage condition gave highest yield of M1-ANS at lower temperature in a shorter time, however it splitted the glycoside linkage almost nonselectively to give substantially no M3. Therefore, the stability to the reaction solvent of the resultant oligosaccharide fragment itself was examined below.

Stability of Oligosaccharides to the Reaction Solvent

The heat stability of M4 to the possible reaction solvents was examined (Fig. 5). In 2-methoxyethanol at 80°C for 75 min, M4 degradated with time. When heated in DMSO-DME (3:7) and DMA-DME (6:4) at 104°C, M4 was stable at least for 45 min. These data indicate that the selection of the solvent in the cleavage reaction is very important because the selectivity of the cleavage reaction may be much influenced by the solvent employed.

Sequential Analysis through Methoxycarbonylation of ANS-aminated Oligosaccharides

Methoxycarbonylation and dilution method were used to improve the reactivity of the sulfonic acid group and increase the selectivity of the reaction.

M3-MOC-ANS (0.9 mM) was submitted to the cleavage reaction in dioxane-DMSO (6:4) at 100°C for 1.5 h, followed by HPLC analysis of the reaction mixture to determine the reducing end (1st cycle). Thereafter, M2 liberated was isolated from the reaction mixture and derivatized with ANS and $NaBH_3CN$ to form M2-ANS. Then M2-ANS was submitted to the cleavage reaction once again after methoxycarbonylation (start of 2nd cycle). In these cases, the

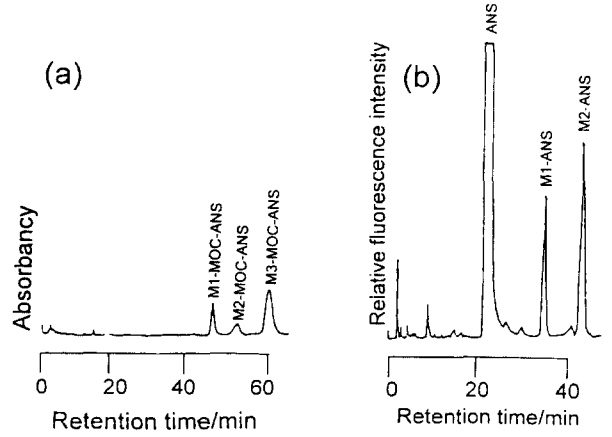


Fig. 6. Chromatograms obtained in the sequential analysis of M3. (a) The cleavage reaction mixture resulting from M3-MOC-ANS. M3-MOC-ANS (0.9 mM) in dioxane-DMSO (6:4, v/v) was heated at 100°C for 1.5 h, and the mixture was injected into the HPLC system. (b) The resultant oligosaccharide fraction recovered from the cleavage reaction mixture and derivatized with ANS/$NaBH_3CN$.

MOC derivatives were detected with a UV detector because they are nonfluorescent. A typical chromatogram of the reaction mixture obtained after the 1st cycle of the cleavage reaction is shown in Fig. 6 (a). On the basis of their peak areas, the reaction yield of M1-MOC-ANS after the 1st cycle was found to be 24.0% and the molar ratio of M1-MOC-ANS to M2-MOC-ANS was 1.9. The ratio obtained here implies that reasonably good selectivity has been achieved under the conditions used for the cleavage reaction, because the ratio would be 1:1 if nonselective reaction occurred. Thus the reducing end of M3 was determined as M1. After the 1st cleavage reaction, the mixture was applied to an anion exchange column to

remove M1-, M2- and M3-MOC-ANS. Oligosaccharide fragments including M2 were eluted from the column and then derivatized with ANS/$NaBH_3CN$ again. A chromatogram of the reaction mixture is shown in Fig. 6 (b). Although M2-ANS and M1-ANS were detected as an expected main product and undesirable by-product, respectively, the molar ratio of M2-ANS to M1-ANS was 1.9. Therefore the resultant oligosaccharide residue was readily determined to be M2. The recovery of M2 was 54.7%, which permits one to perform the next cycle of the sequential analysis.

Furthermore when a much lower concentration of M3-MOC-ANS (45 μM) in dioxane-DMSO (6:4, v/v) was heated at 100°C for 13h, the selectivity of the cleavage reaction was highly improved (Fig. 7(a)), presumably because of a preferential progress of the desirable intramolecular cleavage reaction. The molar ratio of M1-MOC-ANS to M2-MOC-ANS was 6:1, though the reaction yield of the former was not so modified (30%). The recovery of M2 liberated after 1st cleavage reaction was 49.0% and the molar ratio of M2-ANS to M1-ANS was greater than 6 (Fig. 7 (b)). This implies that a fairly good recovery of the sugar fragment has been achieved under the above conditions. However, the procedure requires a long time for the cleavage. Then, the cleavage reaction at higher temperature (110~140°C) and in a shorter time (30 min) was examined using 45 M3-MOC-ANS and dioxane-DMSO (7:3) as the reaction solvent. The reaction did not occurred up to 120°C. At 130°C, the reaction yield of M1-MOC-ANS after 1st cycle of the cleavage reaction was 29% and the molar ratio of M1-MOC-ANS to M2-MOC-ANS was 1.8. The recovery of M2 liberated after the 1st cleavage reaction was 83%. At 140°C, the reaction yield of M1-MOC-ANS after 1st cycle of the cleavage reaction was 75% and the molar ratio of M1-MOC-ANS to M2-MOC-ANS was 5.3. The recovery of M2 liberated after the 1st cleavage reaction was 54.7%. This method gave high reaction yield, good selectivity and good recovery about high temperature and short reaction time.

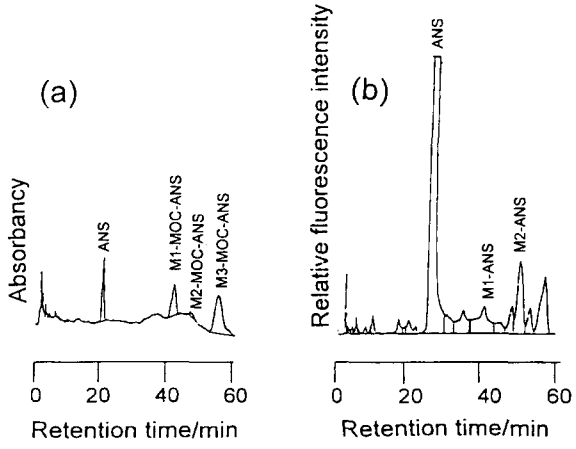


Fig. 7. Cleavage reaction for the sequential analysis of M3 at lower concentration. (a) The cleavage reaction mixture resulting from M3-MOC-ANS. M3-MOC-ANS (45 μM) in dioxane-DMSO (6:4, v/v) was heated at 100°C for 13 h, and the mixture was injected into the HPLC system. (b) The resultant oligosaccharide fraction recovered from the cleavage reaction mixture and derivatized with ANS/$NaBH_3CN$.

Application to Sequential Analysis of Reducing Oligosaccharides

In the sequential analysis of reducing oligosaccharides with M3 and M4, the cleavage reaction in dioxane-DMSO (7:3) to heat at 140°C for 30 min was most prominent from the viewpoint of simplicity, selectivity and recovery. Thus we tried to apply this method to other reducing oligosaccharides such as lactose (Gal-β (1→4)-Glu), 4-O-α-D-galactopyranosyl-D-galactopyranose (Gal-α (1→4)-Gal) and 4'-galactosyl-lactose (Gal-β (1→4)-Gal-β (1→4)-Glu). In all cases, the oligosaccharides were treated with ANS, $NaBH_3CN$

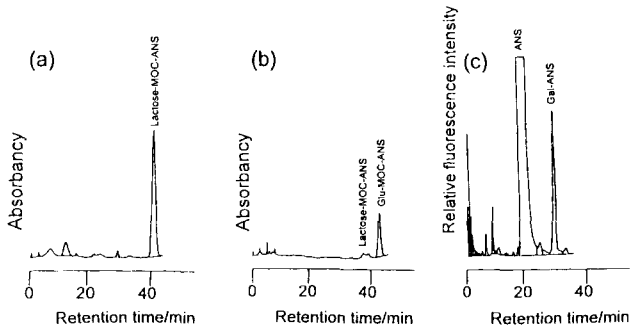


Fig. 8. Sequential analysis of lactose. (a) Chromatogram of lactose-MOC-ANS. (b) Cleavage reaction mixture of lactose-MOC-ANS in the first cycle. Lactose-MOC-ANS (45 μM) in dioxane-DMSO (7:3) was heated at 140°C for 30 min. (c) ANS/$NaBH_3CN$ derivatization products in the second cycle.

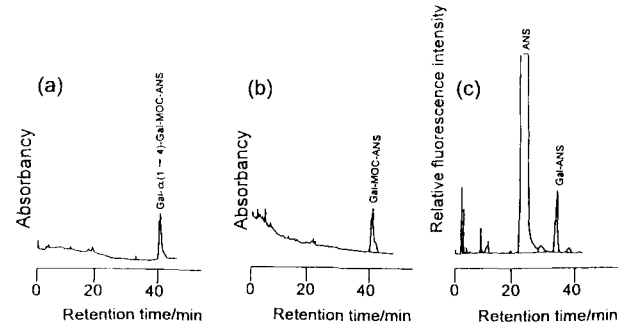


Fig. 9. Sequential analysis of 4-O-α-D-galactopyranosyl-D-galactopyranose. (a) Chromatogram of Gal-α (1→4)-Gal-MOC-ANS. (b) Cleavage reaction mixture of Gal-α (1→4)-Gal-MOC-ANS in the first cycle. Gal-α (1→4)-Gal-MOC-ANS (45 μM) in dioxane-DMSO (7:3) was heated at 140°C for 30 min. (c) ANS/$NaBH_3CN$ derivatization products in the second cycle.

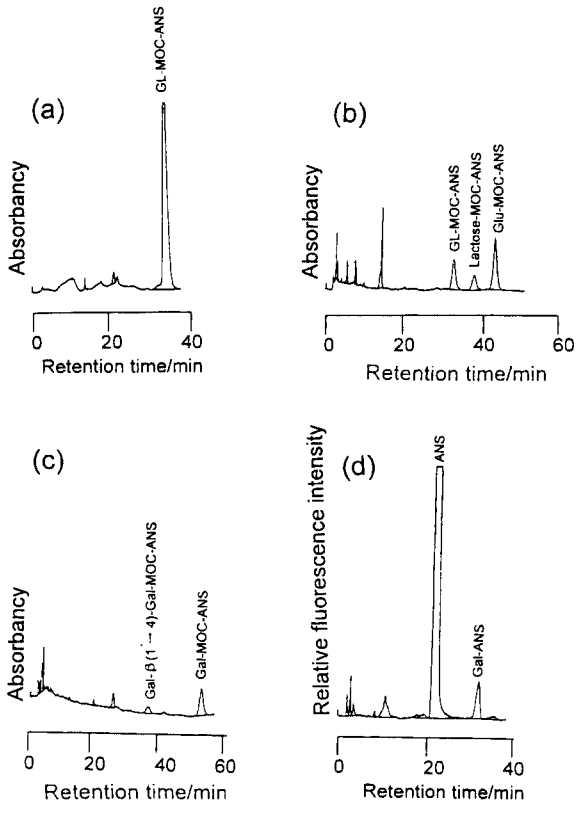


Fig. 10. Sequential analysis of 4'-galactosyllactose. (a) Chromatogram of 4'-galactosyllactose-MOC-ANS. (b) Cleavage reaction mixture of 4'-galactosyllactose-MOC-ANS (45 μM) in dioxane-DMSO (7:3) obtained after heating at 140°C for 30 min. (c) Cleavage reaction mixture in the second cycle. Sample in dioxane-DMSO (7:3) was heated at 140°C for 30 min. (d) ANS/$NaBH_3CN$ derivatization products in the third cycle.

and methyl chloroformate and the concentration of the resultant oligosaccharide-MOC-ANS in dioxane-DMSO (7:3) was adjusted to 45 before the cleavage reaction. After the cleavage reaction of lactose-MOC-ANS, glucose-MOC-ANS appeared with the disappearance of lactose-MOC-ANS (Fig. 8 (b)). Thus the first sugar from the reducing end of lactose was determined to be glucose. After the cleavage reaction, reducing sugar liberated was isolated from the reaction mixture with a QAE Sephadex A-25 column and derivatized with ANS and $NaBH_3CN$. Because the main peak was consistent with the peak of galactose-ANS (Fig. 8(c)), the second sugar from the reducing end was determined to be galactose. By this way, the sequential analysis of lactose was accomplished.

Figure 9 shows the results of the sequencing 4-O-α-D-galacto pyranosyl-D-galactopyranose. Because galactose-MOC-ANS appeared by the cleavage reaction, the reducing end was determined to be galactose. The reducing sugar liberated was derivatized with ANS and $NaBH_3CN$ after pretreatment. Because galactose-ANS was identified as the major product, the second sugar from the reducing end was determined to be galactose. By this way, the sequential analysis of 4-O-α-D-galactopyranosyl-D-galactopyranose was accomplished.

After the first step of sequential analysis of 4'-galactosyllactose (Fig. 10), glucose-MOC-ANS (retention time, 48 min) and lactose-MOC-ANS (retention time, 42 min), were detected in addition to the MOC-ANS derivative of the unreacted 4'-galactosyllactose, indicating that the first sugar from the reducing end is glucose. After the first cleavage reaction, the oligosaccharide liberated was isolated from the reaction mixture with a QAE Sephadex A-25 column, derivatized with ANS/NaBH3CN, methoxycarbonylated with methyl chloroformate and then the second step of the cleavage reaction was carried out (Fig. 10(c)). Because the main peak was consistent with the peak of galactose-MOC-ANS, the second sugar from the reducing end was determined to be galactose. Similarly, the third sugar from the reducing end was determined to be galactose (Fig. 10(d)). By this way, the sequential analysis of 4'-galactosyllactose was accomplished.

We have demonstrated that the present sequential procedure, based on ANS/$NaBH_3CN$ and MOC derivatization, is applicable to some authentic reducing oligosaccharides. Our method is an example showing the possibility of the sequential analysis of oligosaccharides by a chemical approach. The application of the method to elucidate the structures of unknown saccharides is now carried out in our laboratory.

REFERENCES CITED

De Bruyne, C. K. and Carchon, H., Acid hydrolysis of substituted phenyl α-D-galactopyranosides. *Carbohydr. Res.*, 33, 117-125 (1974).

Fife, T. H. and Pellino, A. M., General-acid catalysis of imidazolidine ring opening. The hydrolysis of ethyl-N,N'-[1-(p-(dimethylamino)phenyl) propenediyl]-p-[((2-tetrahydroquinolinyl)methylene)amino]denzoate. *J. Am. Chem. Soc.*, 103, 1201-1207 (1981).

Fife, T. H., Jaffe, S. H. and Natarajan, R., Intramolecular general-acid and electrostatic catalysis in acetal hydrolysis. Hydrolysis of 2-(substituted phenoxy)-6-carboxytetrahydropyrans and 2-alkoxy-6-carboxytetrahydro pyrans. *J. Am. Chem. Soc.*, 113, 7646-7653 (1991).

Fukuda, M., Spooncer, E., Oates, J. E. and Dell, A., Structure of sialylated fucosyl lactosaminoglycan isolated from human granulocytes. *J. Biol. Chem.*, 259, 10925-10935 (1984).

Fukuda, M., Bothner, B., Ramsamooj, P., Dell, A., Tiller, P. R., Varki, A. and Klock, J. C., Structures of sialylated fucosyl polylactosaminoglycans isolated from chronic myelogenous leukemia cells. *J. Biol. Chem.*, 260, 12957-12967 (1985).

Fukuda, M., Carlsson, S. R., Klock, J. C. and Dell, A., Structures of O linked oligosaccharides isolated from normal granulocytes, chronic myelogenous leukemia cells and acute myelogenous leukemia cells. *J. Biol. Chem.*, 261, 12796-12806 (1986).

Furukawa, K., Endo, T. and Kobata, A., Structural analysis of sugar chains of glycoproteins. *Saibo (cell in Japanese)*, 22, 355-362 (1990).

Hase, S., Kikuchi, N., Ikenaka, T. and Inoue, K., Structures of sugar chains of the third component human complement. *J. Biochem.* (Tokyo), 98, 863-874 (1985).

Hong, S. P., Nakamura, H. and Nakajima, T., 8-Amino-2-naphthalenesulfonic acid as a precolumn fluorescence derivatization reagent for reversed-phase high-performance liquid chromatography of reducing sugars. *Anal. Sci.*, 10, 647-648 (1994).

Hong, S. P., Sano, A. and Nakamura, H., A chemical approach for sequential analysis of reducing oligosaccharides. *Anal. Sci.*, 12, 491-493 (1996).

Irimura, T., Tsuji, T., Tagami, S., Yamamoto, K. and Osawa, T., Structure of a complex-type sugar chain of human glycophorin A. *Biochemistry*, 20, 560-566 (1980).

Kocourek, J. and Ballou, C. E., Method for fingerprinting yeast cell wall mannans. *J. Bacteriol.*, 100, 1175-1181 (1969).

Korrel, S. A. M., Clemetson, K. J., van Harbeek, H., Kamerling, J. P., Sixma, J. J. and Vliegenthart, J. F. G., Structural studies on the O-linked carbohydrate chains of human platelet glycocalicin. *Eur. J. Biochem.*, 140, 571-576 (1984).

Mizuochi, T., Amano, J. and Kobata, A., New evidence of the substrate specificity of endo-β-N-acetylglucosaminidase D. *J. Biochem.* (Tokyo), 95, 1209-1213 (1984).

Roy, N. and Timell, T. E., The acid hydrolysis of glycosides. VIII. Synthesis and hydrolysis of three aldotriouronic acids. *Carbohydr. Res.*, 6, 475-481 (1968).

Sano, H., Tsuchiya, T., Ban, Y. and Umezawa, S., Synthesis of 2-amino-2,3-dideoxy-L-ribohexose. *Bull. Chem. Soc. Jpn.*, 49, 313-316 (1976).

Tarentino, A. L., Gomez, C.M. and Plummer T.H., Deglycosylation of asparagine-linked glycans by peptide: N-glycosidase F. *Biochemistry*, 24, 4665-4671 (1985).