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Selective defucosylation by mercaptolysis. A potential step in analyzing branched oligosaccharides

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Abstract

A method of stepwise chemical degradation was elaborated on a μg quantity of 3-*O*-α-L-fucosyllactose. The key step, $TiCl_4$-catalysed dithioacetal formation from the permethylated *N*-4-nitrophenyl triosylamine (**4**) was accompanied by quantitative defucosylation. [^{14}C]Acetylation of the dried mercaptalation mixture gave radiolabelled 3,5-di-*O*-[^{14}C]acetyl-4-*O*-(2,3,4,6-tetra-*O*-methyl-β-D-galactopyranosyl)-2,6-di-*O*-methyl-D-glucose diethyl dithioacetal (**7**) and 5-*O*-[^{14}C]acetyl-2,3,4-tri-*O*-methyl-L-fucose diethyl dithioacetal (**8**). The former was further degraded via the bis(sulfone), and thereby 2,3,4,6-tetra-*O*-methyl-D-galactose (**13**) was expelled. The monosaccharide branches, fucose and galactose, were identified as derivatives **8** and **13**, respectively, by comparison with authentic samples. Isolation of microquantities of products was carried out by preparative TLC.

1. Introduction

In an earlier publication we described the strategy of structurally analyzing oligosaccharides by stepwise chemical fragmentation [1]. Although structures of complex oligosaccharides may be determined by using GLC–MS and high-resolution NMR spectroscopy, this simple and direct chemical method is especially designed for branched oligosaccharides in very small quantities. A key step in the procedure is the formation of an oligosaccharide dithioacetal from the corresponding *O*- and *N*-permethylated *N*-(4-nitrophenyl)-β-D-glycosylamines. Using trifluoroacetic acid as catalyst for mercaptolysing the glycosylamine gave unsatisfactory

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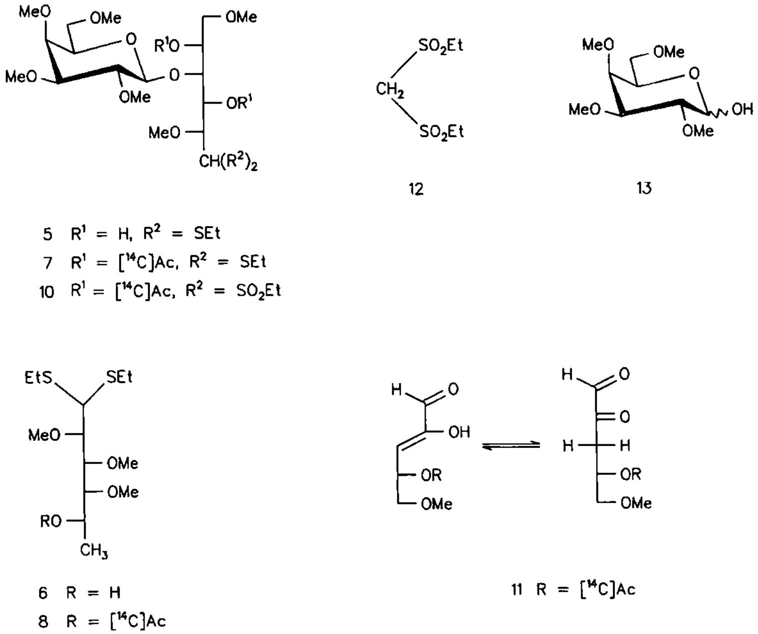
yields of dithioacetals in the case of such "neutral" oligosaccharides as maltose [2], and very sluggish reaction as well as decomposition when such *N*-acylglycosamines as chitobiose were used as model compounds. However, a thioacetalization method by Kumar and Dev [3] with titanium tetrachloride as catalyst was very efficient. In all cases so far investigated by us, formation of dithioacetals from *N*-(4-nitrophenyl)-glycosylamines was rapid and nearly quantitative. Whereas our original procedure dealt with large quantities of oligosaccharides as starting material for detailed structural investigations of fragmentation intermediates, we then wished to conduct the procedure with very small quantities of oligosaccharides, using mainly TLC separations for isolation and identification of products. In a separate publication, methods and equipment for preparative work on micro quantities of products are described [4]. Even without special labelling with chromophores or radioisotopes, a simple sugar may be detected on a HPTLC plate with a concentration zone in quantities of ~ 1 μg. With a chromophore such as *p*-nitrophenylamine, the sensitivity can be enhanced by a factor of 1000. Acetylation of free hydroxyl groups with commercial [^{14}C]acetic anhydride makes a saccharide still more readily detectable. With 3-*O*-α-L-fucosyllactose as a branched model compound, we here elaborate the stepwise degradation on a mg scale.

2. Results and discussion

3-*O*-α-L-Fucosyllactose reacts with 4-nitroaniline in Me_2SO containing a small amount of 10% formic acid at ~60°C to yield *N*-4-nitrophenyl-(3-*O*-α-L-fucopyranosyl-4-*O*-β-D-galactopyranosyl)-β-D-glucopyranosylamine (**1**). Prolonged heating (> 20 min) or higher reaction temperatures led to the appearance of two faster-migrating, UV-light absorbing components, identified by comparison as the *N*-(4-nitrophenyl)-fucosylamine (**2**) and *N*-(4-nitrophenyl)-lactosylamine (**3**). After quenching the reaction by adding water and removal of the precipitated, excess of 4-nitroaniline, compound **1** was freed from all byproducts, especially from Me_2SO, by HPLC [2]. A solid product was obtained from the eluate by centrifugal freeze-drying. Permethylation of **1** was carried out with MeI in Me_2SO and powdered NaOH [2,5]. The methylated compound **4** was extracted with ether from the water-diluted mixture. Purification was carried out by preparative TLC. A sample of **4** was submitted to mercaptolysis and the reaction monitored by TLC (8:1 ethyl acetate–cyclohexane). After 15 min, 6 components could be detected either by their UV absorption or by charring. After another 30 min, only three compounds were left and no further change in composition took place. One compound (R_f 0.57) could immediately be identified by comparison as *N*-methyl-4-nitroaniline. The mixture was dried at 0.01 Torr using a liquid-nitrogen trap, and the residue treated with [^{14}C]acetic anhydride in pyridine. The two remaining compounds (**5**, R_f 0.44; **6**, R_f 0.12) displayed higher mobilities on TLC (**7**,R_f 0.39; **8**, R_f 0.67). In a two-dimensional TLC using two different solvent mixtures, both were well separated from unchanged *N*-methyl-4-nitroaniline (Scheme 1). Scanning the spots for radioactivity gave a ratio of 1:0.4 in favour of the less-mobile

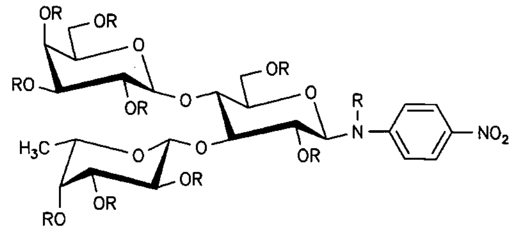


Scheme 1.

compound **7**. Whereas this compound could be clearly detected by charring, the faster migrating acetate **8** was scarcely visible by the same method. As we suspected compound **8** to be 5-*O*-[^{14}C]acetyl-2,3,4-tri-*O*-methyl-L-fucose diethyl dithioacetal, its identity was verified by comparison with a specially synthesised, unlabelled sample. Thus it is clear that the fucosyl residue was released during mercaptolysis of compound **4**. As the labelled compounds **7** and **8** accounted for $> 90\%$ of the detectable radioactivity, the defucosylation must have been almost quantitative. The small deviation from an exact ratio of 1:0.5 for the radioactivities associated with compounds **7** and **8** may be explained by a small amount of ethyl thiofucoside being formed, not detectable by radioacetylation. Such a compound was, however, not detected, when 2,3,4-tri-*O*-methyl-*N*-4-nitrophenyl-α-L-fucosylamine (**9**) was mercaptalized.

Radio-di-*O*-acetylated compound **7**, the remainder of the original trisaccharide derivative, was quantitatively isolated by preparative TLC. The yield was ~ 0.5 mg. Its structure could be verified by ^{1}H NMR spectroscopy to be 3,5-di-*O*-acetyl-2,6,2′,3′,4′,6′-hexa-*O*-methyllactose diethyl dithioacetal. This compound was oxidised by 3-chloroperoxybenzoic acid to yield the bis(sulfone) **10** [1]. Separation from excess reagent and 3-chlorobenzoic acid, and isolation was performed by two-dimensional TLC (Scheme 2).

As expected, this compound underwent MacDonald–Fischer degradation [6], when treated with dilute ammonia in tetrahydrofuran. During the preceding elution process under neutral conditions with 5:1 EtOH–H_2O, partial β-elimina-

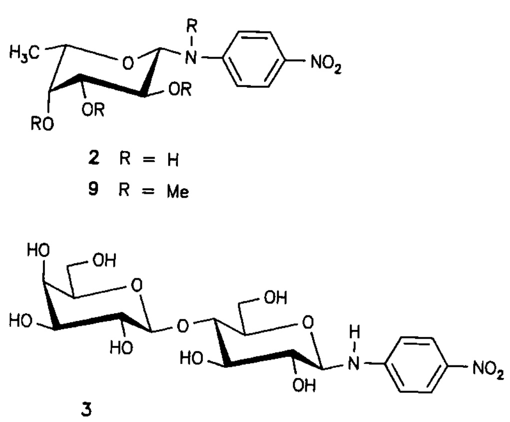


1 R = H
4 R = Me

Scheme 2.

tion of the 2-methoxy group and rehydration must have taken place [1], because the TLC radioactivity pattern was complex. The pattern after treating this mixture with dilute ammonia in tetrahydrofuran showed one homogeneous radioactive compound, which presumably has the structure of 4-*O*-[^{14}C]acetyl-3-deoxy-5-*O*-methyl-D-*glycero*-pent-2-enose (**11**) in equilibrium with its dicarbonyl tautomer. This assumption is plausible, because compared with the starting material, the radioactivity in **11** is decreased by exactly one half. The nonradioactive fragmentation products of **10** could be identified by TLC comparison with authentic samples as bis(ethylsulfonyl)methane (**12**) [6] and 2,3,4,6-tetra-*O*-methyl-D-galactose (**13**). Compound **13** was isolated and treated separately with dansylhydrazine [7]. One spot, strongly fluorescing on TLC coincided with a spot derived from authentic 2,3,4,6-tetra-*O*-methyl-D-galactose after reaction with dansylhydrazine. The mixture consisting of **11**, **12**, and **13** was treated with dansylhydrazine. The product mixture showed another fluorescing spot as well as the hydrazone of **13**. We presume that **11** as a dicarbonyl compound is converted into a dansyl osazone.

All separation and identification procedures, as well as the stepwise degradation, were performed with only 2 mg of a branched trisaccharide as starting



2 R = H
9 R = Me

3

Scheme 3.

material, an amount that may be significantly decreased. The principle of chemical stepwise degradation as described in Ref [1] could be affirmed and experimental procedures improved. The quantitative removal of a fucosyl side-chain by mercaptolysis, and its detection by radiolabelling, is an important step if the method is applied to branched oligosaccharides carrying this peripheral sugar. Further investigations may demonstrate the selective removal of other 6-deoxy sugars by $TiCl_4$-catalyzed mercaptolysis and the potential of $TiCl_4$ as a reagent for cleaving 6-deoxyhexosidic linkages within an oligosaccharide chain. If branches should be ordinary mono- or oligo-saccharides, stable under the mild mercaptolysis conditions, degradation would occur according to the sequence described in Ref [1]. Labelling of the product (in this case two products) of the mercaptolysis with [^{14}C]acetic anhydride is experimentally much easier than with [^{14}C]methyl iodide. The latter would confirm with the procedure described in Ref [1] and lead to radiolabelled products, which can be compared with known compounds.

The stability of an ordinary, secondary acetoxy group under the conditions of the MacDonald–Fischer degradation [6], makes this simple way of sensitively labelling products very useful, when, after mercaptolysis, the chromophoric *N*-methyl-4-nitrophenylamino group is lost. If an oligosaccharide resulting from "feeding" or "pulse-chase" experiments is *a priori* radiolabelled, the chemical procedure described here and in Ref [1] may be superior to any physical method of structural analysis.

3. Experimental

General methods. — For analytical and preparative separations, Silica Gel 60 F_{254} TLC plates (0.25 mm, Merck) and HPTLC plates (0.20 mm with concentration zone, Merck) were used, depending on the amount and concentration of the samples to be resolved. Solutions applied to HPTLC plates should be ~ 0.1%. Ordinary plates can take solutions of ~ 4% which means that on a 9-cm baseline, 1 mg of material can be purified. Limits of detectability by UV-absorption, charring, and iodine absorption of compounds described herein, are listed in Table 2.

The solvent mixtures used, are indicated in Table 1. Unprotected *N*-4-nitrophenyl glycosylamines (detection $\lambda = 375$ nm) are separated from Me_2SO, salts and acids by HPLC (Hypersil ODS 5 μm, 250 × 20 mm, Bischoff). Concentration of nonradioactive solutions to dryness was performed in a SpeedVac Concentrator (Savant Instruments Inc.). Extraction of all compounds from silica gel was carried out with 5:1 $EtOH–H_2O$ as described [4].

Radioactive material was detected either radioautographically (Agfa-Gevaert Curix X-ray film) or with a Berthold automatic TLC-linear analyzer LB 2821. Radioactive samples in solution were assayed in a Berthold BF-815 liquid scintillation counter, using Quickszint 501 (Zinsser). ^{1}H NMR spectra were recorded with a Bruker WM-250 spectrometer at 250 MHz for solutions in $CDCl_3$ (internal Me_4Si). All reactions were carried out in Eppendorf reaction vessels (2 mL).

Table 1
Solvent mixtures used

Compound	Mixture of solvents [a]			
	A	*B*	*C*	*D*
1	0.65			
2		0.36		
3		0.67	0.57	0.54
4		0.60	0.44	
5		0.41	0.12	
6			0.67	
7		0.65	0.39	
8			0.24	
9				0.19
10				0.33
11				0.11
12		0.52		0.12
13		0.58	0.33	0.24
14		0.39		0.09

[a] A, 4:2:1 EtOAc–MeOH–H_2O. B, 27:2:1 EtOAc–MeOH–H_2O. C, 8:1 EtOAc–cyclohexane. D, 3:1 EtOAc–cyclohexane.

Preparation of N-*(4-nitrophenyl)glycosylamines.*—N-*(4-Nitrophenyl-3-*O-*α-*L-*fucosyllactosyl)amine* (**1**). To a solution of 3-*O*-α-L-fucosyllactose (2 mg, 4.1 μmol) in Me_2SO (100 μL) 10:1 formic acid–water (20 μL), and 5 *M* 4-nitroaniline in Me_2SO (60 μL) were added and the mixture was heated (60°C) for ~ 30 min. To monitor the reaction, 1 μL of the solution was diluted in MeOH (10 μL) and analysed using solvent *A* (Table 1). When all of the starting material had disappeared (charring) and the least amount of side products had formed (two UV-absorbing spots, *N*-(4-nitrophenyl)-fucosylamine and *N*-(4-nitrophenyl)-lactosylamine, between the product **1** and 4-nitroaniline) the mixture was diluted with water (10 mL) and 25% ammonia (0.3 mL). The excess 4-nitroaniline was extracted with ether. From the aqueous solution, compound **1** was isolated by HPLC and concentrated to yield an amorphous, yellow coloured solid (1.8 mg, 85%).

Permethylation.—N-*Methyl-*N-*4-nitrophenyl-3-*O-*(2,3,4-tri-*O-*methyl-α-*L-*fucopyranosyl)-4-*O-*(2,3,4,6-tetra-*O-*methyl-β-*D-*galactopyranosyl)-2,6-di-*O-*methyl-β-*D-*glucopyranosylamine* (**4**). To the *N*-(4-nitrophenyl)-glycosylamine **1** (1.8 mg, 2.9 μmol) in Me_2SO (0.3 mL) was added MeI (0.1 mL) and the reaction started by shaking the solution vigorously (vibrator) with freshly powdered NaOH for 15 min. Solid material was separated by centrifugation. The supernatant and one washing

Table 2
Limits of detectability (ng)

Compound	Method			
	Visual	Iodine	Charring	UV
N-(4-nitrophenyl)-galactosylamine	300	300	300	1
Bis(ethylsulfonyl)methane		100		

with Me_2SO (0.1 mL) were diluted with water (1 mL) and then extracted with ether (5 × 0.3 mL). The organic layer was washed with water (0.3 mL) and concentrated to yield an amorphous solid, which was purified by preparative TLC to give **4** (2.07 mg, 87%). ^1H NMR data ($CDCl_3$): δ 8.13, 6.89 (2 d, 2 × 2 H, aryl), 5.43 (d, 1 H, $J_{1',2'}$ 3.75 Hz, H-1′), 4.78 (d, 1 H, $J_{1'',2''}$ 8.85 Hz, H-1″), 4.75 (d, 1 H, $J_{1,2}$ 6.75 Hz, H-1), 3.03 (s, 3 H, CH_3N), 3.60–3,33 (9 s, 27 H, 9 OCH_3).

Mercaptolysis.—4-O-(2,3,4,6-Tetra-O-methyl-β-D-galactopyranosyl)-2,6-di-O-methyl-D-glucose diethyl dithioacetal (**5**) *and 2,3,4-tri-O-methyl-L-fucosediethyl dithioacetal* (**6**). Compound **4** (1.5 mg) was dissolved in dry $CHCl_3$ (0.5 mL) and EtSH (0.5 mL). From a stock solution of dry $CHCl_3$ (1.5 mL) containing $TiCl_4$ (~ 0.2 g), 5 drops (~ 0.1 mL) were added by a syringe. The solution immediately became orange red. After 20 min at room temperature, the mixture, analyzed by HPTLC in solvent *B* revealed along with 4-*N*-methyl-nitroaniline and some starting material one additional UV active component, which was presumed to be 2,6,2′,3′,4′,6′-hexa-*O*-methyl-*N*-(4-nitrophenyl)-lactosylamine. After 1 h, *N*-methyl-4-nitroaniline remained the only UV-active spot, and two products (**5** and **6**) were made visible by charring.

Volatile material was removed in high vacuum and collected in a liquid nitrogen trap. The dry residue was submitted to [^{14}C]-*O*-acetylation.

[^{14}C]-O-Acetylation.—3,5-Di-O-[^{14}C]acetyl-4-O-(2,3,4,6-tetra-O-methyl-β-D-galactopyranosyl)-2,6-di-O-methyl-D-glucose diethyl dithioacetal (**7**) *and 5-O-[^{14}C]acetyl-2,3,4-tri-O-methyl-L-fucose diethyl dithioacetal* (**8**). The dried mixture obtained after mercaptolysis was dissolved in 1 : 3 Ac_2O–pyridine (0.1 mL) containing 250 μCi [1-^{14}C]acetic anhydride (20 mCi/mmol). The radioactive Ac_2O was condensed from an ampoule (Amersham) under high vacuum into the nonradioactive acetylation mixture (dilution of radioactive acetic anhydride was ~ 1 : 20).

After storing for 16 h at 25°C, volatile material was removed from the mixture by collecting it under vacuum in a liquid-nitrogen trap. This acetylation mixture may be used again for radio-*O*-acetylation. The dry residue was dissolved in a sufficient amount of dry CH_2Cl_2, concentrated and again taken up in the same solvent (100 μL). TLC separation gave two main radioactive spots as located by autoradiography; these were eluted separately, isolated (total ~ 0.2 mg, estimated from total radioactivity of ~ 1.2×10^6 dpm), and identified; compound **7** directly by ^1H NMR, and compound **8** by cochromatography with authentic 5-*O*-acetyl-2,3,4-tri-*O*-methyl-L-fucose diethyl dithioacetal.

^1H NMR data for compound **7** ($CDCl_3$):

H-1	4.16 d	H-1′	4.35 d	$J_{1,2}$ 4.50, $J_{1',2'}$ 7.65 Hz
H-2	3.86 dd	H-2′	3.24 dd	$J_{2,3}$ 7.50, $J_{2',3'}$ 9.75 Hz
H-3	5.54 dd	H-3′	3.11 dd	$J_{3,4}$ 2.50, $J_{3',4'}$ 3.20 Hz
H-4	4.17 dd	H-4′	3.63 dd	$J_{4,5}$ 7.60, $J_{4',5'}$ 0.75 Hz
H-5	5.02 ddd	H-5′	3.46 ddd	$J_{5,6a}$ 4.20, $J_{5',6a'}$ 8.10 Hz
H-6a	3.82 dd	H-6a′	3.58 dd	$J_{6a,6b}$ 11.25, $J_{6a',6b'}$ 9.0 Hz
H-6b	3.57 dd	H-6b′	3.50 dd	$J_{5,6b}$ 3.0, $J_{5',6b'}$ 6.0 Hz

3.59, 3.55, 3.52, 3.51, 3.39, 3.33 (6 s, 18 H, 6 OCH_3)
2.73, 2.36 (2 m, 2 OEt)
2.07, 2.06 (2 s, 6 H, 2 OAc).

Preparation of bis-(ethylsulfonyl) derivatives.—3,5-di-O-[^{14}C]acetyl-1,1-bis(ethylsulfonyl)-1-deoxy-4-O-(2,3,4,6-tetra-O-methyl-β-D-galactopyranosyl)-2,6-di-O-methyl-D-glucitol (**10**). Compound **7** (~ 5 × 10^5 dpm) was dissolved in CH_2Cl_2 (0.1 mL) and mixed with a 10% solution of 3-chloroperoxybenzoic acid in CH_2Cl_2 (0.1 mL). After 16 h at 25°C partial separation of **10** from nonradioactive material was accomplished by chromatography in solvent *C* for the bulk amount. Bidimensional TLC in the same solvent established the homogeneity of the radioactive compound. The bulk amount (~ 3 × 10^5 dpm), slightly contaminated with 3-chloroperoxybenzoic acid, was isolated and dissolved in a mixture of THF (40 μL) and 2.5% aq ammonia (60 μL). After storing the mixture for 3 h, volatile material was removed and the dry residue, after dissolution in 5:1 EtOH–H_2O (40 μL), was submitted to TLC separation in solvents *B* and *C*. Two of the three detectable compounds (iodine staining and charring) were identified by comparison with authentic samples as bis(ethylsulfonyl) methane (**12**) and 2,3,4,6-tetra-*O*-methyl-D-galactose (**13**). The third (**11**) still contained half the radioactivity of the starting material. Compounds **11** and **13** could, after isolation, be derivatised with dansylhydrazine. The dansylhydrazone of **13** cochromatographed in solvents *B* and *D* with the dansylhydrazone prepared from authentic 2,3,4,6-tetra-*O*-methyl-D-galactose.

N-*Methyl*-N-*4-nitrophenyl 2,3,4-tri-O-methyl-β-L-fucopyranosylamine* (**9**). L-Fucose (100 mg) was treated as described in the general procedure for the preparation of *N*-(4-nitrophenyl)glycosylamines. After isolating the *N*-(4-nitrophenyl)-β-L-fucopyranosylamine as an amorphous solid (85%), permethylation was performed with 20 mg of material, as described in the general procedure to yield approximately the same amount of permethylated material. ^{1}H NMR data ($CDCl_3$): δ 8.15, 6.87 (2 d, 4 H, aryl), 4.75 (d, 1 H, $J_{1,2}$ 8.85 Hz, H-1), 3.70 (t, 1 H, $J_{2,3}$ 9.75 Hz, H-2), 3.61 (dd, 1 H, $J_{5,6}$ 6.1 Hz, H-5), 3.29 (dd, 1 H, $J_{3,4}$ 3.15 Hz, H-3), 1.32 (d, 1 H, $J_{5,6}$ 6.1 Hz, H-6), 3.62, 3.37 (6 s, 3 H, OCH_3), 3.09 (s, 3 H, NCH_3).

5-O-Acetyl-2,3,4-tri-O-methyl-L-fucosediethyl dithioacetal (**8**). Compound **9** (15 mg) was submitted to mercaptolysis according to the general procedure. The dried mixture was acetylated with unlabelled Ac_2O in pyridine as described before. Purification of ~3 mg was performed by preparative TLC. ^{1}H NMR data ($CDCl_3$): δ 5.15 (dq, 1 H, H-5), 4.13 (d, 1 H, H-1), 3.83 (dd, 1 H, H-4), 3.61 (s, 3 H, OCH_3), 3.6 (dd, 1 H, H-2), 3.56 (s, 3 H, OCH_3), 3.32 (dd, 1 H, H-3), 2.6–2.86 (m, 4 H, 2 SCH_2CH_3), 2.11 (s, 3 H, OAc), 1.38 (d, 3 H, 3 H-6), 1.3 (t, 6 H, 2 SCH_2CH_3). A solution of **8** was used as a standard for cochromatography.

N-*Methyl*-N-*4-nitrophenyl 2,3,4,6-tetra-O-methyl-β-D-galactopyranosylamine* (**14**). The unprotected *N*-4-nitrophenylgalactosylamine was prepared according to the standard method [8]. Permethylation of 0.1 mmol and isolation of the product as described under the general procedure, gave the crystalline, yellow product (82%). Recrystallization from 1-heptane gave compound **14**; mp 103°C; ^{1}H NMR data ($CDCl_3$): δ 8.12, 6.91 (2 d, 2 × 2 H, aryl), 4.75 (d, 1 H, $J_{1,2}$ 8.85 Hz, H-1), 3.73 (t, 1

H, $J_{2,3}$ 8.8 Hz, H-2), 3.72 (d, 1 H, $J_{4,5}$ 0.5 Hz, H-4), 3.55 (m, 1 H, $J_{5,6a}$ 3.0 Hz, H-5), 3.55 (m, 1 H, H-6), 3.53 (dd, 1 H, $J_{3,4}$ 3.0 Hz, H-3), 3.27 (dd, 1 H, $J_{5,6a}$ 9.3 Hz, H-6), 3.62, 3.55, 3.38 (3 s, 9 H, 3 OCH_3), 3.08 (s, 3 H, NCH_3); R_f 0.52 (8:1 EtOAc–cyclohexane). Anal. Calcd for $C_{17}H_{24}N_2O_8$: C, 55.11; H, 7.08; N, 7.56. Found: C, 55.29; H, 7.06; N, 7.38.

For comparison by cochromatography, a 1% solution of **14** in 1 *M* CF_3CO_2H was heated for 15 min at 95°C, and this served as a standard for 2,3,4,6-tetra-*O*-methyl-D-galactose [9] and *N*-methyl-4-nitroaniline.

Acknowledgments

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