Synthetic Studies of Carbohydrate Antibiotics

Part III. Gas-Liquid Chromatography of Trifluoroacetyl Derivatives of Carbohydrates Including Cyclitols¹⁾

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The separation of carbohydrates and cyclitols by gas-liquid chromatography of their trifluoroacetyl (TFA) derivatives is described. TFA derivatives are readily formed in formamide containing sodium trifluoroacetate and trifluoroacetic anhydride without heating so that the chromatographic analyses can be made in a short time. Conditions are described for the chromatography of variety of carbohydrates including the mono- and disaccharides, simple glycosides, aminosugars, and cyclitols. The analyses have been made mostly with a silicone column (SE-52) and a fluorosilicone column (QF-1). Isothermal conditions are usually employed, but for a mixture with components of widely differing molecular weight, linear temperature-programmed procedures are performed.

INTRODUCTION

The gas-chromatographic analyses of carbohydrates have been so far investigated generally by using their O-methyl,²⁾ O-acetyl,³⁾ and trimethylsilyl (TMS)⁴⁾ derivatives. Among these, the most easily prepared TMS derivatives are well separated at the lowest temperatures, and have been considered to be the most satisfactory derivatives for the gas-chromatography.

For the analyses of carbohydrate mixtures including cyclitols, especially their amino derivatives which often appear as a component of the carbohydrate antibiotics, a more con-

venient derivative is desired, since some of the cyclitols are not so easily dissolved in pyridine to produce their TMS derivatives rapidly and quantitatively.

For the amino acids and peptides, Weygand and his coworkers⁵⁾ have found an excellent volatility of their trifluoroacetyl derivatives and performed the gas-chromatographic analyses by using them. In this paper, we have applied their method to the carbohydrates, and found that the TFA derivatives showed the good separations at a much lower temperature than the corresponding methyl or TMS derivatives (see Table I).

Recently, Vilkas and his associates⁶⁾ have also described the versatility of the TFA derivatives for the gas-chromatographic analysis of the sugars. Our procedure is different from theirs in using formamide as the solvent

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5) F. Weygand, B. Kolb, A. Prox, M. A. Tilak and I. Tomida, *Z. Physiol. Chem.*, **322**, 38 (1960).

6) M. Vilkas, Hiu-I-Jan, G. Boussac and M.-C. Bonnard, *Tetrahedron Letters*, **14**, 1441 (1966).

TABLE I. COMPARISON OF THE RETENTION TIME OF THE TFA, METHYL AND TMS DERIVATIVES

Column	SE-52			QF-1	N. G. S.
Temperature (°C)	110	140	200	110	150
Carrier Gas, N ₂ (ml/min)	28.5	25.0	19.5	120.0	30.0
<i>myo</i> -Inositol (TFA)	2.5 min	0.9 min	—	2.7 min	—
Methyl α -D-glucopyranoside (TFA)	8.0	2.7	—	4.3	—
<i>myo</i> -Inositol (Me)	—	8.5	—	—	14.2 min
Methyl α -D-glucopyranoside (Me)	—	7.8	—	—	15.6
<i>myo</i> -Inositol (TMS)	—	—	13.0 min	—	16.3
Methyl α -D-glucopyranoside (TMS)	—	—	5.7	—	11.9

for the trifluoroacetylation and requires a much shorter time for the analysis.

EXPERIMENTAL

Materials. Most of the mono- and disaccharides used in this study were commercially available. 6-Amino-6-deoxy-D-glucose and its derivatives were prepared according to Cramer et al.,⁷⁾ and methyl 3-amino-3-deoxy- β -D-glucopyranoside and methyl 3-amino-3-deoxy- α -D-mannopyranoside were according to Baer et al.⁸⁾ Most of inositols and aminocyclitols were synthesized by the method of Nakajima et al.,⁹⁾ except for *scyllo*- and *epi*-inositol which were prepared according to the procedure of Posternak et al.¹⁰⁾ TFA anhydride was prepared by treating trifluoroacetic acid with phosphorus pentoxide. Sodium trifluoroacetate was prepared by neutralization of 1N trifluoroacetic acid with 1N sodium hydroxide (pH to 7) and by evaporation of the resulting solution. Addition of benzene and evaporation was repeated several times to remove a trace of water. The crystals obtained were dried at 100°C at a reduced pressure and used for the reaction. Penta-O-methyl- α -D-glucose was prepared from methyl α -D-glucopyranoside by

the methylation method of Hakomori.¹¹⁾ Hexa-O-methyl-*myo*-inositol was prepared by the method of Kuhn et al.¹²⁾ TMS derivatives were prepared by the procedure of Sweeley et al.⁴⁾ Solvents were reagent grade and were used as supplied unless specially noted.

The column packings were commercially available preparations. Ten per cent N. G. S. on acid treated and silanized Chromosorb W (80~100 meshes) was obtained from Nishio Kogyo Co., Ltd.; and 3% SE-52 on acid treated and silanized Chromosorb W (80~100 meshes) and 1% QF-1 on Diasolid H (30~50 meshes) were obtained from Nihon Chromato Kogyo Co., Ltd.

Procedures. The general procedure of trifluoroacetylation is as follows. One mg of the dry sample of a free sugar or a related substrate and 0.3 mg of sodium trifluoroacetate were dissolved in 0.05 ml of formamide by heating. The solution was cooled to room temperature, and 0.2 ml of trifluoroacetic anhydride was gradually added with cooling in an ice bath, and the whole mixture was left for 10 minutes at room temperature. Then 1 to 2 μ l of the resulting solution was injected by a microsyringe into the gas chromatographic equipment.

Nature of the trifluoroacetylated products. In order to isolate and characterize the trifluoroacetyl derivatives, a larger amount of the starting material was used. The following five materials were selected as model compounds, and two procedures of trifluoroacetylation were compared: (A) the Bourne's method¹³⁾ and (B) the present method. Bourne and his co-

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9) M. Nakajima, I. Tomida, N. Kurihara and S. Takei, *Chem. Ber.*, **92**, 173 (1959); M. Nakajima, N. Kurihara and A. Hasegawa, *ibid.*, **95**, 141 (1962); M. Nakajima, A. Hasegawa and F. W. Lichtenthaler, *Ann.*, **680**, 21 (1964).

10) T. Posternak, *Helv. Chim. Acta*, **24**, 1045 (1941); D. Reymond, *ibid.*, **40**, 492 (1957); T. Posternak, *ibid.*, **19**, 1333 (1936).

11) S. Hakomori, *J. Biochemistry*, **55**, 205 (1964).

12) R. Kuhn and H. Trischmann, *Chem. Ber.*, **96**, 284 (1963).

13) E. J. Bourne, C. E. M. Tatlow and J. C. Tatlow, *J. Chem. Soc.*, **1950**, 1367; E. J. Bourne, M. Stacey, C. E. M. Tatlow and J. C. Tatlow, *ibid.*, **1951**, 826.

workers have prepared the TFA derivatives for other purposes than gas-chromatographic analysis.

1) **Hexa-O-TFA-*myo*-inositol.** A) In a 10 ml round bottomed flask fitted with a reflux condenser and a calcium chloride tube, 90 mg of dry *myo*-inositol, 1 mg of sodium trifluoroacetate and 2.5 ml of trifluoroacetic anhydride were placed. The mixture was gently refluxed in an oil bath with occasional shaking at 60~70°C for about 30 minutes. The whole materials went into solution, and the heating was continued for additional 10 minutes. After the solution was cooled down to room temperature, it was transferred into an appropriate sublimation apparatus, and excess anhydride and acid formed were removed at a reduced pressure. The sublimation was conducted at 140~160°C (18 mmHg). Two hundred and thirty mg of fine colorless crystals of m.p. 140°C was obtained. Yield 63%. The IR spectrum is shown in Fig. 1.

B) Forty mg of dry *myo*-inositol and 15 mg of sodium trifluoroacetate were dissolved in 0.5 ml of formamide with gently heating. After the solution was cooled down to room temperature, 2.0 ml of trifluoroacetic anhydride was slowly added with ice

cooling. Then a violent reaction occurred and the mixture began to reflux. After the reflux ceased, the solution was kept at room temperature for 10 minutes. After addition of a few ml of *sym*-tetrachloroethane, the solution was concentrated in vacuo. The resulting syrup was dissolved in 1 ml of trifluoroacetic anhydride and set aside in a desiccator over night. Colorless needles (84 mg, m.p. 140°C) were obtained. Mixed m.p. and IR spectra confirmed its identity with the product of (1) (A). The mother liquor (1~2 μ l) was injected into the gas chromatographic equipment. A single peak was observed, the retention time of which was the same as that of the above mentioned crystalline hexa-O-TFA-*myo*-inositol dissolved in tetrahydrofuran (except for the solvent peak having a very short retention time).

2) **Methyl tetra-O-TFA- α -D-glucopyranoside.** A) Four hundred and fifty mg of methyl α -D-glucopyranoside, 150 mg of sodium trifluoroacetate and 5 ml of trifluoroacetic anhydride were treated as described in (1)(A). After distillation at 180°C (bath temperature) and 18 mmHg, 910 mg of a syrup was obtained. Yield 43%. It was dissolved in anhydrous tetrahydrofuran and injected into the gas chromatographic equipment, showing a single peak beside the solvent peak. The IR spectrum is shown in Fig. 1.

B) Methyl α -D-glucopyranoside in formamide was treated as described in (1) (B). The resulting solution was analyzed gas-chromatographically and a single peak was observed on the gas chromatogram having the same retention time as the above identified methyl tetra-O-TFA- α -D-glucopyranoside.

3) **Methyl 6-deoxy-6-trifluoroacetamido-2,3,4-tri-O-TFA- α -D-glucopyranoside.** A) Eighty mg of methyl 6-amino-6-deoxy- α -D-glucopyranoside hydrochloride, 20 mg of sodium trifluoroacetate and 2 ml of trifluoroacetic anhydride were treated as described in (1) (A). After sublimation at 120°C (18 mmHg), 180 mg of needles (85%) were obtained. M.p. 98°C. The IR spectrum is in Fig. 1.

B) In formamide, methyl 6-amino-6-deoxy- α -D-glucopyranoside hydrochloride was trifluoroacetylated as described in (1) (B). The solution was examined gas-chromatographically and showed a single peak at the same retention time as that of the sample prepared by the procedure, (3) (A).

4) **Octa-O-TFA-sucrose.** A) One hundred mg of sucrose, 30 mg of sodium trifluoroacetate, and 20 ml of trifluoroacetic anhydride were treated as described in (1) (A). After sublimation at 180°C, 170 mg of

FIG. 1. IR Spectra of the TFA Derivatives.

colorless crystals (77%) were obtained. The IR spectrum is shown in Fig. 1.

B) In formamide, sucrose was trifluoroacetylated as described in (1) (B). The solution was examined gas-chromatographically, and showed a single peak at the same retention time with that of the sample prepared by the procedure (4) (A).

5) **Octa-O-TFA- α and β -maltose.** A) After 3 hours reflux of a mixture of maltose, sodium trifluoroacetate and trifluoroacetic anhydride, there was obtained a gummy substance. The soluble part was examined gas-chromatographically, but no peak was observed corresponding to octa-O-TFA-maltose.

B) Forty mg of malose monohydrate and 15 mg of sodium trifluoroacetate were dissolved in 0.5 ml of formamide. To the solution, 2 ml of trifluoroacetic anhydride was added causing a violent reaction. After standing 10 minutes at room temperature, the solution was analysed gas-chromatographically. Two peaks having very close retention times were observed which should correspond to the α and β -anomers.

Gas Chromatography. Two instruments were used for the gas-chromatographic analysis: the Hitachi-Perkin-Elmer Model F-6 and the Yanagimoto Model 3D; both of which were attached with hydrogen flame ionization detectors and employed U-shaped stainless steel columns, 1 m length by 3 mm or 5 mm i. d.

As the most useful nonpolar liquid phase, 3% SE-52 was used for the analyses of all types of the derivatives: methyl, TMS and TFA derivatives. One per cent QF-1 was selected as a polar liquid phase for the analyses of TFA derivatives. When a more usual polar liquid phase was used such as N. G. S. or E. G. S., these derivatives were adsorbed and not eluted from the column. For the methyl and TMS derivatives, a 10% N. G. S. column was used without difficulty. A capillary column, 45 m length by 0.5 mm i. d. containing SE-30 (Hitachi-Perkin-Elmer) was also used for the separation of the seven isomers of inositol.

In general, flow rates for the packed column ranged about 20 to 30 ml per minute, except for the QF-1 column, where the rates of 115~120 ml per minute were employed.

RESULTS AND DISCUSSION

The Trifluoroacetylation Reaction

The preparation of the TFA derivatives of some carbohydrates and related substances was first described by Bourne et al., who

reported all of the free hydroxyl groups in the carbohydrates were completely trifluoroacetylated by refluxing for about half an hour with trifluoroacetic anhydride and sodium trifluoroacetate. A TFA derivative of desosamine was reported by Newman,¹⁴⁾ who employed anhydrous ether as a solvent for this reaction. Recently, Vilkas et al.⁶⁾ achieved trifluoroacetylation of carbohydrates with trifluoroacetic anhydride and sodium trifluoroacetate in acetonitrile at 50~60°C, and investigated the gas-chromatographic separation of these TFA derivatives.

The reaction procedure described in this paper does not require so much time as those described so far, and besides, it can be widely applied to various carbohydrates. This procedure is different from others in employing formamide as the solvent. It solubilized all the carbohydrates examined, and the reaction in this solvent occurred very rapidly without heating. During the reaction, trifluoroacetylformamide first formed would be considered to serve as an excellent trifluoroacetylating agent; since Ogata¹⁵⁾ reported that the imides of monobasic acids served often as a very good acylating agent of alcohols.

Since the TFA derivative of an authentic sample examined except for the free reducing sugars showed always a single peak, it appears that all of the free hydroxyl and amino groups of the sample were converted to the corresponding TFA derivatives. This conclusion was also confirmed by the gas-chromatography of the isolated products by the method of Bourne et al. Besides, the isolated TFA derivatives showed reasonable IR spectra, having the absorptions of CF_3 at 1300~1100 cm^{-1} , and ester carbonyl at about 1800 cm^{-1} (and amide absorptions in case of 6-aminoglucose derivative), and no absorption corresponding to OH group(s). (See Fig. 1).

For the TFA derivatives of free reducing

14) H. Newman, *J. Org. Chem.*, **30**, 1287 (1965).

15) T. Ogata, *Bull. Chem. Soc. Japan*, **37**, 1117 (1964).

sugars, two peaks of very close retention times were usually observed, and they are reasonably considered to correspond to those of the α and β -anomers.

Separation of Representative Carbohydrates and Cyclitols

Since each TFA derivative of some carbohydrates showed a good peak at a lower temperature by gas chromatography, we have further investigated some more representative carbohydrates and cyclitols. As shown in Tables II, III and IV, the monosaccharides, inositols and aminocyclitols can be separated at 110°C.

The gas-chromatographic separation of inositol isomers has been studied by two groups of workers who employed the hexaacetyl derivatives¹⁶⁾ and the hexa-TMS derivatives.¹⁷⁾

TABLE II. RELATIVE RETENTION TIME OF THE TFA DERIVATIVES OF MONOSACCHARIDES AND METHYL GLUCOPYRANOSIDES

Methyl α -D-glucopyranoside	3.20	
Methyl β -D-glucopyranoside	3.48	
D-Glucose	1.34	2.07
D-Mannose	1.04	—
<i>myo</i> -Inositol	1.00 (2.5 min.)	

SE-52-110°C; Carrier, N₂ 28.5 ml/min

TABLE III. RELATIVE RETENTION TIME OF THE TFA DERIVATIVES OF INOSITOL ISOMERS

Column	SE-52	SE-30	QF-1
Temperature (°C)	110	110	110
Carrier, N ₂ (ml/min)	28.5	4.35	120
<i>neo</i>	0.37	0.67	0.48
<i>rac</i>	0.40	0.64	0.60
<i>muco</i>	0.55	0.80	1.03
<i>allo</i>	0.64	0.87	1.18
<i>myo</i>	1.00	1.00	1.00
	(2.5 min)	(5.7 min)	(3.0 min)
<i>scyllo</i>	1.56	1.69	1.48
<i>psi</i>	3.33	2.78	2.98

TABLE IV. RELATIVE RETENTION TIME OF THE TFA DERIVATIVES OF AMINOCYCLITOLS

Column	SE-52	QF-1
Temperature (°C)	110	120
Carrier, N ₂ (ml/min)	28.5	115
<i>scyllo</i> -Inosamine	1.06	—
<i>allo</i> -Inosamine-1	3.07	5.37
1-Deoxy- <i>allo</i> -inosamine-5	2.02	3.83
3-Deoxy- <i>epi</i> -inosamine-1	8.73	8.23
Dihydroconduramine-F-4	6.06	5.57
<i>myo</i> -Inositol	1.00	1.00
	(2.5 min)	(1.8 min)

TABLE V. RELATIVE RETENTION TIME OF THE TFA DERIVATIVES OF AMINO SUGARS AND THEIR METHYL GLYCOSIDES

Methyl 6-amino-6-deoxy- α -D-glucopyranoside	1.00	
	(7.6 min)	
Methyl 6-acetamido-6-deoxy- α -D-glucopyranoside	1.08	
Methyl 3-amino-3-deoxy- β -D-glucopyranoside	0.92	
Methyl 3-amino-3-deoxy- α -D-mannopyranoside	0.51	
6-Amino-6-deoxy-D-glucose	0.53	0.62
2-Acetamido-2-deoxy-D-glucose	0.37	—
2-Amino-2-deoxy-D-glucose	0.30	0.50

SE-52 140°C; Carrier, N₂ 28.5 ml/min

TABLE VI. RELATIVE RETENTION TIME OF THE TFA DERIVATIVES OF DISACCHARIDES

Sucrose	1.0	(6.1 min)
Trehalose	1.54	
Lactose	1.61	2.18
Maltose	1.20	1.36
Cellobiose	1.22	1.56

SE-52 160°C, Carrier, N₂ 23.1 ml/min

It is interesting that the elution order of the TFA derivatives (Table III) was very similar to that of the hexaacetyl derivatives (ester), but different from that of the TMS derivatives (ether).

Aminosugars can be analyzed at 140°C (Table V), and disaccharides at 160°C (Table VI). This temperature required for separating the TFA derivatives of disaccharides was much

¹⁶⁾ Z. S. Krzeminski and S. J. Angyal, *J. Chem. Soc.*, **1962**, 3251.

¹⁷⁾ Y. C. Lee and C. E. Ballou, *J. Chromatography*, **18**, 147 (1965).

lower than that used for the TMS derivative.⁴⁾

Linear Temperature-programmed Separation of a Mixture of the TFA Derivatives of Carbohydrates

From the foregoing data of the separation, it was expected that the TFA derivatives of mixtures of monosaccharides, aminosugars, disaccharides, inositols and aminocyclitols could be separated between 100°C and 200°C. A model of a mixed sample containing *myo*-inositol, methyl α -D-glucopyranoside, methyl β -D-glucopyranoside, methyl 6-amino-6-deoxy- α -D-glucopyranoside, sucrose, and trehalose was trifluoroacetylated and examined gas-chromatographically. A chromatogram shown in Fig. 2 was obtained by a programmed operation for temperature increase of 5°C per minute from 100°C to 200°C, and all of the

FIG. 2. Linear Temperature-programmed Separation of the TFA Derivatives of Representative Carbohydrates.

- A. *myo*-Inositol
- B. Methyl α -D-glucopyranoside
- C. Methyl β -D-glucopyranoside
- D. Methyl 6-amino-6-deoxy- α -D-glucopyranoside
- E. Sucrose
- F. Trehalose

peaks in the chromatogram were identified from the data shown in Table VII.

TABLE VII. RELATIVE RETENTION TIME OF MISCELLANEOUS TFA DERIVATIVES (SE-52)

Temperature (°C)	110	140	160
Carrier, N ₂ (ml/min)	28.5	25.0	23.1
<i>myo</i> -Inositol	1.00 (2.5 min)	1.00 (0.9 min)	1.00 (0.6 min)
Methyl α -D-glucopyranoside	3.20	3.00	2.17
Methyl β -D-glucopyranoside	3.48	3.22	2.42
Methyl 6-amino-6-deoxy- α -D-glucopyranoside	—	9.11	5.79
Sucrose	—	—	10.01
Trehalose	—	—	15.38