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GAS–LIQUID CHROMATOGRAPHY AND MASS-SPECTRAL ANALYSIS OF PER-*O*-TRIMETHYLSILYL ACYCLIC KETOXIME DERIVATIVES OF NEURAMINIC ACID

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ABSTRACT

The per-*O*-trimethylsilyl derivatives of the acyclic *O*-methyl- and *O*-(trimethylsilyl)ketoximes of both the methyl and ethyl esters of *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid, and also the acyclic *O*-benzylketoximes of the methyl esters of these *N*-acylneuraminic acids were synthesized. In addition, the per-*O*-trimethylsilyl derivatives of the acyclic *O*-trimethylsilylketoximes and the *O*-trimethylsilyl(^{15}N)ketoximes of the trideuteriomethyl esters of *N*-acetyl- and *N*-glycolylneuraminic acid were prepared. For comparison, the per-*O*-trimethylsilyl derivatives of the cyclic ethyl esters of the two *N*-acylneuraminic acids were synthesized and all compounds were studied by gas–liquid chromatography and combined gas–liquid chromatography–mass spectrometry.

INTRODUCTION

N-Acylneuraminic (sialic) acids are a commonly occurring class of 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acids found in glycoproteins and glycolipids[1,2]. In Nature, the amino group has always been found substituted by an acetyl or glycolyl group. In addition, certain glycoproteins have mono- and di-acetyl esters[3–6] at O-4, -7, -8 and/or -9, and a lactyl ester at O-9 has been identified[7–9]. The identification of the parent compounds, *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGl), by g.l.c.–m.s. has been accomplished by direct trimethylsilylation[10] or by trimethylsilylation of the methyl ester[11] or methyl ester methyl glycosides[12]. Furthermore, the permethylated methyl ester methyl glycosides of NeuAc and NeuGl[13], the per-*O*-acetylated methyl ester methyl glycoside of NeuAc[14], the partially *O*-methylated methyl ester methyl glycosides of NeuAc[15,16], and the acyclic, per-*O*-acetylated methyl ester of borohydride-reduced NeuAc[17] have been analyzed by mass spectrometry.

Recently, this laboratory has reported on the development of a stable, volatile, acyclic derivative of the 2-acetamido-2- deoxyaldohexoses that permits their individual quantification by g.l.c.[18,19]. The acyclic form of the hexosamines was produced by

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treatment with *O*-methylhydroxylamine to produce the corresponding *O*-methyl-aldoxime. These compounds were prepared for g.l.c. analysis by peracetylation or pertrimethylsilylation. In this report, we describe the derivatization and separation of NeuAc and NeuGl as their per-*O*-trimethylsilylated esterified acyclic ketoximes and their identification by g.l.c. and g.l.c.–m.s.

RESULTS AND DISCUSSION

The formation of the methyl esters of NeuAc and NeuGl in anhydrous methanol by using-exchange resin in the H^+ form, as described in the Experimental section, was >98% complete after 2 h at room temperature, and there was no evidence of glycoside formation. Increasing the temperature to 40° reduced the reaction time to one h with no detectable starting material or methyl glycoside observed. The quantitative formation of the ethyl esters in anhydrous ethanol at room temperature and 40° required 3 h and 90 min, respectively. Synthesis of the hydroxy-, *O*-methyl-, and *O*-benzyl-ketoximes of the methyl and ethyl esters of NeuAc and NeuGl was normally complete in <2 min at room temperature with the oximation reagents described. In contrast, attempts to make the acyclic ketoximes of the free acids, or of their ammo-

TABLE I

RETENTION TIMES FOR THE ACYCLIC AND CYCLIC DERIVATIVES OF *N*-ACETYL- AND *N*-GLYCOLYL-NEURAMINIC ACID SEPARATED BY GAS–LIQUID CHROMATOGRAPHY[a]

Per-O-*trimethylsilylated derivative*	1.5% SE-52[b]	3% SP-2550[c]
N-*Acetylneuraminic acid*		
1 Me_3Si ester (cyclic)	6.12	7.06
2 Methyl ester (cyclic)	5.41	7.00
3 Methyl ester *O*-methylketoxime	8.06	9.50
4 Methyl ester *O*-Me_3Siketoxime	8.48(8.36)	9.50
5 Methyl ester *O*-benzylketoxime	11.40	13.52
6 Ethyl ester (cyclic)	5.55	7.05
7 Ethyl ester *O*-methylketoxime	8.12	10.00
8 Ethyl ester *O*-Me_3Siketoxime	8.54	10.15
N-*Glycolylneuraminic acid*		
9 Me_3Si ester (cyclic)	8.18	9.56
10 Methyl ester (cyclic)	7.47	9.50
11 Methyl ester *O*-methylketoxime	10.30	12.06
12 Methyl ester *O*-Me_3Siketoxime	11.15	12.16
13 Methyl ester *O*-benzylketoxime	14.22	16.44
14 Ethyl ester (cyclic)	7.46	9.56
15 Ethyl ester *O*-methylketoxime	10.27	12.58
16 Ethyl ester *O*-Me_3Siketoxime	11.05	13.18

[a]Retention times given in min. [b]1.5% SE-52 on Chromosorb W-HP, 100–120 mesh, 1.83-m nickel column, 2.0 min hold at 200°, programmed to 260° at 6°/min, 24 mL/min nitrogen flow-rate. Injector/detector temperature = 240°. [c]3% SP-2250 (McReynold's constants approximating OV-17) on Suplecoport 100–120 mesh, 1.83-m nickel column, programmed as described in [b].

TABLE II

INTERPRETATION AND RELATIVE INTENSITIES OF SOME IMPORTANT FRAGMENT-IONS IN THE MASS SPECTRA OF VARIOUS ESTERIFIED ACYCLIC KETOXIME-PER(TRIMETHYLSILYL)ATED DERIVATIVES OF *N*-ACETYLNEURAMINIC ACID[a]

Scheme[b] *symbol*	*Fragment*	*Ester (R′)* *Ketoxime (R″)*	*Methyl* O-*methyl* (3) m/z[a]	*Ethyl* O-*methyl* (7) m/z	*Methyl* O-*SiMe*$_3$ (4) m/z	*Ethyl* O-*SiMe*$_3$ (8) m/z	*Methyl* O-*benzyl* (5) m/z
A	M^+		712 (0.2)	726 (0.9)	770 (1.1)	784 (0.6)	788 (1.6)
A − 15	$M - CH_3$		697 (39.6)	711 (32.1)	755 (30.0)	769 (23.9)	773 (45.7)
B	$M - CH_2OSiMe_3$		609 (4.7)	623 (1.9)	667 (1.5)	681 (4.7)	685 (4.2)
L − 15	$M - CO_2R' - OR'' - CH_3$		607 (6.2)	607 (4.1)	607 (8.8)	607 (3.1)	607 (9.2)
B − 90	$M - CH_2OSiMe_3 - CHOSiMe_3$		519 (4.0)	533 (5.0)	577 (6.2)	591 (4.8)	595 (8.7)
C	$M - CH_2OSiMe_3 - CHOSiMe_3$		507 (44.0)	521 (37.9)	565 (33.8)	579 (46.3)	583 (38.8)
G	$(CH{=}N^+HAc)HCOSiMe_3HCOSiMe_3HCOSiMe_3CH_2OSiMe_3$		480 (12.1)	480 (18.7)	480 (14.2)	480 (20.0)	480 (14.0)
B − 131	$M - CH_2OSiMe_3 - (CH_2{=}C\text{-}O) - OSiMe_3$		478 (41.8)	492 (28.3)	536 (28.0)	550 (39.4)	554 (42.9)
C − 90	$M - CH_2OSiMe_3 - CHOSiMe_3 - HOSiMe_3$		417 (17.2)	431 (4.3)	475 (5.3)	489 (6.2)	493 (6.7)
L − 205	$M - CO_2R' - OR'' - CH_2OSiMe_2CHOSiMe_3$		417 (17.2)	417 (3.2)	417 (7.4)	417 (3.9)	417 (5.7)
D	$M - CH_2OSiMe_3 - CHOSiMe_3 - CHOSiMe_3$		405 (21.3)	419 (22.6)	463 (24.8)	477 (20.4)	481 (27.7)
G − 90	$(CH{=}N^+HAc)CHOSiMe_3COSiMe_3{=}CHCH_2OSiMe_3$		390 (17.6)	390 (23.7)	390 (13.2)	390 (14.8)	390 (16.2)
L − 234	$M - CO_2R' - OR'' - CH_2OSiMe_3 - (CH_2{=}C{=}O) - OSiMe_3$		388 (4.2)	388 (2.2)	388 (4.1)	388 (3.7)	388 (2.6)
C − 131	$M - CH_2OSiMe_3 - CHOSiMe_3 - (CH_2{=}C{=}O) - OSiMe_3$		376 (15.7)	390 (23.7)	434 (19.1)	448 (16.2)	452 (17.5)
H − 90	$CH_2OSiMe_3CH{=}COSiMe_3CH{=}O^+SiMe_3$		319 (52.1)	319 (45.5)	319 (47.9)	319 (43.6)	319 (47.0)
L − 307	$M - CO_2R' - OR''CH_2OSiMe_3 - CHOSiMe_3 - CHOSiMe_3$		315 (9.2)	315 (5.6)	315 (8.2)	315 (4.0)	315 (6.1)
I	$CH_2OSiMe_3CHOSiMe_3CH{=}O^+SiMe_3$		307 (20.6)	307 (15.3)	307 (16.1)	307 (13.9)	307 (20.2)
E	$M - CH_2OSiMe_3 - CHOSiMe_3 - CHOSiMe_3 - CHOSiMe_3$		303 (33.8)	317 (46.6)	361 (25.9)	375 (27.8)	379 (24.9)
G − 180	$(CH{=}N^+HAc)COSiMe_3{=}CHCH{=}CHOSiMe_3$		300 (15.4)	300 (23.3)	300 (14.7)	300 (13.6)	300 (18.9)
L − 324	$M - CO_2R' - OR'' - CH_2OSiMe_3 - HOSiMe_3 - (CH_2{=}C{=}O) - OSiMe_3$		298 (2.2)	298 (1.3)	298 (2.0)	298 (1.4)	298 (2.0)
F	$CO_2R'C{=}NOR''CH_2CH{=}O^+SiMe_3$		232 (38.8)	246(100.0)	290 (30.3)	304 (35.3)	308 (44.5)
C − 221	$M - CH_2OSiMe_3 - CHOSiMe_3 - HOSiMe_3 - (CH_2{=}C{=}O) - OSiMe_3$		286 (23.3)	300 (13.3)	344 (17.2)	358 (12.5)	362 (17.1)
L − 336	$M - CO_2R' - OR'' - CH_2OSiMe_3 - CHOSiMe_3 - (CH_2{=}C{=}O) - OSiMe_3$		286 (23.3)	286 (9.4)	286 (14.0)	286 (10.2)	286 (9.1)
G − 234	$480 - (CH_2{=}C{=}O) - OSiMe_3 - CH_2OSiMe_3$		246(100.0)	246(100.0)	246(100.0)	246(100.0)	246(100.0)
	$CHOSiMe_3CH{=}O^+SiMe_3$		217(111.0)	217 (76.9)	217(101.0)	217 (97.0)	217 (82.0)
J	$CH_2OSiMe_3CH{=}O^+SiMe_3$		205 (57.1)	205 (49.4)	205 (51.7)	205 (54.2)	205 (44.6)
	$(CHNHAc){=}CH\text{-}CH{=}O^+SiMe_3$		186 (27.9)	186 (20.2)	186 (25.2)	186 (22.2)	186 (30.1)
K	$CH_2{=}O^+SiMe_3$		103 (44.8)	103 (59.2)	103 (34.8)	103 (72.2)	103 (63.4)

[a]The intensities (in parentheses) of fragment ions of each compound are given relative to that of *m/z* 246 for that compound. [b]Scheme letters correspond to the dsignated fragment-ion letters used in Fig. 1.

TABLE III

INTERPRETATION AND RELATIVE INTENSITIES OF SOME IMPORTANT FRAGMENT IONS IN THE MASS SPECTRA OF VARIOUS ESTERIFIED ACYCLIC KETOXIME-PER(TRIMETHYLSILYL)ATED DERIVATIVES OF *N*-GLYCOLYLNEURAMINIC ACID[a]

Scheme[b] *symbol*	*Fragment*	*Ester (R′) Ketoxime (R″)*	*Methyl* O-*methyl* **(11)** m/z[a]	*Ethyl* O-*methyl* **(15)** m/z	*Methyl* O-*SiMe*$_3$ **(12)** m/z	*Ethyl* O-*SiMe*$_3$ **(16)** m/z	*Methyl* O-*benzyl* **(13)** m/z
A	M^+		800 (0.9)	814 (1.1)	858 (0.7)	872 (1.2)	876 (0.7)
A − 15	M − CH_3		785 (34.3)	799 (28.5)	843 (41.2)	857 (36.8)	861 (32.3)
B	M − CH_2OSiMe_3		697 (8.2)	711 (4.1)	755 (2.6)	769 (1.9)	773 (3.7)
L − 15	M − CO_2R'' − OR'' − CH_3		695 (7.2)	695 (14.4)	695 (13.7)	695 (6.2)	695 (8.9)
B − 90	M − CH_2OSiMe_3 − $HOSiMe_3$		607 (4.1)	621 (3.7)	665 (4.2)	679 (3.4)	683 (3.9)
C	M − CH_2OSiMe_3 − $CHOSiMe_3$		595 (36.3)	609 (40.0)	653 (29.8)	667 (33.8)	671 (37.6)
G	$(CH{=}N^+HCOCH_2OSiMe_3CHOSiMe_3CHOSiMe_3CHOSiMe_3CH_2OSiMe_3$		568 (13.6)	568 (16.1)	568 (14.5)	568 (12.7)	568 (14.1)
B − 131	M − CH_2OSiMe_3 − $(COCH_2OSiMe_3)$		566 (29.2)	580 (23.0)	624 (21.2)	638 (27.0)	642 (32.0)
C − 90	M − CH_2OSiMe_3 − $CHOSiMe_3$ − $HOSiMe_3$		505 (14.3)	519 (5.3)	563 (4.7)	577 (3.2)	581 (5.0)
L − 205	M − CO_2R' − OR'' − CH_2OSiMe_3 − $CHOSiMe_3$		505 (14.3)	505 (7.8)	505 (9.3)	505 (3.3)	505 (8.1)
D	M − CH_2OSiMe_3 − $CHOSiMe_3$ − $CHOSiMe_3$		493 (22.7)	507 (23.2)	551 (19.7)	565 (17.3)	569 (19.9)
G − 90	$(CH{=}N^+HCOCH_2OSiMe_3CHOSiMe_3COSiMe_3{=}CHCH_2OSiMe_3$		478 (14.2)	478 (25.7)	478 (15.2)	478 (14.9)	478 (11.4)
L − 234	M − CO_2R' − OR'' − CH_2OSiMe_3 − $(COCH_2OSiMe_3)$		476 (4.0)	476 (3.3)	476 (2.6)	476 (3.1)	476 (3.1)
C − 131	M − CH_2OSiMe_3 − $CHOSiMe_3$ − $(COCH_2OSiMe_3)$		464 (12.7)	478 (20.0)	522 (19.7)	536 (12.7)	540 (13.1)
H − 90	$CH_2OSiMe_3CH{=}COSiMe_3CH{=}O^+SiMe_3$		319 (41.7)	319 (56.6)	319 (39.7)	319 (46.9)	319 (54.0)
L − 307	M − CO_2R' − $OR''CH_2OSiMe_3$ − $CHOSiMe_3$ − $CHOSiMe_3$		403 (7.3)	403 (4.2)	403 (6.1)	403 (2.5)	403 (3.6)
I	$CH_2OSiMe_3CHOSiMe_3CH{=}O^+SiMe_3$		307 (15.9)	307 (16.1)	307 (11.7)	307 (14.2)	307 (15.7)
E	M − CH_2OSiMe_3 − $CHOSiMe_3$ − $CHOSiMe_3$ − $CHOSiMe_3$		391 (32.6)	405 (41.0)	449 (33.1)	463 (36.9)	467 (29.7)
G − 180	$(CH{=}N^+HCOCH_2OSiMe_3)COSiMe_3{=}CHCH{=}CHOSiMe_3$		388 (16.2)	388 (17.5)	388 (15.2)	388 (16.4)	388 (17.0)
L − 324	M − CO_2R' − OR'' − CH_2OSiMe_3 − $HOSiMe_3$ − $(COCH_2OSiMe_3)$		386 (3.2)	386 (3.8)	386 (2.7)	386 (1.0)	386 (4.7)
F	$CO_2R'C{=}NOR''CH_2CH{=}O^+SiMe_3$		232 (38.8)	246 (41.2)	290 (49.2)	304 (37.5)	308 (41.3)
C − 221	M − CH_2OSiMe_3 − $CHOSiMe_3$ − $HOSiMe_3$ − $(COCH_2OSiMe_3)$		374 (16.1)	388 (17.5)	432 (18.9)	446 (17.9)	450 (10.2)
L − 336	M − CO_2R' − OR'' − CH_2OSiMe_3 − $CHOSiMe_3$ − $(COCH_2OSiMe_3)$		374 (16.1)	374 (11.2)	374 (7.6)	374 (10.9)	374 (8.6)
G − 234	568 − $(COCH_2OSiMe_3)$ − CH_2OSiMe_3		334(100.0)	334(100.0)	334(100.0)	334(100.0)	334(100.0)
	$CHOSiMe_3{=}CHCH{=}O^+SiMe_3$		217 (91.0)	217 (87.2)	217(102.2)	217 (91.4)	217 (97.7)
J	$CH_2OSiMe_3CH{=}O^+SiMe_3$		205 (62.5)	205 (59.1)	205 (79.3)	205 (56.9)	205 (72.9)
	$(CHNHCOCH_2OSiMe_3){=}CHCH{=}O^+SiMe_3$		274 (23.8)	274 (27.3)	274 (24.7)	2774 (25.0)	274 (34.3)
K	$CH_2{=}O^+SiMe_3$		103 (58.6)	103 (82.2)	103 (86.7)	103 (64.4)	103 (65.0)

[a]The intensities (in parentheses) of fragment ions of each compound are given relative to that of *m/z* 334 for that compound. [b]Scheme letters correspond to the designated fragment-ion letters used in Fig. 1.

nium salts, failed. This might be attributed to the negative charge on the C-1 carboxylate anion, which could interfere with the attack of a nucleophile such as hydroxylamine on the C-2 carbonyl group by preventing protonation of the carbonyl oxygen atom. The esterified analogs, however, readily form ketoximes. Upon trimethylsilylation, all acyclic derivatives were readily separated on the g.l.c. liquid phases tested. In Table I, the retention times of all per-*O*-trimethylsilylated cyclic derivatives and acyclic ketoxime derivatives are given for a representative non-polar (SE-52) and an intermediate-polarity (SP-2250) g.l.c. phase. The silylated acyclic ketoxime derivatives of both NeuAc and NeuGl displayed longer retention-times than their non-oximated, cyclic per-*O*-trimethylsilylated methyl, ethyl, or trimethylsilyl (Me_3Si) esters of both sugars. With the exception of the per-*O*-trimethylsilylated methyl ester *O*-Me_3Si ketoxime of NeuAc, when separated on g.l.c. phase SE-52 all of the ketoxime derivatives studied produced single, symmetrical peaks when subjected to g.l.c. analysis. The noted exception displayed two poorly separated chromatographic peaks which evidently reflect presence of the *syn* and *anti* forms of this ketoxime. It is assumed that the *syn* and *anti* forms of the ketoxime structure are also present in the other volatile ketoxime derivatives of NeuAc and NeuGl studied, but, like the *syn* and *anti* forms of the *O*-methylaldoxime derivatives of the hexosamines[18], they were not resolved. As expected, because of the presence of an additional trimethylsilylatable group, all NeuGl derivatives demonstrated longer g.l.c. retention-times than their *N*-acetyl analogs. In general, each ketoxime derivative, as already noted, produced a single g.l.c. peak which did not exhibit the peak tailing that is shown by the cyclic (α and β forms) Me_3Si derivatives of NeuAc when separated on SE-52 and SP-2250 g.l.c. phases. Relative to the per-*O*-trimethylsilylated, cyclic Me_3Si ester Me_3Si glycoside derivatives, which gradually decompose after 3 h under nitrogen at room temperature, all of the ketoxime derivatives, under the same conditions, were stable for >2 weeks, with no evidence of decomposition. Furthermore, as compared with the nonoximated cyclic derivatives, the ketoxime derivatives of an equivalent amount of esterified NeuAc and NeuGl showed an increased g.l.c. detector response. The *O*-benzylketoximes showed the greatest increase in response (38%) followed by the *O*-Me_3Si ketoximes (30%), and the *O*-methylketoximes (15%).

Tables II and III show the major fragment-ions produced by the acyclic per-*O*-trimethylsilyl derivatives of the methyl and ethyl ester *O*-methylketoximes, the methyl and ethyl ester *O*-Me_3Si ketoximes, and the methyl ester *O*-benzylketoximes of NeuAc and NeuGl, respectively. Fig. 1 presents the apparent fragmentation scheme of these esterified ketoxime derivative. Each parent fragment is given a symbol letter in the scheme. These letters correspond to the symbol letters used in Tables I, II and III for fragment ions, thereby relating each fragment ion to the overall molecular-fragmentation scheme in Fig. 1. Further fragmentation of a parent ion by the loss(es) of $HOSiMe_3$, $CH_2=C=O$, $COCH_2OSiMe_3$, and so on, is indicated in Tables II and III by the subtraction of the eliminated mass from the scheme symbol, as well as molecularly by the fragment-ion presented.

R'= CH_3; CH_2CH_3 or CD_3
R"= $-SiMe_3$; $-CH_3$ or CH_2Ph
R'"= $-COCH_3$ or $-COCH_2OSiMe_3$

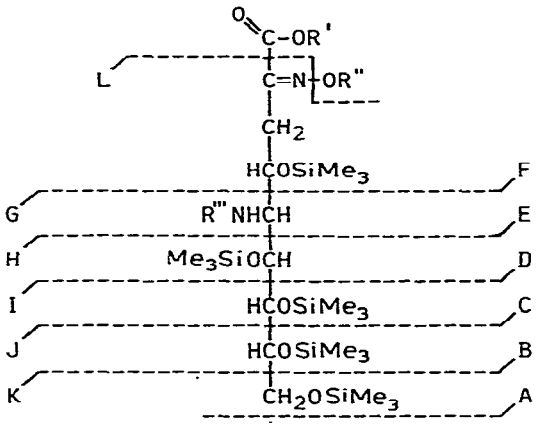

Fig. 1. General mass-spectral fragmentation scheme produced by the per-*O*-trimethylsilylated, acyclic 1-ester-2-ketoxime derivatives of *N*-acetyl- and *N*-glycolylneuraminic acid. Scheme letters are used as symbols for identification of fragment ions in Tables II, III and IV.

All of the acyclic ketoxime derivatives, regardless of the type of ester (at C-1), type of ketoxime (at C-2), or the *N*-acyl group (at C-5) produced the same general fragmentation patterns, as depicted in Fig. 1. Those fragment-ions bearing the foregoing chemical modifications (namely, fragments A–F), however, varied in molecular weight from one derivative to the next. Fragment ions not bearing any of the foregoing chemical differences (that is, fragments H–K) were, as expected, identical. As the fragmentation pattern is the same for all derivatives, as deduced by comparison of their individual mass spectra, the pattern is, for ease of presentation, discussed by examining the fragment ions of the first acyclic derivatives in Table II and III; that is, the per-*O*-trimethylsilyl derivatives of the methyl ester *O*-methylketoxime of NeuAc (Table II) and NeuGl (Table III). By comparing the fragmentation scheme in Fig. 1 with the "scheme symbol" and "fragment" columns of Tables II and III, it is apparent that all parent fragment-ions (fragments A–K) are present, with the exception of fragment H. This last fragment constitutes the C-6–C-9 segment of the acyclic derivatives, which is observed in the mass spectrum after the loss of $HOSiMe_3$ (H − 90). Fragment L in Fig. 1 is discussed later.

The methyl ester *O*-methylketoxime derivative of NeuAc (NeuGl) shows a weak molecular ion (M^+) at *m/z* 712 (800) and an intense M − 15 ion at *m/z* 697 (785). Fragment B (M − CH_2OSiMe_3) is noted as a weak ion at *m/z* 609 (697). This parent fragment-ion appears to be further fragmented by losing $HOSiMe_3$ to produce ion B − 90 at *m/z* 519 (607), or, alternatively, by eliminating $CH_2{=}C{=}O$ (or $COCH_2OSiMe_3$ for NeuGl) and trimethylsiloxide ($OSiMe_3$) to form the intense

ion B — 131 at *m/z* 478 (566). Not shown in these Tables, because of its low relative intensity (<0.1%), is fragment ion B — 221, representing the concomitant loss of $HOSiMe_3$, $CH_2=C=O$, and $SiMe_3$ from the parent B fragment. Fragment C of the acyclic derivative is observed as an intense ion at *m/z* 507 (595) and serves as the parent ion from which is eliminated $HOSiMe_3$ (C-90), $CH_2=C=O$, and $SiMe_3$ (or $COCH_2OSiMe_3$ for NeuGl) (C — 131), or both (C — 221), to produce ions of moderate intensity at *m/z* 417 (505), 376 (464), and 286 (374), respectively. It should be noted that, in this case only, the fragment ions for the methyl ester *O*-methylketoxime of NeuAc (NeuGl) at both *m/z* 417 (505) and *m/z* 286 (374) could each arise from two different fragments of the same mass, that is, L — 205 and C — 90 for *m/z* 417 (505) and, L — 336 and C — 221 for *m/z* 286 (374). Fragment ions of high intensity at *m/z* 405 (493), 303 (391), and notably at 232 (232), represent fragments D, E, and F, respectively.

Ion F is the same for both NeuAc and NeuGl as the derivative segment C-1–C-4 is the same for both compounds. In addition to fragment F (C-1–C-4), cleavage between C-4 and C-5 of the acyclic derivative produces the corresponding fragment G (C-5–C-9) at *m/z* 480 (568). Sequential eliminations of $HOSiMe_3$ from this latter fragment (G) produces fragments C — 90 and G — 180 at *m/z* 390 (478) and 300 (388), respectively. By a different elimination scheme, fragment G — 234 at *m/z* 246 (334), which serves as the base ion in Tables II and III, arises from the parent fragment G via the loss of $CH_2=C=O$, $SiMe_3$, and CH_2OSiMe_3 from NeuAc derivatives, and of $COCH_2OSiMe_3$ and CH_2OSiMe_3 from NeuGl derivatives. As previously noted, fragment H (C-6–C-9) is an ion of low intensity. Upon elimination of $HOSiMe_3$ from fragment H, the resulting fragment H — 90, *m/z* 319 (319), forms one of the most intense ions in the spectrum. Fragment I, representing C-7–C-9 (Fig. 1) of the derivatives, is seen as a moderately intense ion at *m/z* 307 (307). The prominent fragment-ion at *m/z* 217 (217) is apparently formed from fragment I via the elimination of $HOSiMe_3$. Fragment ions at *m/z* 205 (205) and 103 (103) constitute fragment J (C-8–C-9) and fragment K (C-9), respectively. It should be noted that fragmentations at *m/z* 319, 307, 217, 205, and 103 are also generally observed in the mass spectrum of the Me_3Si derivatives of carbohydrates and their corresponding alditols[20,21].

As previously indicated, all acyclic, esterified ketoxime derivatives of NeuAc and NeuGl present essentially the same fragmentation pattern. From derivative to derivative, however, those fragment-ions possessing the varied ester and ketoxime groups show the expected mass-unit (m.u.) shift. In Table II, the fragment ions containing both of these variables for the ethyl ester *O*-methylketoxime, methyl ester *O*-Me_3Si ketoxime, ethyl ester *O*-Me_3Si ketoxime, and methyl ester *O*-benzylketoxime of NeuAc show a shift of +14, +58, +78, and +76 m.u., respectively, when compared with the related fragment-ions from the methyl ester *O*-methylketoxime derivative already discussed in detail. These same shifts are observed for the parallel NeuGl analogs in Table III when compared with the methyl ester *O*-methylketoxime of NeuGl. Lastly, fragment ions in Table III of each NeuGl derivative in which the

TABLE IV

INTERPRETATION AND RELATIVE INTENSITIES OF SOME IMPORTANT FRAGMENT-IONS IN THE MASS SPECTRA OF THE PER(TRIMETHYLSILYL)ATED ACYCLIC TRIDEUTERIOMETHYL ESTER *O*-(TRIMETHYLSILYL)KETOXIME AND TRIDEUTERIOMETHYL ESTER *O*-TRIMETHYLSILYL (N^{15}) KETOXIME DERIVATIVES OF *N*-ACETYL- AND *N*-GLYCOLYL-NEURAMINIC ACID[a]

Scheme[b] *symbol*	*Fragment*	*Ester (R′) ketoxime (R″)*	N-*Acetylneuraminic acid*		N-*Glycolylneuraminic acid*	
			Methyl (d_3) N-*OSiMe*$_3$ (17) m/z[a]	*Methyl* (d_3) N^{15}-*OSiMe*$_3$ (18) m/z	*Methyl* (d_3) N-*OSiMe*$_3$ (19) m/z	*Methyl* (d_3) N^{15}-*OSiMe*$_3$ (20) m/z
A	M^{+}		773 (0.9) (+3)	774 (0.6) (+4)	861 (1.1) (+3)	862 (1.3) (+4)
A − 15	$M - CH_3$		758 (36.2) (+3)	759 (31.3) (+4)	846 (39.2) (+3)	847 (28.2) (+4)
B	$M - CH_2OSiMe_3$		670 (1.9) (+3)	671 (2.2) (+4)	758 (1.7) (+3)	759 (1.0) (+4)
L − 15	$M - CO_2R' - OR'' - CH_3$		607 (9.9) (0)	608 (7.4) (+1)	695 (10.3) (0)	696 (9.0) (+1)
B − 90	$M - CH_2OSiMe_3 - HOSiMe_3$		580 (5.4) (+3)	581 (3.4) (+4)	668 (5.7) (+3)	669 (4.2) (+4)
C	$M - CH_2OSiMe_3 - CHOSiMe_3$		568 (37.0) (+3)	569 (35.9) (+4)	656 (33.3) (+3)	657 (41.6) (+4)
G	$(CH{=}N^{+}HR^{c})CHOSiMe_3CHOSiMe_3CH_2OSiMe_3$		480 (11.7) (0)	480 (13.2) (0)	568 (15.4) (0)	568 (13.6) (0)
B − 131	$M - CH_2OSiMe_3R^{d}$		539 (28.0) (+3)	540 (29.8) (+4)	627 (24.9) (+3)	628 (27.6) (+4)
C − 90	$M - CH_2OSiMe_3 - CHOSiMe_3 - HOSiMe_3$		478 (3.2) (+3)	479 (2.6) (+4)	566 (4.1) (+3)	567 (3.8) (+4)
L − 205	$M - CO_2R' - OR'' - CH_2OSiMe_3 - CHOSiMe_3$		417 (6.4) (0)	418 (7.2) (+1)	505 (7.6) (0)	506 (6.3) (+1)
D	$M - CH_2OSiMe_3 - CHOSiMe_3 - CHOSiMe_3$		466 (26.6) (+3)	467 (23.8) (+4)	554 (21.2) (+3)	555 (22.6) (+4)

G − 90	$(CH{=}N^{+}HR^{c})CHOSiMe_3COSiMe_3{=}CHCH_2OSiMe_3$	390 (13.4)	(0)	390 (20.2)	(0)	478 (16.0)	(0)	478 (15.7)	(0)
L − 234	$M - CO_2R' - OR'' - CH_2OSiMe_3\text{-}R^{d}$	388 (3.3)	(0)	389 (3.9)	(+1)	476 (2.9)	(0)	477 (3.1)	(+1)
C − 131	$M - CH_2OSiMe_3 - CHOSiMe_3\text{-}R^{d}$	437 (15.3)	(+3)	438 (14.4)	(+4)	525 (17.3)	(+3)	526 (12.5)	(+4)
H − 90	$CH_2OSiMe_3CH{=}COSiMe_3CH{=}O^{+}SiMe_3$	319 (52.3)	(0)	319 (49.0)	(0)	319 (44.7)	(0)	319 (50.4)	(0)
L − 307	$M - COOR' - OR''CH_2OSiMe_3CHOSiMe_3CHOSiMe_3$	315 (6.6)	(0)	316 (7.3)	(+1)	403 (5.0)	(0)	404 (5.1)	(+1)
I	$CH_2OSiMe_3CHOSiMe_3CH{=}O^{+}SiMe_3$	307 (13.7)	(0)	307 (15.1)	(0)	307 (14.4)	(0)	307 (15.2)	(0)
E	$M - CH_2OSiMe_3 - CHOSiMe_3 - CHOSiMe_3 - CHOSiMe_3$	364 (31.3)	(+3)	365 (30.6)	(+4)	452 (37.8)	(+3)	453 (36.9)	(+4)
G − 180	$(CH{=}N^{+}HR^{c})COSiMe_3{=}CHCH{=}CHOSiMe_3$	300 (14.4)	(0)	300 (16.1)	(0)	388 (13.7)	(0)	388 (15.2)	(0)
L − 324	$M - COOR' - OR'' - CH_2OSiMe_3 - HOSiMe_3 - R^{d}$	298 (2.1)	(0)	299 (1.9)	(+1)	386 (1.7)	/0)	387 (2.3)	(+1)
F	$CO_2R'C{=}NOR''CH_2CH{=}O^{+}SiMe_3$	293 (31.1)	(+3)	294 (33.4)	(+4)	293 (30.8)	(+3)	294 (33.9)	(+4)
C − 221	$M - CH_2OSiMe_3 - CHOSiMe_3 - HOSiMe_3 - R^{d}$	347 (18.2)	(+3)	347 (19.7)	(+4)	435 (12.7)	(+3)	436 (10.3)	(+4)
L − 336	$M - CO_2R' - OR'' - CH_2OSiMe_3 - CHOSiMe_3 - R^{d}$	286 (8.2)	(0)	287 (7.3)	(+1)	374 (7.7)	(0)	375 (8.8)	(+1)
G − 234	$G - CH_2OSiMe_3 - R^{d}$	246(100.0)	(0)	246(100.0)	(0)	334(100.0)	(0)	334(100.0)	(0)
	$CHOSiMe_3{=}CHCH{=}O^{+}SiMe_3$	217(122.0)	(0)	217(115.2)	(0)	217 (88.3)	(0)	217(106.0)	(0)
J	$CH_2OSiMe_3CH{=}O^{+}SiMe_3$	205 (48.8)	(0)	205 (56.2)	(0)	205 (81.7)	(0)	205 (76.4)	(0)
	$(CHNHR^{c}){=}CHCH{=}O^{+}SiMe_3$	186 (29.9)	(0)	186 (26.2)	(0)	274 (27.0)	(0)	274 (26.5)	(0)
K	$CH_2{=}O^{+}SiMe_3$	103 (49.4)	(0)	103 (56.4)	(0)	103 (73.6)	(0)	103 (67.8)	(0)

[a]The intensities of fragment ions of each compound are given relative to *m/z* 246 for each of the *N*-acetylneuraminic acid derivatives and *m/z* 334 for each of the *N*-glycolylneuraminic acid derivatives. The shift in fragment-ion mass due to the presence of the stable isotope(s) is indicated in parentheses (+) following fragment-ion intensity. [b]Scheme letters correspond to the designated fragment-ion letters used in Fig. 1. Abbreviations: R^{c} = − ($COCH_3$) or − ($COCH_2OSiMe_3$) for *N*-acetyl- and *N*-glycolylneuraminic acid derivatives, respectively. R^{d} = − ($COCH_3$) − $OSiMe_3$ or − ($COCH_2OSiMe_3$) for *N*-acetyl- and *N*-glycolylneuraminic acid derivatives, respectively.

N-acyl function is present show a shift of +88 m.u. when compared with the analogous fragment-ions of NeuAc in Table II.

As shown in Fig. 1, fragment L is formed by the concomitant elimination, from the acyclic derivatives, of the C-1 ester (CO_2R') and the *O*-methyl, *O*-$SiMe_3$, or *O*-benzyl groups (OR″) of the ketoximes. This cleavage produces, in effect, a nitrile from the original carbonyl group (C-2). Fragment ions displaying this type of fragmentation are consistently noted in the mass spectra of the NeuAc and NeuGl derivatives of the acyclic trideuteriomethyl ester *O*-Me_3Si ketoxime and the trideuteriomethyl ester *O*-Me_3Si (^{15}N)ketoxime of both NeuAc and NeuGl were prepared ketoxime and ester groups are not present. By comparing the masses of the type L fragment-ions of Table II (NeuAc) with Table III (NeuGl), it is readily apparent that the difference in the type of *N*-acyl function on C-5 is still manifested. To provide further evidence that the type L fragmentation occurs, the per-*O*-trimethylsilylated derivatives of the acyclic trideuteriomethyl ester *O*-Me_3Si ketoxime and the trideuteriomethyl ester *O*-Me_3Si (^{15}N)ketoxime of both NeuAc and NeuGl were prepared and subjected to g.l.c.–m.s. Compared with the mass spectra of the methyl ester *O*-Me_3Si ketoximes of NeuAc and NeuGl (Tables II and III), the trideuteriomethyl ester *O*-Me_3Si ketoximes of both sugars in Table IV showed no shift in fragment-ion mass, indicating the absence of the ester (CO_2CD_3). Similarly, fragments G, H, I, J, K, and related modified ions, which do not possess the C-1 ester, did not demonstrate a shift in the mass of their recorded fragment-ions (Tables II and III). Fragment ions possessing the trideuteriomethyl ester at C-1 show a shift of +3 m.u. As it is postulated that the nitrogen atom of the ketoximes is not eliminated from the original C-2 carbonyl carbon atom (Fig. 1), the mass spectra of the trideuteriomethyl ester *O*-Me_3Si (^{15}N)ketoxime derivatives of NeuAc and NeuGl were recorded (Table IV). Compared with their respective derivatives in Tables II and III, which do not possess the foregoing stable isotopes, all fragment ions in Table IV that have both the C-1 ester (CO_2CD_3) and C-2 ketoxime (^{15}N-$OSiMe_3$) show a shift of +4 m.u. All fragment ions of the L type show only a shift of +1 m.u., indicating the presence of ^{15}N. As in Tables II and III, all L fragments failed to demonstrate a shift in ion mass because of either the type of substitution on the ketoximes (OR″) or on the type of ester at C-1 (CO_2R'), and that from Table IV, it is apparent that the ketoxime nitrogen atom is still present, it may be deduced that the type L fragments reflect the loss of the C-1 ester and OR″ from the ketoximes. Measurement of metastable transitions might provide further evidence as to the origin of the various fragments.

All fragments of type L are of relatively low intensity, suggesting that the concomitant elimination of CO_2R' and OR″ to produce the L fragment-ion is not primary. This conclusion is supported by the comparison of fragment ions in Tables II, III, and IV. It would appear that the major fragment-ions A — 15, C, B — 131, D, and C — 131, which possess an intact C-1 and C-2 segment, can fragment further and eliminate CO_2R' and OR″ to produce L — 15, L — 205, L — 234, L — 307, and L — 336, respectively. Fragment L itself, without modification, is not observed.

In conclusion, the methyl and ethyl esters of NeuAc and NeuGl readily form acyclic ketoximes which, when trimethylsilylated, are amenable to g.l.c. When subjected to mass-spectral analysis, these per-*O*-trimethylsilylated derivatives fragment in a pattern indicative of their acyclic structure (Fig. 1).

EXPERIMENTAL

Compounds. — *N*-Acetylneuraminic acid and *N*-glycolylneuraminic acid were purchased from Sigma Chemical Company. *N*-Glycolylneuraminic acid often contained a small amount of *N*-Acetylneuraminic acid as a contaminant. This was removed prior to use by a preparative method described by Kamerling *et al.*[11]. Hydroxylamine(^{15}N) hydrochloride was obtained from Prochem, Summit, New Jersey.

Derivatization. — The methyl esters of *N*-acetyl- and *N*-glycolyl-neuraminic acid were prepared by a modification of the method of Kuhn *et al.*[22]. To 5–100 μg of the respective *N*-acylneuraminic acid in a 1.0-mL, tapered vial equipped with a small Teflon stirring bar was added 0.2 mL of abs. methanol-treated and dried Dowex 50 × 8 (H^+ form) 100–120 mesh resin[23]. *myo*-Inositol was added as an internal standard. The mixture was stirred for 2 h at room temperature, or, alternatively, for 1 h at 40°. For the formation of the ethyl esters, abs. ethanol was used in lieu of methanol and the mixture was stirred for 3 h at room temperature or for 90 min at 40°. At the completion of each reaction, the resin and alcohol mixture was transferred to a small glass column, fitted with a small glass-wool plug, which emptied into a 3.0-mL Reactivial® (Pierce Chemical Company). The alcoholic effluent was collected, and the resin and original vial were each washed three times with 0.2 mL of the respective alcohol. The resultant washings were pooled and added to the 3.0-mL Reactivial®. Alcohol was then removed by placing the vial under a stream of dry air.

For the formation of the acyclic ketoximes of the esterified *N*-acylneuraminic acids, the following oximation mixtures were made by dissolving 3.6mM of either *O*-methylhydroxylamine hydrochloride[18,19], hydroxylamine hydrochloride, or *O*-benzylhydroxylamine hydrochloride in anhydrous methanol (1.0 mL) and pyridine (1.8 mL), and then adding 1-dimethylamino-2-propanol (1.8mM) (Aldrich Chemical Company). The prepared reagents are stable for >6 months in glass tubes with Teflon-lined screw caps at room temperature. The desired ketoxime was generated by adding 50 μL of the respective oximation reagent to the Reactivial® containing the esterified sugar. The vial was then capped and heated for 10 min at 50° and then the contents were evaporated to a syrup under a stream of dry air (~5 min). To the vial were then added sequentially pyridine (0.1 mL), trimethylsilylimidazole (0.1 mL), and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (0.1 mL) to form the trimethylsilyl ethers. It should be noted that, after trimethylsilylation, esterified sialic acids possessing unsubstituted hydroxylamine ketoximes form the substituted *O*-trimethylsilyketoxime. Per-*O*-trimethylsilylation of *N*-acetyl- and *N*-glycolylneuraminic acid as the cyclic

free acids or esters was performed as described, without treatment with the oximation reagent.

Analyses. — G.l.c. was performed with a Perkin–Elmer Sigma 3 gas chromatograph equipped with dual flame-ionization detectors and the following nickel columns (0.32 mm × 1.83 m): (*a*) 1.5% GC-SE-52 silicone rubber (Varian Aerograph) on 100–120 mesh Chromosorb W-HP, and, (*b*) 3% SP-2250 on 100–120 mesh Supelcoport (Supelco, Inc.). The same program was used for both columns: 2-min hold at 200° and then the oven temperature was raised to 260° at the rate of 6°/min with a nitrogen flow-rate of 24 mL/min.

For mass-spectral studies, g.l.c. columns were placed in an H and F gas chromatograph interfaced with a CEC model 21-110C mass spectrometer. Mass spectra were recorded at 70 eV with an ionization current of 50 μA, a source temperature of 250°, and a transfer temperature of 218°.

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REFERENCES

1 H. Tuppy and A. Gottschalk, in A. Gottschalk (Ed.), *Glycoproteins. Their Structure and Function*, Part A, Elsevier Publishing Co., Amsterdam/New York, 1972, pp. 403–449.
2 G. Blix and R. W. Jeanloz, in R. W. Jeanloz (Ed.), *Chemistry of Amino Sugars*, Vol. 1A, Academic Press, New York and London, 1969, pp. 213–265.
3 R. Schauer, *Angew. Chem. Int. Ed. Engl.*, 12 (1973) 127–138.
4 H.-P. Buscher, J. Casals-Stenzel, and R. Schauer, *Eur. J. Biochem.*, 50 (1974) 71–82.
5 R. Schauer, H.-P. Buscher, and J. Casals-Stenzel, *Biochem. Soc. Symp.*, 40 (1974) 87–116.
6 J. P. Kamerling, J. F. G. Vliegenthart, C. Versluis, and R. Schauer, *Carbohydr. Res.*, 41 (1975) 7–17.
7 J. Haverkamp, R. Schauer, M. Wember, J. P. Farriaux, J. P. Kamerling, C. Versluis, and J. F. G. Vliegenthart, *Hoppe-Seyler's Z. Physiol. Chem.*, 357 (1976) 1699–1705.
8 R. Schauer, J. Haverkamp, M. Wember, J. F. G. Vliegenthart, and J. P. Kamerling, *Eur. J. Biochem.*, 62 (1976) 237–242.
9 G. Reuter, R. Pfeil, J. P. Kamerling, J. F. G. Vliegenthart, and R. Schauer, *Biochim. Biophys. Acta*, 630 (1980) 306–310.
10 C. C. Sweeley, R. Bentley, M. Makita, and W. W. Wells, *J. Am. Chem. Soc.*, 85 (1963) 2497–2507.
11 J. P. Kamerling, J. F. G. Vliegenthart, and J. Vink, *Carbohydr. Res.*, 33 (1974) 297–306.
12 C. C. Sweeley and D. E. Vance, *Lipid Chromatogr. Anal.*, 1 (1967) 465–495.
13 H. Rauvala and J. Kärkkäinen, *Carbohydr. Res.*, 56 (1977) 1–9.
14 N. K. Kochetkov, O. S. Chizhov, V. I. Kadentsev, G. P. Smirnova, and I. G. Zhukova, *Carbohydr. Res.*, 27 (1973) 5–10.
15 A. K. Bhattacharjee and H. J. Jennings, *Carbohydr. Res.*, 51 (1976) 253–261.
16 H. V. Halbeek, J. Haverkamp, J. P. Kamerling, J. F. G. Vliegenthart, C. Versluis, and R. Schauer, *Carbohydr. Res.*, 60 (1978) 51–62.
17 G. P. Smirnova, N. V. Chekareva, O. S. Chizhov, B. M. Zolotarev, and N. K. Kochetkov, *Carbohydr. Res.*, 59 (1977) 235–239.
18 T. P. Mawhinney, M. S. Feather, J. R. Martinez, and G. J. Barbero, *Carbohydr. Res.*, 75 (1979) C21–C23.

19 T. P. MAWHINNEY, M. S. FEATHER, G. J. BARBERO, AND J. R. MARTINEZ, *Anal. Biochem.*, 101 (1980) 112–117.
20 D. C. DEJONGH, T. RADFORD, J. D. HRIBAR, S. HANESSIAN, M. BIEBER, G. DAWSON, AND C. C. SWEELEY, *J. Am. Chem. Soc.*, 91 (1969) 1728–1740.
21 M. DIZDAROGLU, D. HENNEBERG, AND C. VON SONNTAG, *Org. Mass Spectrom.*, 8 (1974) 335–345.
22 R. KUHN, P. LUTZ AND D. L. MACDONALD, *Chem. Ber.*, 99 (1966) 611–617.
23 G. N. BOLLENBACK, *Methods Carbohydr. Chem.*, 2 (1963) 326–328.