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Note

Thermal degradation of sodium *N*-acetylneuraminate

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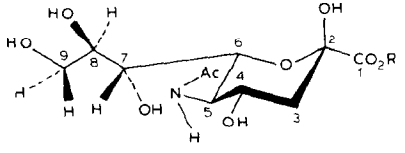
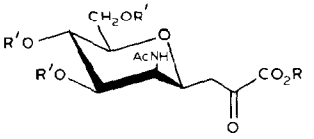
N-Acetylneuraminic acid (Neu5Ac) is widely distributed in Nature as a constituent of glycoproteins, glycolipids, and glycosaminoglycans in various types of cells. The physiological activities[1], biosynthesis[2–4], and chemical synthesis[5–9] of these Neu5Ac-containing compounds have been extensively studied, but there have been few studies on the biological activity[10] and drug efficacy[11] of free Neu5Ac. Because Neu5Ac was found to exhibit expectorant activity[12], we have examined its thermal and physical stability as a part of a drug developmental study on this compound. In the present report, we describe a stereoselective, ring-conversion reaction of sodium Neu5Ac.

Heating of sodium Neu5Ac (**2**) powder for 3 h at 140°, followed by fractionation by chromatography on a column of cellulose, yielded unchanged compound **2** (42.0%), and compound **4** as the main degradation product in a yield of 53.0%.

Compound **4** was purified by recrystallization from methanol, to give colorless needles, m.p. 217° (dec.). The molecular formula of $C_{11}H_{16}NNaO_8$, determined by elemental analysis and mass spectrometry, indicated formation by the dehydration of **2** ($C_{11}H_{18}NNaO_9$). The characterization of the compound as sodium *N*-acetyl-4,8-anhydroneuraminate was based on a detailed comparison of the n.m.r.-spectral data (see Tables I and II) for **4** and its acetylated derivative **6**.





1 R = H
2 R = Na

3 R = R' = H
4 R = Na, R' = H
5 R = Me, R' = H
6 R = Na, R' = Ac

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TABLE I

^{1}H-N.M.R. DATA

Compound	*Chemical shift (p.p.m.)*										
	H-3	*H-3'*	*H-4*	*H-5*	*H-6*	*H-7*	*H-8*	*H-9*	*H-9'*	CH_3CO	OCH_3
2	2.252	1.866	4.066	3.950	4.022	3.549	3.798	3.881	3.649	2.026	
3a	3.060	2.942	4.193	4.302	3.846	3.488	3.357	3.794	3.708	2.041	
3b	2.172	1.798	3.857	4.230	3.796	3.488	3.218	3.727	3.727	2.041	
4a	2.983	2.844	4.188	4.333	3.852	3.496	3.363	3.814	3.734	2.041	
4b	2.143	1.723		4.249		3.439	3.268	3.690		2.041	
5a	3.143	3.023	4.083	4.314		3.514	3.388	3.730		2.063	3.778
5b	2.214	1.810	3.843	4.237	3.798	3.519	3.213	3.789	3.694	2.063	3.778
6	3.095	2.904	4.381	4.563	5.221	5.133	3.927	4.112	4.348	2.002 2.096 2.101 2.118	

	Coupling constant (Hz)									
	$J_{3,3'}$	$J_{3,4}$	$J_{3',4}$	$J_{4,5}$	$J_{5,6}$	$J_{6,7}$	$J_{7,8}$	$J_{8,9}$	$J_{8,9'}$	$J_{9,9'}$
2	13.0	4.9	11.6	10.2	10.1	0.9	9.3	2.8	6.5	11.8
3a	18.0	8.1	4.8	1.8	4.4	9.9	9.9	4.8	2.2	12.5
3b	14.7	10.3	2.2	1.8	4.4	9.9	9.9	2.2	2.2	0–1
4a	17.6	8.4	4.4	1.5	4.4	9.9	9.9	4.8	2.2	12.1
4b	14.3	9.5	2.6	1.5	4.4	9.9	9.9	5.5	2.2	8.4
5a	18.5	7.7	5.3	1.8		9.9	9.9	4.9	2.2	12.3
5b	14.5	10.3	2.2	1.8	4.4	9.9	9.9	4.8	2.2	12.3
6	17.6	8.8	4.4	1.8	4.4	9.9	9.9	4.8	2.2	12.8

TABLE II

^{13}C-N.M.R. DATA

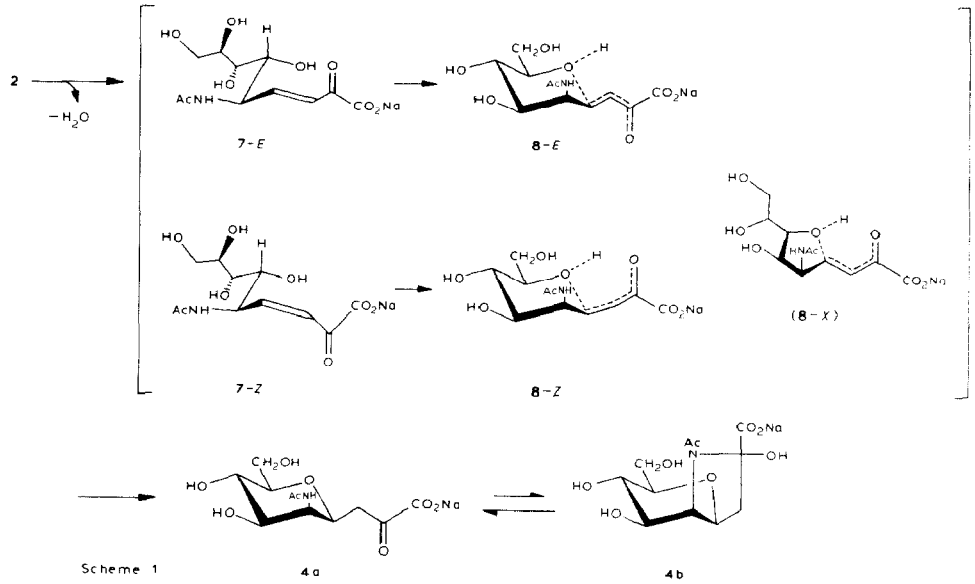
Compound	*Chemical shift (multiplicity)*						
	C-2	*C-3*	*C-4*	*C-5*	*C-6*	*C-7*	*C-8*
2	97.14(s)	40.14(t)	68.05(d)	53.02(d)	70.97(d)	69.32(d)	71.06(d)
3a	195.04(s)	40.43(t)	73.08(d)	53.14(d)	73.69(d)	67.28(d)	80.61(d)
3b	93.49(s)	40.75(t)	73.37(d)	54.04(d)	73.77(d)	67.08(d)	80.24(d)
4a	202.25(s)	41.61(t)	73.48(d)	53.46(d)	73.70(d)	67.40(d)	80.81(d)
5a	192.51(s)	40.59(t)	72.91(d)	53.05(d)	73.25(d)	67.20(d)	80.62(d)
5b	93.45(s)	40.81(t)	73.59(d)	53.47(d)	73.68(d)	66.73(d)	80.01(d)
6	202.56(s)	41.30(t)	73.56(d)	50.93(d)	73.92(d)	66.86(d)	76.32(d)
	C-9	*NCOCH$_3$*	*CO$_2$R or OCOCH$_3$*	*COCH$_3$*	*CO$_2$CH$_3$*		
2	64.04(t)	175.50(s)	177.45(s)				
3a	61.10(t)	163.20(s)	175.66(s)	22.46(q)			
3b	60.78(t)	163.20(s)	174.87(s)	22.46(q)			
4a	61.17(t)	172.06(s)	178.07(s)	24.71(q)			
5a	60.96(t)	161.40(s)	175.48(s)	22.32(q)	53.90(q)		
5b	60.33(t)	161.40(s)	173.45(s)	22.32(q)	53.90(q)		
6	63.35(t)	169.46(s)	173.72(s)	22.36(q)			
			173.85(s)	20.94(q)			
			174.49(s)	20.88(q)			
			175.80(s)				

To confirm the structural assignment, compound **4** was converted into its free-acid form (**3**) by chromatography on a column of Dowex 50 (H^+) ion-exchange resin, and the acid was then identified, from its n.m.r. spectral data[13] and physical constants, as **3**, a compound which had previously been isolated from edible bird's nests by Pozsgay *et al.*[14].

Heating of a solution of **2** in NaOH at a pH >11 for 30 min at 80°, followed by fractionation by chromatography on a column of cellulose, also yielded **4** (38.8%) and unchanged **2** (24.4%). Heating of **2** in Clark–Lubs buffer (pH 2.0), followed by chromatography on a cellulose column also yielded **4** (43.7%) and unchanged **2** (17.1%).

With respect to the formation of **3**, Pozsgay *et al.*[14] speculated that it was derived from 5-acetamido-4-*O*-acetyl-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (Neu4,5Ac$_2$), which is also a constituent of edible bird's nests, because **3** was not formed from Neu5Ac under the conditions they employed for the extraction of sialic acid from edible bird's nests (15mM H_2SO_4 for 2 h at 65–70°). They conjectured that acetyl elimination occurs easily at the acetylated hydroxyl group on C-4, and that the hydroxyl group on C-8 may attack this position nucleophilically to form an anhydro ring. However, we observed that Neu5Ac is converted directly into **4** stereoslectively when it is heated in the dry state, or in an

acidic or alkaline solution. Therefore, we propose the alternative reaction-mechanism depicted in Scheme 1.



Scheme 1

After the formation of an enone intermediate through dehydration between C-3 and C-4 (**7-E** or **7-Z**), a transition state of a thermodynamically stable, chair-form, six-membered ring (**8-E** or **8-Z**) will be attained; this will then be converted into **4** through Michael addition. Through this process, two types of enone, **7-E** and **7-Z**, will be formed; however, if the C_3 unit (=CH–CO–CO_2Na) on C-4 in each form becomes equatorial in the transition state (**8-E** or **8-Z**), which is considered to be thermodynamically more stable, both could eventually produce **4**. Moreover, in Michael addition, a furanose-type five-membered ring might be formed by attack by the 7-hydroxyl group. However, if this is the case, all substituents on C-5, C-6, and C-7 would be *cis* in the transition state (**8-X**); thus, the probability that this type of compound would be produced is low.

As shown in Fig. 1 and Table I, **4** exhibited two patterns for the ^{1}H-n.m.r. spectrum in D_2O. For example, the proton belonging to C-7 of each tautomer appeared as a triplet having J 9.9. Hz at δ 3.496 and 3.439. Similarly, with respect to the protons on C-8 or C-5, peaks having the same multiplicity and spacing were observed at δ 3.363 and 3.268, or δ 4.333 and 4.249, respectively. Because the relative intensities of corresponding lines in the two patterns changed with alteration of the pH, it appeared that **4** might be present in two tautomeric forms (**4a** and **4b**).

Pozsgay *et al.*[14] reported that, when the n.m.r. spectra were measured in D_2O, both the ^{1}H- and ^{13}C-n.m.r. spectra of **3** showed more lines than expected, thus making analysis difficult. However, when they used, as the solvent, dimethyl-d_6 sulfoxide containing 5–10% of trifluoroacetic acid, they obtained simplified

spectra which could be fully assigned. Although they speculated that the additional lines in the D_2O spectra might be caused by keto–enol tautomerism at C-2 and C-3, they did not pursue this hypothesis further. We measured the ^{1}H-n.m.r. spectrum of **3** in D_2O, as had Pozsgay *et al.*[14], and found that all spectral signals were correspondent with those of **4**, but their integration ratios were different, as shown in Table I and Fig. 1.

In this ^{1}H-n.m.r. spectrum, no olefinic proton was observed, but the presence of a different kind of tautomeric compounds was indicated. The structures were deduced as follows.

The abundance ratio of the tautomers was 93:7 (**4a:4b**) for **4**, and 7:15 (**3a:3b**) for **3**, in D_2O solution (3%). Compound **3**, with this increased tautomer ratio, showed the following ^{1}H-n.m.r. features (see Fig. 1): the signals for the protons at C-5, C-6, and C-7 of **3b** were located at δ 4.230, 3.796, and 3.488, with the

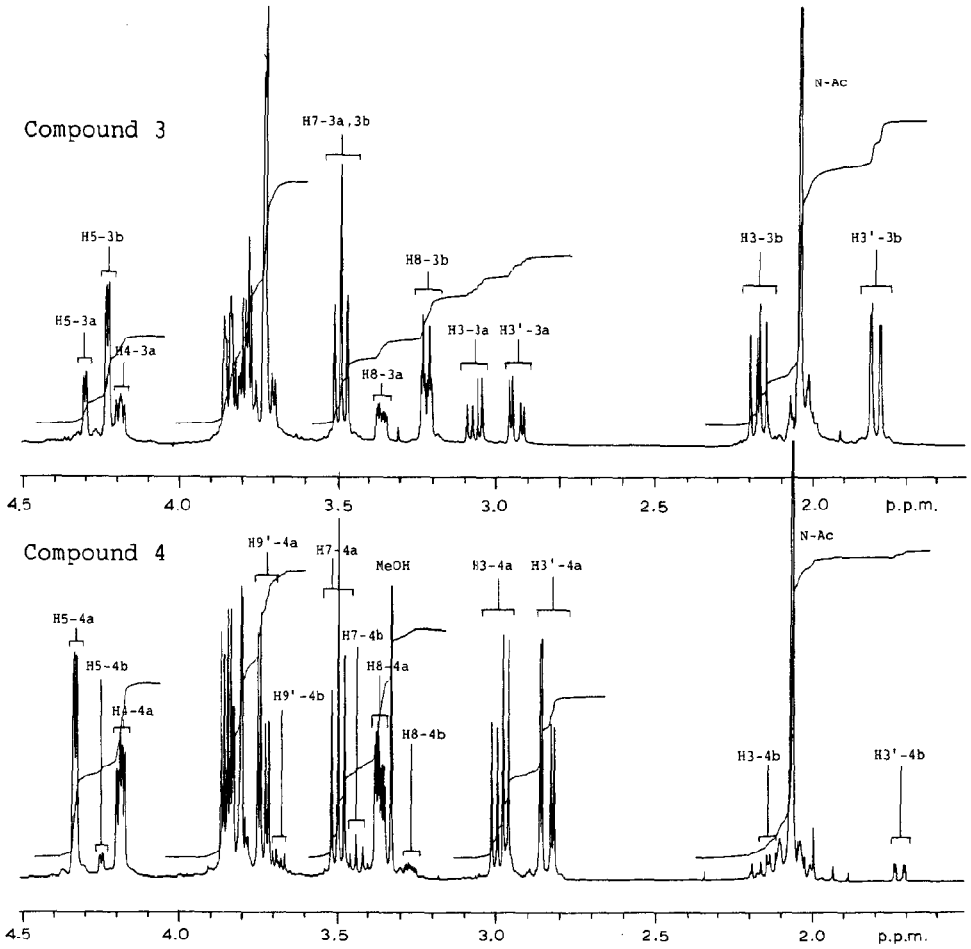


Fig. 1. 500-MHz, ^{1}H-n.m.r. spectra of compounds **3** and **4** in D_2O.

same coupling constants as those of **4a**, and the coupling constants for those at C-4 and C-8 were also about the same. Because C-4, C-5, C-6, C-7, and C-8 are atoms forming a pyranose ring, the $^7C_4$(D) conformation was retained. As to the proton signal at C-3, high-field shifts were observed from δ 2.983 and 2.844 (**4a**) to δ 2.172 and 1.798 (**3b**). Moreover, in the ^{13}C-n.m.r. spectrum, a newly appearing quaternary carbon atom was observed at δ 93.49, and the other signals were observed as pairs. Taking these results into consideration together, this tautomer was deduced to be a compound produced through the bonding of the nitrogen atom in the *N*-acetyl moiety at C-5 and the carbonyl carbon atom at C-2 (**4b**). The occurrence of such a tautomerism of an α-ketocarbonyl-δ-acetamido compound in solution had already been reported by Poisel and Schmidt[15], and the present compound is another example of this sort.

The difference in the abundance ratio of tautomers **3a:3b** and **4a:4b** is considered to be due to differences in the electrophilicity of the carbonyl carbon atom at C-2, which is influenced by the ionization of the adjacent carboxyl group. Therefore, we synthesized the methyl ester **5**, which is completely unable to ionize, and measured its ^{1}H-n.m.r. spectrum. As expected, the abundance ratio of tautomers was 8:92 (**5a:5b**) for **5** in D_2O solution (3%), indicating a preferred formation of **5b** to that of **5a**. The structure of **5** was determined on the basis of the mass, i.r., and n.m.r. spectra.

EXPERIMENTAL

General methods. — Melting points were determined in capillary tubes and are uncorrected. Optical rotations were measured with a JASCO DIP-4 polarimeter. T.l.c. was carried out on Kieselgel 60 HF-254 (Merck), and spots were detected by spraying with sulfuric acid in ethanol followed by heating. I.r. spectra were recorded with a JASCO IR-810 spectrometer. N.m.r. spectra (^{1}H- and ^{13}C-) were recorded with a JEOL GX-500 spectrometer, with Me_4Si as the internal standard for solutions in $CDCl_3$, and with H_2O (at 4.750 p.p.m.) for those in D_2O for ^{1}H-n.m.r. spectra; and with Me_4Si as the internal standard for solutions in $CDCl_3$, and 1,4-dioxane (at 67.40 p.p.m.) for those in D_2O for ^{13}C-n.m.r. spectra. Mass spectra were recorded with a JEOL D300 spectrometer, using fast-atom bombardment.

Sodium N-*acetylneuraminate* (**2**). — To a solution of *N*-acetylneuraminic acid (**1**) (40 g, 0.13 mol) in H_2O (400 mL) was added M sodium hydroxide (130 mL, 0.13 mol) at room temperature. Lyophilization of the mixture gave 42.6 g (quant.) of **2** as a white powder; m.p. 157–182° (dec.), $[\alpha]_D^{20}$ −27.5° (*c* 1.0, H_2O); ν_{max}^{KBr} 3320 (O–H), 1618 (C=O), 1151, 1128, and 1032 (C–O) cm^{-1}. The ^{1}H- and ^{13}C-n.m.r. data are given in Tables I and II: positive-ion f.a.b.-m.s.: *m/z* 354 $(M + Na)^+$, and 332 $(M + H)^+$.

Anal. Calc. for $C_{11}H_{18}NNaO_9$: C, 39.88; H, 5.48; N, 4.23. Found: C, 39.81; H, 5.77; N, 4.21.

Thermal degradation of **2**. — Compound **2** in powder form (5.0 g, 15.1 mmol) was heated for 3 h at 140°. The still friable product was dissolved in methanol (100

mL) and then fractionated by gradient-elution chromatography on a column (diam. 40 mm, l 700 mm) of cellulose with acetonitrile–water, from 7:1 (v/v) to 5:1 (v/v), to give 1.99 g (42.0%) of **4** as a pale-yellow powder, and 2.65 g (53.0%) of unchanged **2**.

Sodium 5-acetamido-4,8-anhydro-3,5-dideoxy-D-glycero-D-galacto-*nonulosonate* (**4**). — Recrystallized from methanol; m.p. 217° (dec.), $[\alpha]_D^{20}$ −34.1° (*c* 0.5, H_2O); ν_{max}^{KBr} 3300 (O–H), 1703, 1655, 1638 (C=O), 1568 (N–H), 1075, 1055, and 1029 (C–O) cm^{-1}. The ^1H- and ^{13}C-n.m.r. data are given in Tables I and II: positive-ion f.a.b.-m.s.: *m/z* 336 $(M + Na)^+$.

Anal. Calc. for $C_{11}H_{16}NNaO_8$: C, 42.18; H, 5.15; N, 4.47. Found: C, 41.90; H, 5.36; N, 4.53.

Degradation of **2** *under alkaline conditions.* — To a solution of **2** (30.0 g, 90.6 mmol) in H_2O (450 mL) was added solid sodium hydroxide (245 mg, 6.1 mmol) at room temperature. The mixture was stirred for 30 min at 80°, cooled, and evaporated, to give a brownish-yellow residue. This was dissolved in methanol (30 mL) and then fractionated by gradient-elution chromatography on a column (diam. 80 mm, l 1000 mm) of cellulose with acetonitrile–water, from 7:1 (v/v) to 5:1 (v/v), to give 11.0 g (38.8%) of **4** as a pale-yellow powder, and 7.33 g (24.4%) of unchanged **2**.

Degradation of **2** *under acidic conditions.* — A solution of **2** (20.0 g, 60.4 mmol) in 200 mL of Clark–Lubs buffer (pH 2.0) was stirred for 30 h at 80°, cooled, and evaporated, to give a brownish-yellow residue, which was dissolved in methanol (34 mL) and then fractionated by gradient-elution chromatography on a column (diam. 60 mm, l 800 mm) of cellulose with acetonitrile–water, from 7:1 (v/v) to 5:1 (v/v), to give 6.20 g (32.8%) of **4** as a pale-yellow powder and 3.41 g (17.1%) of unchanged **2**.

5-Acetamido-4,8-anhydro-3,5-dideoxy-D-glycero-D-galacto-*nonulosonic acid* (**3**). — A solution of **4** (95.2 mg, 0.3 mmol) in H_2O (4 mL) was placed on a column (diam. 14 mm, l 150 mm) of Dowex-50 (H^+) ion-exchange resin and then eluted with water. The acidic fractions were pooled and lyophilized, to yield a pale-yellow powder (**3**; 82.6 mg, 93.3%); $[\alpha]_D^{20}$ −52.5° (*c* 0.7, H_2O) {lit.[14] $[\alpha]_D^{20}$ −38.5° (*c* 0.7, H_2O)}; ν_{max}^{KBr} 3300 (O–H), 1733, 1646, 1540 (C=O), and 1077 (C–O) cm^{-1}. The ^1H- and ^{13}C-n.m.r. data are given in Tables I and II: positive-ion f.a.b.-m.s.: *m/z* 292 $(M + H)^+$.

Methyl 5-acetamido-4,8-anhydro-3,5-dideoxy-D-glycero-D-galacto-*nonulosonate* (**5**). — To a solution of **3** (1.00 g, 3.4 mmol) in 100 mL of methanol was added 13 g of Dowex 50 (H^+) ion-exchange resin at room temperature followed by stirring for 4 d at room temperature. After filtration of the mixture, evaporation of the filtrate gave a pale-yellow powder (**5**; 792 mg, 76.3%); $[\alpha]_D^{20}$ −52.6° (*c* 0.5, H_2O); ν_{max}^{KBr} 3394 (O–H), 1734, 1652, 1542 (C=O), 1268, 1078, and 1046 (C–O) cm^{-1}. The ^1H- and ^{13}C-n.m.r. data are given in Tables I and II; positive-ion f.a.b.-m.s.: *m/z* 306 $(M + H)^+$.

Sodium 5-acetamido-6,7,9-tri-O-acetyl-4,8-anhydro-3,5-dideoxy-D-glycero-D-

galacto-*nonulosonate* (**6**). — To a solution of **4** (13.0 mg, 0.04 mmol) in 3.0 mL of acetic anhydride was added a catalytic amount of pyridine at room temperature, and the mixture was stirred for 14 h at room temperature. After quenching the reaction with methanol, and evaporation of the solvent, the residue was chromatographed on silica gel, to give 19.5 mg (quant.) of **6** as a white powder; $[\alpha]_D^{20}$ −13.0° (*c* 0.5, H_2O); ν_{max}^{KBr} 3378 (N–H), 1748, 1659, 1652, 1633 (C=O), 1372, 1239, and 1052 (C–O) cm^{-1}. The ^1H- and ^{13}C-n.m.r. data are given in Tables I and II; positive-ion f.a.b.-m.s.: *m/z* 440 $(M + H)^+$.

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