208. 4-Methylumbelliferyl 5-Acetamido-3,4,5-trideoxy-α-D-*manno*-2-nonulopyranosidonic Acid: Synthesis and Resistance to Bacterial Sialidases

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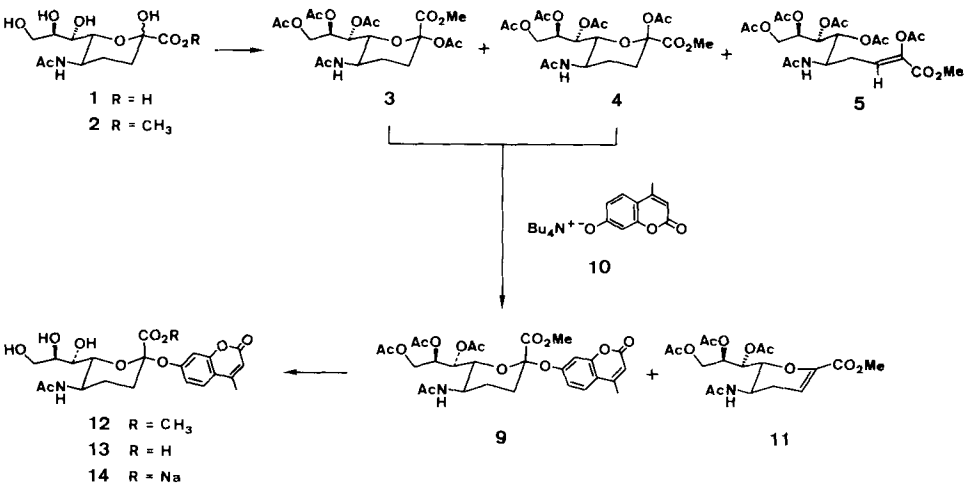
The synthesis of 4-methylumbelliferyl α-D-glycoside **13** of *N*-acetyl-4-deoxyneuraminic acid and its behaviour towards bacterial sialidases is described. *N*-Acetyl-4-deoxyneuraminic acid (**1**) was transformed into its methyl ester **2** and then acetylated to give the anomeric pentaacetates **3** and **4** of methyl 4-deoxyneuraminate and the enolacetate **5** (*Scheme*). A mixture **3**/**4** was treated with HCl/AcCl to give the glycosyl chloride, which was directly converted into the 4-methylumbelliferyl α-D-glycoside **9** of methyl 7-*O*,8-*O*,9-*O*,*N*-tetraacetylneuraminate and into the 2,3-dehydrosialic acid **11**. The ketoside **9** was de-*O*-acetylated to **12** with NaOMe in MeOH. Saponification (NaOH) of the methyl ester **12** followed by acidification gave the free **13**, which was also converted into the sodium salt **14** by passage through *Dowex 50* (Na^+). The 4-deoxy α-D-glycoside **13** is not hydrolyzed at significant rates by *Vibrio cholerae* and *Arthrobacter ureafaciens* sialidase. Neither the free *N*-acetyl-4-deoxyneuraminic acid (**1**), nor the α-D-glycoside **13** inhibit the activity of these sialidases.

Introduction. – The OH group at C(4) of sialic acids seems to be important for the cleavage of sialic-acid glycosides by sialidases as summarized in [1] (*cf.* also [2]). Sialic acids acetylated at C(4) are not released by sialidases. To elucidate, whether the bulkiness of a substituent, *e.g.* an AcO group, at C(4) or the absence of the free OH group is responsible for the resistance of the glycosidic linkage towards sialidase action, we wanted to test the cleavage of an appropriate α-D-glycoside of *N*-acetyl-4-deoxyneuraminic acid (**1**) [1] by sialidases. Among the glycosidase-assay methods based upon the quantification of the liberated aglycone [3–5], the method of *Thomas et al.* [6], using glycosides of 4-methylumbelliferone, has the advantage of allowing a fluorometric evaluation of the liberated aglycone. The higher sensitivity of fluorometric over colorimetric substrates for glycosidase assays has been established by several authors [7–10]. We, therefore, prepared the 4-methylumbelliferyl α-D-glycoside **13** of *N*-acetyl-4-deoxyneuraminic acid to test it in bacterial sialidase activity assays.

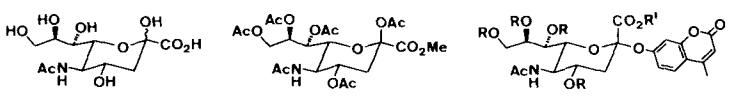
Synthesis of the 4-Methylumbelliferyl α-D-Glycoside of *N*-Acetyl-4-deoxyneuraminic Acid. – Treatment of *N*-Acetyl-4-deoxyneuraminic acid (4-deoxy-Neu5Ac, **1**) [1] with CF_3COOH in anh. MeOH gave the methyl ester **2** [1]. Applying a procedure similar to the one described by *Meindl* and *Tuppy* [11], crude **2** was converted into the anomeric peracetates **3** and **4** in a ratio of 3:7 (89% combined yield from **1**, see the *Scheme*) and into the open-chain enol acetate **5** (6% from **1**).

Scheme



The configuration at the anomeric centre of the peracetates **3** and **4** was assigned by comparison of their molecular optical rotation with the one of 4-deoxy-Neu5Ac (**1**) and of *N*-acetylneuraminic acid (Neu5Ac, **6**) on the one hand, and with the one of the known peracetylated methyl neuraminate **7** [12] on the other hand (see *Table 1*). The major acetylation product (62.5%) of methyl *N*-acetyl-4-deoxyneuraminate (**2**) showed a negative molecular optical rotation at 589 nm ($[M]_D = -68.5°$), the minor product (26.5%) a

Table 1. *Molecular Optical Rotations of Sialic-Acid Derivatives*



Compound	Molecular weight	$[\alpha]_D$ (solv.)	$[M]_D$	$\Delta[M]_D$	Ref.
1	293.3	−47.6° (H_2O)	−139.6°	Δ = − 40.3°	[1]
6	309.3	−32.1° (H_2O)	−99.3°		[34]
4	475.5	−14.4° ($CHCl_3$)	−68.5°	Δ_1 = − 50.9° [a]	
3	475.5	+48.1° ($CHCl_3$)	+228.7°	Δ_2 = +246.3° [b]	
7	533.5	− 3.3° ($CHCl_3$)	−17.6°		[12]
9	591.6	+64.8° ($CHCl_3$)	+383.4°	Δ = + 45.6°	
15	649.6	+52.0° ($CHCl_3$)	+337.8°		[17]
12	465.5	+59.4° ($CHCl_3$)	+276.5°		
13	451.5	+79.3° (H_2O)	+358.0°	Δ = + 30.7°	
16	467.5	+70.0° (H_2O)	+327.3°		[17]

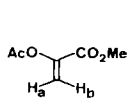
[a]) $\Delta_1 = [M]_D(\mathbf{4}) - [M]_D(\mathbf{7})$.
[b]) $\Delta_2 = [M]_D(\mathbf{3}) - [M]_D(\mathbf{7})$.

positive one ($[M]_D$ = +228.5°). Both Neu5Ac (**6**) and 4-deoxy-Neu5Ac (**1**) showed negative $[M]_D$ values. The known **7** shows also a negative $[M]_D$ value (−17.6°). Hence, we attributed the β-D-configuration to the isomer with the negative $[M]_D$ value, *i.e.* **4**, and the α-D-configuration to the isomer with the positive $[M]_D$ value, *i.e.* **3**.

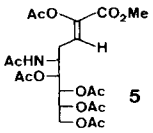
Due to the interaction of the axial H–C(6) with an axial AcO group (see **4**) or an axial COOMe group (see **3**) one should expect a chemical-shift difference for H–C(6) in **3** and **4**. We observed the signal of H–C(6) at 4.71 ppm in the ^{1}H-NMR spectrum of the α-D-anomer **3**, and at *ca.* 4.0 ppm (overlapping with the signal of H–C(5)) in the one of the β-D-anomer **4**.

The enol acetate **5** is characterized by an UV absorption at 213 nm indicating an α,β-unsaturated ester carrying an AcO substituent at C(α) [13]. Signals of an olefinic proton at 6.56 ppm and of 5 AcO groups between 2.27–1.91 ppm in the ^{1}H-NMR spectrum of **5**, as well as a *singlet* for C(2) (140.4 ppm) and a *doublet* for C(3) (126.8 ppm) in its ^{13}C-NMR spectrum confirm the constitution of the enol acetate **5**. The (*Z*)-configuration of **5** was deduced from the (C(1), H–C(3))-coupling constant (see *Table 2*). This coupling constant is significantly different for the (*E*)- and (*Z*)-configuration. A *cis*-coupling is characterized by smaller values (usually 3–8 Hz) than a *trans*-coupling (10–18 Hz) [14] [15]. To simplify the spectrum, we irradiated the proton signal of the MeO group.

Table 2. *C,H-Coupling Constants* [Hz] *of* **5** *and* **8**



8



5

Coupling constant	**8**			**5**
	Fully coupled [Hz]	Sel. decoupled [Hz]	Red. factor	Sel. decoupled [Hz]
$^1J(C,H_a)$	163	138	0.85	^1J = 152
$^1J(C,H_b)$	166	150	0.95	
$^2J(C,H_a)$	6.4	5.5	0.86	^2J = 4.5
$^2J(C,H_b)$	3.6	3.3	0.92	
$^3J(C,H_a)$	10.0	8.2	0.82	^3J = 3.5
$^3J(C,H_b)$	3.5	3.1	0.89	
$^3J(C(1),CH_3)$	2.8			

This causes a reduction of the *J*(C,H)-coupling constants, the extent of which depends on Δδ between the irradiated protons and the coupling partners of the observed ^{13}C-NMR signal. To determine the reduction factor, we measured the *J*(C,H) of methyl 2-acetoxy-2-propenoate (**8**) [16] in the fully coupled and the selectively decoupled ^{13}C-NMR spectrum, irradiating with the same power distribution as in the case of **5**. The coupling constants with H_a were reduced by a factor of *ca.* 0.84 (Δδ = 1.68 ppm), those with H_b by a factor of *ca.* 0.92 (Δδ = 2.24 ppm). In the enol acetate **5**, Δδ between H–C(3) and the MeO group is 2.78 ppm. The reduction factor for **5** should, therefore, be somewhat larger than the corresponding ones for **8**. The observed ^3J(C,H) of 3.5 Hz in the selectively decoupled spectra of **5** should correspond to *ca.* 4 Hz in the fully coupled spectrum, which is too small for a *trans*-coupling. Hence, the enol acetate **5** must be (*Z*)-configurated[1]).

A mixture of the anomeric peracetylated methyl esters **3** and **4** were transformed into the glycosyl chloride (HCl, AcCl, Et_2O) employing a similar procedure as described by *Kuhl et al.* [12] for the preparation of the methyl acetochloroneuraminate. The resulting glycosyl chloride was too unstable to be isolated, and was directly converted into the 4-methylumbelliferyl ketoside **9** by treatment with the tetrabutylammonium salt **10** of 4-methylumbelliferone [17] in the presence of freshly prepared Ag_2CO_3 *(Scheme)*.

[1]) We thank Dr. *R. Kunz* for recording the spectra and for helpful discussions.

Chromatographic purification gave the ketoside **9**[2]) (75% from **3**/**4**) and the 2,3-dehydro-4-deoxyneuraminic-acid derivative **11** (17% from **3**/**4**). As shown by TLC, **11** was already present before the glycosyl chloride was subjected to the *Königs-Knorr* glycosidation [18], where formation of 2,3-dehydrosialic acids is well known [19].

The 4-methylumbelliferyl ketoside **9** was deprotected according to *Zemplen*, as modified by *Baggett* and *Marsden* [17] to give the de-*O*-acetylated glycoside **12** in 95% yield. The methyl ester was saponified with aq. NaOH to give, after chromatography, the 4-methylumbelliferyl α-D-glycoside **13** (MU-4-deoxy-Neu5Ac) of *N*-acetyl-4-deoxy-neuraminic acid in 78% from **9**. The free acid **13** was quite unstable and decomposed progressively, presumably by self-catalyzed hydrolysis [9]. It was, therefore, transformed into the sodium salt **14** by passage through *Dowex 50* (Na^+). Esterification of the free acid **13** with CH_2N_2 in MeOH soln. gave again the methyl ester **12.** Except for the OH signals, the IR- and ^{1}H-NMR spectra of **12** obtained in this way were indistinguishable from those obtained by de-*O*-acetylation of **9** as detailed above.

Determination of the Configuration at C(2) of the Compounds 9 and 12–14. – The molecular optical rotations of 4-deoxy-Neu5Ac **1** and its derivatives on the one hand and of Neu5Ac (**6**) and its derivatives on the other hand are collected in *Table 1*. The acid **1** and its fully acetylated ester **4** (major acetylation product) possess negative $[M]_D$ values like Neu5Ac **6** and the fully acetylated methyl neuraminate **7**. The difference between the $[M]_D$ values of **1** and **6** (−40.3°) and of **4** and **7** (−50.9°) can be rationalized by an additional *'gauche'* ((+)-*sc*) interaction of the OH group at C(4) with the NHAc group at C(5) in the neuraminic-acid derivatives, which is absent in the 4-deoxyneuraminic acid and its derivatives [20] [21]. Inversion of the configuration at C(2) (*cf.* **3**, **15**, and **16**) led to positive $[M]_D$ values. We found positive $[M]_D$ values also for the 4-methylumbelliferyl ketosides **9** and **12**–**14** and considered this as a hint for the α-D-configuration of these ketosides.

Comparison of the ^{13}C-NMR spectrum of the glycoside **13** with the one of the corresponding 4-hydroxy derivative **16** [17] showed similar chemical shifts for all C-atoms with the exception of those for C(3) to C(6). The signal of C(6) in **13** is shifted downfield (2.2 ppm, γ-effect, *cf.* [1] [22]), the signals of C(3), C(4), and C(5) are shifted upfield (*cf.* [1]). This similarity in the ^{13}C-NMR chemical shifts of **13** and **16** constitutes another evidence for the α-D-configuration of the ketoside **13**.

The values of the chemical shift of C(1) of the 4-deoxy-Neu5Ac ketoside **13** (173.8 ppm) and its methyl ester **12** (169.1 ppm) agree well with the corresponding ones of the methyl α-D-ketoside of Neu5Ac **6** (174.3 ppm) and its methyl ester (170.7 ppm) as published by *Eschenfelder* and *Brossmer* [23]; the C(1) signals for the corresponding β-D-ketosides were found at 176.1 ppm (acid) and 171.2 ppm (ester) [23].

A further evidence for the α-D-configuration of neuraminic-acid ketosides is found in the chemical shift of H_{eq}–C(3). *Brossmer* and coworkers [24] have shown that signals of H_{eq}–C(3) of α-D-ketosides of Neu5Ac **6** (D_2O soln.) appear between 2.60 and 2.75 ppm, and those of H_{eq}–C(3) of the corresponding β-D-ketosides between 2.25 and 2.40 ppm. We found H_{eq}–C(3) at 2.54 ppm (**13**) and 2.55 ppm (**14**), respectively. The slight upfield shift in the 4-deoxyneuraminic-acid derivatives may be rationalized by the absence of the OH group at C(4) (absence of a β-effect).

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[2]) No other product containing a 4-methylumbelliferyl group was observed (*cf.* [9]).

Sialidase (EC 3.2.1.18) Experiments. – The MU-4-deoxy-Neu5Ac **13** was tested in sialidase-activity assays with sialidases from *Vibrio cholerae* and *Arthrobacter ureafaciens* (see *Exper. Part*). In addition, the influence of free 4-deoxy-Neu5Ac **1** and of MU-4-deoxy-Neu5Ac **13** on sialidase activity was checked with the 4-methylumbelliferyl α-D-glycoside **16** (MU-Neu5Ac) of *N*-acetylneuraminic acid as substrate.

The MU-4-deoxy-Neu5Ac **13** is hydrolyzed by the bacterial sialidases at extremely low rates. When compared with the inital hydrolysis rate of the corresponding 4-hydroxy derivative **16**, MU-4-deoxy-Neu5Ac **13** is hydrolyzed under the influence of both sialidases only by 1.4% of this rate *(Fig.)*.

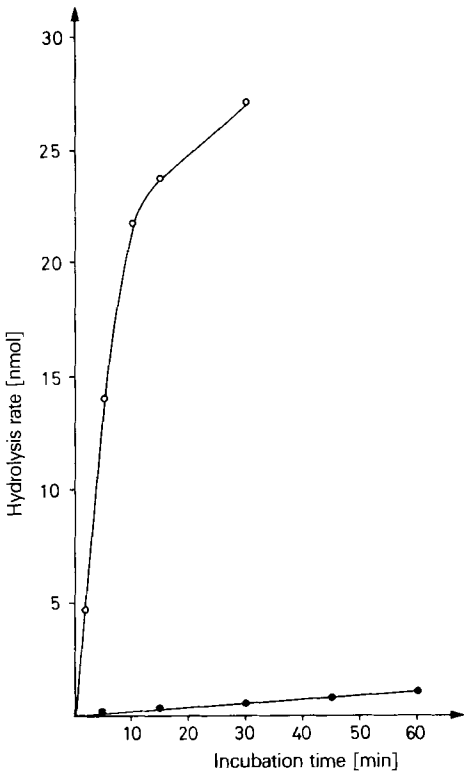

Fig. *Action of* V. cholerae *and* A. ureafaciens *sialidases on 4-methylumbelliferyl* α-D-*glycosides* **13** *and* **16** *of* N-*acetyl-4-deoxyneuraminic* (●) *and* N-*acetylneuraminic acid* (○), *respectively*. The very low activities exhibited by the two sialidases with the *N*-acetyl-4-deoxyneuraminic acid glycoside are drawn in one line, as no significant differences were observed between the hydrolysis rates of these two enzymes. The values are means from 3 experiments each, which gave almost identical results.

Both free 4-deoxy-Neu5Ac **1** and its α-D-glycoside **13** do not inhibit sialidase activity at the concentrations applied, as was tested with **16** as substrate.

These experiments demonstrate the requirement of an OH group at C(4) of the neuraminic acid molecule for hydrolysis of the α-D-glycosidic bond. Absence of this residue seems to prevent binding of sialic acid to the active center of the sialidases tested, as no (competitive) inhibition of the hydrolysis of MU-Neu5Ac **16**, an excellent substrate for sialidases *(Fig.)*, could be observed neither with free 4-deoxy-Neu5Ac **1** nor its α-D-glycoside **13**. In constrast, free Neu5Ac **6** with an equatorial OH group at C(4) is a competitive inhibitor of sialidase at the concentrations used here [2]. Substitution of the OH group at C(4) by an AcO group [2] [25] prevents hydrolysis of the glycosidic groups of

sialic acids by bacterial sialidases, too. In addition, methylation of the OH group at C(4) [26] strongly reduces the hydrolysis rate of this sialic acid by *V. cholerae* sialidase. These residues also seem to prevent or hinder binding of sialic acid to the active center of sialidase, as 4-*O*,*N*-diacetylneuraminic acid does not competitively inhibit sialidase action, similar to the 4-deoxy-Neu5Ac **1** tested here. Epimerization of the OH group at C(4) in neuraminic acid into axial position to give *N*-acetyl-4-epineuraminic acid [27] also reduces the affinity to sialidases. Namely, we could not observe inhibition of the bacterial sialidases by this neuraminic acid isomer at millimolar concentration. It is tempting to investigate more closely the role of the equatorial OH residue at C(4) of neuraminic acid in the mechanism of sialidase catalysis.

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Experimental Part

General. See [1] and [28]. Et_2O was distilled from NaH, AcOEt from K_2CO_3 and then from P_2O_5. CH_3CN was kept over $CaCl_2$ (24 h), distilled from P_2O_5, and stored over molecular sieve 3 Å. $CHCl_3$ was extracted with conc. H_2SO_4, stored over $CaCl_2$, and distilled.

*Methyl 5-Acetamido-2,7,8,9-tetra-*O*-acetyl-3,4,5-trideoxy-α- and -β-*D*-manno-2-nonulopyranosonate* (**3** and **4**, resp.) *and Methyl (Z)-5-Acetamido-2,6,7,8,9-penta-*O*-acetyl-3,4,5-trideoxy-*D*-manno-2-nonenonate* (**5**). A soln. of *N-acetyl-4-deoxyneuraminic acid* (**1**) [1] (500 mg, 1.70 mmol) and CF_3COOH (50 µl) in anh. MeOH (15 ml) was stirred at r.t. (24 h), until TLC (CH_2Cl_2/MeOH 3:1) indicated the disappearance of **1**. The soln. was evaporated to dryness to give crude *methyl* N-*acetyl-4-deoxyneuraminate* [1] (**2**; 549 mg). To an ice-cold suspension of crude **2** in Ac_2O (7.5 ml) was added dropwise (30 min) pyridine (5.75 ml) [11]. The mixture was slowly warmed to r.t. (melting-ice bath) and stirred for 3 d. TLC ($CHCl_3$/MeOH 95:5) indicated then the disappearance of **2**. The solvent was removed, and the residue was co-evaporated with 3 × 20 ml of toluene. FC (75 g SiO_2, $CHCl_3$/MeOH 99:1) yielded **5** (52 mg, 6%) and **3** (215 mg, 26.5%), $CHCl_3$/MeOH 80:1 yielded **4** (505 mg, 62.5%). All 3 compounds were colourless foams.

Data of **3**: $[\alpha]_D^{25} = +48.1°$ (c = 1.1, $CHCl_3$). IR: 3435*w*, 2995*w*, 1746*s*, 1679*m*, 1502*m*, 1435*w*, 1369*s*, 1290*m*, 1240*s* (br.), 1220*m*, 1078*s*, 1044*s*. ^{1}H-NMR (200 MHz): 5.39 (*dd*, J = 6.5, 2.6, H–C(7)); 5.30 (*d*, J = 9.5, NH); 5.21 (*ddd*, J = 6.5, 6.0, 2.8, H–C(8)); 4.71 (*dd*, J = 10.5, 2.5, H–C(6)); 4.39 (*dd*, J = 12.5, 2.8, H–C(9)); 4.08 (*dd*, J = 12.5, 6.0, H–C(9)); 4.11–3.91 (*m*, H–C(5)); 3.73 (*s*, CH_3O); 2.28–1.88 (*m*, 2 H–C(3), 2 H–C(4)); 2.12, 2.10, 2.09, 2.04, 1.93 (5 CH_3). ^{13}C-NMR (50 MHz): 170.70 (*s*); 170.09 (*s*); 170.02 (*s*); 169.66 (*s*); 169.07 (*s*); 168.45 (*s*); 95.80 (*s*); 75.72 (*d*); 70.29 (*d*); 68.00 (*d*); 62.30 (*t*); 52.49 (*q*); 43.64 (*d*); 31.20 (*t*); 26.17 (*t*); 23.36 (*q*); 20.84 (*q*); 20.78 (*q*); 20.72 (*q*). CI-MS: 416 ((*M*+1) – AcOH). Anal. calc. for $C_{20}H_{29}NO_{12}$ (475.46): C 50.52, H 6.15, N 2.95; found: C 50.26, H 6.40, N 2.71.

Data of **4**: $[\alpha]_D^{25} = -14.4°$ (c = 1.1, $CHCl_3$). IR: 3435*w*, 2995*w*, 2955*w*, 1735*s*, 1681*s*, 1507*m*, 1438*m*, 1371*s*, 1290*m*, 1240*s* (br.), 1091*m*, 1040*m*, 1012*m*. ^{1}H-NMR (400 MHz): 5.39 (*dd*, J = 4.5, 1.8, H–C(7)); 5.29 (*d*, J = 7.8, NH); 5.06 (*ddd*, J = 7.0, 4.5, 2.5, H–C(8)); 4.54 (*dd*, J = 12.5, 2.5, H–C(9)); 4.14 (*dd*, J = 12.5, 7.0, H–C(9)); 4.04–3.98 (*m*, H–C(5), H–C(6)); 3.77 (*s*, CH_3O); 2.25–1.74 (*m*, 2 H–C(3), 2 H–C(4)); 2.13 (2×), 2.06, 2.03, 1.92 (5 CH_3). ^{13}C-NMR (50 MHz): 170.54 (*s*); 170.29 (*s*); 169.56 (*s*); 168.34 (*s*); 167.40 (*s*); 96.99 (*s*); 73.74 (*d*); 71.70 (*d*); 68.37 (*d*); 62.33 (*t*); 52.87 (*q*); 43.89 (*d*); 30.31 (*t*); 25.58 (*t*); 23.34 (*q*); 20.88 (*q*); 20.71 (*q*). CI-MS: 416 ((*M*+1) – AcOH). Anal. calc. for $C_{20}H_{29}NO_{12}$ (475.46): C 50.52, H 6.15, N 2.95; found: C 50.27, H 6.41, N 2.78.

Data of **5**: $[\alpha]_D^{25} = +8.4°$ (c = 1.0, $CHCl_3$). UV (EtOH): 213 (10500) ([13]: 211). IR: 3430*w*, 3030 (sh), 2995*w*, 2955*w*, 2925*w*, 2850*w*, 1745*s*, 1680*s*, 1500*m*, 1436*m*, 1368*s*, 1305*m*, 1216*s* (br.), 1130*m*, 1045*m*, 1026*m*. ^{1}H-NMR (200 MHz): 6.56 (*dd*, J = 8.5, 7.5, H–C(3)); 5.54 (*d*, J = 10.0, NH); 5.38 (*dd*, J = 8.4, 3.2, H–C(7)); 5.23 (*dd*, J = 8.5, 3.2, H–C(6)); 5.09 (*ddd*, J = 8.4, 5.8, 3.0, H–C(8)); 4.50–4.32 (*m*, H–C(5)); 4.27 (*dd*, J = 12.5, 3.0, H–C(9)); 4.05 (*dd*, J = 12.5, 5.8, H–C(9)); 3.78 (*s*, CH_3O); 2.50–2.20 (*m*, 2H–C(4)); 2.27, 2.13, 2.11, 2.07, 2.06, 1.91 (6 CH_3). ^{13}C-NMR (50 MHz): 170.56 (*s*); 170.33 (*s*); 170.16 (*s*); 169.93 (*s*); 169.54 (*s*); 161.64 (*s*); 140.44 (*s*); 126.84 (*d*); 70.96 (*d*); 68.61 (*d*); 68.19 (*d*); 61.90 (*t*); 52.56 (*q*); 47.19 (*d*); 29.67 (*q*); 27.39 (*t*); 23.09 (*q*); 20.83 (*q*); 20.75 (*q*); 20.68 (*q*); 20.36 (*q*). EI-MS: 474 (1, $M^{+\cdot}$ – 43), 402 (1), 360 (2), 318 (15), 259 (10), 186 (15), 139 (25), 43 (100). Anal. calc. for $C_{22}H_{31}NO_{13}$ (517.50): C 51.06, H 6.04, N 2.71; found: C 50.90, H 6.09, N 2.59.

Methyl 2-Acetoxy-2-propenoate (**8**) was prepared according to [16] in 36% yield. ^{1}H-NMR ([29], 200 MHz, $CDCl_3$): 5.95 (*d*, *J* = 1.7, H–C(3)); 5.39 (*d*, *J* = 1.7, H–C(3)); 3.71 (*s*, CH_3O); 2.14 (*s*, CH_3). ^{13}C-NMR (50 MHz, $CDCl_3$): 168.69 (*s*); 161.71 (*s*, C(1)); 144.36 (*s*, C(2)); 113.81 (*t*, C(3)); 52.35 (*q*); 20.13 (*q*).

*Tetrabutylammonium Salt of 4-Methylumbelliferone (Tetrabutylammonium 4-Methyl-2-oxo-2*H*-1-benzopyran-7-olate;* **10**). According to [17], to a soln. of NaOH (1.3 g, 32.5 mmol) in H_2O (30 ml) was added 4-methylumbelliferone (5.9 g, 30.4 mmol) and Bu_4NBr (10.7 g, 33.2 mmol). The mixture was shaken for 10 min. Extractive workup with $CHCl_3$ and crystallization from hot $CHCl_3$ gave **10** (2.0 g, 15%) as pale-green cubes. M.p. 177–179° ([17]: 170–172°, pale-green needles). In contrast to [17], where the tetrabutylammonium salt of 4-methylumbelliferone contained 2 coumarin residues per Bu_4N^+ group, **10** contained only 1 coumarin residue per Bu_4N^+ group as have been shown by ^{1}H-NMR. ^{1}H-NMR (80 MHz): 7.18 (*d*, *J* = 8.0, H–C(5)); 6.42 (*dd*, *J* = 8.0, 2.0, H–C(6)); 6.20 (*d*, *J* = 2.0, H–C(8)); 5.51 (*d*, *J* = 0.5, H–C(3)); 3.35–290 (*m*, 4 CH_2N); 2.26 (*d*, *J* = 0.5, CH_3); 1.80–0.80 (*m*, 4 $CH_3CH_2CH_2$).

*Methyl (4-Methyl-2-oxo-2*H*-1-benzopyran-7-yl 5-Acetamido-7,8,9-tri-*O*-acetyl-3,4,5-trideoxy-α-*D*-manno-2-nonulopranosid)onate* (**9**) *and Methyl 5-Acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-3,4,5-trideoxy-*D*-manno-2-nonenonate* (**11**). To a soln. of a 3:7 mixture **3**/**4** (200 mg, 0.420 mmol) in dry Et_2O (12 ml) was added freshly distilled AcCl (0.8 ml). The mixture was cooled to −40°[3]) and saturated with dry HCl[4]). The flask was stoppered and the clear soln. warmed to 0° within 5 h. TLC (AcOEt/acetone 92:8) indicated then the disappearance of **3**/**4** and showed two spots: a faster moving one corresponding to **11** and one for the glycosyl chloride. The solvent was evaporated, the residue dissolved in dry AcOEt (10 ml) and again evaporated to give a colourless foam, which was dried for 15 min at 10^{-2} mbar. To a soln. of the residue in dry MeCN (8 ml) were added ground molecular sieves 3 Å (640 mg), **10** (460 mg, 1.050 mmol), and freshly prepared Ag_2CO_3 [31] (64 mg, 1.680 mmol). The mixture was stirred in the dark at r.t. for 18 h. The soln. was filtered through *Celite*, washed with dry $CHCl_3$ (50 ml) and evaporated. The residue was dissolved in dry AcOEt[5]) (10 ml), stirred at r.t. (15 min), filtered through *Celite*, washed with AcOEt and concentrated. FC (22 g SiO_2, hexane/acetone 6:4) gave **11** (29 mg, 17%), **3** (12 mg, 6%), and **9** (187 mg, 75%; TLC: hexane/acetone 1:1). All compounds were colourless foams.

Data of **9**: $[\alpha]_D^{25}$ = +64.8° (*c* = 1.0, $CHCl_3$). IR: 3430*w*, 3030 (sh), 2995*w*, 2975*w*, 2950*w*, 1737*s*, 1683*m*, 1611*s*, 1558*w*, 1500*m*, 1440*w*, 1385*m*, 1367*s*, 1290*m*, 1220*s* (br.), 1142*m*, 1072*s*, 1040*s*, 855*m*. ^{1}H-NMR (400 MHz, (D_6)benzene): 7.29–7.23 (*m*, H–C(5′), H–C(6′), H–C(8′)); 5.87 (*d*, *J* = 1.1, H–C(3′)); 5.85 (*ddd*, *J* = 7.7, 6.0, 2.6, H–C(8)); 5.72 (*dd*, *J* = 7.7, 2.0, H–C(7)); 5.25 (*d*, *J* = 9.5, NH); 4.76 (*dd*, *J* = 10.7, 2.0, H–C(6)); 4.65 (*dd*, *J* = 12.4, 2.6, H–C(9)); 4.43 (*dd*, *J* = 12.4, 6.0, H–C(9)); 4.31 (*dddd*, *J* = 11.0, 10.7, 9.5, 4.0, H–C(5)); 3.30 (*s*, CH_3O); 2.37 (*ddd*, *J* = 13.7, 4.0, 4.0, H_{eq}–C(3)); 2.18–1.60 (*m*, H_{ax}–C(3), H_{eq}–C(4)); 2.14, 2.02, 1.90, 1.78 (4 CH_3); 1.65 (*d*, *J* = 1.1, CH_3–C(4′)); 1.43–1.27 (*m*, H_{ax}–C(4)); attribution of the signals was achieved by double-resonance spectroscopy. ^{13}C-NMR (50 MHz): 170.52 (*s*); 170.15 (*s*); 170.08 (*s*); 169.73 (*s*); 168.56 (*s*); 160.98 (*s*); 157.14 (*s*); 154.41 (*s*); 152.32 (*s*); 125.61 (*d*); 115.76 (*s*); 115.39 (*d*); 113.16 (*d*); 107.21 (*d*); 100.50 (*s*); 75.64 (*d*); 69.32 (*d*); 67.72 (*d*); 62.11 (*t*); 52.82 (*q*); 44.13 (*d*); 32.68 (*t*); 29.26 (*q*); 26.76 (*t*); 23.39 (*q*); 20.97 (*q*); 20.70 (*q*); 18.65 (*q*); EI-MS: 532 (2, $M^{+\cdot}$ − OAc), 416 (25), 374 (9), 356 (18), 314 (9), 254 (7), 236 (17), 195 (8), 194 (8), 177 (25), 43 (100). Anal. calc. for $C_{28}H_{33}NO_{13}$ (591.59): C 56.85, H 5.62, N 2.37; found: C 56.59, H 5.88, N 2.17.

Data of **11**: $[\alpha]_D^{25}$ = 23.6° (*c* = 1.0, $CHCl_3$). UV (EtOH): 234 (6750) ([13]: 240). IR: 3435*m*, 3030*w*, 3000*m*, 2960*m*, 1740*s*, 1678*s*, 1505*m*, 1439*m*, 1371*s*, 1305*m*, 1230*s* (br.), 1130*m*, 1108*s*, 1045*s*. ^{1}H-NMR (200 MHz): 6.11 (*dd*, *J* = 4.0, 4.0, H–C(3)); 5.71 (*d*, *J* = 9.0, NH); 5.46 (*dd*, *J* = 5.7, 3.7, H–C(7)); 5.25 (*ddd*, *J* = 7.0, 3.7, 3.5, H–C(8)); 4.58 (*dd*, *J* = 12.0, 3.5, H–C(9)); 4.45–4.35 (*m*, H–C(5)); 4.26 (*dd*, *J* = 12.0, 7.0, H–C(9)); 4.18 (*dd*, *J* = 6.0, 5.7, H–C(6)); 3.79 (*s*, CH_3O); 2.64 (*ddd*, *J* = 19.0, 4.2, 4.0, H–C(4)); 2.21 (*ddd*, *J* = 19.0, 4.2, 4.0, H–C(4)); 2.12 (*s*, CH_3); 2.07 (2 CH_3); 1.97 (*s*, CH_3). ^{13}C-NMR (50 MHz): 170.49 (*s*); 170.17 (*s*); 169.83 (*s*); 169.71 (*s*); 162.11 (*s*); 142.54 (*s*, C(2)); 109.46 (*d*, C(3)); 76.19 (*d*); 70.67 (*d*); 68.08 (*d*); 61.69 (*t*); 52.03 (*q*); 41.63 (*d*); 44.63 (*d*); 26.64 (*t*); 22.98 (*q*); 20.68 (*q*); 20.56 (*q*); 20.48 (*q*). CI-MS: 416 (*M*+1), 356 ((*M*+1) − AcOH). Anal. calc. for $C_{18}H_{25}NO_{10}$: C 52.05, H 6.07, N 3.37; found: C 51.98, H 6.30, N 3.17.

*Methyl (4-Methyl-2-oxo-2*H*-1-benzopyran-7-yl 5-Acetamido-3,4,5-trideoxy-α-*D*-manno-2-nonulopyranosid)onate* (**12**). a) *From* **9**. A soln. of **9** (65 mg, 0.110 mmol) in anh. MeOH (2.2 ml) was treated with 100 µl of a 0.5M NaOMe/MeOH. After 4 h, TLC (hexane/acetone 4:6) indicated the disappearance of **9**. The mixture was neutralized with 0.1M AcOH in anh. MeOH and concentrated. FC (7 g SiO_2, CH_2Cl_2/MeOH 95:5) gave **12** (51 mg, > 95%), as a colourless foam. $[\alpha]_D^{25}$ = +59.4° (*c* = 1.0, $CHCl_3$). UV (MeOH): 202 (33650), 282 (7950), 313 (10600).

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[3]) Usually, **3**/**4** precipitated at this temp.

[4]) Freshly prepared from NaCl/conc. HCl and conc. H_2SO_4 according to [30].

[5]) Excess of **10** is soluble in $CHCl_3$, but not in AcOEt.

IR: 3660–3140*m*, 3435*m*, 3030*w*, 2995*m*, 2950*m*, 1728*s*, 1660*m*, 1612*s*, 1508*m*, 1388*m*, 1368*m*, 1264*m*, 1148*m*, 1130*s*, 1072*s*, 1037*m*, 1014*m*, 982*m*, 875*w*, 858*m*. ^{1}H-NMR (400 MHz): 7.52 (*d*, *J* = 8.8, H–C(5′)); 7.15 (*dd*, *J* = 8.8, 2.4, H–C(6′)); 7.02 (*d*, *J* = 2.4, H–C(8′)); 6.41 (*d*, *J* = 8.3, NH); 6.20 (*d*, *J* = 1.0, H–C(3′)); 4.69 (*d*, *J* = 4.2, OH); 4.14–3.58 (*m*, H–C(5), H–C(6), H–C(7), H–C(8), 2H–C(9)); after addn. of D_2O: 4.73 (*ddd*, *J* = 10.5, 10.5, 4.1, H–C(5)); 3.96 (*dd*, *J* = 10.5, 1.4, H–C(6)); 3.93–3.84 (*m*, H–C(8), H–C(9)); 3.77 (*dd*, *J* = 11.3, 4.1, H–C(9)); 3.63 (*dd*, *J* = 9.0, 1.4, H–C(7)); 3.76 (*s*, CH_3O); 3.06 (br. *s*, OH); 2.56 (*ddd*, *J* = 13.0, 3.5, 3.5, H_{eq}–C(3)); 2.41 (*d*, *J* = 1.0, CH_3–C(4′)); 2.29–2.09 (*m*, H_{ax}–C(3), H_{eq}–C(4), OH); 2.03 (*s*, CH_3); 1.68–1.54 (*m*, H_{ax}–C(4)). ^{13}C-NMR (50 MHz): 172.32 (*s*); 169.06 (*s*); 161.04 (*s*); 156.88 (*s*); 154.09 (*s*); 152.62 (*s*); 125.36 (*d*); 117.40 (*d*); 116.10 (*s*); 113.17 (*d*); 108.36 (*d*); 101.30 (*s*); 77.64 (*d*); 70.34 (*d*); 69.18 (*d*); 64.13 (*t*); 53.15 (*q*); 44.32 (*d*); 31.56 (*t*); 26.30 (*t*); 22.87 (*q*); 18.51 (*q*).

b) *From* **13**. A soln. of **13** (20 mg, 0.044 mmol) in MeOH (1.5 ml) was treated with CH_2N_2 in Et_2O until a slightly yellow colour stayed. Solvent was removed, and FC (5 g, SiO_2, *cf. a*) gave **12** (16 mg, 77%). IR and ^{1}H-NMR: indistinguishable from those obtained in *a* with the exception of the chemical shifts of the OH signals.

*4-Methyl-2-oxo-2*H*-1-benzopyran-7-yl 5-Acetamido-3,4,5-trideoxy-*α*-*D*-*inanno*-2-nonulopyranosidonic Acid* (**13**). To a soln. of **9** (100 mg, 0.169 mmol) in anh. MeOH (5 ml) were added 150 µl of 0.5M NaOMe/MeOH. After 2 h at r.t., TLC (CH_2Cl_2/MeOH 4:1) indicated the disappearance of **9**. The solvent was evaporated, the residue dissolved in H_2O (5 ml) and stirred at r.t. until the intermediate methyl ester **12** had disappeared as indicated by TLC (AcOEt/MeOH/0.2M aq. HCOOH 7:2:1). The soln. was acidified to pH 4 by addn. of *Dowex 50* (H^+). The resin was filtered off, washed with H_2O (5 ml), and the filtrate was freeze-dried to give crude **13** (75 mg, 98%). The residue was dissolved in H_2O/MeOH (1:1) and purified by FC (8 g SiO_2, AcOEt/MeOH/H_2O 7:2:1). Fractions containing **13** were combined, concentrated, extracted with H_2O, and freeze-dried to give pure **13** (60 mg, 78%; 72 h at 10^{-5} mbar over P_2O_5). $[\alpha]_D^{22} = +79.3°$ (*c* = 1.0, H_2O). IR (KBr): 3420*s*, 1700*m*, 1612*s*, 1552*m*, 1435*w*, 1390*m*, 1370*w*, 1275*w*, 1135*w*, 1072*m*, 1040*m*, 1017*w*. ^{1}H-NMR (400 MHz, D_2O): 7.71 (*d*, *J* = 8.5, H–C(5′)); 7.17 (*dd*, *J* = 8.5, 2.2, H–C(6′)); 7.16 (*d*, *J* = 2.2, H–C(8′)); 6.26 (*d*, *J* = 0.9, H–C(3′)); 4.16–4.02 (*m*, H–C(5), H–C(6)); 3.91 (*ddd*, *J* = 9.0, 6.5, 2.5, H–C(8)); 3.89 (*dd*, *J* = 12.0, 2.5, H–C(9)); 3.66 (*dd*, *J* = 12.0, 6.5, H–C(9)); 3.63 (*d*, *J* = 9.0, H–C(7)); 2.54 (*ddd*, *J* = 13.0, 4.0, 4.0, H_{eq}–C(3)); 2.43 (*d*, *J* = 0.9, CH_3–C(4′)); 2.21–2.12 (*m*, H_{eq}–C(4)); 2.05 (*ddd*, *J* = 13.0, 10.0, 4.5, H_{ax}–C(3)); 2.02 (*s*, CH_3); 1.69–1.56 (*m*, H_{ax}–C(4)). ^{13}C-NMR (50 MHz, D_2O): 174.68 (*s*); 173.76 (*s*); 164.61 (*s*); 158.13 (*s*); 156.39 (*s*); 153.58 (*s*); 126.41 (*d*); 117.82 (*d*); 115.92 (*s*); 111.71 (*d*); 107.97 (*d*); 103.92 (*s*); 76.56 (*d*); 72.10 (*d*); 68.96 (*d*); 63.26 (*t*); 44.77 (*d*); 32.44 (*t*); 26.70 (*t*); 22.42 (*q*); 18.33 (*q*).

*Sodium (4-Methyl-2-oxo-2*H*-1-benzopyran-7-yl 5-Acetamido-3,4,5-trideoxy-*α*-*D*-manno-2-nonulopyranosid)onate* (**14**). To a suspension of **12** (30 mg, 0.064 mmol) in H_2O (1 ml) were added 130 µl of 0.5M NaOMe/MeOH soln. After 6 h at r.t., TLC (CH_2Cl_2/MeOH 9:1) indicated the disappearance of **12**. The soln. was freeze-dried, and the residue was chromatographed (6 g SiO_2, AcOEt/MeOH/2.5% aq. Et_3N 7:2:1). Fractions containing the Et_3N^+ salt were combined, concentrated, and transformed to the Na^+ salt by passing it through *Dowex 50 W* × *4* (Na^+; 8 ml) to give, after freeze-drying, **14** (25 mg, 83%) as a microcristalline solid, which decomposed at 142–144°. $[\alpha]_D^{25} = +74.9°$ (*c* = 1.0, H_2O). ^{1}H-NMR (400 MHz, D_2O): 7.68 (*d*, *J* = 8.6, H–C(5′)); 7.17 (*dd*, *J* = 8.6, 1.7, H–C(6′)); 7.15 (*d*, *J* = 1.7, H–C(8′)); 6.22 (*s*, H–C(3′)); 4.12–4.03 (*m*, H–C(5), H–C(6)); 3.92 (*ddd*, *J* = 9.5, 6.3, 2.0, H–C(8)); 3.90 (*dd*, *J* = 12.0, 2.0, H–C(9)); 3.67 (*dd*, *J* = 12.0, 6.3, H–C(9)); 3.63 (*d*, *J* = 9.5, H–C(7)); 2.55 (*ddd*, *J* = 13.5, 4.0, 3.5, H_{eq}–C(3)); 2.41 (*s*, CH_3–C(4′)); 2.21–2.13 (*m*, H_{eq}–C(4)); 2.05 (*ddd*, *J* = 13.5, 10.0, 4.5, H_{ax}–C(3)); 1.68–1.57 (*m*, H_{ax}–C(4)).

Sialidase (EC 3.2.1.18) Experiments. The susceptibility of MU-4-deoxy-Neu5Ac **13** was assayed in 1 ml of 0.1M NaOAc buffer containing 0.2 µmol of either **13** or 4-methylumbelliferyl α-D-glycoside **16** of *N*-acetylneuraminic acid (synthesized according to *Warner* and *O'Brien* [32] with a slight modification by *Berg et al.* [33]) and 10 mU of sialidase. The sialidases tested were from *Vibrio cholerae* (*Behringwerke*, Marburg) and *Arthrobacter ureafaciens* (*CalBiochem*), respectively. In the case of the *V. cholerae* enzyme, the acetate buffer was adjusted to pH 5.5 and $CaCl_2$ was added to a final concentration of 1 mM, and in the case of the *A. ureafaciens* sialidase the pH was adjusted to pH 5.0. Blanks were run with both glycosides **13** and **16** containing no enzyme or containing inactivated (5-min heating at 100°) enzyme. The mixture were incubated at 37° and 0.1-ml aliquots were withdrawn after various incubation times between 5 and 60 min followed by monitoring in a fluorimeter (365 nm for excitation and 450 nm for emission). The fluorescence of the blanks was substraced from those of the assays containing sialidase.

To test a possible inhibitory effect of the free acid **1** on sialidase activity, the following incubations were made: 1 ml of incubation mixture of 0.1M acetate buffer, pH 5, contained 5 mU of *V. cholerae* sialidase, 0.2 µmol of **16**, and 1 µmol of the Na^+ salts of **1**. In blanks, the enzyme was omitted. After 0, 5, 10, and 15 min, 0.1-ml aliquots were withdrawn and measured fluorimetrically for liberated 4-methylumbelliferone.

The influence of **13** on sialidase activity was checked by incubation in 1 ml of 0.1M acetate buffer, pH 5.5, 0.20 μmol of **16**, 0.61 μmol of **13**, and 1 mU of *V. cholerae* sialidase. For comparison, corresponding assays were made without **13**. In blanks sialidase was omitted. Liberated 4-methylumbelliferone was monitored after 0, 5, 10, 15, and 30 min by withdrawal of 0.1-ml aliquots from this mixture.

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