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PHASE-TRANSFER-CATALYZED SYNTHESIS OF ARYL α-KETOSIDES OF *N*-ACETYLNEURAMINIC ACID. A 2-METHYLFLUORAN-6-YL GLYCOSIDE OF *N*-ACETYLNEURAMINIC ACID, 2-METHYL-6-(5-ACETAMIDO-3,5-DIDEOXY-α-D-*glycero*-D-*galacto*-NONULOPYRANOSYL-ONIC ACID)XANTHENE-9-SPIRO-1′-ISOBENZOFURAN-3′-ONE, A NEW SUBSTRATE FOR NEURAMINIDASE ASSAY*

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ABSTRACT

Glycosidation of *N*-acetylneuraminic acid by phase-transfer catalysis in chloroform–aqueous alkali gave several known and some new aryl α-ketosides in a short reaction time and in good yields. The 4-methylumbelliferyl α-ketoside, the standard substrate for neuraminidase, was prepared in a yield of up to 70%. New Neu5Ac ketosides were prepared with fluorescein and the fluorescein analog, 2-methyl-6-hydroxyfluoran (2-methyl-6-hydroxyxanthene-9-spiro-1′-isobenzofuran-3′-one) as aglycons, the latter being synthesized from 2-(2-hydroxy-5-methylbenzoyl) benzoic acid and 3-fluorophenol. The α configuration was ascertained by 400-MHz ^{1}H-n.m.r. spectroscopy and by cleavage of the ketosides with neuraminidases from *Vibrio cholerae* and *Clostridium perfringens*. The enzymic hydrolysis of the 2-methylfluoran-6-yl ketoside gave K_m values of 82 μM (*V. cholerae*) and 96 μM (*C. perfringens*).

INTRODUCTION

Sialidase (EC 3.2.1.18) plays an important role in splitting off the α-ketosidic linkage of sialic acids in glycoconjugates that regulate cell-to-cell interactions[1]. For the qualitative and quantitative estimation of this enzyme, widely distributed in viruses, bacteria, and mammalian tissues[2], synthetic α-ketosides of *N*-acetylneuraminic acid (**1**) are required as substrates. The aglycons of these ketosides must have easily detectable properties, such as the characteristic spectroscopic properties of aryl groups.

Since the reports of Meindl and Tuppy[3,4], and Kuhn *et al.*[5], many α-ketosides

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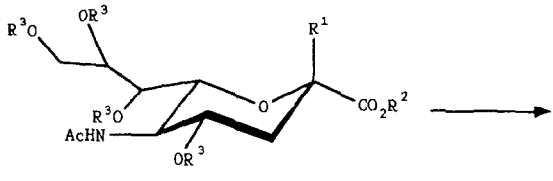
of **1** with aglycon and glycon groups have been synthesized. Some aryl ketosides were used in the neuraminidase assay. The first of these chromogenic substrates was the phenyl α-ketoside (**11**) synthesized by Meindl and Tuppy[6]. The released phenol is detected, after derivatization, with the Folin–Ciocalteu reagent. Neuraminidase hydrolysis of the *p*-nitrophenyl ketoside[7,8] (**12**), first synthesized by Privalova and Khorlin[7], releases *p*-nitrophenol, which can be detected without further derivatization. Tuppy and Palese[9] synhesized the 3-methoxyphenyl ketoside (**13**). The 3-methoxyphenol released forms a chromophore with 4-amino-2,5-dimethoxy-4-nitrobenzene[9] or 4-aminoantipyrine[10,11]. A further chromogenic substrate, the 5-bromo-3-indolyl ketoside, was prepared by Eschenfelder and Brossmer[12,13]. Enzymically released 5-bromoindoxyl spontaneously forms green 5,5′-dibromoindigo. The most sensitive assay developed up to now uses the 4-methylumbelliferyl ketoside[14–18] (**14**) as substrate. Liberated 4-methylumbelliferone is determined by fluorimetry to a concentration down to the nmol.

The number of methods for the stereoselective preparation of ketosides of **1** is limited. The disadvantages of the Koenigs–Knorr method[19] are the long reaction time, the expensive catalyst, and often the low yields, and that of the Williamson method[8,17] is the low solubility of the aryl salt in organic solvents. The ketosides described herein could not be prepared with these methods. Therefore, we used phase-transfer catalysis[20] to prepare several aryl α-ketosides. In this reaction, the aglycon compound forms, in aqueous alkali solution, a reactive nucleophilic anion which reacts with the carbohydrate halide in an organic phase in the presence of a tetralkyl- or aryltrialkyl-ammonium salt as phase-transfer catalyst.

Baggett and Marsden[18] prepared the tetrabutylammonium salt of 4-methylumbelliferone (7-hydroxy-4-methylcoumarin) by a phase-transfer-like reaction, but this salt was condensed with **3** in a separate reaction. We describe herein the direct condensation of **3** with 4-methylumbelliferone and other compounds, such as phenol, *p*-nitrophenol, and 3-methoxyphenol, in a one-step, phase-transfer reaction. Using this method, we also succeeded in preparing new ketosides of *N*-acetylneuraminic acid (**1**) with fluorescein (**21**) and the fluorescein-analog chromophore, 6-hydroxy-2-methylfluoran (**19**).

RESULTS AND DISCUSSION

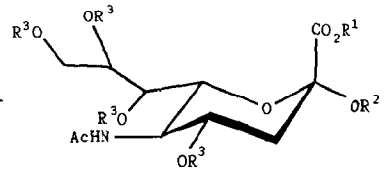
The high stereoselectivity of phase-transfer catalysis as a new effective, one-step procedure to synthesize aryl α-ketosides of *N*-acetylneuraminic acid (**1**) was shown by ^{1}H-n.m.r.-spectroscopy of several known and unknown ketosides, and by use of these ketosides as substrates for neuraminidase. The known phenyl (**10**), *p*-nitrophenyl (**11**), 3-methoxyphenyl (**12**), and methylumbelliferyl ketoside (**13**) were synthesized with this method, and comparison of their properties with those of the literature confirmed the α configuration. Chemical shifts at δ 2.84–2.88 for H-3*e*, as well as positive rotation values were observed[21,22]. Further, the ketosides could be cleaved by sialidase which is known to hydrolyze α-ketosides of **1**.



1 R^1 = OH, R^2 = R^3 = H

2 R^1 = OH, R^2 = Me, R^3 = H

3 R^1 = Cl, R^2 = Me, R^3 = Ac



4 R^1 = Me, R^2 = a, R^3 = Ac

5 R^1 = Me, R^2 = b, R^3 = Ac

6 R^1 = Me, R^2 = c, R^3 = Ac

7 R^1 = Me, R^2 = d, R^3 = Ac

8 R^1 = Me, R^2 = e, R^3 = Ac

9 R^1 = Me, R^2 = f, R^3 = Ac

10 R^1 = H, R^2 = a, R^3 = H

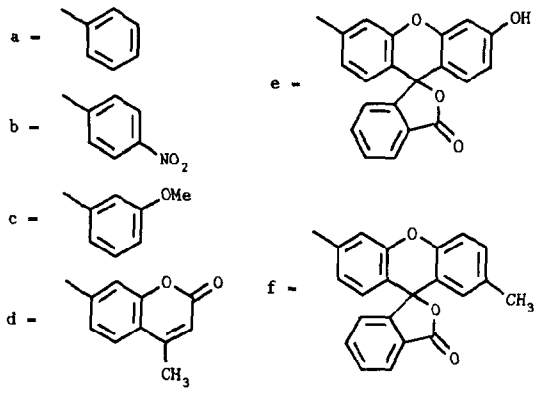
11 R^1 = H, R^2 = b, R^3 = H

12 R^1 = H, R^2 = c, R^3 = H

13 R^1 = H, R^2 = d, R^3 = H

14 R^1 = H, R^2 = e, R^3 = H

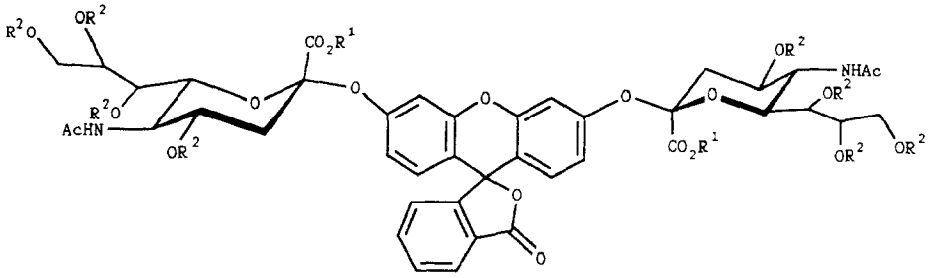
15 R^1 = H, R^2 = f, R^3 = H



Liberated *p*-nitrophenol was spectrophotometrically detected at 400 nm, and 4-methylumbelliferone by fluorescence spectrophotometry (excitation at 360 nm, emission at 450 nm). The cleavage of the phenyl (**10**) and methoxyphenyl ketoside (**13**) was determined by t.l.c.

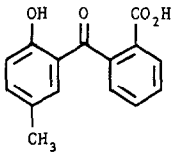
The synthesis of the 4-methylumbelliferyl ketoside was carried out as an optimized procedure with a yield of up to 70% in the coupling step. Use of other aryl aglycons, although nonoptimized, gave yields of 55–65% in the coupling step from **3** to give **4**, **5**, and **6**. These yields are comparable with others or even higher than those described in the literature. By further varying the concentration of sodium hydroxide, the amount of catalyst, or the concentration of the aryl alcohols, the synthesis may give still higher yields.

The sodium hydroxide concentration of 0.1M was optimal for the synthesis of **13** because hydrolysis of **3** was largely suppressed and the destruction of the methylumbelliferone prevented. At higher sodium hydroxide concentrations, the dark-red color of the aqueous layer showed the destruction of 4-methylumbelliferone.

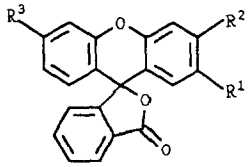


16 R^1 = Me, R^2 = Ac

17 R^1 = R^2 = H



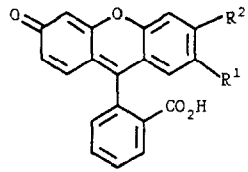
18



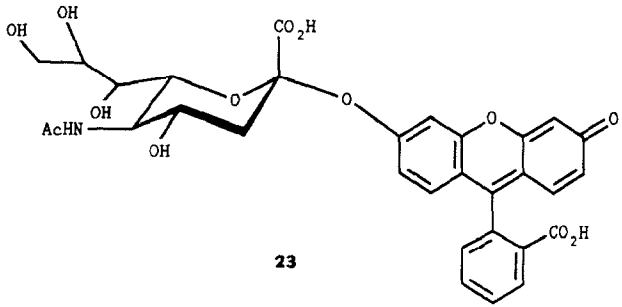
19 R^1 = Me, R^2 = H, R^3 = OH

20 R^1 = Me, R^2 = H, R^3 = F

21 R^1 = H, R^2 = R^3 = OH



22 R^1 = Me, R^2 = H



23

New ketosides of *N*-acetylneuraminic acid could be prepared with fluorescein as aglycon. The monoketoside **8** was obtained in a yield of 40%, whereas the Koenigs–Knorr procedure gave a yield lower than 10%. The di(*N*-acetylneuraminic acid) ketoside **16** could be isolated as a byproduct in low yield; it could not be isolated in the Koenigs–Knorr method. The purification of **16** was not possible, because this compound is very unstable, and the largest part of the product was destroyed during deacetylation and deesterification. In t.l.c. examinations of the

TABLE I

SPECTROSCOPICAL DATA FOR 6-HYDROXY-2-METHYLFLUORAN (**19**), 6-FLUORO-2-METHYLFLUORAN (**20**), 2-METHYL-6-HYDROXYFLUORYL KETOSIDE (**15**), MONOFLUORESCEIN KETOSIDE (**14**), AND DIFLUORESCEIN KETOSIDE (**16**)

Compound	*Fluorescence*	λ_{max} *(nm)*	ε	*Excit.*$_{max}$ *(nm)*	*Emiss.*$_{max}$ *(nm)*
14	+	473		480	518
		453			
15	–	<300			
16	–	<300			
19	+	457	16 301	458	528
		364	13 116		
20	–	<300			

hydrolysis products of **16** and **17**, **8** and fluorescein were always detectable as byproducts. Thus, exact analytical data could not be obtained, but ^{1}H-n.m.r. examinations and the behavior of the isolated compound against mineral acid supported the existence of structure **16**. Because of its instability and the low yield of its synthesis, **17** was not considered a good substrate for neuraminidase assay. However, the clear formation of a fluorescent compound by neuraminidase treatment of impure **17** indicated the α-configuration of the substrate.

The monofluorescein compound **14** is also not a useful substrate to detect neuraminidase activity because of its high fluorescence (Table I). The free hydroxyl group enables the formation of a quinoid structure **23** which is one condition for the fluorescence. The ketoside **14** showed the same u.v. spectrum as the monoethyl ether of fluorescein[23] with maxima at 453 and 473 nm over a wide pH range (Table I). This is characteristic for monosubstituted fluorescein compounds. It showed a bright yellow fluorescence with excitation maxima at 480 and emission maxima at 518 nm.

Therefore, we synthesized 2-methyl-6-hydroxyfluoran (**19**) according to Gronowska *et al.*[24]; it has the same basic structure as fluorescein but only one phenolic group to form the quinoid system **22**. By substitution of this group, the formation of the quinoid system is blocked and the fluorescence suppressed. On cleavage of the glycosidic bond, the chromophore **22** is liberated and can be detected fluorimetrically.

A useful and effective method to prepare monohydroxyfluorans was developed by Gronowska *et al.*[24]. They prepared the basic compound 3-hydroxyfluoran by the reaction of 2-(2-hydroxybenzoyl)benzoic acid with 3-fluorophenol in the presence of zinc chloride. We used 2-(2-hydroxy-5-methylbenzoyl)benzoic acid (**18**) as intermediate component because the synthesis of this compound is more efficient than the synthesis of 2-(2-hydroxybenzoyl)benzoic acid. By use of *p*-cresol in the Friedel–Crafts reaction with phthalic anhydride, only the ortho position can be substituted and **18** is the major component formed. The methyl group of C-5

does not influence the further reaction with 3-fluorophenol and the properties of the formed 2-methyl-6-hydroxyfluoran. Although the nonfluorescent fluorofluoran **20** (Table I) was formed as a byproduct, the yield of **19** is much higher than in the synthesis of Ghatak and Dutt[25] who first prepared this compound. Compound **19** can be excited at 458 nm and the emission measured at 528 nm. The extinction coefficient at 458 nm has a value of 16 301 at pH 11 (Table I). Quantitative measurement can be carried out down to concentrations of 10^{-12}M.

A nonfluorescent α-ketoside of **19** with *N*-acetylneuraminic acid (Table I) could be prepared by phase-transfer catalysis, but no ketoside could be isolated with the Koenigs–Knorr method. The ^{1}H-n.m.r.-value of δ 2.9, the positive rotation value of $[\alpha]_D^{20}$ +16°, and the neuraminidase cleavage of the compound showed the α configuration. By incubation of the ketoside with *V. cholerea* or *C. perfringens* neuraminidase, a strong increase of fluorescence could be observed.

Kinetic studies were carried out with *V. cholerae* and *C. perfringens* neuraminidase. K_m values of 82μM for *V. cholerae* and 96μM for *C. perfringens* neuraminidase were obtained. These values are lower than the values obtained with the 4-methylumbelliferyl ketoside which are[14,16] in the range of 0.1mM. The low K_m values of the ketoside **14** demonstrated a higher affinity of this substrate for the tested neuraminidase than the affinity of **13**. The high fluorescence of the free fluoran compound makes possible a quantitative determination down to a concentration of 10^{-12}M, and so the substrate reaches the same sensitivity as the ketoside **13**. Furthermore, **14** showed no rest-fluorescence and the fluoran **19** is less sensitive to photochemical decomposition than 4-methylumbelliferone[26]. These observations showed that the newly synthesized ketoside **14** is a useful substrate for neuraminidase assay and offers an alternative to other synthetic substrates.

The one-step, phase-transfer-catalyzed glycosidation of *N*-acetylneuraminic acid (**1**) with 4-methylumbelliferone, instead of a separate isolation of the tetraalkylammonium salt of 4-methylumbelliferone, and then separate glycosidation reaction as described by Baggett and Marsden[18], has the advantage of shorter reaction times. This and the fact that no expensive silver salt is necessary, as for the Koenigs–Knorr method, are also very important for the synthesis of the other aryl ketosides. The short reaction times together with the small formation of byproducts could make the phase-transfer procedure interesting for commercial production.

Phase-transfer catalysis is a very efficient synthetic method for the preparation of aryl α-ketosides of *N*-acetylneuraminic acid (**1**). The preparation of several known and unknown α-ketosides of this compound, as described in this paper, demonstrates the possibility of various applications of the procedure. Generally, organic compounds that are able to form ions under mild conditions can be glycosidically coupled with *N*-acetylneuraminic acid (**1**).

EXPERIMENTAL

General methods. — Melting points are uncorrected. Optical rotations were

determined with a Perkin–Elmer 241 automatic polarimeter. Fluorescence was measured with a Perkin–Elmer 650 10LC fluorescence spectrophotometer. I.r. spectra were recorded with a Beckman Acculab 1-spectrometer and u.v. spectra with a Beckman DU 50 spectrophotometer. ^{1}H-N.m.r. spectra were recorded with a Bruker AM 400-spectrometer. *N*-Acetylneuraminic acid was isolated from edible birds nests. *Vibrio cholerae* (0.22 U/mL) and *Clostridium perfringens* (3 U/mg) neuraminidase were obtained from Sigma (USA). T.l.c. was performed on Silica Gel Merck 60 F_{254} with detection by u.v. light or by charring with H_2SO_4 in (*A*) ethyl acetate, (*B*) 1:1 (v/v) ethyl acetate–petrol ether, (*C*) 1:9 (v/v) methanol–chloroform, and (*D*) 2:1:1 (v/v) propanol–butanol–water. Column chromatography was conducted on Silica Gel Merck 60.

*Methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-β-*D*-glycero-*D*-galacto-2-nonulopyranosyl chloride)onate* (**3**). — Methyl 5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate (**2**) was prepared by the method of Kuhn *et al.*[5]. Treatment of **2** with acetyl chloride (10 mL) in anhydrous glacial acetic acid in a closed vessel for 24 h at room temperature gave **3** as a colorless syrup. The syrup was used for subsequent reactions without further purification.

General glycosidation procedure. — Compound **3** (510 mg, 1.0 mmol) was dissolved in chloroform (25–30 mL) and the aryl compounds (2–5 mmol) were dissolved in aqueous NaOH (25–30 mL) of various concentrations. The two-phase system was refluxed for 0.5 h in the presence of benzyltriethylammonium chloride (0.5 g, 2.2 mmol) as phase-transfer catalyst. The organic layer was separated and twice washed with 0.1M NaOH solution saturated with NaCl (30 mL), and then washed with saturated NaCl solution (30 mL). It was dried ($MgSO_4$) and then concentrated to dryness under low pressure at room temperature. The residue was chromatographed on a silica gel column with ethyl acetate as solvent. The fractions containing the ketoside (controlled by t.l.c.) were pooled and evaporated. The ketosides could be crystallized from ethyl acetate–petrol ether.

*Methyl (phenyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-α-*D*-glycero-D-galacto-2-nonulopyranosid)onate* (**4**). — Phenol (470 mg) was dissolved in aqueous NaOH (25 mL, 0.2M) and the reaction was carried out according to the general glycosidation procedure. The yield was 330–350 mg (57–62%), m.p. 102–106°; ^{1}H-n.m.r. ($CDCl_3$): δ 1.91–2.12 (15 H, OAc, NAc), 2.20 (m, 1 H, H-3*a*), 2.72 (dd, 1 H, $J_{3e,4}$ 4.6, $J_{3e,3a}$ 12.75 Hz, H-3*e*), 3.64 (s, 3 H, $COCH_3$), 4.08 (m, 1 H, H-5), 4.12 (m, 1 H, H-9a), 4.30 (m, 1 H, H-9b), 4.39 (m, 1 H, H-6), 7.05 (m, 3 H, arom.), and 7.25 (m, 2 H, arom.).

*(Phenyl 5-acetamido-3,5-dideoxy-α-*D*-glycero-D-galacto-2-nonulopyranosid)-onic acid* (**10**). *General deacetylation procedure.* — Compound **4** (280 mg, 0.5 mmol) was dissolved in anhydrous methanol (50 mL) and 0.1M methanolic sodium methoxide solution (1 mL) was added. The mixture was stirred at room temperature for 2 h. Dowex 50W-X8 (H^+) cation-exchange resin was added to neutralize the base. The resin was filtered off and twice washed with methanol. The solvent was evaporated at room temperature and the residue dissolved in aqueous 0.1M

NaOH solution (100 mL). After being stirred for 0.5 h at room temperature, the mixture was made neutral again with Dowex 50W-X8 (H^+) resin and lyophilized to yield 150 mg (76%) of chromatographically pure **10** as a colorless powder, m.p. 141–143°, $[\alpha]_D^{20}$ +21.0° (*c* 1.2, methanol); ν_{max}^{KBr} 3550–3300 (OH), 3100 (C–H, arom.), 2950 (C–H, aliphat.), 1730, 1710 (C=O), 1600 (C–C, arom.), 1585, 1490 (CH_3), 1230 (COC, phenol), 1070, and 755 cm^{-1} (monosub. benzene); ^{1}H-n.m.r. (D_2O): 1.96 (m, 1 H, $J_{3a,3e}$ 12.32 Hz, H-3*a*), 2.03 (s, 3 H, NAc), 2.87 (dd, 1 H, $J_{3e,4}$ 4.60 Hz, H-3*e*), 3.56–3.90 (m, 7 H, H-4,5,6,7,8,9a,9b), 6.90 (m, 1 H, arom.), 7.17–7.21 (m, 2 H, arom.), and 7.29–7.38 (m, 2 H, arom.); lit.[6] m.p. 132–135°, $[\alpha]_D^{24}$ −13.5° (*c* 0.55, dimethyl sulfoxide).

*Methyl (4-nitrophenyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosid)onate* (**5**). — 4-Nitrophenol (700 mg, 5 mmol) was dissolved in aqueous NaOH (25 mL), and the reaction was carried out according to the general glycosidation procedure to give **5** (340–370 mg, 55–60%), m.p. 107–112°; ^{1}H-n.m.r. ($CDCl_3$): δ 1.94–2.18 (5 s, 15 H, 5 OAc, NAc), 2.30 (m, 1 H, $J_{3a,3e}$ 12.68 Hz, H-3*a*), 2.76 (dd, 1 H, $J_{3e,3a}$ 13.08, $J_{3e,4}$ 4.70 Hz, H-3*e*), 3.66 (s, 3 H, $COCH_3$), 4.08 (m, 1 H, H-5), 4.12 (m, 1 H, H-9a), 4.21 (m, 1 H, H-9b), 4.64 (m, 1 H, H-6), 4.97 (m, 1 H, H-4), 5.37 (m, 1 H, H-7), 5.38 (m, 1 H, H-8), 7.16 (m, 2 H, arom.), and 8.12–8.21 (m, 2 H, arom.); lit.[8] m.p. 104–108°.

*(4-Nitrophenyl 5-acetamido-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosid)onic acid* (**11**). — Deacetylation of **5** as described under the general deacetylation procedure gave **11** in 75% yield, m.p. 128–131°, $[\alpha]_D^{20}$ +53.5° (*c* 1.08, methanol); ν_{max}^{KBr} 3560 (NH), 3500–3300 (OH), 1750, 1730 (C=O), 1600 (C–C, arom.), 1520, 1360 (NO_2), 1240 (C–O–C), 1050, 890, 870, 740, and 660 cm^{-1}; ^{1}H-n.m.r. (D_2O): δ 1.90 (m, 1 H, $J_{3a,3e}$ 12.10 Hz, H-3*a*), 2.05 (s, 3 H, NAc), 2.84 (dd, 1 H, $J_{3e,3a}$ 12.80, $J_{3e,4}$ 4.54 Hz, H-3*e*), 3.54–4.21 (m, 7 H, H-4,5,6,7,8,9a,9b), 6.96–7.29 (m, 2 H, arom.), and 8.14–8.21 (m, 2 H, arom.); lit.[8] m.p. 162–164°, $[\alpha]_D^{25}$ +48.5° (*c* 1.3, methanol).

*(3-Methoxyphenyl 5-acetamido-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosid)onic acid* (**12**). — Reaction of 3-methoxyphenol (620 mg, 5 mmol), dissolved in 0.2M aqueous NaOH (25 mL) with **3** gave methyl (3-methoxyphenyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-α-*D*-*glycero*-*D*-*galacto*-2-nonulopyranosid)onate (**6**) (330–350 mg, 55–60%), m.p. 115–119°; ^{1}H-n.m.r. ($CDCl_3$): δ 1.98–2.16 (m, 16 H, H-3*a*, OAc, NAc), 2.62 (dd, 1 H, $J_{3e,3a}$ 12.92, $J_{3e,4}$ 4.68 Hz, H-3*e*), 3.66 (s, 3 H, $COCH_3$), 3.73 (s, 3 H, $C_6H_4OCH_3$), 4.04 (m, 1 H, H-5), 4.15 (m, 1 H, H-9a), 4.30 (m, 1 H, H-9b), 4.36 (m, 1 H, H-6), 4.92 (m, 1 H, H-4), 5.32 (m, 1 H, H-7), 5.33 (m, 1 H, H-8), 6.59–6.65 (m, 3 H, arom.), and 7.09–7.13 (m, 1 H, arom.).

The general deacetylation procedure applied to **6** gave **12** in 70–75% yield, m.p. 133–137°, $[\alpha]_D^{20}$ +25° (*c* 1.35, methanol); ν_{max}^{KBr} 3500–3300 (OH), 2980 (CH–, aliph.), 1730, 1650 (C=O), 1600 (C–C arom.), 1490 (CH_3), 1375 (C–O–C, phenol), 1280, 1200, 1145, and 1040 cm^{-1}; ^{1}H-n.m.r. (D_2O): δ 1.97 (m, 1 H, $J_{3a,3e}$ 12.32 Hz, H-3*a*), 2.04 (s, 3 H, NAc), 2.86 (dd, 1 H, $J_{3e,3a}$ 12.66, $J_{3e,4}$ 4.68 Hz, H-3*e*), 3.80 (s,

3 H, $C_6H_4OCH_3$), 3.58–4.11 (m, 7 H, H-4,5,6,7,8,9a,9b), 6.55 (m, 1 H, arom.), 6.62–6.77 (m, 2 H, arom.), and 7.25 (m, 1 H, arom.); lit.[9] m.p. 127–129°, $[\alpha]_D^{24}$ −31° (*c* 4.5, dimethyl sulfoxide).

Methyl(4-methylcoumarin-7-yl5-acetamido-4,7,8,9-tetra-O-*acetyl-3,5-dideoxy*-α-D-glycero-D-galacto-*2-nonulopyranosid)onate* (**7**). — Reaction of 4-methylcoumarin-7-yl sodium salt (1 g, 5 mmol), dissolved in 0.1M aqueous NaOH (25 mL), according to the general glycosidation procedure gave **7** (420–450 mg, 65–69%), m.p. 94–98°; ^{1}H-n.m.r. ($CDCl_3$): δ 1.93–2.19 (5 s, 15 H, OAc, NAc), 2.26 (m, $J_{3a,3e}$ 12.68 Hz, H-3*a*), 2.42 (s, 3 H, CH_3), 2.74 (dd, 1 H, $J_{3e,3a}$ 13.02, $J_{3e,4}$ 4.68 Hz, H-3*e*), 3.69 (s, 3 H, $COCH_3$), 4.06 (m, 1 H, H-5), 4.14 (m, 1 H, H-9a), 4.30 (m, 1 H, H-9b), 4.54 (m, 1 H, H-6), 5.00 (m, 1 H, H-4), 5.38 (m, 1 H, H-7), 5.39 (m, 1 H, H-8), 6.21 (s, 1 H, olefinic), 7.01 (m, 1 H, arom.), 7.08 (m, 1 H, arom.), and 7.53 (m, 1 H, arom.); lit.[16] m.p. 82–83°, lit.[17] m.p. 98–100°, lit.[18] 98–100°.

(4-Methylcoumarin-7-yl 5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-*2-nonulopyranosid)onic acid* (**13**). — Deacetylation of **7** (400 mg, 0.6 mmol) according to the general procedure gave **13** (230 mg, 80%), m.p. 178–181°, $[\alpha]_D^0$ +48.5° (*c* 0.95, water); ν_{max}^{KBr} 3500 (NH), 3490–3300 (OH), 2975 (CH–, aliph.), 1700, 1645 (C=O), 1615 (C–C, arom.), 1570 (CH_3), 1390 (C–O–C, phenol), 1280, 1140, 1075, and 1025 cm^{-1}; ^{1}H-n.m.r. (D_2O): δ 2.00 (m, 1 H, $J_{3a,3e}$ 12.32 Hz, H-3*a*), 2.04 (s, 3 H, NAc), 2.38 (s, 3 H, CH_3), 2.88 (dd, 1 H, $J_{3e,3a}$ 12.62, $J_{3e,4}$ 4.70 Hz, H-3*e*), 3.59–4.90 (m, 7 H, H-4,5,6,7,8,9a,9b), 6.19 (m, 1 H, olefinic), 7.13 (m, 1 H, arom.), 7.16 (m, 1 H, arom.), and 7.65 (m, 1 H, arom.); lit.[17] m.p. 162°, lit.[18] m.p. 178°, lit.[14] $[\alpha]_D^{25}$ −9.7° (water), lit.[15] $[\alpha]_D^{22}$ +59° (water), lit.[16] $[\alpha]_D$ +51° (*c* 0.9, water), lit.[18] $[\alpha]_D$ +70° (*c* 0.8, water).

3-Hydroxy-6-[methyl(5-acetamido-4,7,8,9-tetra-O-*acetyl-3,5-dideoxy*-α-D-glycero-D-galacto-*2-nonulopyranosyl)onate]xanthene-9-spiro-1'-isobenzofuran-3'-one* (**8**). — Fluorescein (**21**) (1 g, 3 mmol) reacted with **3**, according to the general glycosidation procedure, to give **8** (420 mg) as the main product in 40% yield, m.p. 123–129°; ν_{max}^{KBr} 3400 (NH), 2980 (CH, aliph.), 1750, 1660 (C=O), 1605 (C=C, arom.), 1465, 1430, 1375, 1230 (C–O–C, phenol), 1100, 1080, 1035, 860, and 755 cm^{-1}; ^{1}H-n.m.r. ($CDCl_3$): δ 1.95–2.19 (5 s, 15 H, OAc, NAc), 2.21 (m, 1 H, H-3*a*), 2.82 (m, 1 H, H-3*e*), 3.68 (s, 3 H, $COCH_3$), 4.08 (m, 1 H, H-5), 4.16 (m, 1 H, H-9a), 4.29 (m, 1 H, H-9b), 4.53 (m, 1 H, H-6), 5.00 (m, 1 H, H-4), 5.35 (m, 1 H, H-7), 5.38 (m, 1 H, H-8), 6.05 (m, 1 H, arom. OH), 6.57–6.81 (m, 5 H, arom.), 7.04 (m, 1 H, arom. H), 7.19 (m, 1 H, arom.), 7.80–7.89 (m, 2 H, arom.), and 8.02 (m, 1 H, arom.).

Anal. Calc. for $C_{40}H_{39}NO_{17}$: C, 59.63; H, 4.88; N, 1.74. Found: C, 59.83; H, 4.94; N, 1.72.

3-Hydroxy-6-(5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-*2-nonulopyranosylonic acid)xanthene-9-spiro-1'-isobenzofuran-3'-one* (**14**). — Deprotection of **8** according to the general procedure gave **14** as a chromatographically pure, bright-yellowish powder, m.p. 165–170°, $[\alpha]_D^0$ +7.7° (*c* 1.56, methanol), t.l.c. R_F

0.42 (*C*); ν_{max}^{KBr} 3500–3300 (OH), 2970 (CH, aliph.), 1740, 1640 (C=O), 1490 (CH_3), 1430, 1380, 1250 (C–O–C, phenol), 1175, 1035, and 710 cm^{-1}; ^{1}H-n.m.r. [D_2O–$(CD_3)_2SO$]: δ 2.01 (s, 3 H, NAc), 2.16 (m, 1 H, $J_{3a,3e}$ 12.65 Hz, H-3*a*), 2.83 (d, 1 H, $J_{3e,3a}$ 12.88, $J_{3e,4}$ 4.40 Hz, H-3*e*), 3.38–4.29 (m, 7 H, H-4,5,6,7,8,9a,9b), 6.60–6.94 (m, 5 H, arom.), 7.22 (m, 1 H, arom.), 7.68 (m, 1 H, arom.), 7.77 (m, 1 H, arom.), 7.84 (m, 1 H, arom.), and 8.06 (m, 1 H, arom.).

Anal. Calc. for $C_{31}H_{29}NO_{13}$: C, 59.71; H, 4.69; N, 2.25. Found: C, 59.45; H, 4.51; N, 2.01.

*2-Methyl-6-[methyl(5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosyl)onate]xanthene-9-spiro-1'-isobenzofuran-3'-one* (**9**). — Glycosidation of **19** (830 mg, 2.5 mmol), dissolved in 0.7M aqueous NaOH (40 mL), with **3** according to the general procedure gave **9** (280–320 mg, 35–40%), m.p. 120–125°, $[\alpha]_D^{20}$ +1.9° (*c* 1.0, methanol), t.l.c. R_F 0.36 (*A*); ν_{max}^{KBr} 3400 (NH), 2975 (CH, aliph.), 1760, 1690 (C=O), 1610 (C=C, arom.), 1490 (CH_3), 1430, 1370, 1235 (C–O–C, phenol), 1110, 1080, 1040, 820, and 750 cm^{-1}; ^{1}H-n.m.r. ($CDCl_3$): δ 1.97–2.15 (m, 16 H, H-3*a*, OAc, NAc), 2.19 (s, 3 H, CH_3), 2.69 (dd, 1 H, $J_{3e,3a}$ 13.04, $J_{3e,4}$ 4.98 Hz, H-3*e*), 3.73 (s, 3 H, $COCH_3$), 4.06 (m, 1 H, H-5), 4.15 (m, 1 H, H-9a), 4.28 (m, 1 H, H-9b), 4.47 (m, 1 H, H-6), 5.00 (m, 1 H, H-4), 5.30 (m, 1 H, H-7), 5.36 (m, 1 H, H-8), 6.55 (m, 1 H, arom.), 6.75 (m, 1 H, arom.), 7.04–7.27 (m, 5 H, arom.), 7.61–7.70 (m, 2 H, arom.), and 8.04 (m, 1 H, arom.).

Anal. Calc. for $C_{41}H_{41}NO_{16}$: C, 61.27; H, 5.14; N, 1.75. Found: C, 61.02; H, 5.10; N, 1.64.

*2-Methyl-6-(5-acetamido-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosylonic acid)xanthen-9-spiro-1'-isobenzofuran-3'-one* (**15**). — Deprotection of **9** according to the general procedure gave **15** as a colorles powder (75% yield), m.p. 169–174°, $[\alpha]_D^{20}$ +14.6° (*c* 1.2, methanol); ν_{max}^{KBr} 3500–3300 (OH), 2975 (CH, aliph.), 1780, 1640 (C=O), 1610 (C–C, arom.), 1490 (CH_3), 1435, 1250 (C–O–C, phenol), 1300, 1400, and 1120 cm^{-1}; ^{1}H-n.m.r. (D_2O): δ 1.96 (m, 1 H, H-3*a*), 2.04 (s, 3 H, NAc), 2.15 (s, 3 H, CH_3), 2.89 (m, 1 H, H-3*e*), 3.53–4.04 (m, 7 H, H-4,5,6,7,8,9a,9b), 6.70 (m, 1 H, arom.), 6.86 (m, 1 H, arom.), 7.14–7.34 (m, 5 H, arom.), 7.75–7.82 (m, 2 H, arom.), and 8.08 (m, 1 H, arom.).

Anal. Calc. for $C_{32}H_{31}NO_{12}$: C, 61.83; H, 5.03; N, 2.25. Found: C, 61.27; H, 5.34; N, 2.22.

2-(2-Hydroxy-5-methylbenzoyl)benzoic acid (**18**). — This compound was prepared according to Ullman and Schmidt[27] with some modifications. The addition of $AlCl_3$ was completed after 5–10 minutes. When the reaction was completed, the mixture was hydrolyzed with M HCl. The organic layer was separated and evaporated to dryness under reduced pressure. The resulting yellow-brown mass was dissolved in warm, M aqueous NaOH and precipitated with conc. HCl, and then **18** crystallized from 30% ethanol–water to give yellowish needles (81%), m.p. 194–196, t.l.c. R_F 0.8 (*A*), 0.5 (*B*), and 0.3 (*C*); ν_{max}^{KBr} 3300–3000 (OH), 2700, 2600 (arom.), 1690 (C=O, acid), 1650 (C=O), 1600 (C–C, arom.), 1490, 1430 (CH_3), 1340 (OH), 1300, 1230 (C–O–H, phenol), 1150, 1090, 970, 875 and 800 (disubstit.

benzene), and 750 cm^{-1} (monosubstit. benzene); ^{1}H-n.m.r. ($CDCl_3$): δ 2.14 (s, 3 H, CH_3), 6.81, 6.95, 7.27, 7.35, 7.59, 7.60, and 8.14 (m, 7 H, arom.).

Anal. Calc. for $C_{14}H_{12}O_4$: C, 68.85; H, 4.95. Found: C, 68.70; H, 4.78.

6-Hydroxy-2-methylxanthen-9-spiro-1'-isobenzofuran-3'-one (**19**). — Compound **18** (2.4 g, 0.01 mol) was ground with anhydrous $ZnCl_2$ (3 g, 0.02 mol) and heated with 3-fluorophenol (1.4 g, 0.0125 mol) at 130° for 7 h. The dark-red, brown melt was dissolved in boiling glacial acetic acid (50–70 mL) and the solution poured into water (800 mL). The amorphous pink solid thus obtained was filtered off and dried. The raw product contained mainly **19** and, as byproduct, the fluoro compound **20**. To separate **20**, the solid was extracted several times with M aqueous NaOH (**19** was soluble, **20** was not soluble). On addition of conc. HCl, **19** (2.2–2.5 g, 64–70%) was obtained as a yellow-pink precipitate. For preparative use, **19** was esterified with acetic anhydride, recrystallized several times from ethanol and hydrolyzed with ethanolic KOH. To obtain chromatographically pure **19**, the product was chromatographed on a silica gel column with ethyl acetate as solvent, m.p. 230–233°, t.l.c. R_F 0.85 (*A*) and 0.55 (*B*); ν_{max}^{KBr} 3400 (OH), 2970 (CH, aliph.), 1740 (C=O), 1610 (C–C, arom.), 1504, 1460 (C=C), 1290, 1230 (C–O–C, phenol), 1120 (C–O–C, lactone), 980, 820, 750, and 700 cm^{-1}; ^{1}H-n.m.r. ($CDCl_3$): δ 2.18 (s, 3 H, CH_3), 4.9 (s, 1 H, OH), 6.51–6.60 (m, 3 H, arom.), 6.75 (m, 1 H, arom.), 7.13–7.26 (m, 3 H, arom.), 7.59–7.68 (m, 2 H, arom.), and 8.04 (m, 1 H, arom.).

Anal. Calc. for $C_{20}H_{14}O_4$: C, 75.46; H, 4.44; O, 20.10. Found: C, 75.60; H, 4.46; O, 19.80.

Enzymic hydrolysis. —Enzymic hydrolysis of the synthesized ketosides was carried out with neuraminidase from *C. perfringens* and *V. cholerae*. Incubations were conducted at 37° in a total volume of 1 mL of 0.1M sodium acetate buffer, at pH 5.1 for *C. perfringens* and 5.8 for *V. cholerae*. Aliquots (1 mL) were treated with 0.1M Na_2CO_3 (pH 11.5) or glycine–NaOH buffer (pH 10.5) to stop hydrolysis. Enzymic cleavage of the phenyl and the methoxyphenyl ketosides was controlled by t.l.c. Absorbance of liberated *p*-nitrophenol was measured at 400 nm. Fluorescence of 4-methylumbelliferone was measured at 450 nm (excitation at 360 nm), that of fluorescein at 520 nm (excitation at 490 nm), and that of 6-hydroxy-2-methylfluoran at 528 nm (excitation at 460 nm). Kinetic studies were also carried out with neuraminidases from *C. perfringens* in 0.1M acetate buffer (pH 5.1) and *V. cholerae* in 0.1M acetate buffer (pH 5.8) with **15** as substrate. K_m values were estimated with a Lineweaver–Burk and a direct-linear plot. Both gave K_m values of 82μM for *V. cholerae* neuraminidase and 96μM for *C. perfringens* neuraminidase. The blank reaction without enzyme in the appropriate solution showed a hydrolysis rate of 5–7% of the ketoside within 1 h.

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