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PHOTOREACTIVE CMP-SIALIC ACIDS AS SUBSTRATES FOR α2,6-SIALYLTRANSFERASE[#]

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Abstract: Chemo-enzymatic synthesis afforded a series of CMP-activated sialic acids carrying a photoreactive group at C-9 or C-5, and in part a radiolabel as well. They were found to be accepted by α2,6-sialyltransferase. - Differing amino group reactivity of novel 5,9-diamino sialic acid enabled us to attach sequentially a benzophenone photophore at C-9 and a radiolabel at C-5.

Sialyltransferases exert an important role in glycoprotein and glycolipid biosynthesis in catalysing the incorporation of sialic acids *via* the respective CMP-glycosides into terminal positions of glycoconjugate glycans. We have previously demonstrated the feasibility of the transfer of synthetic sialic acid analogs modified at C-4, C-5 and C-9 employing different sialyltransferases and various acceptor glycoproteins.[1, 2, 3] These results stimulated us to synthesize CMP-activated sialic acids carrying a photolabel in the sialic acid part of the molecule. In this report the aryl azide and the benzophenone photophor were chosen as photoactivatable groups. An additional radiolabel should greatly enlarge the potential application for crosslinking studies, after transfer of the sialic acid to the acceptor.

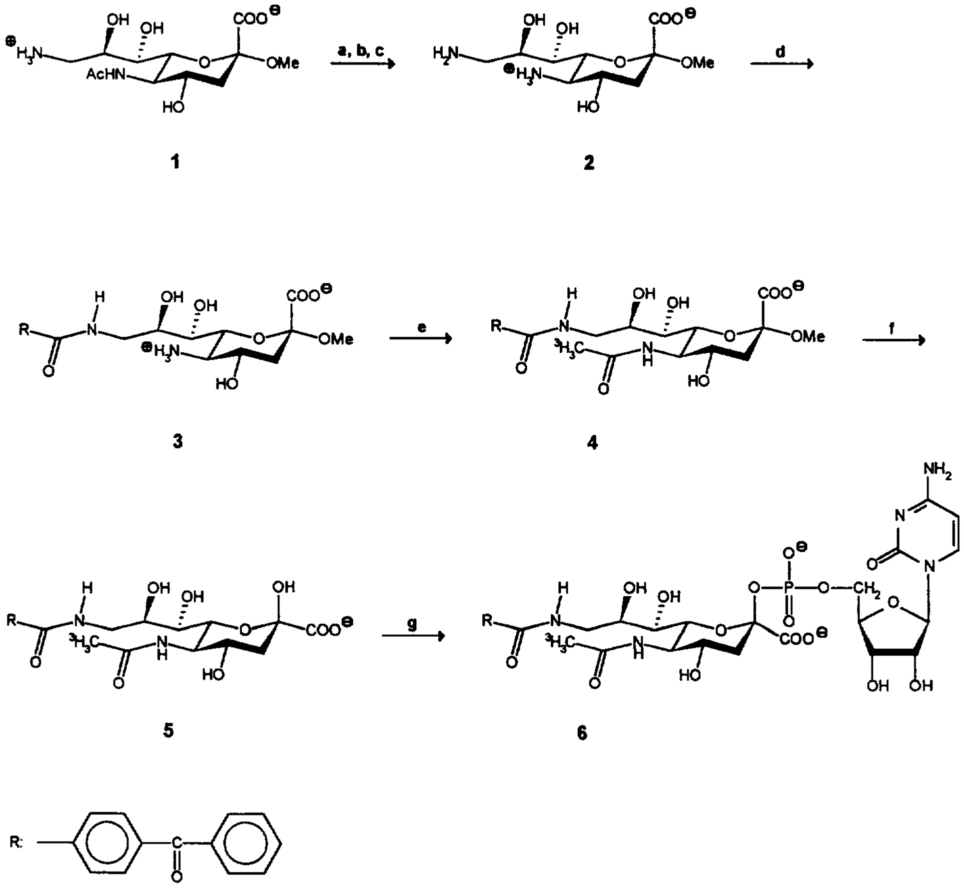
CMP-9-(4-benzoylbenzoylamino)-9-deoxy-Neu5[^{3}H]Ac (Scheme 1)

Key step in the synthesis of CMP-9-(4-benzoylbenzoylamino)-9-deoxy-Neu5[^{3}H]Ac was the ready *N*-deacetylation of 9-amino-9-deoxy-Neu5Ac methyl α-glycoside (**1**)[4] by treatment with aqueous barium hydroxide[4, 5]. In agreement with the data of other sialic acids possessing a free amino function at C-5[4], the ^{1}H NMR spectrum of 9-deoxy-5,9-diamino-neuraminic acid methyl α-glycoside (**2**) showed an upfield shift of the proton H-5 (**1**: H-5, 3.82 ppm; **2**: H-5, 3.24 ppm). The two amino groups of derivative **2** exhibited different reactivities. In contrast to the amino function at C-9 the one at C-5 could not be substituted by aromatic residues using the respective heterobifunctional *N*-hydroxysuccinimide ester. This might be due to the sterical hindrance at the secondary carbon C-5 of sialic acid as opposed to the primary carbon C-9. Thus, selective reaction of the *N*-hydroxysuccinimide (NHS) ester of 4-benzoylbenzoic acid[6] afforded exclusively 9-(4-benzoylbenzoylamino)-9-deoxyneuraminic acid methyl α-glycoside **3**[7], unambiguously proven by ^{1}H NMR spectroscopy as follows: i. the intensity of the signals of the aromatic protons exactly corresponded to *one* benzoylbenzoyl group; ii. the chemical shift of proton H-5 of **2** and **3** showed similar values (**2**: 2.78 ppm; **3**: 2.87 ppm), revealing that the amino function at C-5 of compound **3** had not been modified; iii. the protons at C-9 of **3** were shifted significantly downfield (**2**: H-9 3.04 ppm, H-9' 3.46 ppm; **3**: H-9 3.62 ppm, H-9' 3.86 ppm), indicating acylation of the amino group at this position.

Radiolabeling sialic acid analog **3** at C-5 by reacetylation employing [^{3}H]acetic anhydride afforded **4** which was identified by scanning the radioactivity after thin layer chromatography.[8]

[#] Dedicated to Professor Richard Neidlein on the occasion of his 65th birthday.

Scheme 1

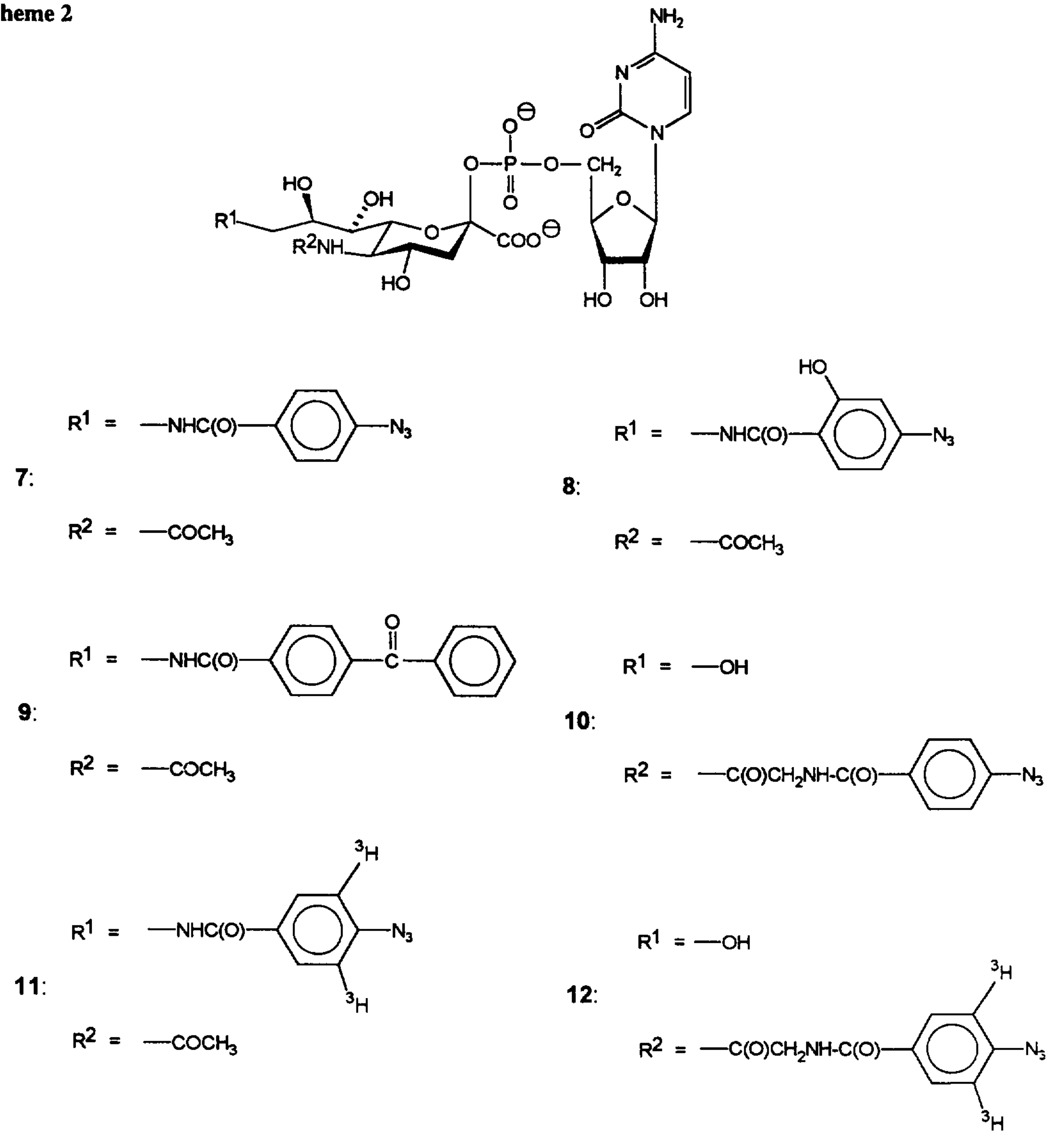


Reagents: a: $Ba(OH)_2$, 12 h, 100 °C; b: CO_2; c: IR-120 (H^+), aq. NH_3; d: NHS-benzoylbenzoic acid, $NaHCO_3$, DMF; e: $[^3H]Ac_2O$, $NaHCO_3$, H_2O/MeOH; f: 0.02 N HCl, 4 h, 80 °C; g: 3.5 µmol of 5, 15 µmol CTP, 0.12 mmol Tris-HCl pH 9.0, 28 µmol $MgCl_2$, 0.2 mg BSA, 1.4 µmol DTE, 700 mU CMP-Neu5Ac synthase (37 °C, 5 h)

The R_f-value of the labeled compound coincided with that of unlabeled **4** synthesized from **1** in conventional way. **4** was separated from sodium $[^3H]$acetate by gel filtration. Difference in charge of **3** and **4** allowed the final purification by anion exchange chromatography affording **4** in 85% yield (specific activity 3.05 Ci/mmol). Cleavage of the glycoside by acid hydrolysis produced 9-(4-benzoylbenzoylamino)-9-deoxy-Neu5$[^3H]$Ac **5**[9]. HPLC analysis showed no further purification to be necessary. Favourable kinetic activation data of **5** for partially purified CMP-Neu5Ac synthase of bovine brain[1] at pH 9.0 (K_M 0.25 mM, V_{max} 0.14; Neu5Ac: K_M 1.2, V_{max} 1.0) allowed us to perform the enzymatical synthesis of CMP-9-(4-benzoylbenzoylamino)-9-deoxy-Neu5$[^3H]$Ac (**6**) on a preparative scale with 44% yield[10].

CMP-9-(4-azidobenzoylamino)-9-deoxy-Neu5Ac (CMP-9-ABA-Neu5Ac), CMP-9-(4-azidosalicoylamino)-9-deoxy-Neu5Ac (CMP-9-ASA-Neu5Ac), and CMP-5-N-(4-azidobenzoyl)-aminoacetyl-neuraminic acid (CMP-5-N-ABAA-Neu)

Scheme 2



The synthesis of the photoreactive CMP-glycosides with an aryl azide group in the sialic acid moiety was performed[12] by chemical coupling of the heterobifunctional NHS-azidosalizylic acid and NHS-azidobenzoic acid to CMP-activated 9-amino-9-deoxy-Neu5Ac,[1, 4, 11] and of NHS-azidobenzoic acid to CMP-5-*N*-aminoacetyl-Neu, both prepared by enzymatic synthesis. In analogy, CMP-9-(4-benzoylbenzoylamino)-9-deoxy-Neu5Ac (CMP-9-BBA-Neu5Ac) was prepared using the NHS ester of benzoylbenzoic acid.[12]

CMP-9-azidobenzoylamino-9-deoxy-Neu5Ac and CMP-5-*N*-(azidobenzoyl)-aminoacetyl-neuraminic acid [Neu] were also synthesized in a radioactive form using the respective tritiated *N*-hydroxysuccinimide ester[13, 14] whereas for the synthesis of CMP-9-(4-benzoylbenzoylamino)-9-deoxy-Neu5[^{3}H]Ac a combination of chemical and enzymatic steps was employed (see above).

Purification was achieved primarily by HPLC. Since the radiolabeled CMP-glycosides were present in smaller amounts, an analytical column was used for HPLC. Desalting of the preparations was achieved by gel filtration on Bio-Gel P-2. The final yield of CMP-9-[^{3}H]ABA-Neu5Ac and CMP-5-*N*-[^{3}H]ABAA-Neu was 49% and 39% in contrast to that of the unlabeled compounds (about 80%).

The UV spectra of CMP-9-ASA-Neu5Ac, CMP-9-ABA-Neu5Ac and CMP-5-ABAA-Neu in aqueous solutions showed at 274 nm the characteristic peak of the photoreactive aryl azide group. The photosensitivity of these CMP-glycosides was demonstrated by irradiation for 5 min at 254 nm, whereupon the aryl azide peak disappeared.

Kinetic data of the sialyl transfer

CMP-9-ABA-Neu5Ac, CMP-9-ASA-Neu5Ac, CMP-9-BBA-Neu5Ac, and CMP-5-ABAA-Neu were examined as substrates for pure Galβ1-4GlcNAc α2,6-sialyltransferase from rat liver.[15] The transfer assay was performed using asialo-α_1-acid glycoprotein as acceptor substrate. Incorporation of the Neu5Ac derivatives was linear with time up to 45 min at 50 µM CMP-glycoside which represented the lowest concentration used in the kinetic assays. K_M values determined for the CMP-glycosides modified at C-9 of sialic acid ranged from 1.5 to 1.8-fold the value obtained with CMP-Neu5Ac. Also V_{max} values for each of these CMP-Neu5Ac analogs were of comparable magnitude. In contrast, K_M for CMP-5-ABAA was 3.6-fold higher and V_{max} only half compared to that of CMP-Neu5Ac.

Table 1: **Apparent kinetic data of rat liver Galβ1,4GlcNAc α2,6-sialyltransferase for the four unlabeled photoactivatable CMP-glycosides described in this study, employing asialo-α_1-acid-glycoprotein as acceptor.**

K_M und V values were obtained by the graphical method of Lineweaver-Burk[16]. V values are expressed relative to that determined for CMP-Neu5Ac (= 1.0).

Donor substrate	K_M(µM)	V_{max}rel	V_{max}/K_M(1/mM)
CMP-Neu5Ac	45	1.0	22.2
CMP-9-(4-benzoylbenzoylamino)-9-deoxy-Neu5Ac	68	0.8	11.8
CMP-9-(4-azidosalicoylamino)-9-deoxy-Neu5Ac	80	1.1	13.8
CMP-9-(4-azidobenzoylamino)-9-deoxy-Neu5Ac	66	0.9	13.6
CMP-5-*N*-(4-azidobenzoyl)-aminoacetyl-Neu	161	0.5	3.1

In summary, we describe a number of CMP-sialic acids carrying a photoactivatable group in the sialic acid moiety which, in part, were also radiolabeled. The syntheses were achieved by starting from a CMP-activated sialic acid analog or employing a Neu5Ac derived 5,9-diamino sialic acid which, after attaching the different labels, was activated. The latter compound is of potential interest as the two amino groups, differing in reactivity, allow the modification of sialic acid to proceed in sequence at the primary and secondary amino group.

We are currently studying the application of the photolabeled CMP-sialic acids for sialyltransferases of other specificity and the synthesis of photoreactive sialic acids with different properties.

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5. **Methyl 5,9-diamino-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (2):** To a solution of methyl 5-acetylamino-9-amino-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (**1**) (200 mg, 0.60 mmol) in water (20 ml) $Ba(OH)_2 \cdot 8H_2O$ (2.0 g, 6.34 mmol) was added and the mixture refluxed for 5 h. After neutralization with dry ice, $BaCO_3$ was filtered off and the filtrate lyophilized. Remaining Ba^{2+} were removed by chromatography on Amberlite IR-120 (H^+) (10 ml) and **2** was eluted with a gradient of 0-0.5 N aq. NH_3. Fractions containing the product were collected and freeze-dried; yield 146 mg (87%) of **2** as a colourless powder. R_f 0.09 (1:1 (v/v) n-PrOH/H_2O); $[\alpha]_D$ -14.2° (c 0.5, H_2O);
   ^1H NMR (300 MHz, D_2O): δ 1.57 (dd, $J_{3ax,3eq} = J_{3ax,4}$ = 12.2 Hz, 1H, H-3_{ax}), 2.65 (dd, $J_{3eq,4}$ 4.6 Hz, 1H, H-3_{eq}), 2.78 (dd, $J_{4,5} = J_{5,6}$ 9.9 Hz, 1H, H-5), 3.04 (dd, $J_{8,9}$ 9.8 Hz, $J_{9,9'}$ 13.1 Hz, 1H, H-9), 3.31 (s, 3H, OCH_3), 3.41-3.51 (m, 2H, H-4, H-9'), 3.64 (dd, $J_{6,7}$ 1.9 Hz, 1H, H-6), 3.76 (dd, $J_{7,8}$ 8.8 Hz, 1H, H-7), 4.08 (ddd, $J_{8,9'}$ 3.0 Hz, 1H, H-8). Negative FAB MS: m/z = 279 [57%, $(M-H)^-$], 277 [100%].
   Anal. calc. for $C_{10}H_{20}N_2O_7 \cdot H_2O$ (298.29): C 40.27, H 7.43, N 9.39. Found: C 39.96, H 7.33, N 8.82.
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7. **Methyl 5-amino-9-(4-benzoylbenzoylamino)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (3):** To **2** (44 mg, 0.15 mmol) in water (1 ml) were added 0.5 M $NaHCO_3$ (0.5 ml) and the NHS ester of 4-benzoylbenzoic acid (50 mg, 0.15 mmol) in DMF (5 ml) and the mixture was stirred at room temperature for 1 h. After evaporation at ambient temperature, the residue was taken up in water and insoluble by-products were removed by centrifugation. Preparative thin layer chromatography on silica gel (1:4:1 (v/v) methanol/ethyl acetate/water) and elution of the product with methanol was followed by centrifugation and repeated wash. After evaporation, **3** was dissolved in water and freed of traces of silica gel by passing through a Millipore-filter. Lyophilisation yielded 64 mg (81%) of **3**.
   R_f 0.2 (1:4:1 (v/v) MeOH/ethyl acetate/H_2O); $[\alpha]_D$ -5.6° (c 0.5, H_2O); λ_{max} = 263.5 nm (ε_M 16400 $M^{-1} \cdot cm^{-1}$); ^1H NMR (300 MHz, D_2O): δ 1.60 (dd, $J_{3ax,3eq} = J_{3ax,4}$ 12.1 Hz, 1H, H-3_{ax}), 2.67 (dd, $J_{3eq,4}$ 4.6 Hz, 1H, H-3_{eq}), 2.87 (dd, $J_{4,5} = J_{5,6}$ 9.8 Hz, 1H, H-5), 3.33 (s, 3H, OCH_3), 3.54 (m, 1H, H-4), 3.62 (dd, $J_{8,9}$ 8.0 Hz, $J_{9,9'}$ 14.7 Hz, 1H, H-9), 3.74 (dd, $J_{6,7}$ 1.0 Hz, 1H, H-6), 3.80 (dd, $J_{7,8}$ 8.3 Hz, 1H, H-7), 3.86 (dd, $J_{8,9'}$ 2.3 Hz, 1H, H-9'), 4.13 (ddd, 1H, H-8), 7.57-7.91 (m, 9H, Ar). Negative FAB MS: m/z = 487 [100%, $(M-H)^-$]. Anal. calc. for $C_{24}H_{28}N_2O_9 \cdot 2H_2O$ (524.52): C 54.96, H 6.15, N 5.34. Found: C 54.89, H 6.09, N 5.27.
8. **Methyl 5-[^3H]acetylamino-9-(4-benzoylbenzoylamino)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (4):** To the solution of **3** (4.8 mg, 9.8 μmol) in water (80 μl) and methanol (340 μl) was added 1 M $NaHCO_3$ (40 μl), the mixture transferred into a glass ampoule containing precooled (0°C) [^3H]acetic anhydride (25 mCi, specific radioactivity 6.1 Ci/mmol). After 2 h at room temperature, **4** was purified by chromatography on Bio-Gel P-2 (elution with water). Further

purification was achieved employing chromatography on DEAE-Sephadex A-25 (HCO_3^-) (10 ml), which was washed with water and eluted using a gradient of 0-0.5 M NH_4HCO_3. Radioactive fractions were pooled and evaporated yielding 10.7 mCi (85%) of **4** (specific radioactivity 3.05 Ci/mmol).

R_f 0.18 (15:1 (v/v) acetone/water).

9. **5-[^{3}H]Acetylamino-9-(4-benzoylbenzoylamino)-3,5,9-trideoxy-D-*glycero*-β-D-*galacto*-2-nonulopyranosonic acid (5): 4** in 0.02 N HCl (10 ml) was maintained at 80°C with stirring for 4 h. When TLC (1:4:1 (v/v) methanol/ethylacetate/water) indicated complete cleavage of the glycosidic bond, the solution was evaporated and codistilled with water until neutral pH.
10. **Enzymatic synthesis of CMP-9-(4-benzoylbenzoylamino)-9-deoxy-Neu5[^{3}H]Ac (6):** Reaction mixture (400 μl) contained **5** (3.5 μmol, 10.7 mCi), Tris-HCl pH 9.0 (0.08 mmol), $MgCl_2$ (20 μmol), bovine serum albumin (0.2 mg), dithioerythritol (1 μmol), CTP (7 μmol). The reaction was started by addition of partially purified CMP-sialic acid synthase from bovine brain[1] (500mU) and incubated at 37°C. After 2 h, and 3 h, resp., another Tris-HCl pH 9.0 (0.02 mmol), $MgCl_2$ (4 μmol), dithioerythritol (0.2 μmol), CTP (4 μmol) and CMP-sialic acid synthase (100 mU) were added (final volume 700 μl). The reaction was monitored by TLC (3:7 (v/v) ethanol/1 M ammoniumacetate pH 6.5), and after completion of the reaction (5 h) the protein was precipitated by addition of cold acetone (855 μl). The reaction tube was placed on ice for 30 min, centrifuged at 6000 x g for 20 min, and the supernatant carefully decanted . The pellet was re-extracted twice with a mixture of water (100 μl) and cold acetone (122 μl) and treated as above. Combined supernatants were evaporated to a final volume of ~100 μl. **6** was purified by HPLC on an aminopropyl-phase column (5 μm particle size; 50:50 (v/v) acetonitrile/15 mM KH_2PO_4) co-injecting 1 μl of a CMP-9-(4-benzoylbenzoylamino)-9-deoxy-Neu5Ac standard. Pooled CMP-glycoside fractions were neutralized and then evaporated. Further purification was achieved by Bio-Gel P-2 chromatography with water as eluens. Fractions containing **6** were evaporated to a final volume of 50 μl yielding 4.68 mCi (44%) (specific radioactivity 3.05 Ci/mmol).
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12. **General procedure for the synthesis of CMP-9-(4-azidobenzoylamino)-9-deoxy-Neu5Ac (7), CMP-9-(4-azidosalicoylamino)-9-deoxy-Neu5Ac (8), CMP-9-(4-benzoylbenzoylamino)-9-deoxy-Neu5Ac (9), and CMP-5-*N*-(4-azidobenzoyl)-aminoacetyl-neuraminic acid (10):** To the solution of CMP-9-amino-9-deoxy-Neu5Ac (for **10**: CMP-5-*N*-aminoacetyl-Neu) (20 μmol) in water (100 μl), DMF (1.5 ml), 0.5 M $NaHCO_3$ (250 μl) and the corresponding *N*-hydroxysuccinimide ester (40 μmol) were added. The mixture was kept at room temperature for 1.5 h and the reaction monitored by analytical HPLC (50:50 (v/v) acetonitrile/15 mM KH_2PO_4). After completion of the reaction and evaporation at ambient temperature to a final volume of approximately 0.2 ml, CMP-glycosides were separated by semipreparative HPLC on an aminopropyl-phase column (5 μm particle size; 65:35 (v/v) acetonitrile /15 mM KH_2PO_4). Inorganic phosphate was precipitated by diluting the CMP-glycoside to the sixfold volume with cold ethanol, subsequent ice-cooling for 30 min and centrifugation at 6000 x g for 15 min. The pellet was extracted twice with a cold mixture of water (1 ml) and ethanol (5 ml) and treated as above. Combined supernatants were evaporated and further purified by gel filtration on Bio-Gel P-2 (200-400 mesh, 2.5 cm x 80 cm) eluting with water. Fractions containing the CMP-glycoside were collected and evaporated at 25°C.
13. **CMP-9-(4-azido-[^{3}H]benzoylamino)-9-deoxy-Neu5Ac (11):** To CMP-9-amino-9-deoxy-Neu5Ac (1.6 μmol) in water (50 μl) were added dimethylsulfoxide (150 μl), 0.5 M $NaHCO_3$ (25 μl), and the succinimide ester of 4-azido-[^{3}H]benzoic acid (50 μCi, 48.8 Ci/mmol) dissolved in 2-propanol (50 μl) and the mixture was allowed to stand at room temperature for 1.5 h. The turnover of the reaction (80%) could be determined by analytical HPLC (see above) co-injecting 1 μl of a CMP-9-(4-azidobenzoylamino)-9-deoxy-Neu5Ac standard. **11** was purified by analytical HPLC and Bio-Gel P-2 as described for the preparation of **6** affording 24.5 μCi (49% relative to the starting succinimide ester, specific radioactivity: 48.6 Ci/mmol).
14. **CMP-5-*N*-(4-azido-[^{3}H]benzoyl)-aminoacetyl-neuraminic acid (12):** To CMP-5-*N*-aminoacetyl-neuraminic acid (1.13 μmol) in water (180 μl) were added 0.5 M $NaHCO_3$ (50 μl), dimethylsulfoxide (350 μl) and the succinimide ester of 4-azido-[^{3}H]benzoic acid (70 μCi, 48.8 Ci/mmol) dissolved in 2-propanol (70 μl). After 2 h at room temperature the turnover of the reaction was 60%. Quantification and purification of **12** were done as described for **6** and **11**, giving 27.1 μCi (39% relative to the starting succinimide ester, specific radioactivity: 48.6 Ci/mmol).
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