D. Synthesis of Sialic Acids and Sialic Acid Derivatives

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I. Introduction

Sialic acids as such, sialic acid derivatives, analogues, glycosides and sialo-oligosaccharides have been subject of many synthetic investigations. These studies were aimed at a further exploration of the properties of sialic acids, the preparation of substrates and inhibitors for sialidases, sialyltransferases or for sialic acid converting enzymes, and to make compounds accessable for analytical purposes or for studies related to metabolism or biological functions of sialic acids.

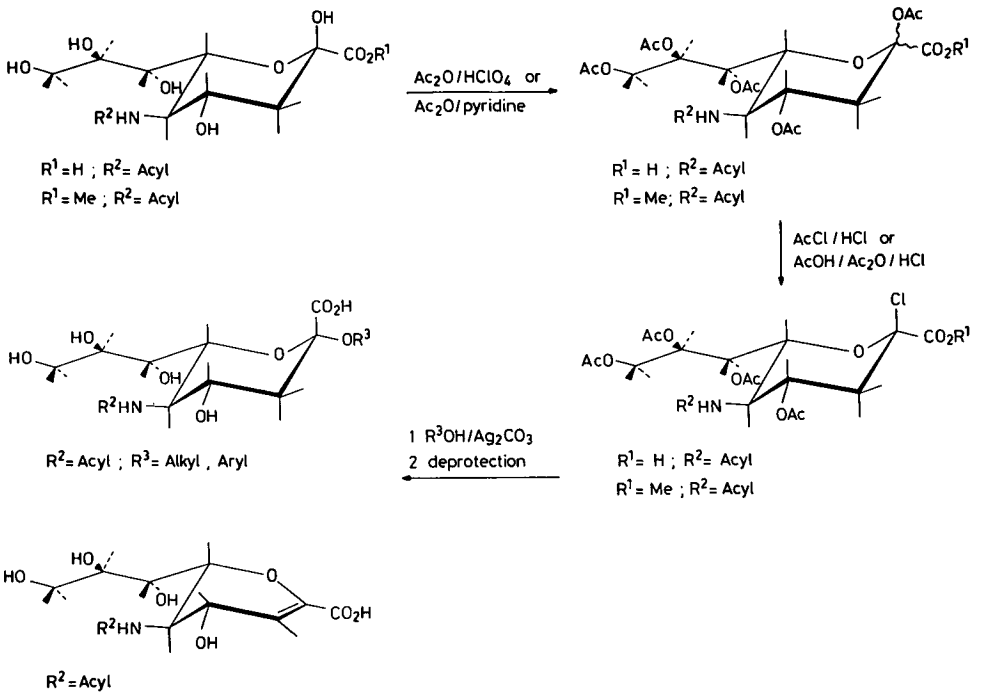
In comparison to the synthesis of analogous compounds from simple aldoses or ketoses, the situation for sialic acid is more complicated, due to the occurrence of several different functional groups. Synthetic studies on sialo-compounds have been reviewed by BLIX and JEANLOZ (1969), TUPPY and GOTTSCHALK (1972), HOLMQUIST (1975), and VAN DER VLEUGEL (1981).

II. Glycosides of Sialic Acids

For the synthesis of alkyl and aryl α-glycosides of sialic acids often Koenigs-Knorr-like procedures have been applied. A large number of these glycosides have been obtained by silver carbonate-promoted condensation of 5-acylamino-4,7,8,9-

tetra-O-acetyl-2-chloro-2,3,5-trideoxy-β-**D**-*glycero*-**D**-*galacto*-2-nonulopyranosonic acids or the corresponding methyl esters with the appropriate alcohol, followed by removal of protecting groups. This reaction sequence is summarized in Scheme 1. The yields vary from 20-60%. Low reaction rates influence the yield unfavourably, since concomitantly the elimination of hydrogen chloride from the sialic acid 2-chloride derivative takes place, which becomes then quantitatively

Scheme 1



more important (see section IV). Although the replacing of silver carbonate by silver oxide or mercury(II) cyanide (MEINDL and TUPPY 1965 a) does not lead to a significant improvement in the outcome of the reaction, the nature of the condensation promoting agent is important. The 2-pyridyl α-glycoside of N-acetylneuraminic acid has been prepared by condensation of 4,7,8,9-tetra-O-acetyl-2-chloro-2-deoxy-β-N-acetylneuraminic acid methyl ester with the silver salt of 2-hydroxypyridine. The aglycon in this reaction acts also as a promotor. The nucleophilicity of the oxygen atom of 2-hydroxypyridine is increased by substitution of silver for hydrogen (HOLMQUIST and BROSSMER 1972 b, HOLMQUIST 1975). Higher yields in the preparation of α-glycosides were obtained by ESCHENFELDER *et al.* (1975) and ESCHENFELDER and BROSSMER (1979, 1980), by application of polymeric promotors like silver polymaleate or silver polymethacrylate. The underlying principle of this catalyst is the observation that glycosyl halides react readily with alcohols in the presence of insoluble silver salts

Table 1. *Survey of alkyl (aryl) α-glycosides of N-acylneuraminic acid(s) (methyl esters)*

N-Acyl group	α-Glycoside	References
Formyl	benzyl	Brossmer and Nebelin 1969
Acetyl	methyl	Kuhn *et al.* 1966(*), Meindl and Tuppy 1965a, 1966b*, Van der Vleugel *et al.* 1982a*, Yu and Ledeen 1969*, Beau and Schauer 1979
Acetyl	ethyl	Eschenfelder and Brossmer 1979(*), Van der Vleugel *et al.* 1982a*
Acetyl	isopropyl	Van der Vleugel *et al.* 1982a*
Acetyl	pentyl	Meindl and Tuppy 1965a, b
Acetyl	neopentyl	Van der Vleugel *et al.* 1982a*
Acetyl	hexyl	Meindl and Tuppy 1965a, b
Acetyl	decyl	Meindl and Tuppy 1965a
Acetyl	allyl	Brossmer *et al.* 1974
Acetyl	carboxymethyl	Holmquist and Brossmer 1972a
Acetyl	2-aminoethyl	Eschenfelder and Brossmer 1980, Holmquist and Brossmer 1972b
Acetyl	2-aminoethylamino-carbonylmethyl	Holmquist 1974
Acetyl	2-hydroxyethyl	Brossmer *et al.* 1974
Acetyl	3-hydroxypropyl	Meindl and Tuppy 1965a
Acetyl	4-hydroxybutyl	Brossmer *et al.* 1974
Acetyl	5-hydroxypentyl	Meindl and Tuppy 1965a
Acetyl	cyclohexyl	Meindl and Tuppy 1965a
Acetyl	cyclohexylmethyl	Meindl and Tuppy 1965a
Acetyl	phenyl	Meindl and Tuppy 1967(*)
Acetyl	*m*-methoxyphenyl	Tuppy and Palese 1969
Acetyl	*p*-nitrophenyl	Privalova and Khorlin 1969
Acetyl	benzyl	Faillard *et al.* 1966, Meindl and Tuppy 1965a, 1966b(*)
Acetyl	*p*-methoxybenzyl	Meindl and Tuppy 1965a
Acetyl	*m*-nitrobenzyl	Meindl and Tuppy 1965a
Acetyl	*p*-nitrobenzyl	Gielen and Uhlenbruck 1969
Acetyl	*m*-chlorobenzyl	Meindl and Tuppy 1965a
Acetyl	*m*-bromobenzyl	Meindl and Tuppy 1965a
Acetyl	*m*-iodobenzyl	Meindl and Tuppy 1965a
Acetyl	4-methylumbelliferyl	Myers *et al.* 1980, Potier *et al.* 1979, Thomas *et al.* 1978, Warner and O'Brien 1979
Acetyl	2-pyridyl	Holmquist and Brossmer 1972b
Acetyl	5-bromo-3-indolyl	Gossrau *et al.* 1977
Glycolyl	methyl	Meindl and Tuppy 1966a, b(*)
Glycolyl	pentyl	Meindl and Tuppy 1966a
Glycolyl	decyl	Meindl and Tuppy 1966a
Glycolyl	benzyl	Meindl and Tuppy 1966a
Glycolyl	*m*-nitrobenzyl	Meindl and Tuppy 1966a
Propionyl	benzyl	Meindl and Tuppy 1966c

Table 1 (continued)

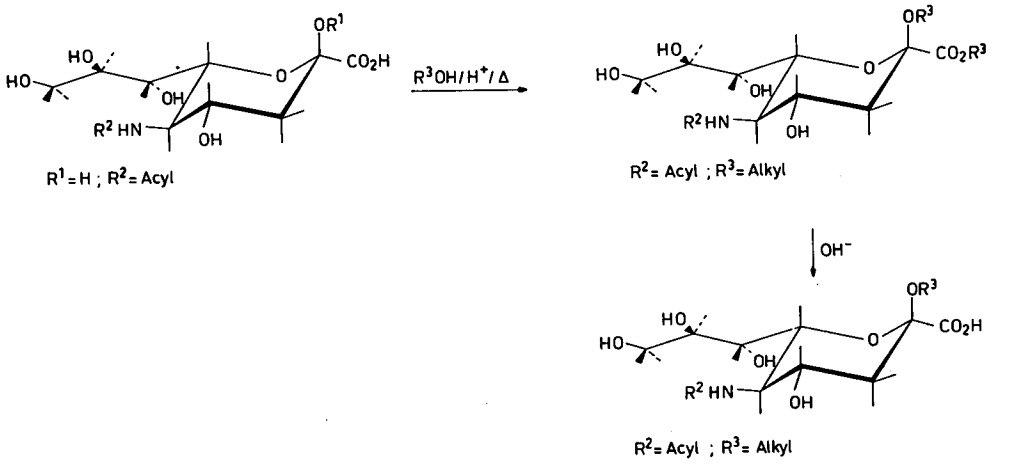
N-Acyl group	α-Glycoside	References
Propionyl	2-aminoethylamino-carbonylmethyl	HOLMQUIST and NILSSON 1979
Butyryl	benzyl	MEINDL and TUPPY 1966c
Succinyl	benzyl	BROSSMER and NEBELIN 1969
Benzoyl	methyl	MEINDL and TUPPY 1966c(*)
Benzoyl	benzyl	MEINDL and TUPPY 1966c
Benzyloxycarbonyl	benzyl	FAILLARD *et al.* 1969

* Methyl ester instead of free acid.

of dicarboxylic acids or hydroxycarboxylic acids (HELFERICH and MÜLLER 1970, WULFF *et al.* 1970, 1972, WULFF and RÖHLE 1974). The preparation of alkyl α-glycosides was also conveniently carried out with silver salicylate as promotor, affording high yields of sialic acid derivatives (VAN DER VLEUGEL *et al.* 1982a). Table 1 summarizes a series of alkyl (aryl) α-glycosides of N-acylneuraminic acid(s) (methyl esters), prepared by several investigators.

β-Linked simple glycosides of sialic acid can be obtained by heating of a solution of sialic acid in the appropriate alcohol in the presence of an acid catalyst, followed by saponification of the formed ester (Scheme 2). This procedure is an

Scheme 2



R^1 = H ; R^2 = Acyl

R^2 = Acyl ; R^3 = Alkyl

R^2 = Acyl ; R^3 = Alkyl

example of alcoholysis according to the FISCHER method (see BOCHKOV and ZAIKOV 1979). Under the thermodynamically controlled reaction conditions only a few percent of the α-anomer is formed. The method is limited to sialic acids which are

Table 2. *Survey of alkyl (aryl) β-glycosides of N-acylneuraminic acid(s) (methyl esters)*

N-Acyl group	β-Glycoside	References
Free amino	methyl	BÖHM and BAUMEISTER 1955, GIELEN 1965, 1967, KLENK and LAUENSTEIN 1952
Acetyl	methyl	BLIX *et al.* 1956*, KUHN *et al.* 1966(*), MCGUIRE and BINKLEY 1964*, WIRTZ-PEITZ *et al.* 1969, YU and LEDEEN 1969*
Acetyl	ethyl	ESCHENFELDER and BROSSMER 1979(*)
Acetyl	pentyl	MEINDL and TUPPY 1965 b
Acetyl	hexyl	MEINDL and TUPPY 1965 b
Acetyl	carboxymethyl	HOLMQUIST and BROSSMER 1972 a
Acetyl	phenyl	MEINDL and TUPPY 1967
Acetyl	benzyl	FAILLARD *et al.* 1966
Acetyl	2-pyridyl	HOLMQUIST and BROSSMER 1972 b
Acetyl	CMP	KEAN and ROSEMAN 1966, SCHAUER *et al.* 1972, SCHAUER and WEMBER 1973, CORFIELD *et al.* 1979 b, HAVERKAMP *et al.* 1979 a, b
Acetyl	2-aminoethylamino-carbonylmethyl	HOLMQUIST and ÖSTMAN 1975
Fluoroacetyl	methyl	SCHAUER *et al.* 1970
Trifluoroacetyl	methyl	KLENK *et al.* 1956
Chloroacetyl	methyl	SCHAUER *et al.* 1970
Glycolyl	methyl	BLIX *et al.* 1956*, WIRTZ-PEITZ *et al.* 1969, YU and LEDEEN 1970*
Glycolyl	CMP	KEAN and ROSEMAN 1966, SCHAUER *et al.* 1972, SCHAUER and WEMBER 1973, CORFIELD *et al.* 1979 b
Glycyl	methyl	DEREVITSKAYA *et al.* 1965 a*
N-Benzyloxy-carbonylglycyl	methyl	DEREVITSKAYA *et al.* 1965 a*
Benzoyl	methyl	MEINDL and TUPPY 1966 c(*)
Benzyloxycarbonyl	methyl	GIELEN 1965, 1967(*)

* Methyl ester instead of free acid.

stable under the acid conditions. Glycosides from complex alcohols cannot be prepared along this route. Acid catalysts in this reaction can be e.g. Dowex 50 (H^+ form) (20–48 h; 60–70 °C), or 0.1 N HCl (2–3 h; 60–70 °C). In the latter case long reaction times may lead to extensive N-deacylation. It should be noted that the methanolysis procedure which is currently used for sugar analysis of carbohydrates or glycoconjugates containing sialic acid, leads to complete N-deacylation (see chapter F). However, the removal of the N-acyl group followed by N-reacylation opens the way for preparing sialic acids bearing different N-acyl groups in the free as well as in the glycosidically bound form (see section IX). The

benzyl β-glycoside of N-acetylneuraminic acid has been obtained by FAILLARD *et al.* (1966) in 40% yield (besides 4% of the α-anomer) by heating 2,4,7,8,9-penta-O-acetyl-N-acetylneuraminic acid with benzyl alcohol in the presence of $ZnCl_2$, followed by O-deacetylation. β-Glycosides of sialic acids have sometimes been obtained as more or less important side-products in syntheses aimed at the preparation of α-anomers. The β-glycosides have been summarized in Table 2.

Besides the O-glycosides mentioned so far, also attention has been paid to the synthesis of N- and S-analogues. The *p*-nitrophenyl N-glycoside of α-N-acetylneuraminic acid has been prepared by silver carbonate-promoted reaction of 4,7,8,9-tetra-O-acetyl-2-chloro-2-deoxy-β-N-acetylneuraminic acid methyl ester with *p*-nitroaniline and subsequent deprotection (PRIVALOVA and KHORLIN 1969). Treatment of the same precursor with sodium azide, followed by de-esterification, led to the formation of 2-azido-2-deoxy-α-N-acetylneuraminic acid (SUPP *et al.* 1980). The same authors reported also the preparation of the β-form. The azides can readily be converted into the 2-amino derivatives of N-acetylneuraminic acid. Subsequently, the amino function has been benzoylated. Data on the syntheses of the methyl and *p*-nitrophenyl S-glycosides of α-N-acetylneuraminic acid have been presented by PRIVALOVA and KHORLIN (1969), whereas PONPIPOM *et al.* (1980) have described a method for the synthesis of 5-acetamido-2-S-[6-(5-cholesten-3β-yloxy)hexyl]-3,5-dideoxy-2-thio-β-**D**-*glycero*-**D**-*galacto*-2-nonulopyranosonic acid.

III. Sialodisaccharides

In the synthesis of sialodisaccharides most studies were directed to α-glycosidically linked compounds, because virtually all naturally occurring sialo-compounds contain sialic acid in α-glycosidic linkage. It is clear that the coupling reaction is more complicated than for the preparation of simple glycosides. By consequence side-reactions may become more important, leading to rather low yields.

The compounds N-acetyl-**D**-neuraminyl-α(2→6)-**D**-glucose, N-acetyl-**D**-neuraminyl-α(2→6)-**D**-galactose, N-acetyl-**D**-neuraminyl-α(2→6)-N-acetyl-**D**-glucosamine, N-acetyl-**D**-neuraminyl-α(2→3)-**D**-glucose, and N-acetyl-**D**-neuraminyl-α(2→3)-N-acetyl-**D**-glucosamine have been obtained in 8-18% yield by coupling methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-2-chloro-2,3,5-trideoxy-β-**D**-*glycero*-**D**-*galacto*-2-nonulopyranosonate with the appropriately protected derivatives of **D**-glucose, **D**-galactose and N-acetyl-**D**-glucosamine under silver carbonate-promoted Koenigs-Knorr conditions (KHORLIN *et al.* 1971). It has to be emphasized that the choice of the protecting groups is an essential part of the synthesis strategy. Removal of such groups must be possible without disruption of the newly formed glycosidic bond.

The disaccharides N-acetyl-**D**-neuraminyl-α(2→6)-1,2 : 3,4-di-O-isopropylidene-α-**D**-galactopyranose, methyl N-acetyl-**D**-neuraminyl-α(2→6)-α-**D**-galactopyranoside, N-acetyl-**D**-neuraminyl-α(2→6)-**D**-glucose, N-acetyl-**D**-neuraminyl-α(2→2)-N-acetyl-α-**D**-neuraminide, and N-acetyl-**D**-neuraminyl-α(2→2)-N-acetyl-β-**D**-neuraminide have been prepared by condensation of the abovementioned

N-acetylneuraminic acid synthon with suitable protected derivatives of **D**-galactose, **D**-glucose and N-acetylneuraminic acid, respectively, in the presence of a polymer-bound silver salt as promotor. The latter catalyst afforded sialodisaccharides in yields of 10–40%, thereby showing the influence of the type of catalyst (BROSSMER *et al.* 1978 a, b).

Condensation of the same N-acetylneuraminic acid synthon with benzyl 2,3,4-tri-O-benzyl-β-**D**-galactopyranoside in the presence of silver salicylate afforded after deprotection the potassium salt of N-acetyl-**D**-neuraminyl-α(2→6)-**D**-galactose in 48% yield (VAN DER VLEUGEL *et al.* 1982 b). During the coupling reaction also a few percent of the β-isomer was formed.

VAN DER VLEUGEL *et al.* (1982 c) also reported on the synthesis of a β-linked sialodisaccharide, namely, N-acetyl-**D**-neuraminyl-β(2→6)-N-acetyl-**D**-glucosamine. This product was obtained by the silver triflate-promoted coupling of the already mentioned N-acetylneuraminic acid synthon with benzyl 2-acetamido-2-deoxy-3,4-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-α-**D**-glucopyranoside followed by removal of the protecting groups. It has to be noted that after the condensation reaction the fully protected β-isomer was contaminated, among other products, with the corresponding α-isomer (ratio $\beta : \alpha = 4 : 1$). When silver salicylate was used instead of silver triflate, the fully protected 2-O-salicyloyl derivative of N-acetylneuraminic acid was formed almost exclusively.

The aforementioned results demonstrate clearly that for the synthesis of sialo-oligosaccharides a generally applicable route has still to be developed.

Also attention has been paid to the synthesis of sialodisaccharides, having sialic acid in reducing position. By condensation of tetra-O-acetyl-α-**D**-glucopyranosyl bromide or tetra-O-acetyl-α-**D**-galactopyranosyl bromide with methyl 5-acetamido-2,4,7,8-tetra-O-acetyl-3,5-dideoxy-9-O-trityl-**D**-*glycero*-**D**-*galacto*-2-nonulopyranosonate in nitromethane under the influence of silver perchlorate, followed by O-deacetylation, the methyl esters of the corresponding β(1→9) disaccharides were obtained in 67 and 46% yield, respectively (KHORLIN and PRIVALOVA 1968).

IV. 2-Deoxy-2,3-dehydro-N-acylneuraminic Acids

2-Deoxy-2,3-dehydro-N-acylneuraminic acids have been applied in several competitive inhibition studies with neuraminidases from different sources (see for instance: MEINDL and TUPPY 1969 b, MEINDL *et al.* 1971, 1974, PALESE and COMPANS 1976, VEH and SCHAUER 1978, KUMAR *et al.* 1981).

2-Deoxy-2,3-dehydro-N-acetylneuraminic acid has been synthesized by treatment of 4,7,8,9-tetra-O-acetyl-2-chloro-2-deoxy-β-N-acetylneuraminic acid (MEINDL and TUPPY 1965 a) with triethylamine (10 min, 20 °C) or silver carbonate (60–90 min, 80–90 °C) in dioxan or acetone, and subsequent alkaline O-deacetylation (MEINDL and TUPPY 1969 a). This compound has also been detected frequently as a by-product in the synthesis of α-glycosides (MEINDL and TUPPY 1965 a) and of α-linked sialodisaccharides (VAN DER VLEUGEL *et al.* 1982 b, 1982 c).

The unsaturated sialic acid could also be obtained by prolonged heating (5 h, 90 °C) of 2,4,7,8,9-penta-O-acetyl-N-acetylneuraminic acid in dioxan, followed by O-deacetylation (MEINDL and TUPPY 1969 a).

Using the two routes mentioned above, also a series of other unsaturated sialic acids with different N-acyl groups have been prepared (MEINDL and TUPPY 1969 a). Methyl esters, prepared by esterification with diazomethane, can be hydrogenated very easily with a Pd catalyst (MEINDL and TUPPY 1969 a). An additional series of unsaturated N-acylneuraminic acids was synthesized using 2-deoxy-2,3-dehydroneuraminic acid (obtained from the N-benzyloxycarbonyl-analogue by hydrogenolytic cleavage with $Pd/BaSO_4/H_2$) or its benzhydryl ester as precursors (MEINDL and TUPPY 1973). The various N-acyl groups reported by MEINDL and TUPPY (1969 a, 1973) are summarized in Table 3; for recent analytical data of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid, see chapters E, F, and G.

Table 3. *Survey of N-acyl groups of 2-deoxy-2,3-dehydro-N-acylneuraminic acid(s) (methyl esters)* (MEINDL and TUPPY 1969 a, 1973)

Formyl	aminoacetyl	β-(N′-benzyloxycarbonyl)aminopropionyl
Acetyl	acetylaminoacetyl	β-carboxypropionyl
Fluoroacetyl	dimethylaminoacetyl	butyryl
Difluoroacetyl	mercaptoacetyl	carbamoyl
Trifluoroacetyl	glycolyl	β-carboxyacrylyl
Chloroacetyl	propionyl	thiodiacetyl
Iodoacetyl	β-aminopropionyl	benzoyl
Cyanoacetyl	β-acetylaminopropionyl	benzyloxycarbonyl

Another approach for the synthesis of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid has been reported by BEAU and SCHAUER (1979). 4,7,8,9-Tetra-O-*p*-nitrobenzoyl-2-bromo-2-deoxy-N-acetylneuraminic acid methyl ester could be converted quantitatively into the unsaturated analogue using triethylamine or molecular sieves 4 Å in dichloromethane. After deprotection of the latter derivative the free unsaturated N-acetylneuraminic acid was obtained. These authors also mentioned the synthesis of a 2-deoxy-2,3-dehydro-N-acetylneuraminic acid methyl ester, in which the primary hydroxyl group had been replaced by a Cl-atom.

When N-acetylneuraminic acid methyl ester was treated with sulfuric acid and acetic anhydride, after O-deacetylation a mixture of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid methyl ester and 2-deoxy-2,3-dehydro-4-epi-N-acetylneuraminic acid methyl ester was obtained. The ratio of the two compounds was influenced by the reaction temperature. A minor by-product of the acetylation reaction showed to be 2-methyl-(methyl 7,8,9-tri-O-acetyl-2,6-anhydro-2,3,5-trideoxy-**D**-*glycero*-**D**-*talo*-non-2-enonate)-[4,5-*d*]-2-oxazoline (KUMAR *et al.* 1981).

V. Methylated Sialic Acids

Condensation of 2-acetamido-3-O-benzyl-4,6-O-benzylidene-2-deoxy-**D**-manno-pyranose with potassio-di-*tert*-butyloxaloacetate, followed by methylation with methyl iodide/silver oxide gave 6-O-benzyl-7,9-O-benzylidene-8-O-methyl-N-acetylneuraminic acid γ-lactone. After removal of the benzyl and benzylidene

groups, and opening of the lactone ring, 8-O-methyl-N-acetylneuraminic acid was obtained (KHORLIN and PRIVALOVA 1970). This sialic acid has been found to occur in glycolipids (KOCHETKOV *et al.* 1973, SUGITA 1979).

Reaction of 3-acetamido-3-deoxy-4,5:6,7-di-O-isopropylidene-2-O-methyl-*aldehydo*-**D**-*glycero*-**D**-*galacto*-heptose with [ethoxy(ethoxycarbonyl)methylene]-triphenylphosphorane, followed by an ethoxymercuration-demercuration reaction and acid hydrolysis resulted in the formation of the ethyl esters of 4-O-methyl-N-acetylneuraminic acid and 4-O-methyl-4-epi-N-acetylneuraminic acid (BEAU *et al.* 1978). By oxymercuration of ethyl 5-acetamido-3,5-dideoxy-2-O-ethyl-4-O-methyl-**D**-*glycero*-**D**-*galacto*-non-2-enonate with mercury(II) trifluoroacetate, followed by borohydride-demercuration, 4-O-methyl-N-acetylneuraminic acid ethyl β-glycoside was obtained (BEAU *et al.* 1980). 4-O-Methyl-N-acetylneuraminic acid has been used in metabolism studies, as reported by BEAU and SCHAUER (1980). Using tritiated sodiumborohydride, the corresponding 4-O-methyl-N-acetyl-[3-^{3}H]neuraminic acid was obtained (BEAU and SCHAUER 1980).

A set of partially O-methylated derivatives of methyl N,N-acetyl,methyl-β-**D**-neuraminate methyl glycoside for methylation analyses studies have been prepared by VAN HALBEEK *et al.* (1978) and BRUVIER *et al.* (1981) (see chapter F). VAN HALBEEK *et al.* (1978) started from well-defined partially O-acetylated methyl N-acetyl-β-**D**-neuraminate methyl glycosides. After treatment with methyl vinyl ether and methyl iodide/sodium methylsulfinylmethanide/methylsulfoxide, removal of the 1-methoxyethyl groups yielded the partially O-methylated derivatives (N-methylated). BRUVIER *et al.* (1981) carried out a partial methylation of methyl N-acetyl-β-**D**-neuraminate methyl glycoside using methyl iodide/silver oxide/N,N-dimethylformamide. For the preparation of the 4,7,8,9-tetra-O-methyl derivatives of the methyl esters of N-acetylneuraminic acid benzyl α- and β-glycoside, see LUTZ *et al.* (1968).

VI. Isotopically Labelled Sialic Acids

For the study of biochemical processes several labelled sialic acids and sialic acid derivatives have been prepared.

Several approaches have been reported for the synthesis of N-[1-^{14}C]acetylneuraminic acid: i. Hydrogenolysis of N-benzyloxycarbonylneuraminic acid in the presence of [1-^{14}C]acetic anhydride (WESEMANN and ZILLIKEN 1966); ii. Acylation of neuraminic acid methyl β-glycoside with [1-^{14}C]acetic anhydride followed by mild acid hydrolysis (WIRTZ-PEITZ *et al.* 1969); iii. Acylation of neuraminic acid methyl ester methyl β-glycoside with [1-^{14}C]acetic anhydride and subsequent removal of protecting groups (SCHAUER and BUSCHER 1974). N-[1-^{14}C]Glycolylneuraminic acid has been prepared by acylation of neuraminic acid methyl β-glycoside with [4-^{14}C]1,3-dioxolan-2,4-dione followed by mild acid hydrolysis (SCHAUER *et al.* 1970). N-[1-^{14}C]acetyl-neuraminic acid has been obtained also enzymatically by condensation of N-[1-^{14}C]acetyl-mannosamine and phosphoenolpyruvate (WARREN and GLICK 1966), and by incubations of surviving slices of submaxillary salivary glands of ox and horse with [1-^{14}C]acetate followed by isolation of the glycoprotein fraction and mild acid hydrolysis (SCHAUER 1970, SCHAUER *et al.* 1972). Using the latter approach also O-[1-^{14}C]acetylated sialic

acids were obtained and N-[1-^{14}C]glycolyl-neuraminic acid. See also BRUNETTI *et al.* (1962), WARREN and FELSENFELD (1962), and BLACKLOW and WARREN (1962).

Reaction of neuraminic acid methyl ester methyl β-glycoside with [^{3}H]acetic anhydride followed by release of the methyl groups led to the formation of N-[^{3}H]acetyl-neuraminic acid (SCHAUER and BUSCHER 1974). Starting from 2-acetamido-2-deoxy-**D**-mannose and using Na^{13}CN, BENZING-NGUYEN and PERRY (1978) reported a stepwise synthesis of N-acetyl-[1-^{13}C]neuraminic acid. N-acetyl-[1-^{14}C]neuraminic acid or N-glycolyl-[1-^{14}C]neuraminic acid have been prepared also enzymatically using [1-^{14}C]pyruvate and N-acetylmannosamine or N-glycolylmannosamine, respectively (KEAN and ROSEMAN 1966). Enzymatic coupling of N-acetyl-[1-^{14}C]mannosamine and phosphoenolpyruvate led to the formation of N-acetyl-[4-^{14}C]neuraminic acid (WARREN and GLICK 1966). N-acetyl-[3-^{3}H]neuraminic acid can easily be prepared by treatment of N-acetyl-neuraminic acid with alkaline $^{3}H_2O$. Depending on the pH only H3ax or both H3eq and H3ax are exchanged (FRIEBOLIN *et al.* 1981, DORLAND *et al.* 1982, see also chapter G). The synthesis of 4-O-methyl-N-acetyl-[3-^{3}H]-neuraminic acid (BEAU and SCHAUER 1980) has already been mentioned in section V. The same authors reported also the acylation of 2-deoxy-2,3-dehydro-neuraminic acid with [1-^{14}C]acetic anhydride, yielding 2-deoxy-2,3-dehydro-N-[1-^{14}C]acetyl-neuraminic acid (BEAU and SCHAUER 1979).

The doubly labelled sialic acids N-acetyl-[2-^{14}C,9-^{3}H]neuraminic acid (NÖHLE and SCHAUER 1981) and N-glycolyl-[2-^{14}C,9-^{3}H]neuraminic acid (NÖHLE *et al.* 1982) were prepared from sodium[2-^{14}C]pyruvate and either N-acetyl-[6-^{3}H]mannosamine or N-glycolyl-[6-^{3}H]mannosamine, with the aid of the N-acetylneuraminate lyase from *Clostridium perfringens*. The metabolic fate of these compounds was studied after oral and intravenous application to mice and rats.

A number of these labelled sialic acids have been converted into their CMP-analogues, and subsequently incorporated into glycoconjugates (chapter I). Sialoglycoconjugates labelled in sialic acid can also be prepared by incubations of surviving tissue slices with isotopically labelled precursors of sialic acid, as N-[^{3}H]acetyl-mannosamine.

By using periodate oxidation/tritiated borohydride reduction (see also section VIII), sialoglycoconjugates can be labelled very easily. VAN LENTEN and ASHWELL (1972) and SCHAUER *et al.* (1976) reported the use of this technique for the modification of sialoglycoproteins. VEH *et al.* (1977) have described this degradation for gangliosides; see also PFANNSCHMIDT and SCHAUER (1980) for additional data.

VII. Substrates for Sialidase Investigations

For the tracing of sialidase activities in biological materials, a great variety of natural and synthetic sialyl-substrates have been reported. After incubation two approaches have been worked out: i. Determination of the liberated (modified) sialic acid (e.g. spectrofotometrically or radiochemically); ii. Determination of the released aglycon. Concerning the second approach, besides the use of radio-labelled substrates of sialo-oligosaccharides (BHAVANANDAN *et al.* 1975, STRECKER *et al.* 1976) or gangliosides (SCHRAVEN *et al.* 1977, GHIDONI *et al.* 1981) obtained by

treatment with tritiated borohydride, also some useful synthetic glycosides of α-N-acetylneuraminic acid have been developed.

PRIVALOVA and KHORLIN (1969) reported the synthesis of the *p*-nitrophenyl glycoside of α-N-acetylneuraminic acid. The substrate was obtained by glycosylation of *p*-nitrophenol with 4,7,8,9-tetra-O-acetyl-2-chloro-2-deoxy-β-N-acetylneuraminic acid methyl ester in chloroform in the presence of silver carbonate and Drierite, followed by O-deacetylation and saponification. In enzymatic reactions, released *p*-nitrophenol can be determined spectrofotometrically.

The chromogenic substrate α-N-acetylneuraminic acid 3-methoxyphenyl glycoside has been described by TUPPY and PALESE (1969). This substrate was synthesized by reaction of 4,7,8,9-tetra-O-acetyl-2-chloro-2-deoxy-β-N-acetylneuraminic acid (MEINDL and TUPPY 1965a) with 3-methoxyphenol in the presence of silver carbonate, followed by O-deacetylation. For the determination of the liberated 3-methoxyphenol in enzymatic reactions two methods have been described, namely, coupling with the diazonium salt of 4-amino-2,5-dimethoxy-4'-nitroazobenzene (TUPPY and PALESE 1969) yielding a red coloured product, and, coupling with 4-aminoantipyrine in the presence of the oxidizing agent potassium ferricyanide yielding a coloured quinone (SANTER *et al.* 1978).

The synthesis of a fluorogenic substrate, namely, the 4-methylumbelliferyl glycoside of α-N-acetylneuraminic acid has been reported by four groups. In all approaches a similar main strategy was followed. THOMAS *et al.* (1978) described the coupling of 4,7,8,9-tetra-O-acetyl-2-chloro-2-deoxy-β-N-acetylneuraminic acid methyl ester with 4-methylumbelliferone in distilling toluene using $CdCO_3$ as a catalyst. The substrate was obtained after O-deacetylation and saponification, $[\alpha]_D^{25} = -9.7°$ (H_2O). WARNER and O'BRIEN (1979) used the sodium salt of 4-methylumbelliferone, whereas the coupling was carried out in acetonitrile in the presence of silver carbonate. The ammonium salt of the substrate obtained after deblocking had $[\alpha]_D^{22} = +59.8°$ (H_2O, pH 5.0). POTIER *et al.* (1979) advised the use of 4-methylumbelliferone with acetonitrile in the presence of silver carbonate and activated molecular sieves. The obtained sodium salt had $[\alpha]_D^{20} = +51°$ (c 0.99, H_2O). Finally, MYERS *et al.* (1980) used N,N-dimethylformamide as a solvent for the coupling of the sialic acid derivative and the sodium salt of 4-methylumbelliferone.

VIII. C_7- and C_8-Analogues of Sialic Acids

For the preparation of 5-acetamido-3,5-dideoxy-**D**-*galacto*-octulosonic acid (C_8-Neu5Ac) two approaches have been developed. The compound has been prepared by alkaline condensation of 2-acetamido-2-deoxy-**D**-lyxose with potassio-di-*tert*-butyloxaloacetate in methanol (MCLEAN and BEIDLER 1969, MCLEAN *et al.* 1971). Furthermore, periodate oxidation/borohydride reduction of the glycerol side-chain of sialic acids has been applied. Using varying amounts of periodate, C_8-Neu5Ac as well as 5-acetamido-3,5-dideoxy-**L**-*arabino*-heptulosonic acid (C_7-Neu5Ac) are obtained. Although in principle many sialoglycoconjugates can be used, procedures have been worked out for the mild periodate oxidation followed by borohydride reduction of *Collocalia* mucoid. After mild acid

hydrolysis of the degraded mucin, C_8-Neu5Ac and C_7-Neu5Ac can be isolated via column chromatography (MCLEAN *et al.* 1971, VEH *et al.* 1977). It is also possible to start with N-acetylneuraminic acid methyl ester methyl β-glycoside. In this case, to prevent reduction of the ester function of the periodate-oxidized product, Dowex 1/borohydride was used as a reducing agent (MCLEAN *et al.* 1971). For sialic acid-modifications in glycoproteins, glycolipids and oligosaccharides, and their use in biological studies, see for instance SUTTAJIT and WINZLER (1971), SUTTAJIT *et al.* (1971), VEH *et al.* (1977), and PFANNSCHMIDT and SCHAUER (1980).

The periodate oxidation/reduction approach has also been used to differentiate between the methyl α- and β-glycosides of N-acetylneuraminic acid. Only the C_7-analogue of the β-anomer could be lactonized (YU and LEDEEN 1969). The preparation of the C_7-analogue of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid using periodate oxidation/borohydride reduction was reported by MEINDL and TUPPY (1970).

IX. Miscellaneous Sialic Acids and Sialic Acid Derivatives

The preparation of sialic acids with different N-acyl groups has already got attention in the sections II (including Tables 1 and 2), IV and VI. More details can be obtained from the review of TUPPY and GOTTSCHALK (1972). Surveys of the known reactions involving the carboxyl group and the alcoholic groups of sialic acid have been presented by TUPPY and GOTTSCHALK (1972) and HOLMQUIST (1975). See also HOLMQUIST (1971), BROSSMER and HOLMQUIST (1971), BROSSMER and HOLMQUIST (1974), and BROSSMER *et al.* (1974). For studies on the stability of simple derivatives of sialic acid, see KARKAS and CHARGAFF (1964).

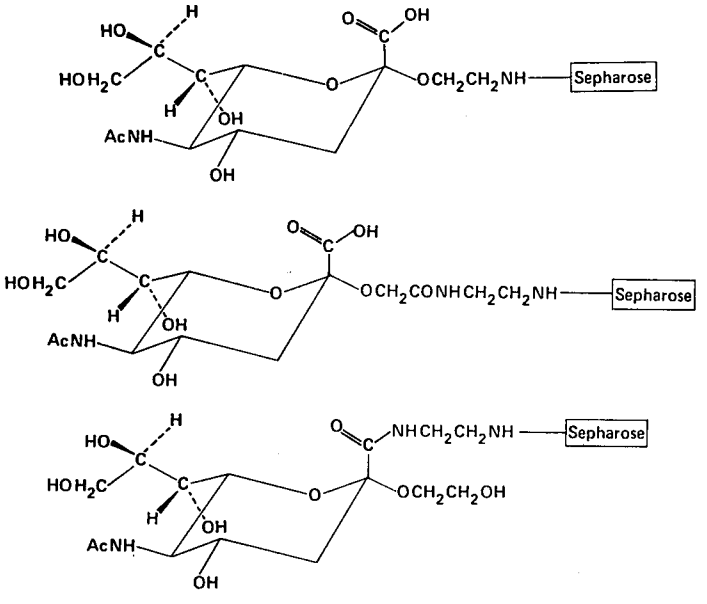
The 9-azido-9-deoxy derivative of N-acetylneuraminic acid has been prepared both chemically and enzymatically. For the chemical synthesis, N-acetylneuraminic acid methyl ester methyl α-glycoside was converted into the corresponding 9-O-tosyl derivative as intermediate and then in the 9-azido-9-deoxy compound by reaction with NaN_3. After removal of methyl groups, 5-acetamido-9-azido-3,5,9-trideoxy-**D**-*glycero*-**D**-*galacto*-2-nonulosonic acid was obtained (BROSSMER *et al.* 1979). The enzymatic preparation was carried out by condensing phosphoenolpyruvate and 6-azido-6-deoxy-N-acetylmannosamine in the presence of N-acetylneuraminate synthase (BROSSMER *et al.* 1980). In principle the 9-azido-9-deoxy derivative of N-acetyl-[1-^{14}C]neuraminic acid can be prepared using [1-^{14}C]phosphoenolpyruvate. Also the CMP-analogue has been reported (BROSSMER *et al.* 1979). For another synthetic approach of azido-derivatives, see BRANDSTETTER *et al.* (1982). Furthermore, the syntheses of the 9-amino-9-deoxy and 9-iodo-9-deoxy derivatives of N-acetylneuraminic acid have been mentioned (SUPP *et al.* 1980).

The synthesis of the methyl ester of 9-O-glycyl-N-acetylneuraminic acid has been reported by DEREVITSKAYA *et al.* (1965 b). The methyl ester of N-acetylneuraminic acid was coupled with N-benzyloxycarbonylglycine in the presence of dicyclohexylcarbodiimide; subsequently the benzyloxycarbonyl group was removed by hydrogenolysis. The 9-phosphate esters of N-acetylneuraminic acid and N-glycolylneuraminic acid were prepared enzymatically (ROSEMAN *et al.* 1961, WARREN and FELSENFELD 1961, WATSON *et al.* 1966 a, b). Also a chemical

synthesis has been mentioned (BROSSMER *et al.* 1974). The preparation of partially O-acetylated derivatives of the methyl ester methyl β-glycoside has been reported by HAVERKAMP *et al.* (1975) and VAN HALBEEK *et al.* (1978).

N-acetyl-[3-OH]neuraminic acid was synthesized by reaction of N-acetylmannosamine with bromopyruvate or hydroxypyruvate (DEVRIES and BINKLEY 1972). The synthesis of N-acetyl-[3-F]neuraminic acid by condensing fluoropyruvic acid and N-acetylglucosamine or N-acetylmannosamine in alkaline solution has been published by GANTT *et al.* (1964).

Scheme 3



A method for the incorporation of fluorescent probes in sialoglycoproteins has been described by INGHAM and BREW (1981). After mild periodate oxidation the resulting aldehyde functions of the degraded side-chains of sialic acids were condensed with dansylhydrazine, dansylethylenediamine or fluoresceinamine, followed by reduction. Compared with the other derivatives, dansylhydrazine conjugates are relatively unstable. ABRAHAM and LOW (1980) used fluorescent probes for the investigation of human erythrocyte membranes.

To study glycoconjugates by e.p.r. spectroscopy, techniques have been reported for the spin-labelling of sialic acids. For the attachment of the spin-label 4-amino-2,2,6,6-tetramethylpiperidin-1-oxyl, the carboxyl group (APLIN *et al.* 1979 b) as well as the periodate-oxidized side-chain (APLIN *et al.* 1979 a, FEIX and BUTTERFIELD 1980) of sialic acid were chosen. See also DAVOUST *et al.* (1981).

Sialic acids have also been coupled to proteins. To investigate the haptenic properties of sialic acid, the *p*-nitrobenzyl α-glycoside of N-acetylneuraminic acid

has been reduced to the corresponding *p*-aminobenzyl glycoside, which was diazotized and coupled to ovalbumin (GIELEN and UHLENBRUCK 1969).

It has been demonstrated that α-glycosides of sialic acid(s) (derivatives), when immobilized on solid supports, can be used for affinity chromatography of sialidases. HOLMQUIST (1974) reported the preparation of the 2-aminoethyl and the 2-aminoethylaminocarbonylmethyl α-glycosides of N-acetylneuraminic acid, and of the 2-aminoethylamide of the 2-hydroxyethyl α-glycoside of N-acetylneuraminic acid. These derivatives were coupled to cyanogen bromide-activated Sepharose 2B or 4B via the amino group of the α-glycoside (N-acetylneuraminic acid-Sepharose) or the amide (N-acetylneuraminamide-Sepharose) (Scheme 3). The matrix-bound derivatives were also treated with periodate/borohydride to afford the 7-carbon analogues. It was demonstrated that especially N-acetylneuraminamide-Sepharose did not significantly adsorb *Vibrio cholerae* sialidase. In this context also the behaviour of the 2-aminoethylaminocarbonylmethyl β-glycoside of N-acetylneuraminic acid coupled to Sepharose has been studied (HOLMQUIST and ÖSTMAN 1975). For additional data, see HOLMQUIST and NILSSON (1979). Promising results have also been obtained using other sialic acid-Sepharose coupling products (CORFIELD and SCHAUER, unpublished results). Sepharose 4B was activated by cyanogen bromide or periodate. To connect the sialic acids with the support material, two types of spacers were studied, namely, adipic acid dihydrazide and polymethylacrylic acid hydrazide. Binding of neuraminic acid methyl β-glycoside and 2-deoxy-2,3-dehydro-neuraminic acid was carried out via the free amino group after activation of the matrix with HNO_2. Binding of the N-acetyl analogues was performed via the side-chain, after periodate oxidation, in the presence of cyanoborohydride. For the coupling of sialoglycoconjugates to insoluble supports, see CORFIELD *et al.* (1979 a).

Acknowledgement

The studies from the authors' laboratory were supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

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