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Immobilized enzymes in preparative carbohydrate chemistry

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Abstract. The scope of a galactosylation procedure with five immobilized enzymes has been examined with oligosaccharides bearing terminal, non-reducing βGlcNAc residues. Tri-, penta- and hexasaccharides related to glycolipids or glycoproteins have been prepared. Cytidine monophosphate was converted to the triphosphate with phosphoenol pyruvate, ATP (catalytic) and two immobilized enzymes, and utilized in a synthesis of CMPNeu5Ac with an immobilized synthetase. Acylneuraminate pyruvate-lyase immobilized on agarose gave a gel which catalysed the synthesis of representative sialic acids from pyruvate and mannosamine derivatives.

INTRODUCTION

Glycosidation with glycosyl nucleotides and the highly specific glycosyltransferases is a very common biochemical practice, which has been applied to free oligosaccharides, glycoconjugates, and even whole cells, and would dispense the organic chemist from the tedious protection - deprotection strategy. However, its very low scale - from the nano- to the micromole - is a major drawback. Enzymes and glycosyl nucleotides are costly reagents, not easily recoverable. This may reflect only temporary economic conditions, but there is a more fundamental problem: the stoichiometric use of a glycosyl nucleotides accumulates in the medium the corresponding nucleotide, which may be inhibitory to the transferase at mM concentrations (Ref. 1). The now classical solution to these problems is to attach the enzyme to a suitable insoluble polymer which is used as an aqueous suspension. When the reaction is finished, the enzyme is separated from the products by filtration, and may be used again many times in favourable circumstances. Only catalytic quantities of glycosyl nucleotides are necessary, as they are constantly regenerated in the medium by the interplay of appropriate substrates with other enzymes, also present in the immobilized state.

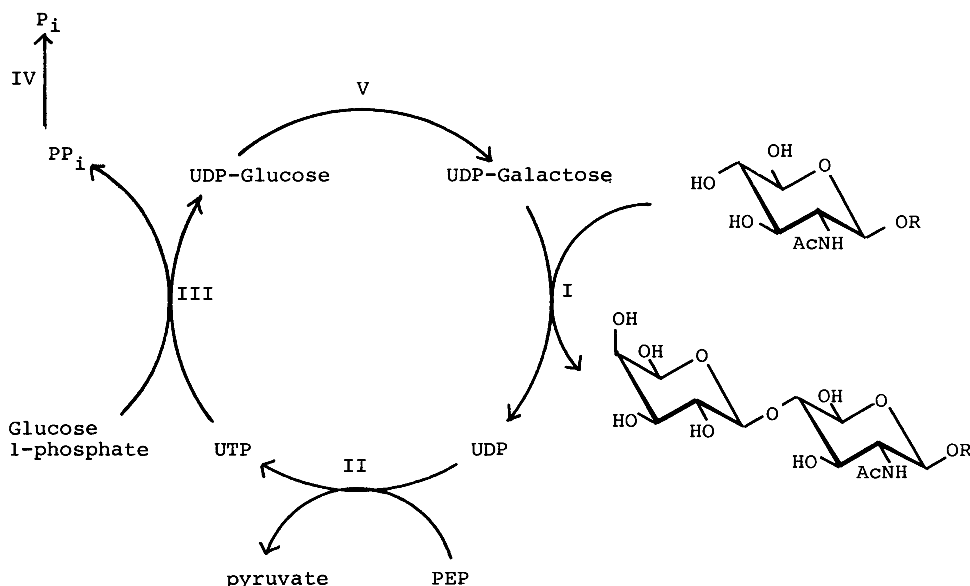
The objection may be raised that many useful glycosyltransferases are poorly available, being found only in mammals, sometimes indeed only in human blood or milk. The presently rapidly developing cloning techniques may soon put an end to these shortages, so that it seems strongly advisable that organic chemists being at once to train themselves in the manipulation of these new reagents, which may be on the market in a not too distant future.

The transferase properties of the *Escherichia coli* β-galactosidase have been used for the synthesis of a disaccharide (Ref. 2). The immobilized β-galactosidase, acting on a mixture of lactose and N-acetylglucosamine, gave a mixture of products which contained 20% βGal*p*-(1→6)-GlcNAc. In view of the very low price of most simple sugars, the use of such glycosidases for disaccharide synthesis should be considered, especially for the preparation of starting material, whenever the product is not too difficult to separate from the reaction mixture on the 10-100 mmol scale. For later steps, the association of a specific transferase with the enzymes of glycosyl nucleotides regeneration appears more useful. We shall first describe the syntheses of oligosaccharides related to the Ii system of blood groups with the enzymes of the Leloir pathway, a method first used for a synthesis of lactosamine (Ref. 3).

We shall next discuss problems of sialylation : the syntheses of CTP and CMPNeu5Ac, and the high scale preparations of sialic acids.

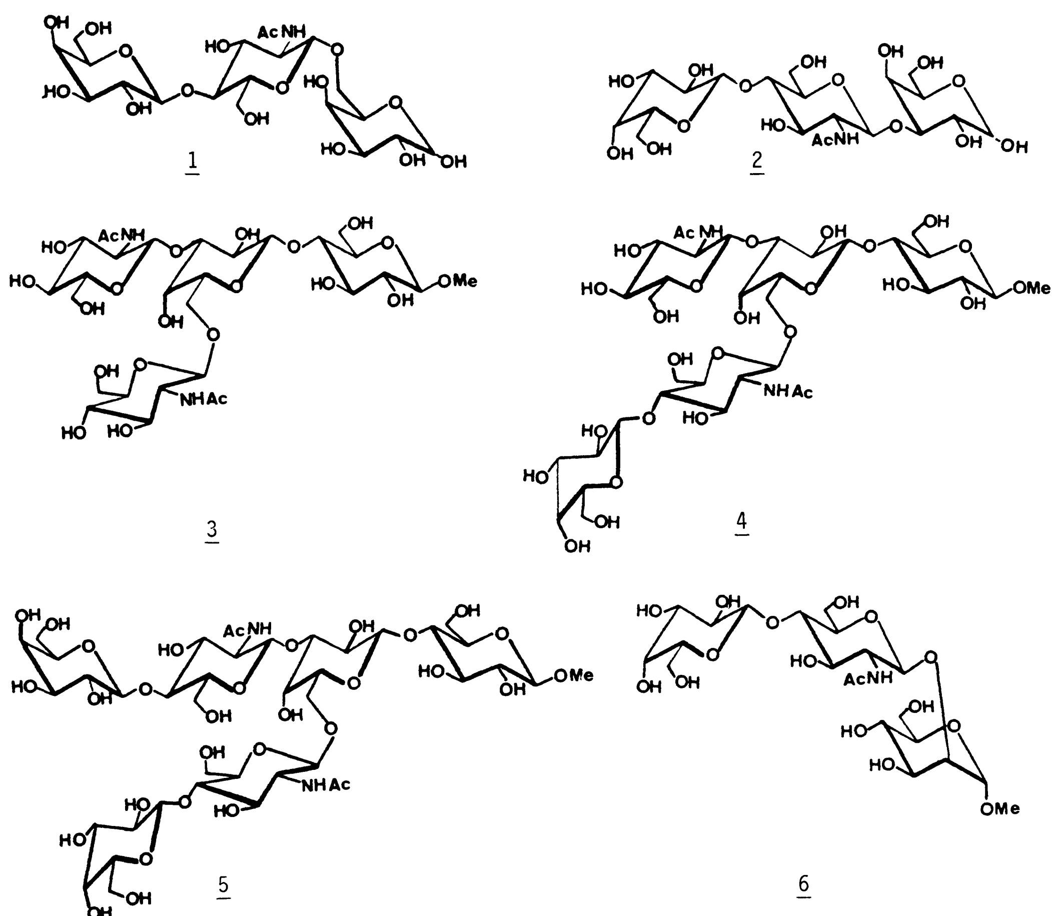
GALACTOSYLATIONS WITH *N*-ACETYLGLUCOSAMINE β-(1 → 4)-GALACTOSYLTRANSFERASE

The galactosylation cycle is shown in Scheme 1. The transfer of β-D-galactopyranosyl unit from uridine diphosphate galactose to the O-4 position of a terminal, non-reducing residue of β-N-acetylglucosamine, catalysed by transferase I, releases an equimolecular quantity of uridine diphosphate. This is enzymatically phosphorylated to uridine triphosphate by phosphoenolpyruvate in the presence of pyruvate kinase II. Another specific transferase, III, catalyses the synthesis of the glycosyl nucleotides, uridine diphosphate glucose, from uridine triphosphate and α-D-glucose 1-phosphate. This is a reversible reaction which must be displaced in the synthetic direction by the destruction of its other product, pyrophosphate, which is hydrolysed to inorganic phosphate with the help of pyrophosphatase IV. The last step is the conversion of uridine diphosphate glucose to uridine diphosphate galactose, catalysed by epimerase V. Broadly speaking, the system must be fed with α-D-glucose 1-phosphate and the "source of energy" (phosphoenolpyruvate) and releases inorganic phosphate and pyruvate as by-products.



Scheme 1. The β-D-galactopyranosylation cycle.- Complete system (final concentrations, mM) : oligosaccharide substrate (6.7); α-D-glucose 1-phosphate (7); phosphoenolpyruvate, PEP (7); UDP-glucose (0.17); NAD^+ (1); $MnCl_2$ (2); $MgCl_2$ (4); KCl (70); dithiothreitol (10); NaN_3 (1.5). The pH is adjusted to 8, and the immobilized enzymes are added : I, β-D-galactosyltransferase (E.C. 2.4.1.22) (3.8 U); II, pyruvate kinase (E.C. 2.7.1.40 (34 U); III, UDP-glucose pyrophosphorylase (E.C.2.7.7.9) (5 U); IV, inorganic pyrophosphatase (E.C.3.6.1.1) (25 U); V, UDP-galactose 4-epimerase (E.C. 5.1.3.2) (3.7 U). Final volume 100 mL. Temperature : 30°C.

Enzymes I-V are commercially available; enzymes II, III and IV are relatively inexpensive. Nevertheless, we preferred to prepare galactosyltransferase in our laboratory. For this, the only necessary addition to the usual equipment of the organic chemistry laboratory was a refrigerated centrifuge. Our experience is that the carbohydrate chemist, trained to work with water-soluble substances, needs no extensive practical knowledge in enzymology to concentrate 180 U of this enzyme from 2 L of cow colostrum (Ref. 1). The five enzymes are immobilized separately as already described (Ref. 4 and 5). The nature of the support does not appear to be critical (see, for example, Ref. 3). The agarose gels are suspended in water, and the pH is maintained at its optimum value, 8.0, with pH-stat equipment. A 0.1 M Tris buffer, pH 8.0, may also be used for small scale preparations, when an excess of salts may be tolerated in the work up. The system is gently stirred at 30°C. The complete reaction requires a few days with 2 U of immobilized transferase per mmol of substrate. After it has stopped, the product is separated from the gels, which can generally be utilized again, either on the same substrate or another one. The oligosaccharide is recovered from its solution by ion-exchange de-ionization followed by freeze-drying. Starting material, if still present, is removed by silica gel column chromatography. The reaction may slow down at 70% completion. The reason is the accumulation of an ionic inhibitor, maybe phosphate. In such a case, the solution separated from the gel is deionized, and mixed again with the same gel, and, of course, a fresh batch of ionic cofactors.



Oligosaccharides 1, 2, 4, 5 and 6 were prepared by mixed-type synthesis, enzymatic galactosylation being the last step in an otherwise traditional sequence (Ref. 6, 7, 8 and 9). The identification of 1, 2 and 5 rests on the comparison of their properties, especially the NMR spectra, with those of samples already prepared in our laboratory by classical means (Ref. 8 and 10). Trisaccharide 1 was first recognized as the epitope of one of the I-antigens in man, I(Ma); but it is likely that it has a more fundamental significance, the I(Ma) antigen being expressed on mouse embryos from the single cell stage until after the sixth day of development (Ref. 11). Trisaccharide 2 is a fragment of the main chain of glycolipids. The free hexasaccharide corresponding to glycoside 5 is a trace component of human milk (5 mg/L) (Ref. 12).

Galactosylation of the branched trisaccharide-glycoside 3 raised an interesting problem. In principle, there are two reactive positions, one on each terminal non-reducing β-N-acetylglucosamine residue. The residue linked to the primary position of galactose appears to be more reactive (but only marginally more) than the other one, as was shown in delicate kinetic experiments from the group of Van den Eijnden, with the soluble enzyme (Ref. 13). Moreover, the reaction of 3 (1 μmol) with excess uridine diphosphate galactose (4 μmol) and soluble transferase (0.1 U), afforded hexasaccharide 5 (Ref. 14). On the other hand, our immobilized enzyme system in the presence of one equivalent each of α-D-glucose 1-phosphate and phosphoenolpyruvate gave only traces of hexasaccharide 5, even after 6 days. The only product which was practically obtained was a pentasaccharide. The two-dimensional COSY ^{1}H NMR spectrum of the derived peracetate could be interpreted in a completely consistent manner. Assignment of chemical shifts to each of the 35 ring protons showed that, on the βGlcNAc residue linked to position 3 of galactose, proton H-4 was geminal to an acetoxy group, and in consequence, this residue was not galactosylated. This was confirmed by the comparison on the mppm scale of the chemical shifts of the anomeric protons of the pentasaccharide with those of tetrasaccharide 3 and hexasaccharide 5, already interpreted in our laboratory (Ref. 3). Thus, this pentasaccharide 4 was obtained with a selectivity probably unattainable by any current method of organic chemistry. Doubling the proportion of reagents changed nothing. Hexasaccharide 5 could only be prepared - in modest yield - with long reaction times and addition of fresh enzymes.

Trisaccharide 6 characterized by its 400 MHz ^{1}H-NMR spectrum in D_2O was prepared as a possible substrate for sialytransferase. The corresponding peracetate (Ref. 15) and free sugar (Ref. 16) were already known.

The 6-O-acetyl and 3-O-allyl derivatives of N-acetylglucosamine were not substrates. On the other hand, it seems that any unsubstituted, terminal, non-reducing β-N-acetylglucosamine residue can be galactosylated with our system. Long reaction times and renewal of immobilized enzymes may help in the case of sluggish reactions. Finally, the reactions may be scaled up as much as desired. Then, the problem is the introduction of the penultimate β-N-acetylglucosamine residue. The relevant transferases, although they are known (Ref. 17), are available only with difficulty for the time being. In this respect, we may mention a new chemical coupling procedure which has been developed in our laboratory and which utilizes the common 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl chloride with stannous triflate as promotor. Both products are stable and inexpensive. For example, this halogenose, "diacetone-galactose" (1 eq.), stannous triflate (1.5 eq.), and tetramethylurea (2 eq.) in dichloromethane solution at room temperature gave, after 4 h, a 77% yield of pure, protected disaccharide βGlcNAcp-(1→6)-Gal (94% based on recovered galactose - no α anomer detected). Yields are good to excellent with primary alcohols, and although less satisfactory at the 3-position, are nevertheless preparatively useful, since no exchange of acyl on nitrogen is necessary, as in phthalimido-based procedures.

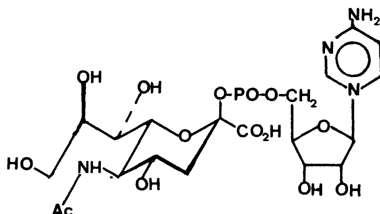
PROBLEMS ASSOCIATED WITH SIALYLATION

This is obviously the field where the quest for high scale enzymatic techniques is most warranted, as the organic chemistry of sialic acids is fraught with problems. For instance the building of the sequence αNeup5Ac-(2→6)-βGalp-(1→4)-βGlcpNAc-(1→2)-Man, common in glycoproteins, is an arduous task (Ref. 19). Even more difficult is the sialylation at position 3 of galactose (Ref. 20). Moreover, in many cases - maybe in most cases - the sialic acid residue in the native antigen is present as a highly labile acetic or L-lactic ester (Ref. 21). The synthesis of such sialosides by the techniques of organic chemistry would involve a still uncertain adaptation of glycosidic coupling methods. Finally, we may forecast that in the near future, the problem will arise to sialylate glycoproteins without denaturation on a scale compatible with commercialization, and immobilized enzymes may help in this connection.

The sialyltransferases are a family to enzymes which catalyse the transfer of a sialic acid residue from the glycosyl nucleotides cytidine monophosphate-N-acetylneuraminic acid (7, CMPNeu5Ac) to a specific position in a specific sugar [reaction (2)]. Free, unphosphorylated N-acetylneuraminic acid is directly converted to its active derivative 7 by cytidine triphosphate (CTP) [reaction (1)] in the presence of a synthetase:

CTP + Neu5Ac → CMPNeu5Ac + PPi (1)

CMPNeu5Ac + ROH → sialoside + CMP (2)



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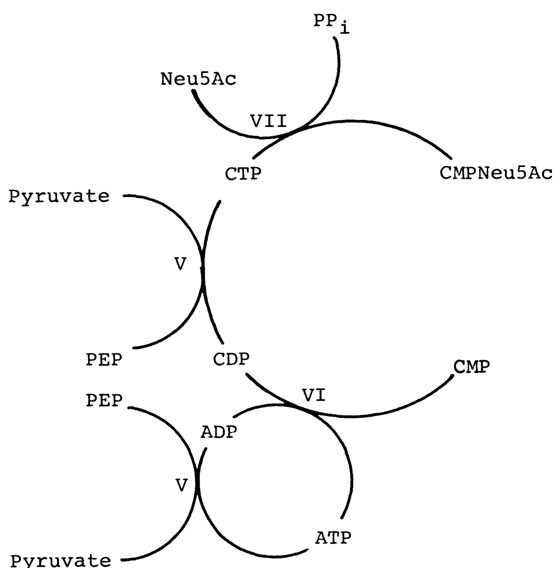
These two enzymes are neither commercially available nor easily prepared. Still, several enzymatic preparations of 7 on the 100 μmol scale have been reported by biochemists (Ref. 22 and literature cited there). This is already one order of magnitude higher than the final scale of some reported oligosaccharides syntheses in this field. We shall now describe a system of three immobilized enzymes for the preparation of CMPNeuAc (Scheme 2).

The not unduly expensive cytidine monophosphate (CMP) is phosphorylated to its diphosphate (CDP) in the presence of immobilized nucleoside monophosphate kinase, VI. The phosphate donor is ATP, which is regenerated as in Scheme 1 with phosphoenolpyruvate (PEP) and immobilized pyruvate kinase, V. Conversion of CDP to CTP must also be catalysed by the same system, that is, PEP and pyruvate kinase, and this creates a small problem for this enzyme.



Scheme 2. Preparation of cytidine monophosphate N-acetylneuraminic acid from CMP and N-acetylneuraminic acid. Synthesis of CTP : to the immobilized enzymes, VI, nucleoside monophosphokinase (E.C.2.7.4.4) (0.3 U) and V, pyruvate kinase (10 U), suspended in Tris buffer (either 50 mM, pH 9, or 100 mM, pH 7) (15 mL) were added to the final concentrations given (mM), CMP (Na salt, 13.3); PEP (K salt, 27); ATP (Na salt, 1.3); $MgCl_2$ (4); KCl (50); thymol (1). Temperature 37°C. Synthesis of CMPNeuAc : to the immobilized CMPNeu5Ac synthetase (E.C.2.7.7.4.3) (0.6 U) in Tris buffer, pH 7.0 containing 3.75 mM mercaptoethanol were added, to the final concentrations given (mM), CTP (13); Neu5Ac (3.3); $MgCl_2$ (4); $MnCl_2$ (6.25); thymol (1). Temperature 37°C.

As the optimum pH of the synthetase is 9 in the presence of Mg^{++} ions, and that of sialyltransferase is in the vicinity of 7, any attempt to make them work together in the same vessel would be hopeless. Fortunately, Higa and Paulson, looking for suitable conditions to activate the alkali-labile acetates of N-acetylneuraminic acid, with the same enzyme in the soluble state, made the very pertinent observation that replacement of Mg^{++} ions lowers the optimum pH to 7, with only 30% loss of activity (Ref. 22). On the other hand, although there are no pH incompatibilities, enzyme V, VI and VII cannot work together because the synthetase is inhibited by CMP (K_i 20 mM). In any case, we desired to avoid conditions which might slow down reaction 2, as CMPNeu5Ac is reported not to be very stable in the medium (we have not noticed any degradation at pH 7 for 48 h). Thus the synthesis of CMPNeu5Ac is a two-step reaction, where every component is readily available except the synthetase. Higa and Paulson reported the extraction of 63 U from three calf brains, maybe in very favourable conditions. In principle, this would allow the preparation of 1.3 g of CMPNeuAc with our system, which could be utilized again.

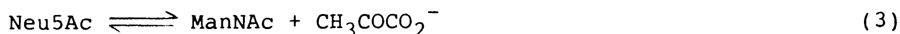
In a very recent report (Ref. 25) describing the preparation of a collection of sialosides in the range of 10-20 µmol with soluble transferases, the authors stress the fact that the availability of CMPNeu5Ac might be a limiting factor in such approaches.

We are currently working on the immobilization of sialyltransferase from cow colostrum.

SYNTHESES OF SIALIC ACIDS

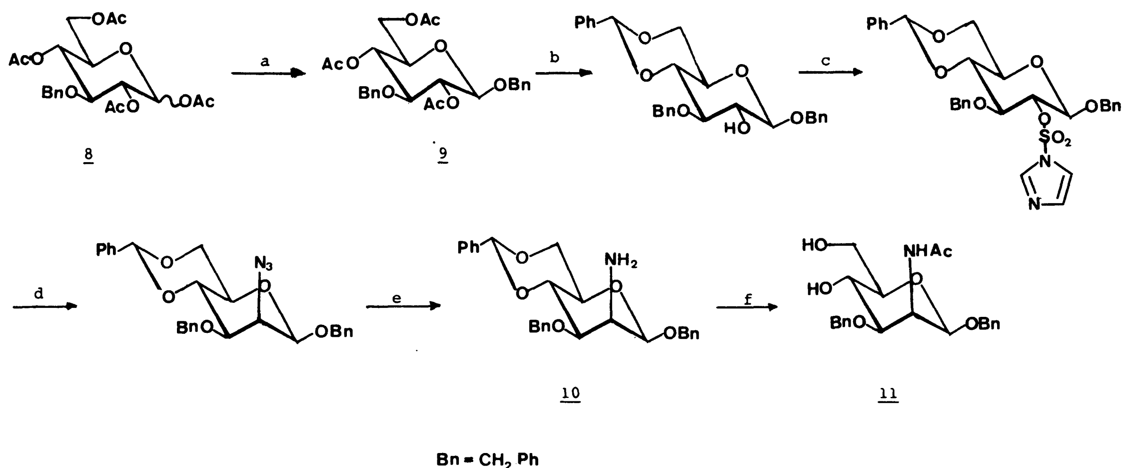
A list of natural sialic acids may be found in Schauer's review in 1982 (Ref. 21). Most are esters, primarily acetates, of the alcoholic functions at positions 4, 7, 8 and 9 of N-acetyl- or N-glycolylneuraminic acid. N-Acetylneuraminic acid itself, which has been obtained by synthesis (Ref. 26) or extraction from the urine of patients suffering from some rare diseases, is not a very accessible compound. All its congeners have been obtained by extraction from natural sources, for example, the submaxillary mucin of some domestic mammals (Ref. 22); they are even less easily available, but at least as important from the physiological point of view.

This state of affairs led us to examine the possibilities of enzymatic synthesis. A well-known, commercial aldolase, acylneuraminate pyruvate-lyase catalyses the reversible reaction (3) :



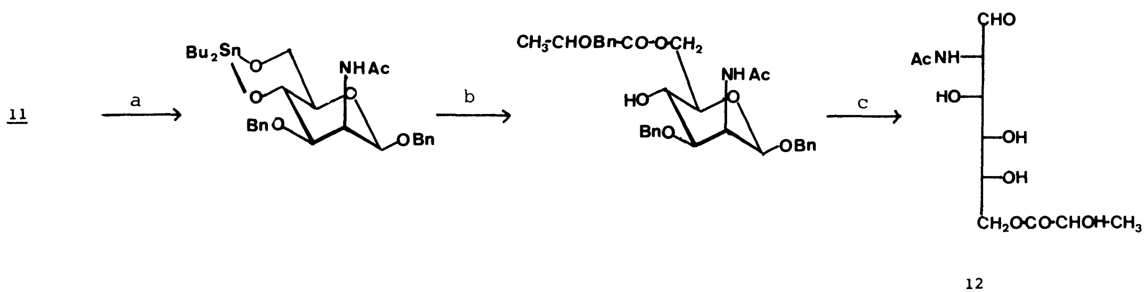
$$\text{Neu5Ac} \rightleftharpoons \text{ManNAc} + CH_3COCO_2^- \quad (3)$$

The enzyme in water solution has already been used in the preparation of small amounts of labelled Neu5Ac (Ref. 27). The affinity of the bacterial enzyme from Clostridium perfringens for Neu5Ac (K_m 1.85 mM) is not very different from its affinity for the 9-O-acetyl derivative (K_m 2.00 mM) or the 7-O-acetyl derivative (K_m 4.55) (Ref. 28). Against the use of this enzyme, the objection may be raised at once that the difficulty is only shifted, because of the high price of N-acetylmannosamine itself. Actually, when we used this sugar as a starting material, we restricted ourselves to short reaction sequences with high yield: thus N-glycolylmannosamine was prepared by reaction of mannosamine with p-nitrophenyl 2-benzyloxyacetate followed by hydrogenolysis over palladium. The ester, 2-acetamido-6-O-acetyl-2-deoxy-D-mannose (13, R^1 = Me, R^2 = Ac), was obtained by selective acetolysis of the pertrimethylsilyl derivative of N-acetylmannosamine at positions 1 and 6, followed by hydrolytic removal of the remaining trimethylsilyl protecting groups. Finally the anomeric acetate was removed with concomitant conversion into the benzyl glycosylamine (Ref. 29). This method, elaborated by A. Veyrières, could also be used in an efficient synthesis of the D-gluco isomer (Ref. 30).

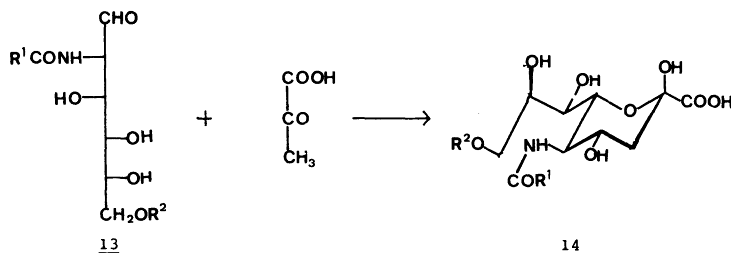


Scheme 3.
a) i: NH_3-MeCN; ii : CCl_3CN, K_2CO_3: iii : $PhCH_2OH$,BF_3-EtO;
b) i : MeONa-MeOH; ii : PhCHO, $ZnCl_2$;
c) i : NaH-DMF; ii : N,N'-sulfuryldiimidazole;
d) $Bu_4N^+N_3^-$, PhMe, 80°C, 2 h; e) $LiAlH_4$;
f) i : Ac_2O-pyridine; ii : AcOH-H_2O, 100°C, 0.5 h.

However, our general route to mannosamines substituted at positions other than 5 (Scheme 3) starts from the peracetate of 3-O-benzyl-D-glucose, 8, which can be prepared in 200 g batches. Selective anomeric de-acetylation according to a recent reported procedure (Ref. 31) allowed utilisation of the Schmidt procedure (Ref. 32) for the preparation of the benzyl glucoside 9. For azidation with inversion of configuration, we have used the new imidazolylsulfonyl leaving group discovered by Hanessian and Vatèle (Ref. 33). The reagent needed, N,N'-sulfuryldiimidazole, is very easily prepared and can be stored indefinitely without special precautions. Azidation of the sugar derivative is practically quantitative in mild conditions. Yields are excellent for all the reactions of Scheme 3. The highly crystalline protected mannosamine 10 precipitated directly on concentrating the ether solution after removal of inorganic material at the end of step e (Scheme 3). The function of this amine as a turn-table in our general route is illustrated by the preparation of the lactyl ester 12 (Scheme 4).



Scheme 4. a) Bu_2SnO-benzene; b) CH_3-CHOBn-COCl; c) Pd-H_2.



Scheme 5. Preparation of sialic acids.- Complete system : To the immobilized acylneuraminate-pyruvate lyase (E.C.4.1.3.3) (2.7 U) suspended in 50 mM phosphate buffer, pH 7.2 (36 mL), were added, to the final concentrations given (mM) : the N-acyl-mannosamine substrate (100); Na pyruvate (1000); dithiothreitol (1); NaN_3 (1.5). Temperature 37°C.

Commercial aldolase was immobilized on agarose, with a 51% yield of enzymatic activity, to give a gel with a specific activity of 1.25 U/mL. The conditions of the reactions are summarized in Scheme 5. We may note as a general comment that these synthetic pathways are completely unphysiological. The aldolase we use has only catabolic functions in cells. In nature, N-acetylneuraminic acid is built from phosphorylated precursors, and modifications only happen later on, by enzymatic oxidation of the N-acetyl to the N-glycolyl group, or enzymatic esterification.

N-Acetylneuraminic acid (14, R^1= CH_3, R^2=H).
With our enzymatic technique, it is not necessary to start from the costly pure N-acetylmannosamine. N-Acetylglucosamine is first epimerized in alkaline medium. Excess N-acetylglucosamine is then removed by one crystallization from the equilibrium mixture; and the enriched mother-liquor, with a 1:1 D-gluco/D-manno ratio, is directly treated with aldolase. Only the D-manno configuration is recognized by the enzyme, and the D-gluco epimer is not inhibitory. Furthermore, the carboxylic acid function of the product allows an easy separation from the unchanged neutral sugars in the medium. The yield is 1 mmol of N-acetylneuraminic acid per enzymatic unit (Ref. 34).

N-glycolylneuraminic acid (14, R' = CH_2OH, R^2 = H).
This acid, apparently absent in man, is very common in other mammals, up to 90% of the sialic acids fraction in some tissues. The mixture obtained by the alkaline epimerization of N-glycolylglucosamine may also be used in the enzymatic synthesis.

9-O-Acetyl-N-acetylneuraminic acid (14, R^1 = CH_3, R^2 = Ac).
This is an ester of common occurrence (Ref. 35). Interestingly, according to some recent reports, a N-acetyl-9-O-acetyl-neuraminic acid residue is present in the antigenic epitope of a ganglioside found in the developing rat embryonic neuroectoderm and in human melanoma cells recognized by a monoclonal antibody, Mab D1-1, prepared against the rat B49 cell lines (Ref. 36, 37).

9-O-Lactyl-N-acetylneuraminic acid (14, R^1 = CH_3, R^2 = $CH_3CHOHCO$).
The presence of the L-lactyl diastereoisomer in natural sources has been mentioned several times. The DL-lactic acid ester of N-acetylmannosamine 12 was found to be a substrate of the aldolase. However, the corresponding sialic acid was readily hydrolyzed at pH 7.2 during the enzymatic reaction, so that the product was contaminated with about 30% of N-acetylneuraminic acid. Partial resolution could be achieved with the reported chromatographic systems (Ref. 38). Its ready hydrolysis at pH 7.2 during the enzymatic reaction is a cause of low yield.

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