Carbohydrate Natural Products as a Scaffolding for the Preparation of Potential Neuraminidase Inhibitors

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Summary

Compound **10b**, 6-acetamido-6,8-dideoxy-D-*erythro*-β-D-*galacto*-octopyranosyl-1-oxyacetic acid sodium salt, was synthesised by hydrazinolysis of Lincomycin, acetylation of methylthiolincosaminide (MTL) **9a**, and by subsequent glycosylation of acetate **9b** with methyl glycolate under mild conditions (NIS/TfOH). The methyl ester **10a** was hydrolysed by treatment with Amberlite Ira-400 (OH^-) resin and aqueous sodium hydroxide, followed by neutralisation with Dowex-50 W × 8 (H^+) resin and lyophilisation to give **10b**. This carboxylate may represent the first derivative in a novel series of sialidase inhibitors utilising carbohydrate natural products. The phosphonate **11c**, prepared under the same experimental conditions with dibenzyl(hydroxymethyl) phosphonate as acceptor, also displays an inhibitory activity towards *Clostridium perfringens* sialidase (K_i in mM range as with Neu5Ac).

Introduction

As a target, inhibition of influenza neuraminidase is highly attractive due to its location on the surface of the virion and its point of action in the viral life cycle[1, 2]. It is well known that neuraminidase cleaves terminal Neu5Ac from glycosides to produce free Neu5Ac. Thus a number of workers have been involved in the design of potential NA inhibitors based on the substrate and on the product of the enzymatic reaction.

Approaches to the discovery of NA inhibitors, as reviewed by Bamford[3], have been primarily based on the knowledge of the substrate and of the mechanism of the enzymatic reaction, of transition state analogues, and then on more detailed information of the enzyme active site.

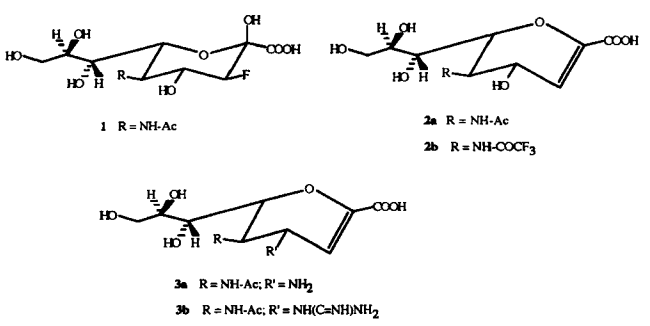

Chart 1. Early and target NA inhibitors.

The most important structures found in the three categories mentioned, representing the main neuraminidase inhibitors (3FNA **1**, 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid DANA **2a**, 5-trifluoroacetamido analogue FANA **2b**, and 4-amino and 4-guanidino-DANA derivatives **3a–3b**) are described in Chart 1.

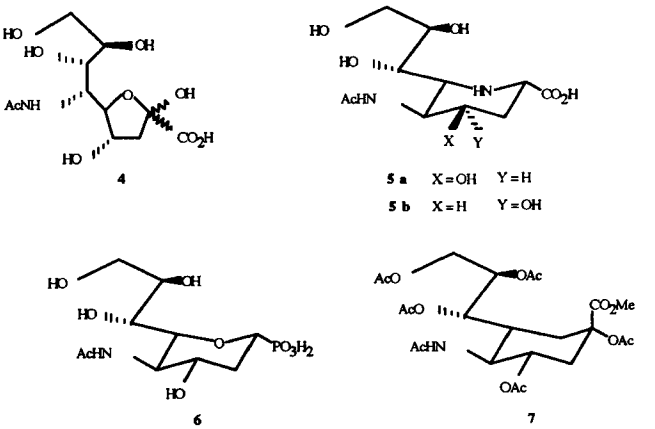

Chart 2. Empirical approach to NA inhibitors.

A few NA inhibitors deriving from an empirical approach are illustrated in Chart 2:

a) compound **4** is a furanose isomer of sialic acid and shows the NHAc group in the side chain rather than in the sugar ring. This compound was synthesised by the aldol condensation of D-glucose with oxalacetic acid and has an activity comparable with that of Neu5Ac2en[4]

b) piperidine derivatives **5**, analogues of Siastatin B, possess activity similar to Neu5Ac2en itself[5], while piperidine analogues with shortened side chain are weak inhibitors, thus evidencing the importance of an intact 1, 2, 3-trihydroxy propyl side chain[6]

c) compound **6** shows a remarkable effect of the phosphonate group on enzyme binding[7]

d) the carbocyclic analogue of Neu5Ac **7**, obtained from the Dies-Alder *endo*-adduct of furan and acrylic acid, is not active towards sialidase from *Arthrobacter ureafaciens*[8].

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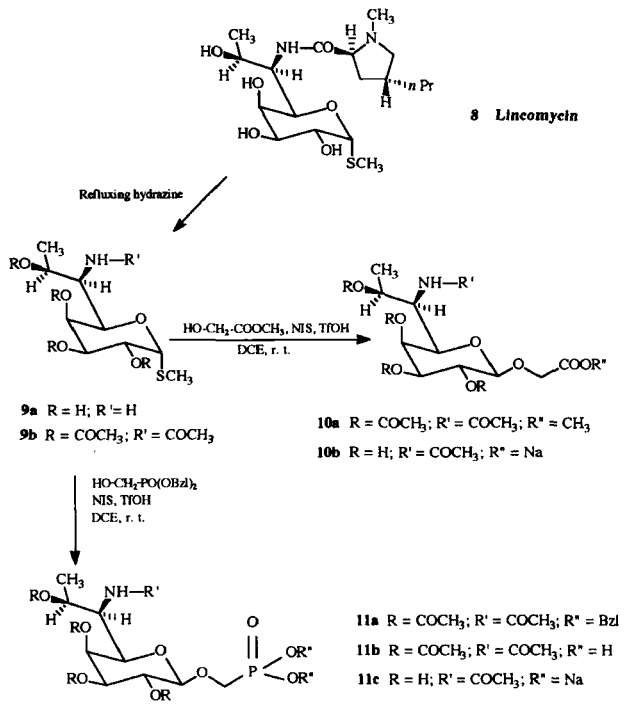
The above reported compounds are only few examples of the numerous synthetic NA inhibitors; they were all obtained by a random screening, and by means of a synthesis that requires many reaction steps, thus revealing a lack of simple chemical modifications of natural substrates that may lead to active derivatives. In fact, an alternative approach to chemical modification of Neu5Ac type structures by using a simple carbohydrate starting material has been barely exploited.

Chemistry

Lincomycin **8** (Scheme 1), a thioglycoside antibiotic, contains an α-D-*galacto*-octopyranoside moiety and yields methylthiolincosaminide MTL **9a** by cleavage at the amide bond on refluxing with hydrazine [9]. Compound **9a**, containing the easily 1-methylthio-activatable group and NHAc in the C-6 glycerol side chain, can be suitable for a development of Neu5Ac analogues. In fact, an appropriate activation of the carbohydrate moiety[10–15] could allow introduction of a carboxylic group, which, although not directly bound to the anomeric carbon, could optimise the interaction with the enzyme active site that needs a pseudo-equatorial conformation of the carboxylate group in the transition state.



Scheme 1: Synthesis of 6-acetamido-6,8-dideoxy-D-*erythro*-β-D-*galacto*-octopyranosyl-1-oxy-acetic and phophonic acids.

Here we report the synthesis of 6-acetamido-6, 8-dideoxy-D-*erythro*-β-D-*galacto*-pyranosyl-1-oxyacetic acid **10b** and phosphonic acid derivatives **11c** obtained from Lincomycin **8**[9] (Scheme 1). The key feature to obtain the carboxylic derivative **10b** from pentacetyl methylthiolincosaminide (MTL) **9b** is represented by an appropriate activation of the carbohydrate portion. Inasmuch as thioglycosides have been largely used to 'store' and then later activate the anomeric center for the synthesis of disaccharides[10], a great variety of activation conditions for these glycosyl donors are known[11,12]. The thiomethyl functionality in nucleosides can be readily activated with *N*-iodosuccinimide (NIS) and catalytic trifluoromethanesulphonic acid (TfOH) to give glycoside linkages in the presence of alcohols[13–15]. We followed Knapp's method[14] for the glycosylation of MTL pentacetate **9b** with the primary alcohols [methyl glycolate and dibenzyl (hydroxymethyl) phosphonate[7]], to introduce the loose carboxylic and phosphonic groups in the D-*galacto* sugar moiety (Scheme 1). By virtue of the participating acetoxy group at C-2, and regardless of the starting anomeric configuration, the glycosylation products, (i.e. the methyl ester **10a** and dibenzyl phosphonate **11c**) were formed exclusively as β-anomers[14]. The methyl ester **10a** was readily converted to carboxylic acid derivative **10b** by sequential treatment with Amberlite-IRA 400(OH^-) resin and aqueous sodium hydroxide, followed by neutralisation with Dowex 5W × 8(H^+) resin, and lyophilisation[16]. The phosphonic acid derivative **11c** was obtained by deprotection of *O*-benzyl groups performed by catalytic hydrogenation of **11a** with 10% palladium/carbon and ammonium formate as the hydrogen donor[17] and by lyophilisation after the hydrolysis of **11b** led in the same conditions above reported for methylester **10a**.

Results

Inhibitory activity of **10b** was tested on the bacterial and viral sialidases. Under the incubation conditions reported in the experimental section, a concentration of 75 mM of the acetic derivative sodium salt **10b** produced an inhibition of 60% of the bacterial activities and of 32% of the viral sialidase respectively.

Kinetic analyses of the inhibition of sialidase from *Clostridium perfringens* by phosphonate derivative **11c** was performed as reported in the experimental section. As shown in Figure 1, the inhibition of 4-MU-Neu Ac hydrolysis by **11c** was unexpectedly non-competitive with an apparent K_i value of 90 mM.

The compounds **10b** and **11c** may represent the first example in a novel series of potential sialidase inhibitors derived by simple chemical modifications of a carbohydrate natural

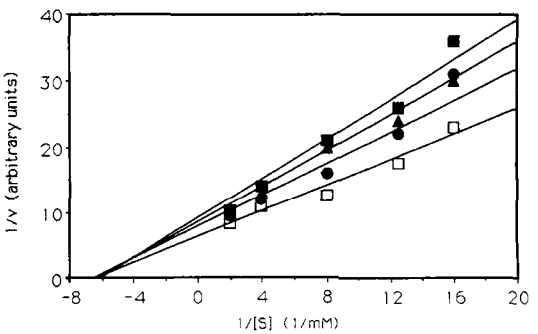


Figure 1. Lineaweaver-Burk plot of the inhibition of sialidase from *Clostridium perfringens* by phosphonic derivative. 4-MU-NeuAc was used as substrate. Phosphonate compound was added at final concentration of 0 (□), 20 (●), 40 (▲), and 60 (■) mM. Each value represents the mean of duplicate experiments.

product. On considering structure-activity relationships, it is obvious that the selectivity for NA is likely to be enhanced by a rational design to improve the interactions with the active site of the enzyme[18–20].

In fact the derivatives **10b** and **11c** are interesting candidates for further development and may be considered as neuraminic acid 'analogues' containing a carboxylic and a phosphonic group joined to the anomeric carbon by a glycoside linkage and the NHAc group in the C-6 glycerol side chain.

Acknowledgment

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Experimental

Materials and Methods

Chemical Synthesis

Melting points were determined using a Kofler hot-stage apparatus and are uncorrected. Methylthiolincosaminide was obtained by hydrazinolysis of Lincomycin base according to Schroeder et al.[9]. Methylglycolate was purchased from Fluka. Dibenzyl (hydroxymethyl) phosphonate was prepared according to Czollner et al.[7]. MTL was acetylated with pyridine and acetic anhydride at room temperature. Analytical TLC was performed on precoated plates of Merck silica gel (60 F-254). Silica gel 60 (200–400 mesh) was used for column chromatography. ^{1}H-NMR spectra were recorded at 300 MHz (Varian XL-300) using $CDCl_3$ as solvent and TMS as internal standard. Elemental analyses were within ± 0.04% of theoretical values and were determined in the Laboratory of Central Analysis Service of C. N. R. (Montelibretti, Italy).

Biology

2′-(4-Methylumbelliferyl)-αDN-acetylneuraminic acid (4-MUNeuAc) and 2-(3′-methoxyphenyl)N-acetyl α neuraminic acid (MPN) were from Sigma Chemical Co. Sialidase from *Clostridium perfringens* (1U/mg) was purchased from Fluka; sialidases from *Vibrio Cholerae* (20U/mg) and from *Newcastle disease virus* (5U/mg) (NDV) were from Boehringer Mannheim. All other chemical were analytical grade.

*General Procedure for Compounds (**10a** and **11a**)*

Trifluoromethanesulphonic acid (1.2 equiv) is added over several min to a stirred solution of the acetylated thioglycoside **9b** 0.46 g (1 mmol), *N*-iodosuccinimide (2.5 equiv) and methylglycolate (1.5 equiv) or dibenzyl (hydroxymethyl) phosphonate[7] (1.5 equiv) in dry 1,2-dichloroethane at room temperature, under nitrogen atmosphere. When TLC analysis indicates the disappearance of the thioglycoside (120 min), the reaction is treated with 10% aqueous sodium thiosulphate until the purple colour is discharged.

The organic layer is washed with water, dried over magnesium sulphate, concentrated and chromatographed on silica gel by using 7/3 ethylacetate/toluene as eluant to afford the carboxylate derivative **10a** (0.25 g, 49%); mp 91–92 °C (ethyl acetate-*n*-hexane).– ^{1}H NMR: δ = 1.25 (d, *J* = 6.4 Hz, 3H, CH_3), 1.94, 1.99, 2.02, 2.05, 2.18 (s, 15H, 4 $COCH_3$, CH_3CONH), 3.63 (d, *J* = 7.2, 1H, N*H*$COCH_3$), 3.80 (s, 3H, CO_2CH_3), 4.4 (s,2H, O$CH_2CO_2CH_3$), 4.5–5.5 (m, 4H, CH envelope), 6.25 (d, *J* = 6.0 Hz, 1H, anomeric H).–$C_{21}H_{31}NO_{13}$, Anal. C, H, N.

In the same experimental conditions with dibenzyl(hydroxymethyl) phosphonate from acetylated glycoside **9b** (1 mmol) was obtained the phosphonate derivative **11a** (0.35 g, 50%); mp 171–173 °C (ethyl acetate).– ^{1}H NMR: δ = 1.29 (d, *J* = 6.6 Hz, 3H, CH_3), 1.95, 1.99, 2.02, 2.07, 2.19 (s, 15H, 4 $COCH_3$, CH_3CONH), 3.65 (d, *J* = 7.5, 1H, N*H*$COCH_3$), 3.95–4.25 (m, 2H, CH), 4.55–4.70 (m, 2H, OCH_2PO(OBn)$_2$), 5.0–5.10 (m,1H, CH), 5.15–5.20 (m,4H, 2CH_2Ph), 5.25–5.35 (m, 1H, CH), 6.25 (d, *J* = 7.8 Hz, 1H, anomeric H), 7.35–7.50 (m, 10 H, aromatic H).– $C_{33}H_{42}NO_{14}P$, Anal. C, H, N.

*Cleavage of O-Benzyl Derivative (**11a**)*

A mixture of the *O*-benzyl derivative **11a** 0.35 g (0.5 mmol), ammonium formate[17] (0.15 g), 10% palladium on carbon (Fluka) 1 g for two benzyl groups, and methanol (15 mL) was refluxed until TLC indicated complete cleavage of the benzyl ether (30 min). The catalyst was filtered off and washed with the solvent. The filtrate was evaporated to give a crystalline product **11b** (0.22 g, 84%), in its ^{1}H NMR the absence of benzylic and aromatic protons was evident.

*Hydrolysis of Compounds (**10a** and **11b**)*

To a solution of the methyl ester **10a** (0.50 g) or phosphono derivative **11b** 0.53 g (1 mmol) in anhydrous MeOH (31 mL) was added dried Amberlite IRA-400 (OH^-) resin (2g)[16]. The mixture was stirred for 3h at room temperature and then filtered.The resin was washed with MeOH (2 × 15 mL), and the filtrate and washings were concentrated to dryness to afford a few mgrams of a solid residue.The solid resin was then taken up in 0.15 M aq NaOH (11mL), stirred at room temperature for 2h, and finally the pH of the mixture was adjusted to 7.0/7.5 with Dowex-50 W × 8 (H^+) resin.

After filtration,the filtrate was lyophilised to afford the sodium salt of the acid acetic derivative **10b** (0.23; 66%).$C_{12}H_{20}NO_9Na.H_2O$, Anal. C, H, N. The phosphonic acid derivative **11c** (0.27 g, 63%) was obtained in the same experimental conditions. $C_{11}H_{20}NO_{10}PNa_2.2H_2O$, Anal. C, H, N.

Biological Assays

The enzymatic assays were carried according to Palese et al.[21]. The assay mixtures contained, in a final volume of 0.2 mL, 25 mU of sialidase from bacterial sources (*Clostridium perfringens* and *Vibrio Cholerae*), or 20 mU of viral enzyme from *NDV*, 0.1M sodium phosphate buffer (pH 5.5), 1.5 mM MPN. After 30 min of incubation at 37 °C, the reaction was stopped by the addition of 1.5mL of 10% Na_2CO_3 and 0.2 mL of 2N Folin Ciocalteau reagent. After 20 min the colour development of the solution was read at 750 nm. Control reactions were carried out at the same substrate concentration without the enzyme.

Kinetic analyses were conducted with sialidase from *Clostridium perfringens* using the fluorometric method according to Myers et al.[22]. The incubation mixtures contained, in a total volume of 0.2mL, 0.5 mU of sialidase, 0.1M sodium acetate buffer pH 5.0, between 0.5 and 0.06 mM 4-MU-NeuAc and between 20 and 60 mM phosphonate derivative **11c** as inhibitor. In the blanks the enzyme was omitted. After starting the reaction by addition of the substrate, the mixtures were incubated at 37 °C for 10 min. The reaction was stopped by addition of 1.8 mL of a buffer containing 0.133 M glycin, 0.042 M sodium carbonate and 0.06 M sodium chloride (pH 10.0). Released 4-methylumbelliferone was determined fluorometrically at λ = 360 nm for excitation and at λ = 460nm for emission.

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