INHIBITION OF BIOSYNTHESIS AND BIOCHEMICAL MODULATION OF *N*-ACYLNEURAMINIC ACID (BIOCHEMICAL ENGINEERING OF SIALOCONJUGATES). A REVIEW

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Dedicated to Professor Miloslav Černý on the occasion of his 75th birthday.

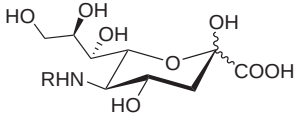
The key enzyme of sialic acid biosynthesis is the bifunctional UDP-GlcNAc 2-epimerase/ManNAc kinase. Novel inhibitors of this enzyme have been synthesized. The *N*-acyl side chain of sialic acid can be biochemically engineered by incubating cells with non-natural *N*-acylmannosamine analogues such as *N*-propionylmannosamine and related compounds. These modified sialic acids lead to various biological changes, such as stimulation of T-lymphocyte proliferation, inhibition of the uptake of influenza A virus, stimulation of neuritic growth, increased expression of sialyl-Lewis^x and altered adhesion. A review with 41 references.

Keywords: UDP-*N*-acetylglucosamine 2-epimerase/*N*-acylmannosamine kinase inhibitors; *N*-Acetylneuraminic acid; *N*-Propionylneuraminic acid; *N*-Propionylmannosamine; Influenza viruses; T-lymphocytes; Neurite growth; Sialyl-Lewis^x; Recombinant glycoproteins; Glycans; Sialic acid; Sialylation.

1. BIOLOGICAL IMPORTANCE OF SIALYLATION

Glycans of glycoproteins are synthesized in the Golgi apparatus by specific glycosyltransferases, which attach nucleotide-activated monosaccharides to specific saccharide residues of glycoproteins. Although these glycans have a common core structure, a combination of different monosaccharides and different linkage types allows the cell to create an enormous number of different structures. Note that the terminal monosaccharide of most glycoproteins is a sialic acid (Fig. 1). The main structural features of this acetamidononulosonic acid are the carboxylic group at C1 (from pyruvate), the hydroxy groups at the glycerol-type tail (C7 to C9) and its *N*-acyl side chain at C5. The carboxylic group is responsible for its low p*K* value 2.8. The hydroxy groups at the glycerol tail are recognized by influenza C viruses. A very prominent constituent of sialic acid is the *N*-acyl side chain at C5. In addition to *O*-acetyl derivatives at C7–C9, the animal kingdom also possesses two other major constituents: *N*-acetylneuraminic acid in humans and *N*-glycolylneuraminic acid in apes. Thus the *N*-side chain appears to distinguish humans from apes[1].

Glycosylation is the most common posttranslational modification of proteins. One of the most prominent monosaccharides is *N*-acetylneuraminic acid (sialic acid). The sialic acids represent a family of amino sugars, which are essential components of complex *N*- and *O*-glycans of glycoproteins and glycolipids (gangliosides). Sialylation of these glycoconjugates plays an important role in development, regeneration and pathogenesis of several diseases[2,3].



R =	acetyl (Ac)	-CO-CH_3
	propionyl (Prop)	-CO-CH_2CH_3
	butanoyl (But)	-CO-$CH_2CH_2CH_3$
	pentanoyl (Pent)	-CO-$CH_2CH_2CH_2CH_3$
	hexanoyl (Hex)	-CO-$CH_2CH_2CH_2CH_2CH_3$
	crotonoyl (Crot)	-CO-CH=CH_3
	levanoyl (Lev)	-CO-CH_2CH_2-CO-CH_3
	glycolyl (Gc)	-CO-CH_2OH
	azidoacetyl (Ac-azido)	-CO-CH_2N_3

FIG. 1
Structure of sialic acids

Their biological functions are closely related to their terminal position in glycoproteins or gangliosides, either as part of a membrane constituent or of a serum glycoprotein. Sialic acids are intimately connected with different receptor functions of glycoconjugates, acting as receptors for certain hormones and growth factors. They are also involved in the interaction of homotypic or heterotypic cells in cell adhesion, as well as in organ development, where interaction with components of the biomatrix is essential. Cell surface saccharides, in particular *N*-acetylneuraminic acid, are intimately associated with the onset of inflammatory diseases. Sialylated receptors recognize defined viruses, bacteria or protozoa. The recognition of influenza viruses, e.g. influenza A virus requires the presence of sialic acid in the respective receptor. Chronic inflammatory diseases are maintained by the interaction of sialylated receptors (selectins) and ligands at the surface of endothelial cell and their counterparts, blood leukocytes. The migration of leukocytes is guided via their surface glycans when tissues are damaged by mechanical or thermal processes, e.g. during the reperfusion syndrome after myocardial infarction or during the development of atherosclerosis, in autoaggressive diseases or organ loss after transplantation.

Cancer cells are known to show an increased degree of sialylation of surface constituents, which protect them for immune surveillance. Moreover, the endowment of cell surface of tumor cells with different monosaccharides is closely related to tumor initiation and tumor progression.

2. BIOSYNTHESIS OF SIALIC ACID

The biosynthesis of *N*-acetylneuraminic acid is a multistep process starting with the amidation of the glycolysis intermediate, fructose 6-phosphate, to form glucosamine 6-phosphate. The enzymes of this pathway have been described[4,5]. The key step in the subsequent pathway is the conversion of UDP-*N*-acetylglucosamine (UDP is uridine diphosphate), the specific intracellular substrate, into *N*-acetylneuraminic acid. The key enzyme is the bifunctional UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase[6–8]. This enzyme epimerizes the UDP-bound *N*-acetylglucosamine by loss of UDP to release *N*-acetylmannosamine, which is immediately phosphorylated to *N*-acetylmannosamine 6-phosphate. *N*-Acetylneuraminic acid 9-phosphate is formed by aldol condensation of phosphoenol pyruvate and *N*-acetylmannosamine 6-phosphate (Fig. 2).

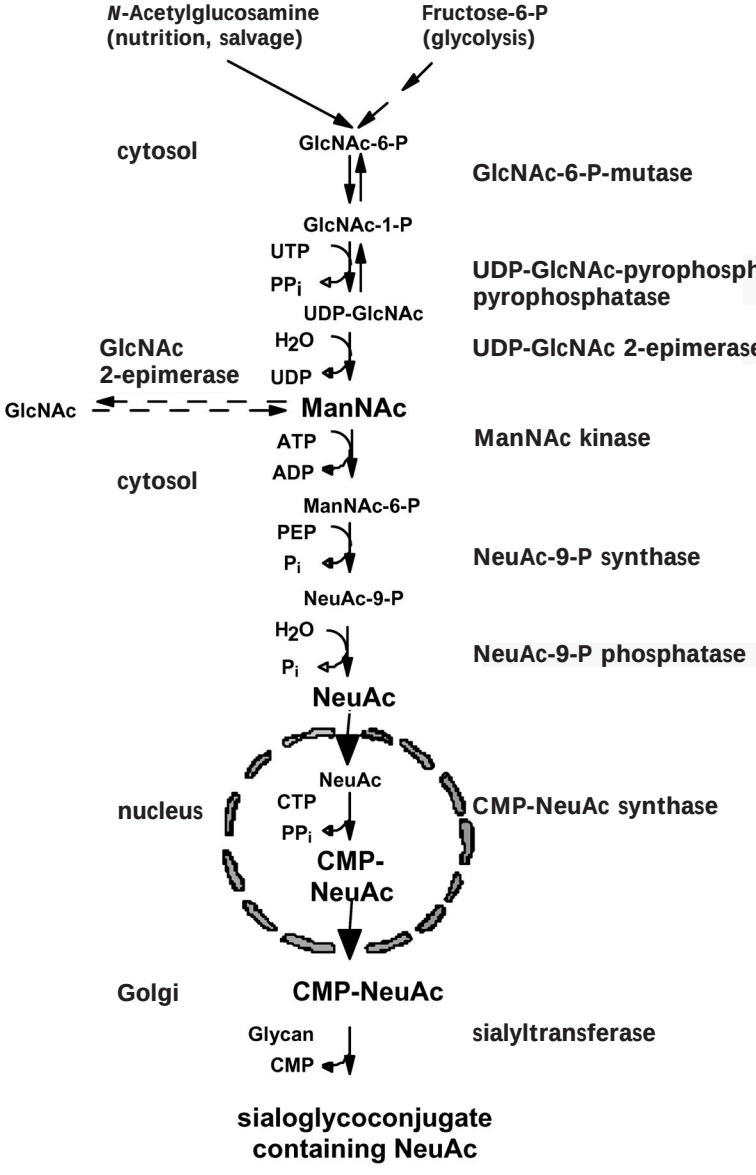


FIG. 2
Biosynthesis of sialic acid

2.1. Inhibitors of Sialic Acid Biosynthesis

The diversity of individual substructures correlates with the function of *N*-acetylneuraminic acid in glycoconjugates. In view of its great biological importance, especially with respect to its high concentration in the plasma membrane of cancer cells, there is a pressing need for inhibitors of the biosynthesis of sialic acid. Our first attempts to inhibit UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase were made in cooperation with M. Černý[9]. It was shown that *N*-propionylglucosamine 6-phosphate and, surprisingly, to a lesser extent *N*-propionylmannosamine 6-phosphate, inhibits the epimerase activity. In 1992, Zeitler et al. demonstrated that 3-*O*-methyl-*N*-acetylglucosamine inhibits *N*-acetylmannosamine kinase[10]. Furthermore, an irreversible inhibitor of the 2-epimerase activity was synthesized by periodate oxidation of the ribose ring of UDP-*N*-acetylglucosamine[11]. New reversible inhibitors of 2-epimerase have been synthesized by Stolz et al.[12] They comprise two groups, one including analogues of the substrate, UDP-*N*-acetylglucosamine, while the second group contains analogues of acetamidoglucal, the reaction intermediate.

3. BIOCHEMICAL ENGINEERING OF THE *N*-ACYL SIDE CHAIN OF SIALIC ACID AND ITS BIOLOGICAL IMPLICATIONS

In recent years the human genome project has produced an enormous quantity of data. This flood of information has been further increased by the development and extended use of DNA-chip technologies. There has been parallel progress in the analysis of the proteome, in particular through improvements in analytical methods.

The main goal of biochemical engineering in the life sciences is to use the enzymatic machinery of cells to achieve chemical synthesis of novel biomolecules. Such engineered biomolecules may have novel properties and are potentially useful in both science and medicine. Biochemical engineering faces the challenge to obviate the limited potential of the gene by supplying non-DNA-coded products for basic research and industrial production. The present account focuses on the production of novel glycoproteins by biochemical engineering, leading to new (and unexpected) biological characteristics. Some applications of this methodology in biology with potentials in medicine are outlined.

Application of the above mentioned synthetic sialic acid precursors to different biological systems has revealed important and unexpected functions of the *N*-acyl side chain of sialic acids. Furthermore, the introduction

of chemically reactive ketone and azido groups into cell surface glycoconjugates, using the respective *N*-acyl-modified sialic acid precursors[13], offers a variety of applications including the generation of artificial cellular receptors for viral gene delivery. Novel sialic acid precursors have enabled studies of sialic acid modifications on the surface of living cells and have improved our understanding of saccharide receptors in their native environment. Biochemical engineering therefore provides new tools for studying the biological relevance of sialic acid and for exploiting it as a tag for therapeutic and diagnostic applications.

Biochemical engineering of the *N*-acyl side chain of sialic acid has opened a new field of sialic acid research with a potential for biological applications. As described below, one primary outcome of this new methodology is the demonstration that the small *N*-acyl side chain has considerable biological importance and a high potential for exploitation. It should be noted that the first direct precursor of sialic acid is *N*-acetylmannosamine. The point of the biochemical engineering is to use, instead of normal *N*-acetylmannosamine, the non-physiological *N*-propionylmannosamine (ManNProp), whose side chain is extended by a methylene group. This non-physiological mannosamine analogue is converted by the same enzymes as described for *N*-acetylmannosamine to the corresponding *N*-acetylneuraminic acid. The first attempt at such a conversion was reported in 1981 by Grünholz et al.[9] Using a cell-free system of rat liver, it could be shown by paper chromatographic analysis that *N*-propionylmannosamine is converted to *N*-propionylneuraminic acid. Ten years later this new *N*-acylneuraminic acid was clearly identified by gas chromatography and mass spectrometry[14]. This represented the birth of the technique, the biochemical engineering of the side chain of *N*-acylneuraminic acid, as a new tool for biochemical modification of surface and soluble sialoglycoproteins, resembling a kind of biochemical microsurgery. Instead of *N*-propionylmannosamine, it was found that other extended side chains like *N*-butanoyl, *N*-pentanoyl, *N*-hexanoyl or *N*-but-2-enoyl (*N*-crotonoyl) could be applied, leading to dramatic biological effects. A milestone in this kind of biochemical engineering was reached by C. Bertozzi and her group in Berkeley, when they introduced a 4-oxopentanoyl (*N*-levulinoyl) group, bearing a reactive ketone group[13]. *N*-Levulinoylmannosamine is also converted into the respective *N*-levulinoylneuraminic acid and was inserted into the plasma membrane glycoproteins. Ketone groups are chemically orthogonal to all other native functional groups in the cell. Accordingly, ketones can serve as unique chemical targets that react selectively with aminooxy- or hydrazide-functionalized molecules, thereby permitting the

covalent attachment of a wide range of structures to the cell surface under physiological conditions, offering the possibility to bind drugs or magnet resonance imaging (MRI) contrast reagents[15].

Several different *N*-acyl side chains have been tested and demonstrated not to hinder the biosynthetic enzymes. In contrast, there is good evidence that the degrading enzymes, the sialidases, are sensitive to any changes of the *N*-acyl side chain. First evidence of this was obtained in 1969 by Faillard et al.[16], when they showed that the α-benzyl glycoside of *N*-(benzyloxycarbonyl)neuraminic acid is not split by sialidase. Brossmer and Nebelin found that the benzyl glucoside of *N*-succinylneuraminic acid is also not split by sialidase[17].

3.1. Engineering of Cell Surface Sialic Acid Interferes with Virus Infections

Recognition of viruses and binding to an appropriate receptor on the surface of the host cell by a virus is the first step of viral infection. Despite the ubiquity of sialic acid on the cell surface, sialylated oligosaccharides are essential receptor components for many animal viruses from different virus families, such as influenza A and C viruses, SARS viruses, Newcastle disease virus, cardioviruses, as well as murine and primate polyomaviruses[18]. If 18–64% of the sialic acids on host cells were biochemically engineered by treatment with the respective analogues (usually ManNProp and especially ManNPent), binding and/or infection by different primate polyomaviruses, which depend on the cell surface of sialic acids for entry, were usually markedly reduced[19,20]. For human polyomavirus BK, however, elongation of the *N*-acyl side chain by one methylene group (from *N*-acetyl to *N*-propionyl) resulted surprisingly in an up to seven-fold enhancement of infection. Further extension to *N*-pentanoyl, however, drastically reduced infection to less than 5%. Binding and infection by the African green monkey B-lymphotropic papovavirus were decreased about ten-fold by incorporation of the *N*-propionyl side chain. In contrast, the sialidase-independent infection of closely related simian virus 40 was, as expected, unaffected[19].

3.2. Stimulation of Glia Cells

Modified sialic acids have striking biological consequences for glia cells of the mammalian central nervous system. Application of ManNProp to primary glial cells in culture leads to the expression of *N*-propionylneuraminic acid in glycoproteins on the cell membrane[21]. This biochemical engineer-

ing of the *N*-acyl side chain of sialic acid stimulated the proliferation of astrocytes and microglia *in vitro*. Furthermore, oligodendrocytes showed increased signs of a non-mature cell stage if ManNProp was applied. Mature oligodendrocytes are the myelin-forming cells in the central nervous system. They develop from oligodendrocyte progenitor cells, which can be immunologically identified by their expression of specific ganglioside-epitopes, such as the A2B5-epitope. This A2B5-epitope is regarded as a specific marker for a subset of rat oligodendrocyte progenitor cells. Application of ManNProp leads to a dramatic increase of A2B5-expression *in vitro*. Since the A2B5-epitope is considered to be a functional marker of cells of the early oligodendrocyte lineage, ManNProp has to be considered as a potent regulator of the lineage progression of oligodendrocytes at early stages of their development. Only oligodendrocyte progenitor cells and not mature oligodendrocytes are proliferative and migratory. These properties play an important role not only during the development but also in the regeneration of the adult nervous system, since they develop constitutively into myelin-forming cells *in vitro* and *in vivo*. Oligodendrocytes are functionally impaired in a number of severe neurological diseases. In the important disease, multiple sclerosis, oligodendrocytes are lost, followed by demyelination. These results underline the important role of biochemically engineered sialic acid in neural development and regeneration. The molecular mechanism underlying the stimulation of oligodendrocytes might consist of signal transduction events. The incorporation of *N*-propionylneuraminic acid, followed by the application of GABA, leads to calcium oscillations in oligodendrocytes. It has been proposed that biochemical engineering of the *N*-acyl side chain of sialic acid in conjunction with the activation of GABA receptors, which are sialylated glycoproteins, modulates, e.g. increases, the intracellular calcium concentration in oligodendrocytes[22]. The prolonged increase of intracellular calcium concentration after calcium oscillations could be responsible for the occurrence of A2B5-positive oligodendrocyte precursor cells.

Further data underlining the potency of biochemically engineered sialic acid in myelination have been reported by Schnaar and co-workers. They used *N*-glycolylmannosamine as a synthetic precursor and demonstrated the conversion of neuronal sialic acids from *N*-acetyl- to *N*-glycolylneuraminic acid. The result was an inhibition of the binding of myelin-associated glycoprotein (MAG) to neuronal cells[23]. MAG is expressed on myelin in the central nervous system and is known to be a potent inhibitor of neurite regrowth after nerve damage. This inhibitory capacity is mediated by the interaction of oligodendrocyte-expressed MAG with ganglio-

sides of neurons, since MAG is a sialic acid-binding molecule of the siglec family. This means of interfering with MAG binding to nerve cells might enhance the possibility of post-traumatic nerve regeneration.

In this context it should be noted that also in human lung fibroblast cultures, the contactinhibin-regulated cell growth is influenced by chemically modified *N*-acylmannosamines. Treatment of these cells with ManNProp for 7 days results in the disappearance of the density-dependent inhibition of growth[24].

These examples demonstrate the potency of synthetic D-mannosamines as possible therapeutic agents in nerve regeneration.

3.3. Stimulation of Neurons

Rat PC12-cells have been widely used as a standard system for the study of neurite outgrowth. These cells respond to nerve growth factor (NGF) by extending neurites via a ras-dependent pathway. We quantified neurite outgrowth of PC12-cells grown in the absence or presence of ManNProp on different substrates.

PC12 cells were cultured on poly-D-lysine, collagen I or laminin. The best neurite outgrowth was observed on laminin. In the presence of ManNProp, PC12-cells extended up to over 60% longer neurites on laminin compared to control cultures in the absence of ManNProp. Similar results were obtained with primary neurons. This stimulation of neurite outgrowth was concentration-dependent[25]. We also showed that re-establishment of the perforant pathway was stimulated in brain slices when the slices were cultured in the presence of ManNProp [25]. In addition, several cytosolic proteins with regulatory functions were identified, which are differently expressed after treatment with ManNProp. Since sialic acid is the only monosaccharide activated in the nucleus, we hypothesise that transcription is modulated by the unnatural cytidine-monophosphate-*N*-propionylneuraminic acid and that sialic acid activation might serve as a general tool for regulating cellular functions, such as neurite outgrowth[25]. This indicates the fundamental role of sialic acids in general and in particular the role of the *N*-acyl side chain of the biochemically engineered sialic acid in neurite outgrowth.

3.4. Activation of Human T-lymphocytes by N-Propionylmannosamine

Sialic acids are implicated in the differentiation and maturation of lymphocytes[26]. Preliminary studies revealed that application of ManNProp leads to

incorporation of *N*-propionylneuraminic acid into human T-cells. These biochemically engineered T-cells show several hallmarks of activation, including proliferation, secretion of interleukin-2 and expression of the IL-2-receptor α-chain. This stimulation is dose-dependent and in the same range as that observed with the commonly used toxic plant lectins, concanavalin A or wheat germ agglutinin. ManNProp, however, did not induce cytotoxicity, even at high concentrations. The ManNProp-induced stimulation is accompanied by an increase in the peptidase activity of CD26 [27], a costimulator of lymphocytes, and this increase is paralleled by the incorporation of *N*-propionylneuraminic acid into CD26 [28].

3.5. Immunotargeting of Tumor Cells Expressing Unnatural Polysialic Acids

Immunotargeting of tumor cells by creating vaccines based on cancer-specific cell surface glycoconjugate antigens has long been proposed, but many tumors fail to express unique markers. Polysialylation, e.g. α(2-8)-linked polysialic acid sequences located on the outer chains of an *N*-linked oligosaccharide, is a unique and functionally important property of the neural cell adhesion molecule[29,30]. Its expression is regulated in embryogenesis, with maximal expression in the perinatal phase and restriction in the adult nervous system to plastic regions[31]. However, overexpression of polysialic acid is found in a number of cancers, including small cell lung carcinomas and Wilms tumors[32,33], and is thought to promote tumor cell metastasis[34,35]. Using ManNProp, Jennings and co-workers successfully generated polysialic acid containing *N*-propionylneuraminic acid[36]. Since a monoclonal antibody to polyNeuProp is available (13D9)[37], this biochemically engineered polysialic acid expressed on leukemic tumors can be used for antibody-mediated cell killing[36]. Furthermore, in an *in vivo* study this antibody effectively controlled metastasis of a solid tumor model in mice, which had been given ManNProp.

Bertozzi and co-workers have demonstrated that even ManNLev is incorporated into polysialic acids[38]. Using ManNLev, it is possible to introduce reactive ketone groups onto cell surfaces; this also offers novel opportunities for specifically attacking polysialylated tumor cells. The same group reported recently that ManNBut is a potent inhibitor of polysialylation[39]. Application of ManNBut resulted in biosynthesis of the respective *N*-acyl-modified sialic acid (*N*-butanoylneuraminic acid), but this biochemically engineered sialic acid seems to induce termination of polysialylation. If ManNBut or ManNPent are specific terminators of polysialylation, these sugars would also be potent drugs for fighting polysialylated tumors.

Future work will have to address the applicabililty of these strategies to the targeting of polysialic acid-expressing human cancers and assess the potential adverse effects of biochemical engineering.

3.6. Increased Expression of Sialyl-Lewis^x Structures

HL60-I cells do not express UDP-GlcAc 2-epimerase/ManNAc kinase, the key enzyme of the biosynthesis of sialic acids. Therefore they do not express sialyl-Lewis^x structures and, consequently, do not bind to selectins. Application of *N*-acetyl-D-mannosamine leads to the expression of sialyl-Lewis^x structures and to binding to selectins. Surprisingly, incorporation of *N*-propionylneuraminic acid into glycoconjugates of these cells leads to a dramatic increase of sialyl-Lewis^x structures and to increased adhesion to selectins[40].

3.7. Stability of Glycoproteins is Increased After Incorporation of N-Acyl-Modified Sialic Acids

The biological half-life time of many glycoproteins is regulated via terminal sialic acids. We found that the half-life of the highly sialylated CEACAM1, a member of the immunoglobulin superfamily, is increased by more than 50% by replacement of the *N*-acetylneuraminic acid by the novel, engineered *N*-propionylneuraminic acid. This demonstrates that biochemical engineering not only modulates the structure of cell-surface sialic acids, but also influences the biological stability of a defined glycoprotein[41]. The application of this finding to the expression of recombinant glycoproteins, e.g. erythropoietin, could lead to a decrease in the amount of the respective drug, needed for treatment of the patient.

Some of the observed effects of ManNProp on cells might be explained by the prolonged expression of specific cell surface receptors.

4. CONCLUSION

1. Several inhibitors of sialic acid biosynthesis have been obtained: *N*-propionylglucosamine, metabolically converted to its 6-phosphate and 3-*O*-methyl-*N*-acetylglucosamine, both reversible inhibitors of *N*-acetylmannosamine kinase, periodate oxidized UDP-*N*-acetylglucosamine as irreversible inhibitor of UDP-*N*-acetylglucosamine-2-epimerase and a set of UDP-GlcNAc analogues as reversible inhibitors of this 2-epimerase.

2. Biochemical engineering of the *N*-acyl side chain of *N*-acylneuraminic acid (belonging to the family of about 50 sialic acids) is a new method to reveal the biological importance not only of sialic acids themselves, but also of their *N*-acyl side chains. This new chemical/biochemical procedure could simply be achieved by chemically modifying the *N*-acyl side chain of the natural metabolic precursor *N*-acetyl-D-mannosamine. The replacement of the *N*-acetyl group by *N*-propionyl, *N*-butanoyl, *N*-pentanoyl or *N*-hexanoyl groups is accepted by the biosynthetic enzymes, thus leading to the synthesis of respective *N*-acylsialic acids in cell cultures or *in vivo*. On the basis of these results, *N*-levulinoyl group was introduced by C. Bertozzi yielding *N*-levulinoylneuraminic acid with the advantage of the chemically reactive carboxyl group. The biological implication of the biochemical introduction of these unnatural *N*-acylneuraminic acids in membrane glycoproteins are widespread, e.g., the inhibition of the uptake of polyoma and influenza A viruses, stimulation of neurite outgrowth, activation of T-lymphocytes, or increase of the expression of sialyl-Lewis^x. The prolongation of the half-life of CEACAM would be of special importance, since it is due to the decreased activity of sialidases; in contrast to the biosynthetic enzymes, the sialidases are sensitive to variations of the *N*-acyl side chain. This new procedure offers a new possibility to study the biochemical and biological properties of sialic acids and especially of its *N*-acyl side chain.

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