EVALUATION OF THE INHIBITORY ACTIVITY
OF VARIOUS UNREPORTED CMP-Neu5Ac
DONOR CONGENERS ON GM3 SYNTHASE

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Summary

A class of sialosides (down reported 3a-e, 4a-c, 5a-c and 5e), designed to inhibit the biological transfer of sialic acid 1 to the terminal portion of glycoconjugates, mediated by the enzymes sialyltransferases, was synthesized and their inhibitory activities was tested on a α-2,3 sialyltransferase, from Pasteurella multocida, and on GM3 synthase, present both as a cellular homogenate and in a cell line of HEK293 (Human Embryonic Kidney).

The synthesized compounds 3a-e are congeners of the natural sialyl donor CMP-Neu5Ac 2 and incorporate some structural properties of the compound 3a, the only known inhibitor of GM3 synthase enzyme reported in a short note. Moreover, we synthesized also the lactones 4a-c and the peracetylated derivatives 5a-c and 5e in order to improve the cell permeability of their parent hydroxyl acids.

The GM3 synthase activity was evaluated detecting the formation of the labeled GM3 in process using lactosyl-ceramide labelled ([3-3H(sphingosine)]LacCer) as acceptor, or labeled sphingosine ([3-3H] SPH) as unsialylated ganglioside precursor. Last procedure allowed to evaluate also the
complete pattern of gangliosides formed in the cell in the presence or absence of inhibitors. All synthesized compounds were tested on GM3 synthase of HEK cellular homogenate. The more active acidic compounds were also tested on intact cell lines, improving their lipoflicity, by methyl esterification and after peracetylation. The free compounds 3b-c, 3e and 4c show an inhibitory activity always higher than 3a. Similarly the peracetylated methylester 5b-c and 5-e are more activity than 5a in intact cell lines.

All compounds show an inhibitory activity higher on the cell homogenates than that on HEK cells. The lactones 4a-c were not active in cell lines probably since they do not cross the cell membrane as the peracetylated methyl ester 5a-e that are actives.

The α-sialoside 5e resulted the most active. This is a very interesting result since evidences that the geometry at the anomeric centre of the glycosyl donor is not an exential requisite.

However, all results together extend the knowledge on inhibitors of GM3 synthase and provide new structural information for the development of other novel STs inhibitors.
1. INTRODUCTION
1. INTRODUCTION

Sialyltransferases (STs) are an enzyme family able to transfer the N-acetyleneuraminic acid (Neu5Ac; SA) 1 or its companions, activated as cytidine 5'-monophosphate-N-acetyleneuraminic acid (CMP-Neu5Ac) 2, (Figure 1) to the terminal portion of a glycoconjugate, where a glycoconjugate (glycolipid, glycoprotein, and lipopolysaccharide) is a compound in which one or more monosaccharide or oligosaccharide units are covalently linked to a noncarbohydrate moiety (the aglycone).4-6

\[ \text{R} = \text{NHAc N-acetyleneuraminic acid or Neu5Ac; SA} \]

Companions of SA
\[ \text{R} = \text{NHCOCH}_3 \text{OH N-glycolyneuraminic acid or Neu5Gc} \]
\[ \text{R} = \text{OH 3-deoxy-nonulosonic acid or KDN} \]

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**Figure 1:** A) Neu5Ac 1a (SA) with main companions and activated SA (CMP-Neu5Ac) and B) biological sialylation.

The sialic acid molecule, leaving the donor and bonding the acceptor, undergoes a reversal of its beta configuration at the anomeric carbon, thus forming an alpha bond.

The process is of relevance since sialylated glycolproteins, glycolipids are involved in various biologically important processes as cellular interactions during cell development, cell differentiation, inflammation processes and binding of virus or bacteria.4, 5, 7, 8

*More often the name SA is used to signify one of the more studied members of sialic acid family (N-acetyleneuraminic acid (Neu5Ac), N-glycolyneuraminic acid (Neu5Gc) and 3-deoxy-nonulosonic-acid (KDN).*
In fact, due to its strategic terminal position, Neu5Ac plays an important role in recognition and signaling phenomena occurring in a large number of cellular events (cell-cell interaction, immune response, cellular differentiation, apoptosis and migration). CMP-sialic acid derives from the sialic acid, present in the cell nucleus, that is activated as CMP-SA by catalysis of a synthetase. Then, the formed CMP-SA migrates to the Golgi apparatus, where allows the sialyl transfer, assisted by a ST. In this way the formation of a sialylate-glycoconjugate or, specifically, of the GM3 occurs. From there, they are transferred outside of the cell, on the cell membrane.

Of particular interest it appears a specific ST called GM3 synthase (CMP-N-acetylneuraminic acid: lactosyl-ceramide (LacCer) $\alpha-2,3$ ST) responsible for the synthesis of GM3, the simplest member of a family of sialylatedglycosfingolipids, called gangliosides, particularly abundant on neuronal cell surface. GM3 is the precursor for most of the more complex ganglioside species and is synthesized by transfer of a Neu5Ac molecule from CMP-Neu5Ac to a terminal galactose residue of a LacCer through the $\alpha$-2,3 glycosidic bond formation. The involvement of sialylglycoconjugate, such as GM3, in some important physiological and pathological events, as well as the relationship between the alteration of ST activity and pathological states (tumor, metastatic and inflammatory processes), suggests that this enzyme may be of value as a therapeutic target.

However, despite the interest for inhibitors of STs no efficient inhibitors of GM3 synthase has, until now, been reported, apart from the de-phosphonate derivative 3a (Figure 2) described, in a short paper, as specific for the GM3 synthase.

![Figure 2: Inhibitor of GM3 synthase reported in literature.](image)

In order to find insights on the biological sialylation process using compounds potentially able to regulate these pathways, in my PhD work, I decided to perform a study of possible inhibitors of GM3 synthase. In particular, I focused my interest on inhibitors having, as 3a, a structure that mimes that of the CMP-Neu5Ac 2.

Thus, I programmed and performed the synthesis of eight acidic sialosides, analogues of CMP-Neu5Ac, and of their 1,7 lactone congeners. Moreover, I tested the newly synthesized compounds
on a bacterial α-2,3 ST, using an universal kit, and on GM3 synthase evaluating the formation of labeled GM3 obtained from labeled sphingosine ([3-3H(sphingosine)] LacCer). In this final comprehensive report on my PhD thesis work, I summarized and discussed the main obtained results.
1.1 Sialyltransferases (STs)

STs are a subset of glycosyltransferase enzymes (GTs), a family that catalyzes the biological transfer of monosaccharides from an activated donor to a specific acceptor molecule forming a glycosidic linkage. To perform their catalytic activity STs use CMP-Neu5Ac as a sialyl donor and in their action these enzymes mediate the transfer of a molecule of Neu5Ac to the terminal non-reducing end of a growing carbohydrate chain linked glycoconjugate (glycoproteins, glycolipids and lipopolysaccharides) (Figure 3). Usually, they bond the Neu5Ac molecule by a $\alpha$-glycosidic linkage to terminal residue of glycoconjugates.\(^4,6,7,12\)

![Chemical structure](image)

**Figure 3:** Biological sialylation.

The terminal sugar, accepting Neu5Ac, can be a molecule of galactose, that forms the bond at C-3 or C-6 position, a residue of N-acetyl galactosamine that forms $\alpha$-2,6 linkages or another residue of Neu5Ac bonded to the C-8 position.

STs are commonly (CAZy database) classified in six different families: GT29 comprising eukaryotic and viral ST sequences and GT4, GT38, GT42, GT52 and GT80 comprising bacterial ST sequences.\(^4,6\)

Moreover on the basis of crystallographic and fold data available, STs can be grouped into two structural superfamilies that represent variations of the canonical glycosyltransferase (GT-A and GT-B) folds. These two superfamilies differ in the nature of their active site residues, notably the catalytic base (a histidine or an aspartate residue). In the following paragraphs I report a short comment on the ST family in eukaryotic, viral and prokaryotic.\(^4\)

1.1.1 Eukaryotic and viral STs

All eukaryotic and viral ST sequences are grouped into the GT29 CAZy family,\(^4,6\) while, to date, 20 different STs, acting on glycoproteins and/or glycolipids, have been characterized in human. They are classically split into the following four groups, depending on the type of linkage formed and of the nature of the sugar acceptor:
• **ST6Gal family** comprises only two protein members (ST6Gal-I and ST6Gal-II) that catalyze the transfer of Neu5Ac residues to the hydroxyl group in C-6 of a terminal galactose residue of type 2 disaccharide (Galβ1-4GlcNAc), and potentially to the N-acetylgalactosamine (GalNAc) residue of LacdiNAc motif (GalNAcβ1-4GlcNAc);

• **ST6GalNAc family** comprises six different members (ST6GalNAc-I to VI) that catalyze a similar reaction but they use as acceptor a GalNAc residue found on O-glycosylproteins (ST6GalNAc-I, II and IV) or on glycolipids (ST6GalNAc-III, V and VI);

• **ST3Gal family** comprises six protein members of the ST3Gal group (ST3Gal-I to VI) catalyze the formation of a α-2,3 linkage between Neu5Ac and terminal galactose residues found on glycoproteins and glycolipids. The subfamilies I and II use as a specific acceptor the type 3 oligosaccharides structure Galβ1-3GalNAc-R as asialo-GM1 and GM1a. The ST3Gal III, IV, V and VI recognize as acceptor these oligosaccharide structure: Galβ1-3/4Glc(NAc)-R. In particular, only subfamily V uses as specific acceptor the LacCer affording the ganglioside GM3.  

• **ST8Sia family** comprises six enzymes (ST8Sia-I to VI) that mediate the transfer of Neu5Ac to the hydroxyl group in C8 of another terminal Neu5Ac residue, found on glycoproteins and glycolipids.

Vertebrate STs (GT29) predominantly reside in the Golgi compartment and they have, similarly with the other Golgi-resident GTs, a type II architecture consisting (Figure 4) in:

![Figure 4: Vertebrate STs architecture.](image)

- A short N-terminal cytoplasmatic tail with high variable amino acid composition and no essential for the catalytic activity, but its role, is still not clarified. It has been proposed that it could define subcellular localization and could provide stability to ST in intracellular dynamics;
• a unique transmembrane domain that is known as Golgi membrane anchor and it could play a role in localization of the ST;

• a variable stem domain length from 20 to 200 amino acids. This is the region that inactive the catalytic domain when is not necessary for the catalysis. This region often displays high sequence variability and little secondary organization and is therefore predicted to be highly flexible.\(^\text{13}\) However, the role of the stem is not only this, but it has been proposed that it could play a role in both the enzyme retention in appropriate sub-compartment (Golgi apparatus) and in enzyme activity. Moreover, the mechanism of this activation is not yet clarified;\(^\text{14}\)

• a large C-terminal catalytic domain facing in the lumen of the Golgi compartment.\(^\text{14}\)

A special feature of GT29 enzymes, considered hallmarks for the identification of ST gene, is the presence in their catalytic domain of four conserved peptide sequences, termed sialylmotifs such as sialylmotif L (large), S (small), motif 3 and VS (very small). The functional significance of the sialylmotifs L and S, assessed by site-directed mutagenesis, using the human ST6Gal-I as a model, led to reach the following conclusion:\(^\text{4, 6}\)

• the sialylmotif L is devoted to the recognition of sugar donor;

• the sialylmotif S aimed to the binding of both donor and the acceptor substrates;

• the sialylmotif L and S are believed important for the catalytic activity of ST, in fact they form a disulfide linkage between two cysteine residues;

• motifs 3 and VS contribute to the binding of the acceptor.

Except for these peptide motifs, the various GT29 groups show few sequence similarities. Considering only the twenty distinct human ST sequences, that have been cloned to date, we can observe that they have in common the presence of only 10 invariant residues (five in motif L, two in motif S and VS and one in motif 3).

### 1.1.2 Prokaryotic STs

Bacterial STs are very important since are expressed by pathogenic bacteria to mimics sialylated human glycan structures on their cell surface. Thus, the enzymes that synthesize these structures represent attractive targets for therapeutic development.\(^\text{4, 6}\)

Bacterial STs, as evident from the above classification, appear to have more flexible substrate specificity than their eukaryotic counterparts. In fact, these enzymes differ from mammalian ones, which are commonly monofunctional, since have multiple functions, including ST activities responsible for forming different sialyl linkages with or without additional sialidase and trans-sialidase activities.
All the known bacterial STs have been classified into five different CAZy families (GT4, GT38, GT42, GT52 and GT80).6

- **Family GT4** includes highly homologous capsular polysaccharide (CPS) polymerases (SiaDs), such as *N. meningitidis* serogroups W135 and Y which have both hexosyltransferase (α1,4 galactosyltransferase activity for SiaDW135 and α1,4 glucosyltransferase activity for SiaDY) and sialyltransferase activities responsible for the synthesis of sialic acid-containing heteropolymeric CPSs [−6Gal/Glcα1,4 Neu5Aca2−]n.

- **Family GT38** includes polySTs (NeuS, SiaD) from *E. coli* K1 or K92 and *N. meningitides* serogroup C.6,15

- **Family GT42** comprises mono-functional α-2,3 STs and bifunctional α-2,3/α-2,8 STs from *C. jejuni* and *H. influenza* (Cst-I, Cst-II, Cst-III, Lic3A, Lic3B);16, 17

- **Family GT52** includes mono-functional α-2,3 STs and bifunctional α-2,3/α-2,6 STs from *Neisseria* spp. (Lst), but also an α-1,2 glucosyltransferase that uses UDP-α-D-Glc from *Salmonella enterica* subsp. *Arizonae*.17, 18 The presence of both inverting and retaining enzymes in the same family is somewhat unexpected and highlights the difficulty in assigning a precise biochemical function to the yet uncharacterized protein sequences that classify into family GT52.

- **Family GT80** comprises α-2,3 STs, α-2,6 STs and bifunctional α-2,3/α-2,6 STs. One peculiar feature is that some bacterial STs are multifunctional.

### 1.1.3 Structures 3D of STs

Currently, some structural information is available for seven STs, belonging to families GT4, GT29, GT42, GT52 and GT80. On the contrary, no structural data of the seven crystallized ST are available for the bacterial ST family GT38.4,6

Observing the 3D structures, it appears that STs, whatever their eukaryotic or prokaryotic origin, fall into the two main folding groups: GT-A and GT-B folds. The folds of GT29 and GT42 families are considered as variants of the GT-A architecture, but they lack the DxD motif that is one characteristic feature of most GT-A enzymes. They do not require the metal ion for their catalytic activities, whereas STs of families GT38 and GT52 an also adopt a GT-B fold. These two ST structural super-families not only differ in their spatial arrangement but also in the nature of their activate site residues, notably the catalytic base (a histidine or an aspartate residue), thus suggesting that they evolved independently.
1.2 Sialylation and sialylglycoconjugate

From a chemical point of view, the sialylation is any reaction that introduces a sialyl group into a molecule. As I discuss in next paragraph, no definitive conclusion has been reached on its mechanism, but some hypotheses can be formulated.

1.2.1 Catalytic mechanism of STs

On the bases of its result, the sialylation is a nucleophilic substitution reaction operated by a nucleophilic carbohydrate hydroxyl on the anomeric carbon of the sialyl donor bearing a good leaving group activated by a phosphate ester function.⁴, ⁶, ¹⁹

\[
\text{Acceptor-OH} + \text{CMP-β-SA} \xrightarrow{\text{ST}} \text{Sialyl-α-glycoconjugate} + \text{CMP}\]

**Figure 5:** ST mediated sialylation reaction.

Considering the stereochemistry of the sialosyl donor and that of sialic fragment bonded to the end of the glycoconjugate, an inversion of the configuration (β to α) at the anomeric center occurs. Indeed, STs are classified as GTs inverting metal-ion independent enzymes, where the reaction occurs by the inversion of anomeric carbon configuration β, in CMP-Neu5Ac, to α in sialylglycoconjugate.⁴, ⁶

This inverting mechanism, catalyzed by GTs appears, to support a possible S\(_{N}2\) mechanism, whereby a general base deprotonates the incoming nucleophile of the acceptor sugar, thus enabling the direct displacement of the nucleoside diphosphate.⁴-⁶

Moreover, a retaining GT catalyzed mechanism is also proposed that should proceed via a double-displacement reaction with a covalently bound glycosyl-enzyme intermediate. In this mechanism, a suitably positioned amino acid within the active-site functions as a nucleophile to catalyze the reaction is necessary.¹⁹ In comparison to inverting glycosyltransferases, retaining glycosyltransferase reactions also proceed through oxocarbenium ion-like transition states.
Despite this, several evidences (inhibition and kinetic studies) appear to support the hypothesis that is S\textsubscript{N}1 the mechanism for ST reaction.\textsuperscript{20, 21} However, both the conjectures are in agreement with the formation oxacarbeniumione transition states in the ST reactions (Figure 6).

**Figure 6:** Schematic representation of (a) inverting and (b) retaining catalytic mechanisms via oxacarbeniumione transition state and ST oxacarbeniumione.

Despite of this, the mechanism for retaining glycosyltransferases is still being explored in order to identify any intermediate in its support that at the moment appear only highly speculative. At the moment, in absence of appropriate mechanism evidences, any choose between on S\textsubscript{N}1 or S\textsubscript{N}2 mechanism is not possible. Despite the knowledge of the catalytic enzyme are still limited, however the catalytic role of some aminoacidic residues located on the active site appear important. For example, both aminoacidic residues of histidine (His) family or aspartic acid family (Asp) are important for their action as
catalytic base in different ST. Some other amino acidic residues have been also found to facilitates the elimination of the phosphate leaving group and their action on specific ST has been demonstrated.\textsuperscript{4-6}

However, structural data to understand the precise molecular base that account for the acceptor substrate specificities are still scarce. Differences in acceptor specificity are attributed to the lid domain region that probably plays a major role in selecting different acceptor molecules.\textsuperscript{4}

Thus, additional structural studies (modelling and mutation) are needed to further understanding the mechanism action.

1.2.2 Biological synthesis of the CMP-Neu5Ac sialosyl donor

The biosynthesis of CMP-Neu5Ac, is strictly correlated to that of Neu5Ac (Figure 7). Herein, I will consider both.

In vertebrate system Neu5Ac derived by condensation of ManNAc-6-phosphate (ManNAc-6-P) with phosphoenolpyruvate (PEP). The precursor of ManNAc-6-P is glucose which is transformed in the cytosol, in several steps, into Uridine Diphosphate \textit{N}-Acetylglucosaminine (UDP-GlcNAc). This substrate is converted in ManNAc-6-phosphate (ManNAc-6-P) by a bifunctional enzyme called GlcNAcepimerase (GNE) / ManNAc kinase (MNK). Then, the ManNAc-6-P is the substrate of a specific aldolase to give the Neu5Ac-9-P that, in turn, is transformed by specific phosphatase (encoded by \textit{NANP}), to free Neu5Ac in the cytoplasmic compartment.\textsuperscript{22}

At this point Neu5Ac is transferred within the nucleus where it is activated, as CMP-Neu5Ac, by the action of specific enzyme called CMP-synthase. In eukaryotic cells, there is a feedback inhibition system contributing to govern the levels of sialic acid in the cell: free CMP-Neu5Ac acts as allosteric inhibitor of GNE. These topological issues are not applicable to prokaryotes, where CMP-Neu5Ac are synthesized in the cytoplasm and directly used in the coordinated assembly of cell-surface glycans, before their delivery to the surface.

Finally, the CMP-Neu5Ac is transferred in the Golgi apparatus where the Neu5Ac is linked by specific STs to an appropriate acceptor molecule. Then, the oligosaccharide of nascent glycoconjugate is carried outside on the cell membrane.\textsuperscript{22}
12

1.2.3 Acceptor family

The main acceptors of sialic acid, transferred by specific ST using CMP-SA as high-energy donors, are the penultimate sugars of glycoconjugates (glycolipids, glycoproteins and lipopolysaccharides).

The most common linkages of Neu5Ac are to the C-3 or C-6 positions of galactose residues or to the C-6 position of N-acetylgalactosamine residues. However, Neu5Ac can also take up internal positions within glycans, the most common being when one SA residue is attached to another, often at C-8 position.\(^{22}\) In addition, internal SA can occur in the repeating units of some bacterial polysaccharides and echinodermal oligosaccharides.

**Figure 7:** Intracellular sialic acid metabolism. CTP, cytidine triphosphate; PEP, phosphoenolpyruvate; OGS, oligosaccharides. The sialic acid pathway beginning with UDP-GlcNAc proceeds by the sequential action of (a, b) UDP-GlcNAc 2-epimerase/ManNAc 6-kinase; (c) sialic acid 9-phosphate synthase; (d) sialic acid 9-phosphatase; (e) CMP-sialic acid synthetase. Then, CMP-sialic acid Golgi transporter and sialyltransferase (several in humans) install a sialic acid on glycoconjugates destined for the cell surface.
1.2.4 Function of sialylglycoconjugates under physiological and pathological conditions.

Sialylglycoconjugates being expressed on the cell membrane, are involved in many biological functions as cell-cell talking, molecular recognition events, cell differentiation, immune response modulation and many others.\textsuperscript{4-6, 23} In fact, many evidences show that sialylglycoconjugates are recognized from specific glycoprotein for example as, lectines, that act as regulating factor in the activation of lymphocyte B and in the homeostasis of lymphocytes T. Thus, they modulates in this way, the immune response and the inflammatory processes.

Moreover the sialylglycoconjugates play important roles in cell adhesion, survival and proliferation (see GM3 section). On the other hand, the SAs of a glycoconjugate can mask the antigenic recognized site located at the penultimate galactose residue of the glycoconjugate.

Furthermore, the high expression of SAs on outer cell membranes appears to suggest that they have roles in the stabilization of molecules and membranes, as well as in modulating interactions with the environment. In particular, the electronegative charges of sialylglycoconjugates, due to negative charge at the sialic acid residues in the terminal portion, allow the modulation of some functions such as the binding and transport of ions and drugs, the stabilization protein conformation, including enzymes and enhancement of the viscosity of mucins.

On the other hand many evidences have suggested a correlation between the alteration of ST activity and of sialylglycoconjugate presence in several diseases. Indeed an alteration of the sialyltransferase activity causes the characteristic composition of glycoproteins in transformed cells, as shown in human colorectal cancer, breast carcinoma, leukemic cell lines and metastatic tissue.\textsuperscript{4-6, 24}

Thus, the elevated plasma sialyltransferase activities can be used as tumor markers for cancer diseases. These alterations in the ST activity and in sialylglycoconjugated composition in sick cells have been observed also in: inflammatory pathology, autoimmune illness, metabolic disorderand many others.
1.3 Sialyltransferase inhibitors

In recent years much attention has been devoted to develop inhibitors of STs due to the fact that, inhibition of these enzymes represents an useful tool in elucidating the biological functions of the sialylation process and possibly in improving therapeutic applications. In fact, these enzymes are considered potential therapeutic targets in many illnesses and in particular in some tumours (as breast carcinoma, metastatic tissue and colorectal cancer) in pathological inflammation processes, in metabolic disorder and many others. As a consequence, in spite of the scarce structural information on the enzymes structure, a number inhibitors have been developed following different lines. A suitable accepted classification may be:

1 donor-based inhibitors (CMP-Neu5Ac);
2 acceptor-based inhibitors;
3 transition state analogues;
4 bisubstrate analogues.
5 other ST inhibitors

It will be herein shortly discuss them.

1.3.1 Donor-based inhibitors

These inhibitors mime the CMP-Neu5Ac, the common donor substrate of all known STs, and compete with it. In particular, they should be synthesized considering the structure of the natural donor substrate and of the previously data reported, concerning the consequences of its modification or substitution to its three molecular portions:

- cytidine portion
- phosphate bridge
- sialic acid moiety

The cytidine portion is essential for the competitive inhibition activity. Indeed, CMP (cytidine monophosphate), CDP and CTP are potent inhibitors of human serum α-2,6 ST ($K_i = 16$ to $50 \mu M$), while the replacement of the cytidine nucleotide with another nucleotide (AMP, UDP), affords to a drastic reduction of antisialyltransferase activity.

The contribution of the phosphate bridge to the binding, is not clear. In fact, the substitution of this portion with different groups does not afford a remarkable inhibition, as shown by derivatives I-II ($K_i = 250$ to $780 \mu M$), thiophosphonate III and amino acid derivative IV ($IC_{50} = 1000 \mu M$ towards α-2,6 ST) or the dephosphonate derivative 3a (21% toward α-2,3 ST and 30% inhibition at 21mM toward α-2,6 ST) (Figure 8).
No conclusion can be made, in our opinion, concerning the stereochemistry of the phosphonate group linking to the sialic portion. In fact, few epimers has been synthesized and/or tested and the only reports, concerning phosphite or aminoacidic congeners afford contrast result, (a low decreasing and inalteration).

**Sialic acid portion** is not an essential requirement for high binding affinities.\(^{28,29}\) In fact, interesting result was obtained replacing the SA portion with another acidic sugar moiety. Indeed, the substitution with derivatives of quinic acid, V-IX compounds, affords a \(K_i\) value between 20-1400 \(\mu\)M against an \(\alpha\)-2,6 ST from rat liver.\(^{29-31}\) Of interest it appears the introduction of a fluorine atom in the SAs molecule that improve eight time \(K_i\) value (\(X \ K_i = 5.7 \ \mu\)M) (Figure 8). As already known from other glycosyltransferase inhibitors, fluorine substituted glycosyl donors are potent inhibitors of these enzyme. Indeed, the electronegative fluorine atom prevents the cleavage of the glycosidic bond by destabilization of the oxacarbenium ion transition state.\(^{26,32}\) As a final comment, we can say that to design a rational formulation of donor inhibitor analogues, only few literature information are still available.
Acceptor-based inhibitors

The programming of inhibitors according to this approach is based on the knowledge of the interactions involved in the enzyme-substrate recognition process. Acceptor analogues compete with the natural glycoconjugate in the active site of the enzyme, without being glycosilated, due to the lack of the accepting group in their structure.

Several compounds, which mimics the structure of the terminal glycan portion of the sugar acceptors, have been reported in the literature. However, herein, I report the compounds that in my opinion, afford the relatively most interesting results. As showed in the Figure 9, compounds XI-XIII and the dimeric compound XIV, that mime the structure of N-acetyllactosamine, show values of $K_i = 760-4140 \mu M$ towards $\alpha$-2,6 ST from rat liver, very similar to that corresponding to the endogenous acceptor ($K_M = 900 \mu M$). Furthermore kinetic analyses on these compounds show some interesting features:

1. the binding of CMP-SA with enzyme do not affect the bond between acceptor, or its analogues, and STs;
2. their inhibition mode is not purely competitive;
3. the hydroxyl group at C-6 of N-acetyl lactosamine is not important for the enzyme substrate binding. Indeed, compounds XV-XVII, showed similar inhibition data ($K_i = 760$ to $1700 \mu M$).

The values of $K_i$ of all these inhibitors are very lower than those of donor analogues. This result could be explained considering that the designed analogs are based on the small terminal unit of the sugar acceptor, while STs recognize not only the terminal residues, but also more extended portions...
of their natural acceptors. In fact, kinetic studies toward rat liver α-2,6 ST show that they form multivalent binding sites.\textsuperscript{35}

![Chemical structures of acceptor-based inhibitors.](image)

**Figure 9:** Acceptor-based inhibitors.

### 1.3.3 Transition state-based inhibitors

The structures of those inhibitors have been devised considering the possible formation of an oxacarbenium ion in the transition state XVIII (Figure 10), of the sialylation with inversion of the anomeric carbon configuration. This intermediate has a distorted half-chair conformation and a positive charge that is delocalized between the anomeric carbon and the oxygen atom of the ring. In particular, Smith and coworkers, on the basis of the proposed transition state formation, have noticed the following essential features, that the transition state inhibitor should possess:

- an anomeric carbon with sp\(^2\) hybridization;
- an appropriate distance between the anomeric carbon and the CMP leaving group;
- two negative charges of oxygen atom with a distance of about five bonds;
- an essential cytidine portion, while the sialic portion may be substituted by other groups.

According to this, the first transition-state mimetic inhibitors were the 2-deoxy-2,3-didehydro-\(N\)-acetylneuraminic acid (DANA) derivatives. These DANA derivative show a sialic portion\textsuperscript{20} IXX-XX and a good values of \(K_i\) (0.04 to 6.0 \(\mu\)M) and in addition XX and XXI, having an exocyclic double bond. \textsuperscript{21,36} Subsequently, Smith et al. developed other new inhibitors of transition state by the replacement of sialic motif (very expensive as starting material) with sialyl-mimetics, such as D-glucosamine. In this way, the new compounds XXII-XXVI, differing only for the simplified side chain, showed inhibitory activity further improved. According with my knowledge, the compounds
XXIV-XVI exhibit the best inhibition activity, against \(\alpha-2,6\) ST, until now reported. Another class of transition-state analog inhibitors was developed containing: a CMP moiety essential for activity, a carboxylate, or, alternatively, a phosphonate group as a spacer, while the sugar or glycosidic portion (Neu5Ac or D-glucosamine) is replaced with a stable “non-glycosidic” bond to CMP. A double bond is introduced in the pyranose sialic ring to obtain a flattened ring system which mimics the transition-state. Major exponents of this class are derivatives of cycloexanone XXVII and compounds XXVIII-XXX with endocyclic and exocyclic double bonds, synthesized staring from D-quinic acid. All these compounds show an important antisialyltransferase activity. In particular, compounds XXIX and XXX are potent inhibitors against \(\alpha-2,3\) (\(K_i = 4-10\ \mu\text{M}\)) and \(\alpha-2,6\) (\(K_i = 0.27-1.6\ \mu\text{M}\)).

Starting from these results, new specific inhibitors were synthesized against \(\alpha-2,6\) STs. \textit{Inter alia}, the sp\(^2\) hybridization of anomeric carbon of the transition state is simplified by the replacement with planar or hetero-aromatic system. The phosphonate group displaces the carboxyl group of sialic acid portion that allows the presence of two negative charges and it must be spaced again five bonds and the bond to the essential cytidine portion. Some other \(\alpha\)-hydroxyacetate and \(\alpha\)-hydroxyphosphonate analogues, with various aromatic rings, show a good inhibition activity against \(\alpha-2,6\) ST. For example, compounds XXXI-XXXIII have a \(K_i\) value between 7-23 \(\mu\text{M}\) as well as XXXIV and XXXV that contain different aromatic rings and conserved the antisialyltransferase activity (\(Ki= 0.6-0.13\ \mu\text{M}\)).

As described above, all the powerful sialyltransferase inhibitors known are polar and charged. However compounds of this type are difficult to exert their functions in cells or organisms due to their low membrane permeability. The design and discovery of inhibitors that can easily permeate the cell membrane remain a major challenge.
Figure 10: Transition state-based inhibitors.

1.3.4 Bisubstrate-based inhibitors

The bisubstrate inhibitors are characterized by the presence, in the same molecule, of elements that mime both donor and acceptor portions. For this, as evident from the formulas, of the more active inhibitors of this type (Figure 11), their design take advantage from the knowledge acquired in the studies of transition-state analogs.
Herein, I report the more interesting compounds showing inhibitory activity\textsuperscript{34} (XXXVI and XXXVII and XXXVIII; Figure 11).

In another approach the natural CMP-Neu5Ac donor was attacked via a short $\text{SCH}_2\text{S}$ spacer to the $6'$-position of a lactosamine derivative. The resulting bisubstrate analog XXXVIII exhibits an inhibition activity ($K_i = 10\mu\text{M}$) towards $\alpha$-2,3 and $\alpha$-2,6 STS which is in the range of the corresponding donor substrate analogs.\textsuperscript{39}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure11}
\caption{Bisubstrate-based inhibitors.}
\end{figure}

1.3.5 Other ST inhibitors

In addition to the more common strategy examined for the development of the inhibitors of STs, some other structures of relatively small endogenous molecules, antibiotic antisense-oligonucleotides, and natural products show an inhibitory activity against STs.

For example the $\alpha_1$macroglobuline is a small endogenous protein reported as a selective inhibition against $\alpha$-2,6 ST but the mechanism of inhibition action is not clear.\textsuperscript{40}

Another exemple is the $N$-butylmannosamine that is a small molecule able to get in the metabolism of sialic acid and it is converted in CMP-SABut. This compound is transferred less efficiently than the natural CMP-Neu5Ac, showing an inhibition effect toward the $\alpha$-2,8 STs.\textsuperscript{41}

Moreover, the treatment with specific antisense-oligonucleotide, complementary to the region upstream of the initial codon of Gal$\beta$1,4GlcNAc$\alpha$-2,6 ST, shows the decrease of the expression of enzyme and of its mRNA level.\textsuperscript{42}
Furthermore, by screening of natural products and microbial extracts (about 7500 samples) it was possible to individuate the sayasaponin I, the first competitive inhibitor of CMP-Neu5Ac for ST3Gal I, with a value of $K_i = 2.1 \mu M$, that shows the most potent antisyialyltransferase activity.\textsuperscript{43}
1.4 GM3 synthase

Of particular relevance it appears GM3 synthase (ST I, ST3Gal V, CMP-NeuAc:LacCer-α-2,3-sialyl transferase; EC 2.4.99.9) that catalyzes the transfer of Neu5Ac, to the terminal galactose residues of LacCer, thus affording GM3, the metabolic precursor for the biosynthesis of ganglioside series (Figure 12). This enzyme is highly specific for their lipid substrate and it recognizes only LacCer as acceptor substrate. Studies on genes encoding STs, including those that synthesized gangliosides have permitted to predict the amino acid sequences of these enzymes and of GM3 synthase and to have many insights on the regulatory mechanism for their expression. The isolation of the cDNA of GM3 synthase appears to depend on the type of tissue, dominating in brain, muscles, testes, and placenta. Moreover same successful cloning of the gene encoding GM3 synthase makes it possible to prepare the recombinant enzyme both as full sequence and fragments. This possibility has allowed some preliminary studies developed to understand the mechanism regulating GM3 contents in tissues and cells that, however, are not completely clarified.

Figure 12: Biosynthesis of a, b and c series of gangliosides. Cer, ceramide; SA, sialic acid; ST I, GM3 synthase; ST II, GD3 synthase; ST III, GT3 synthase; ST IV, GD1a synthase; ST V, GT1a synthase; GlcT, GlcCer synthase; GalT I, LacCer synthase; GalT II, GM1 synthase; GalNAcT, N-acetylgalactosylaminyltransferase, GM2 synthase.
The gene encoding human GM3 synthase is located on the second chromosome and consists of nine exons containing the coding region in exons 4-9. Functional analysis of the 5′-flanking region by the transient express method revealed that the –177 to –83 region from the transcription initiating site functions as the core promoter essential for transcriptional activation of the GM3 synthase gene in cells of human neuroblastoma and hepatoma.

Several signal pathways (PKA, PKC, stress-activated protein as kinase/c-JunN-terminal kinase, and p38MAPK) are able to regulate its expression by the activation of CREB. Indeed, within promoter region of gene for GM3 synthase, there is CRE (cAMP response elements), that is recognizes from CREB increasing of GM3 synthase levels as well as GM3.

Moreover, also Sp-1 and AP2 transcription factors have a binding site within gene promoter of enzyme, with enhance the basal activity of the GM3 synthase.

Intracellular location of GM3 synthase is a factor regulating its synthesis and that of higher gangliosides that occurs, mainly in cells and tissues, of both endoplasmic reticulum and the Golgi apparatus. Recently it has been proposed the presence of two isoforms of GM3 synthase, a short and long isoforms. The first is mainly located in the “proximal” (cis/medial/trans) Golgi apparatus and it should be involved in synthesis of simple gangliosides GM3 and GD3, while the second one is located in Golgi apparatus (distal, trans-net) and should be mainly involved in synthesis from GM3 of more complex gangliosides (GD1a, CT1a, GT1b, and GQ1b see Figure 12).
1.5 GM3

GM3 is an essential component of plasma membrane lipid ganglioside-enriched microdomains (GEM) or rafts. This topological distribution has been found in the majority of extra neural tissues of vertebrata and represents an interesting and fruitful research field. The main function of GEM is in signal transduction where it concentrates receptor and signaling molecules on the membrane. This maximize the effect of the ligand–receptor binding during the signal transduction and prevent undesirable “talks” between different signaling pathways. In particular, GM3 is involved in regulation of all cellular processes (cell proliferation, differentiation, apoptosis, embryogenesis, oncogenesis and many others).

Herein I report a short mention of the GM3 role in some of these processes.

1.5.1 GM3 in cell proliferation and oncogenesis

Several literature data on the ability of GM3 to inhibit the growth of tumor cells and tumor development are reported.\(^2\) This ability is due to a fundamental property of this ganglioside to suppress tyrosine phosphorylation of some growth factor receptors (as fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF)) present in membranes of tumoral cells. This action has been detected in some human tumors, such as neuroblastome, lymphoma and ovary cancer.\(^2\) In contrast with this general behavior, has been suggested that GM3 induces the proliferation of carcinoma in the presence of urochinas.\(^50\)

However, GM3 can also influence proliferation by another mechanism: this ganglioside can inhibit the cell population growth by prolonging the G1 phase of the cell cycle in fibroblast cultures.

However, it should be noted that the ratio of GM3 to complex gangliosides determines their influence on tumor growth and angiogenesis.

1.5.2 GM3 in apoptosis

GM3 plays a dual role in apoptosis: since in on one hand, it suppresses the effect of apoptotic cytokines (antiapoptotic effect), and in the other one causes apoptosis due to inhibition of cell proliferation (pro-apoptotic effect).

- **Antiapoptotic effect** is correlated with an increase of the sialidase activity, that transforms GM3 in SA and lactosylceramide (LacCer). This compound has an anti-apoptotic effect by an up-regulation on Bcl-2, that one turn inhibits the apoptosis machine. In this way, the levels of GM3 decrease while the cell motility and cell growth increase.\(^51\)
• **Pro-apoptotic effect** is mediated by two different actions. The first one is an inhibition of the cell proliferation that especially occurs in metastatic cells. The second effect involves the relationship between the GM3 and $\alpha$–TNF levels. Last one is a multifunctional cytokine with a well-established role in immunity modulation and inflammation. Moreover, it is a known inducer of insulin resistance and apoptosis.\textsuperscript{52} Indeed, increase the GM3 level induces the correlative enhancement to expression of $\alpha$-TNF affording a pro-apoptotic effect.\textsuperscript{9}

1.5.3 GM3 in cell adhesion

Many literature reports suggest that the role of GM3 in the cell adhesion consists in an enhancement of the cell extracellular matrix interaction. For instance it has been observed that adhesiveness of melanoma B 16 on endothelia cells is due to a specific interaction between GM3, expressed on the melanoma B 16 cells, and LacCer located on the cell endothelia surface. The adhesion degree is in relationship with the expression level of GM3 on the cell surface of melanoma cells.\textsuperscript{53} These data suggest that the adhesion of GM3 occurs by action on integrine receptors.\textsuperscript{54} These conclusions are consistent also with data reporting the involvement of GM3 in suppression of cell mobility, invasiveness and apoptosis.

1.5.4 GM3 in cell differentiation

The ganglioside GM3 seems to be important also in modulating the cells differentiation. Many evidences of this activity have been showed by the relationship between differentiation and levels of GM3. For example the high level of GM3 (tenfold increase) have been found in differentiation of human polypotent leukemic cells (HL60) to monocyte/macrophage phenotype induced by mitogenic agent as phorbol ester (Protein kinase C (PKC)).\textsuperscript{55} Moreover, the GM3 is able to induce the differentiation of HL 60 cells also without the presence of proliferation inducer as mitogenic agents. In fact the accumulation of GM3 in the plasma membrane induces a variation of the ganglioside pattern, exposed on the cell membrane that is needful for the cellular differentiation.\textsuperscript{55, 56}
1.5.5 GM3 in insulin resistance

GM3 level are also regulated by the expression of α-TNF and, in the adipocyte cells, the enhancement of the α-TNF expression leads an increase of GM3 levels with consequently suppression of the transduction signals mediated by the insulin receptors GM3 mediate.\textsuperscript{11,57} Indeed, in this way, the adipocyte is not able to respond to insulin signals conducing to the insulin resistance mediated by GM3 since causes on uncoupling of the insulin receptor activity towards the insulin receptor substrate with suppression of the insulin-sensitive glucose transport. Moreover has been reported that, using inhibitors against the biosynthesis of GM3, the effect of α-TNF on adipocyte cells is suppress, resolving the insulin resistance.\textsuperscript{11,57} Thus the serum level of GM3 may be useful marker for the management of metabolic syndrome, including insulin resistance, as well as, for the early diagnosis of atherosclerosis.\textsuperscript{10} Recently inhibitors GM3 biosynthesis are proven to have therapeutic value by oral administration in diabetic rodent model.\textsuperscript{58}

1.5.6 Other processes

Other studies that relate the GM3 values and the occurrence of pathologies have been reported. As an example, some observation demonstrate a direct link between GM3 synthase and hearing functions. Indeed, mice lacking of GM3 exhibit complete hearing loss due to selective degeneration of the organ of Corti. However, also the relationship between over expression of GM3 and some viral diseases has been suggested.\textsuperscript{9,10} Thus, the involvement of GM3 synthase, in many biological and pathological function, suggests that this enzyme may be of value as therapeutic or biological tool.
2. AIM OF THE WORK
2. AIM OF THE WORK

With the aim to find suitable inhibitors of GM3 synthase, in my thesis work I decided to study the synthesis of the glycoside 3a, the first congeners, reported in a short note, as the only active inhibitor of GM3 synthase and of other unreported analogues. Indeed, I devised to synthesize also some perfluorinated analogues 3b-c and lactones 4a-c, confident that these compounds could have an inhibitions activity improved in respect to that 3a. Moreover, I devised to setup a synthetic procedure that could allow to establish, without doubts, the stereochemistry of the final compounds that, in the case of 3a, had been only empirically established.

![Chemical structures](image)

Figure 13: Donor inhibitors based on CMP-Neu5Ac designed and synthesized.

For the selection of the structure I considered that the presence of the perfluoroacylamido groups could increase the inhibition activity of the compounds as occurs for the 3 α fluorine CMP-Neu5Ac in respect to the natural non fluorinate compound 2 (Figure 8). In my mind, the presence of the fluorinated acyl groups in glycoside 3b-c and 4b-c should growth the tendency of the carbonyls to their tetrahedral hydrate form, a characteristic know to expand the activity of carbonyls against some enzymes activity. Similarly, the inner esterification of the polar carboxylic group with the 7 hydroxyl group, could increase the inhibition activity of the compounds 4a-c in respect to the parent
acids. Moreover I devised to synthesized also the perfluorinated glycoside $\alpha$–epimers at anomeric center 3d-e. Their testscould support or contrast the conjecture that the stereochemistry of this center is an essential requirement for the inhibitory activity on ST.27 Furthermore I synthesized the peracetylated derivatives 5a-c and 5e in order to facilitate the cell permeability of the glycosides in experiments performed on cells.

I could test these compounds on cell homogenate and on cell lines in order to verify their action. After the selection of the active compounds, I could refine their synthesis and their structure.

Herein, I report the successful synthesis of all programmed compounds: acids, lactones, $\alpha$–epimers, and acetylated analogues and encouraging results on their inhibitory activity on GM3 synthase.
3. RESULTS AND DISCUSSION
3. RESULTS AND DISCUSSION

In this section I will discuss all the experimental results starting from the description of the synthetic protocols I set up to obtain some new glycoside derivatives, Neu5Ac-CMP donor analogues. Then, I will continue the discussion reporting some biological assays on STs and GM3 synthase performed to evaluate the inhibitory activity of these new compounds.

3.1 Chemical results

We decided to synthesize the glycosides 3a-e and their 1,7-lactones 4a-c taking advantage from some works, recently done in the Prof. Mario Anastasia laboratory, both on the $N$-transacylation of amides and on the 1,7-lactonization of Neu5Ac 1. So, we started our synthetic work from the known cytidine aldehyde 6 that, in a relatively shorter way than that reported in literature afforded the alcohol 9, i.e. the cytidine portion of the required glycosides 3a-e (Scheme 1). The alcohol 9, by glycosidation with the nucleophilic 5-acylamido-4,7,8,9-tetra-O-acetyl-2-chloro-2,3,5-trIDEOxy-β-D-glycero-D-galacto-2-nonulopyranosates 11a-c, afforded β-glycosides 12a-c, in satisfactory yields (39, 36 and 27% respectively), accompanied by variable, but always minor, amounts of their 2α-epimers 13a-c (<1, 20 and 24% respectively) (Scheme 1).

We performed the reaction with the chlorinated donors 11a-c since we were interested to obtain the β-glycosides 12a-c and the α-epimers 13a-c, not only to test the influence of the α and β geometry in the inhibition, but also to interpret the chemical and physical properties of these compounds having both isomers.
Scheme 1: Synthesis of: A) cytidine portion, B) sialosyl donors and C) glycosidic reaction. Reagent and conditions: A) i: (Ph)_3P=CHCHO, CH_2Cl_2, 23°C, overnight, ii:H_2, Pd/C, MeOH, rt, 1h; iii: NaBH_4, MeOH, -20°C, 15 min, 45%; B) iv: AcCl, MeOH, 0°C to 23°C, overnight, 77, 81 and 80%; C) v: AgOTf, toluene/CH_3NO_2, rt, 3h, β-anomers yields (39, 36 and 27%) and α-anomer yields (<1, 20, 24%).

In the work, the aldehyde 6 was transformed into the homologue alcohol 9 by a Wittig reaction with (triphenylphosphoranylidene) acetaldehyde, followed by the a catalytic hydrogenation of the formed α,β-unsaturated aldehyde 7 and by a successive chemical reduction of the saturated aldehyde 8 with NaBH_4 to the alcohol 9. We attempted also a direct reduction of the aldehyde 7, using various chemical or catalytic methods that, however, afforded unsatisfactory results, due to the cleavage of the acetamido and of the cytidine base of the unsaturated or saturated aldehydes 7 or 8. The aldehyde 6 could be isolated, without significant loss, by simple column chromatography on silica, on the contrary, the saturated and unsaturated aldehydes 7 and 8 showed an evident decomposition, during their purification on silica, reason for which we successively reacted in crude form, limiting the purification to a sample used for their complete characterization. At the end, we isolated the alcohol 9, in pure form as a stable compound in 45% yield from the aldehyde 6. This alcohol showed the expected physico-chemical properties that were identical to those in part reported for compound 9 prepared by a different route.\(^1\)

In parallel, we prepared the 2-chloroderivatives 11a-c (77, 81, 80%) useful to perform the glycosidation of the alcohol 9, treating the appropriate peracetylated sialic acid methyl ester 10a-c
with hydrogen chloride in methanol (Scheme 1). Then we performed the glycosidation of the alcohol 9 with the 2-chlorine of Neu5Ac 11a, under the Koenig-Knorr like conditions, and we obtained the desired β-glycoside 12a in satisfactory yields (39%), that we consider satisfactory since some starting alcohol could be recovered by column chromatography. The unfluorinated β-glycoside 12a was accompanied by trace amounts of the α-epimer 13a (< 1%). Then, we performed the reaction with the fluorinate compounds 11b-c and observed the glycosides 12b-c (36% and 27% respectively) accompanied by more consistent amounts of their 2α-epimers 13b-c (20% and 24% respectively). This confirmed the literature observation according to which, the presence of a perfluorinated acylamido group at C-5 of sialic acid donors decreases the β-stereoselectivity of the glycosidation.61 The obtained glycosides showed the correct elemental analyses and chemical and physical properties in agreement with their structure. However, in spite to our expectations their complete NMR analysis did not allow to assign rationally their α or β-geometry that, at this point of the work, we assigned on the basis of the empirical rules for the sialosides,62 (this assignment was also in the reported short note disclosing the synthesis of 3a) i.e. we assigned the β-configuration to the anomers showing a relatively lower coupling constant between H-7 and H-8 sialic protons ($J_{H7,H8} = 1.6-1.9$ Hz for β-anomers and 8.7 Hz for α-anomers) and a relatively larger chemical shift difference between the two H-9 protons (around 0.87 ppm) in respect to the α-anomers ($\delta < 0.1$). Moreover, in the course of our work we unequivocally confirmed this assignment by chemical way, showing that only the sialosides deriving by an appropriate deprotection of the sialosides 12a-c, of assigned β-geometry, were able to form 1,7-lactones.

At this point, after some unsuccessful attempts to improve the yields of our glycosylation reactions, we decided to experiment the selective preparation of the glycoside 12a-c following an two step procedure, i.e. performing the glycosylation of the alcohol 9 with dibromine derivatives 14a-c, (92, 93, 95%) prepared by simple addition of bromine to the sialyl glycals methyl esters 15a-c. We were confident that, as first reported by T. Goto et al.,63 a dibromine sialosyl donor 14a-c, as a consequence of the trans geometry of its bromine atoms, reacts preferentially from the β-side. If, in our case, this effect exceeded the opposite influence of the perfluorinate acylamido groups at C-5, we ideally could obtain a quantitative selectivity in favor of the β-glycosides 16a-c (51, 48, 32%). This, by reductive elimination with (n-Bu)3SnH of the 3-bromine atom, could afford the desired β-glycoside 12a-c (81, 83, 87%) (Scheme 2).

In effect, the sialylation, performed at 23 °C, occurred in all cases in a short time affording exclusively the expected β-glycosides 16a-c that, by successive debromination, gave the glycosides 12a-c identical in all respects to those obtained above.

However, the overall yields of the two steps process resulted only slightly higher of those observed
in the one step preparation of the β-glycoside 12a-c, thus we considered this procedure as an alternative not at all attractive.

Scheme 2: Syntheses of: A) dibromine sialosyl donors 14a-c and alternative glycosylation reactions. Reagent and conditions: A) i: Br₂, CH₂Cl₂, 0°C to rt, 92, 95, 93%; B) ii: AgOTf, toluene/CH₂NO₂, rt, 3h, 51, 48, 32%; iii: (Bu)₃SnH, THF, reflux, 2h, 81, 83, 87%.

With the synthetized protected sialosides 12a-c, we performed the synthesis of the desired lactones using as common key intermediates the acids 3a-c obtained by a sequential regeneration of the protected functions according to the reaction sequence reported in the scheme (Scheme 3). This sequence was selected experiencing some less satisfactory results obtained reverting the regeneration of the protected functions. The sequence merits a few key observations concerning first of all the hydrolytic conditions found to remove the methyl ester function keeping the 5-acylamido group. This was easy in the case of the acetyl group that allowed, as expected, the use of the aqueous sodium hydroxide (on the contrary NaHCO₃ does not work, contrast with reference)¹ and in the case of the heptafluorobutyric amide that tolerates the methanolic K₂CO₃, in moist methanol. On the contrary, it was critical in the case of the trifluoroacetyl group that occurs in a suitable way only in the presence of triethylamine in aqueous methanol under strictly controlled conditions.

The obtained sialosides 3a-c showed physical-chemical properties (Mass spectra, NMR) in agreement with the assigned structures. In particular appear indicative for their β-structure the NMR analysis with a total proton and carbon resonance assignments, achieved by combination of 1D and 2D NMR experiments. These evidenced show clear proton signals shifted to relatively high fields (δ = 2.40-2.43 ppm) for the 3-equatorial hydrogen atoms of sialic acid portion, diagnostic for the β-sialoside bond in the molecules.⁶⁴ The relatively high fields shift was evident on comparing the chemical shift observed in the ¹H-NMR spectrum of the α-glicosides 3d and 3e (δ = 2.81-2.79
(ppm), obtained by regeneration of the protected functions of the α-glicosides 13b and 13c (Scheme 3). Indeed we prepared also α-isomers, 3e and 3d, and we controlled their $^1$H-NMR spectra, their inability to form an 1,7-lactone and their anti GM3 syntase activity.

The $^1$H-NMR attributed to the known glycoside 3a was also identifiable to that reported for the same compound, provided that some mistakes and erroneous attributions of the literature are carried out (compare experimental data).

Scheme 3: Synthesis of free sialyl-glycosides and 1,7 lactones: Reagent and conditions: i: moistly TFA, CH$_2$Cl$_2$ reflux 30 min-1h, 72-79%; ii: NaOMe, MeOH, rt, 1h, 88-91%; iii a: NaOH aq 1M, rt, 40 min, 90-92%; iii b: Et$_3$N, MeOH/H$_2$O, rt, 12h, 86-88%; iii c: K$_2$CO$_3$ H$_2$O/MeOH, rt, 92%; iv: CbzCl, Et$_3$N, THF/DMF (4:3; v/v), 0 °C to 23 °C, 1h, 70-71%; v: TFA aq (95% v/v), rt, 30 min, 75%.

With the appropriated hydroxyl acidic glycosides 3a-c in the hands, we attempted their direct 1,7-lactonization, using our protocol recently set-up for the preparation of 1,7-lactone of Neu5Ac 1 (Scheme 3). 65 The reaction afforded the desired 1,7-lactones 4a-c with different yields that were good for 4b-c, while were unsatisfactory for 4a. No lactones were obtained in the parallel reaction of the α-glycosides 3e and 3d. We considered these results in keeping with the geometry assigned to the starting glycosides that in this way was supported by chemical evidences. Moreover, considering that the unsatisfactory yields in the reaction of 3a was due to the lower solubility of this
glycoside in the reaction solvent, we decided to attempt the lactonization on the corresponding acetonide 17 (Scheme 3). In this case, the reaction occurs in satisfactory yield and affords the protected 1,7 lactone 18 in good yields. The regeneration of the hydroxylic groups of cytidine system of the lactone 4a with different acids, appeared not completely selective and difficult to control.

Thus, we accepted as the best conditions those using aqueous CF$_3$COOH (95%, 30 min, 23 °C) affording, in 75% yields, a mixture (65-10%) of the desired final lactone 4a and of its parent acid 3a. Moreover, we could separate the desired by reverse-phase preparative column chromatography. On the other hands, sample of all final compounds were purified by reverse-phase preparative column chromatography and lyophilisation this way before their use in biological assays.

Then, in order to achieve a further objective, which is to develop of cell-permeable inhibitors, I accomplish also the synthesis of the corresponding analogues protected as acetyl derivatives, compounds 5a-e.

In fact, we were supported by some literature studies, which demonstrated the effectiveness of administering the peracetylates derivatives of sialic acid as precursors readily converted to the corresponding free donor substrate analogs intracellularly in cell culture.$^{66}$ Thus, we prepared the protected compounds 5a-e, in agreement with the scheme below (Scheme 4), by simple deprotection of the hydroxylic cytidine portion of the sialosides 12a-e and reacetylation. The reactions work well and afforded the desired compounds in high yields (around 70%).

![Scheme 4: Synthesis of peracetylated analogues 5a-e.](image)

Scheme 4: Synthesis of peracetylated analogues 5a-e. *Reagents and conditions: i: mostly TFA, CH$_2$Cl$_2$ reflux 30 min-1h, 77-79%; ii: Ac$_2$O, Py, 23 °C, 3h 89-91%).*
Tested compounds

Acidic sialosides

1,7-lactone sialosides

Peracetylated sialosides
3.2 Biological results and discussion

Considering our decision to study donor-based ST inhibitors, we decided, at first, to test the ability to inhibit the enzyme activity of the acidic compounds 3a-e and of the lactones 4a-c on a bacterial α-2,3 sialyltransferase (from *Pasteurella multocida*). For this purpose we used a very simple and universal non-radioactive commercial assay that is versatile for the evaluation of the inhibition activity of compounds on purified STs. Then, in order to find new inhibitors of GM3 synthase, we decided to test our synthesize compounds on cell homogenate fraction containing GM3 synthase of Human Embryonic Kidney, HEK cells. For this we devised to use an HPTLC radioactivity imaging assay according to the procedure reported by Prinetti *et al.* since this assay was also applicable to non-purified enzymes. In the following section, I will describe the results obtained using all the synthesized compounds at 1 mM concentration and for the most promising compounds to a further concentration of 10 µM. Finally, we also report the data obtained performing assays on HEK cell cultures finalized to determine the variation of sphingolipid pattern in the presence of our inhibitors, using their peracetylated derivatives, compounds 5a-c and 5e.

Herein, I first discuss the results on a commercial α-2,3 sialyltransferase then the biological assays on GM3 synthase.

3.2.1 Inhibition on bacterial α-2,3 ST from *Pasteurella multocida*

We first tested the anti ST inhibitory activity of the acidic compounds 3a-e and of the lactones 4a-c on a bacterial α-2,3-ST. All compounds, were first purified, by preparative HPLC, and then tested for their possible anti-sialytrasferase activity using *N*-acetylactose amine as acceptor. I devised used a colorimetric assay reported by L. Wu *et al.* based on the evaluation of inorganic phosphate formed in the hydrolysis of the nucleotidic leaving group lost by CMP-sialic acid in the sialyltransferase reaction. The malachite green phosphate detection reagents that turn inorganic phosphate to a green colored complex allow us to evaluate the amount of inorganic phosphate released, that is equal to the CMP-sialic acid consumed or the sialyl-conjugate produced (see experimental section).

Compounds 3a-e and 4a-c afforded the inhibition results reported in the following table (Table 1).
Table 1: Inhibition of the bacterial ST from Pasteurella multocida by free acidic sialosides 3a–e and lactones 4a–c.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m$ (µM) or $K_i$ (µM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP-Neu5Ac</td>
<td>422 ± 9</td>
</tr>
<tr>
<td>3a</td>
<td>1312 ± 14</td>
</tr>
<tr>
<td>3b</td>
<td>402 ± 7</td>
</tr>
<tr>
<td>3c</td>
<td>109 ± 4</td>
</tr>
<tr>
<td>3d</td>
<td>406 ± 6</td>
</tr>
<tr>
<td>3e</td>
<td>128 ± 3</td>
</tr>
<tr>
<td>4a</td>
<td>602 ± 8</td>
</tr>
<tr>
<td>4b</td>
<td>215 ± 4</td>
</tr>
<tr>
<td>4c</td>
<td>65 ± 3</td>
</tr>
</tbody>
</table>

$^a$Each value represents the mean ± standard deviation of two or three independent experiments carried out in triplicate.

As evident from the $K_i$ of the tested compounds 3b-e and 4a-c, all of them are always noticeable lower than the $K_i$ of 3a and of the $K_m$ obtained in our experiment for CMP-Neu5Ac. Interesting, the fluorinated congeners 3b and 3c are three times or more than one order of magnitude active than 3a. Apparently, the 5N-heptafluorobutyrrate compounds 3c, 3e and 4c are the most actives. An additional increase of the inhibitory activity is observed in all lactones compounds 4a-c when compared with their respective acid analogues. Even more interesting, the $\alpha$-epimers 3d-e retain a comparable activity suggesting, in agreement with the few literature data, that the geometry at this center is not an essential requirement for the inhibitor-enzyme interaction.$^{27}$

Thus, we can conclude that both the presence of fluorine atoms and lactonization improve the inhibitory activity of the synthesize sialosides.
3.2.2 Inhibition of GM3 synthase

Then, considering the final aim of our work, we decided to test all the new compounds on the GM3 synthase.

Since, we were interested to confirm and evaluate the inhibitory activity of compound 3a, the only reported inhibitor of GM3 synthase, and to compare its inhibition activity with other prepared congeners, we performed an initial screening evaluation of all the synthesized compounds at the concentration of 1 mM. This concentration is lower than that (21 mM) used in the literature to obtain with the compound 3a 20% inhibition of the GM3 synthase in tissues.

In our experiments, almost initially, we could use a crude GM3 synthase obtained from HEK cells.\(^1\) In this way, we could select the most active compounds that in a second moment could be tested on a purified enzyme. Thus, we evaluated our compounds on cell homogenate fraction containing GM3 synthase using as acceptor substrate of the enzyme labeled LacCer, at the position three of sphingosine, according to Prinetti \textit{et al.}\(^3\)

In this way the activity of the obtained GM3 could be evaluated from the radioactivity incorporated in the molecule. As a scale we decided to consider as maximum (100%) the radioactivity associated to the GM3 formed in absence of inhibitors. We considered as the minimum (0%) the situation in which no reactivity was associated to GM3 and, in the meantime, all radioactivity was associated to the starting LacCer. The intermediate values were established by a mathematical extrapolation. Herein, I reported the results obtained on the free and lactonecompounds, I avoided to describe the results on protected compounds that result inactive at a concentration of 1 mM.

As we can observe from the results, reported in the table (Table 2), as % inhibition extent, compound 3a, its fluorinated analog 3b and the \(\alpha\)-heptafluorinate 3es how at 1 mM the 100% inhibition.
Table 2: Inhibition of cell homogenate fraction containing GM3 synthase of HEK cells by free acidic sialosides 3a–e and lactones 4a–c at a 1mM concentration.

Each value represents the mean ± standard deviation of two or three independent experiments carried out in triplicate, expressed as percentage of GM3 on positive control.

Data were average from a triplicate inhibition assay performed at 37° C for 3 hours. Moreover I repeated again the experiments with 3c and 3d thus confirming their behavior, that, surprisingly, was different from that of 3b and 3d.

Also lactones 4a-c show an unexpected behavior when compared with their acid analogues, in fact the unfluorinated lactone 4a is less active than the acid 3a, as in the case of the lactone 4b and its acid analogue 3b; on the contrary the lactone 4c is more active than 3c.

Moreover very interesting data are obtained with their 2α isomers 3e-d. In particular compound 3e displays a 100% inhibition activity, higher than its β-epimer.

Thus, all the synthesized compounds, having an inhibition activity lower than 10%, were tested at two order of magnitude lower concentration (10µM).
Table 3: Inhibition of cell homogenate fraction containing GM3 synthase of HEK cells by selected free acidic sialosides 3a, 3b, 3e and lactone 4c at a 10 µM concentration.

<table>
<thead>
<tr>
<th>GM3 synthase inhibitors (10 µM)</th>
<th>CT+</th>
<th>3a</th>
<th>3b</th>
<th>3e</th>
<th>4c</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM3 % on CTRL</td>
<td>100</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
</tbody>
</table>

Each value represents the mean ± standard deviation of two or three independent experiments carried out in triplicate, expressed as percentage of GM3 on positive control.

As evident from the data reported in table (Table 3), all the compounds tested at this concentration (10 µM), independently from their acid or lactonic structure, show an inhibitory activity in the range of 50% compared to the positive control. Interesting, all the tested compounds show an inhibitory activity higher than that of compound 3a, the most active compound being the 2α-isomer 3e.

3.2.3 Metabolic evaluation of sphingolipid pattern by treatment with peracetylated GM3 synthase inhibitors

Encouraged by the good results obtained on GM3 synthase of HEK cell homogenates, we decided to test the active substances in cell cultures suitable to determine also the sphingolipid pattern of HEK cells. For this we treated with inhibitors and [3-³H] sphingosine ([3-³H] SPH), a natural precursor of sphingolipids. With this assay, it could be possible to evaluate the cellular content of sphingolipids in terms of both quality and quantity, in presence or absence of our inhibitors, simply by an extraction of the metabolic radioactive products and HPTLC separation. In particular, we decided to evaluate the inhibitory activity of both lactones 3a-c and peracetylated methyl esters derived from free acids, compounds 5a-e. We tested the protected analogs considering that these molecules have an improved increased lipophilicity useful to cross membranes. The results obtained are reported in the table below (Table 4) and they are once again expressed considering 100% the GM3 formed in absence inhibitors.
Herein I reported are the results that, to date, are most promising, obtained for the protected derivatives of acids. However, further biological evaluation is necessary and merit additional work.

Table 4: Evaluation of radiolabelled GM3 on HEK cells treated by selected peracetylatedmethyl esters sialosides 5a-c, 5e at a 10 µM concentration versus the control.

![Radiolabeled GM3](image)

Each value represents the mean ± standard deviation of two or three independent experiments carried out in triplicate, expressed as percentage of GM3 on positive control.

As evident from the results reported in the picture and referred to triplicate experimentally all the tested compounds are actives. In particular, the peracetylated 5c, derivative of the fluorinated inhibitor 3c, shows the lower activity followed by the unfluorinated 5a. This result is in agreement with the trend of values found inhibition assay on homogenate fraction containing GM3 ST. On the contrary an evident improvement of inhibitory activity is observed for the perfluoroacetate 5b and for the heptafluorobutirrat α-epimers 5e. Thus, this result suggest that, once again, the most active inhibitors of GM3 at the moment known is the derivative 3e.

In the same experiments, in addition to the GM3 inspection, we also checked the levels of the other known gangliosides (GD1a, GM1 and GM2) present in the aqueous phase (Table 5a).
Table 5 (a) and (b): Radiolabeled sphingolipid distribution, aqueous (a) and organic phases (b), of HEK cells treated with inhibitors by metabolic labeling with sphingosine ([3-^3^H] SPH).

(a)

Radiolabeled Sphingolipid Distribution
(Aqueous Phase)

(b)

Radiolabelled Sphingolipid Distribution
(Organic Phase)

Each value represents the mean ± standard deviation of two or three independent experiments carried out in triplicate, expressed as percentage of GM3 on positive control.
The data reported for the treatment with the compounds synthesized 5a-c and 5e, show that, in our conditions, only the synthesis of ganglioside GM3 is significantly decreases (p< 0.0001). This suggests that the inhibition obtained is specific for GM3 synthase. Moreover, inspecting the data concerning the GM2, we could observe, almost in some cases, an a statically significant increase of the levels of GM2. This is in agreement with a literature showing that decreasing the GM3 biosynthesis causes a parallel increase of GM2. Also in this aspect in our opinion further studies and experimentations are required.

We evaluated also the incorporation of labeled sphingosine in soluble organic fraction of cells (Table 5b). This in order to inspect the complete sphingolipidic pattern, as reported in the picture on organic fraction containing neutral lipids sphingomyelin (SM), globoside 3(Gb3), glucosylceramide (GlcCer), ceramide (Cer). As evident from the results, unchanged picture of natural lipids is observed. This appears to suggest that all our inhibitors, independently from their activity level, are all specific for GM3 synthesis. This is a result noticeable for future researches.
4. CONCLUSIONS
4. CONCLUSIONS

In conclusion, I have synthesized, by an unreported procedure, eight sialoside congeners of the natural sialyl donor CMP-Neu5Ac.

The synthetic protocol, set up for the glycosidation, allows to assign the structure of all compounds beyond any reasonable doubt using both NMR evidences of α and β epimers and the successive exclusive 1,7 lactonization of the β-epimers. The preparation of the reported inhibitor 3a allowed also to set up some capricious reaction conditions reported in the synthesis and to correct NMR assignment of the few reported intermediate and of the final compound.

Interesting, the availability of the α-epimers allowed us to evidence that the α and β geometry of the glycoside bound of the CMP-Neu5Ac mimic inhibitors is irrelevant or even ameliorative of the inhibitory activity of the compounds.

This observation, together with the final results showing that an acetylation of the hydroxyl and amine group of the cytidine portion and the saccharide portion facilitates the cell permeability provide interesting structural formation for the development of new STs inhibitors.

The set up of the synthetic general method for the preparation of the active inhibitors appear useful for a rational programming of new inhibitory, even with computational chemistry support.

Concerning biological experiments devoted to ascertain the inhibitory activity of the compounds, efforts are still necessary both to purify GM3 synthase and to avoid the use of radioactivity. In this respect, deuterium labelled precursory offer more convenient opportunity for further studies.
5. EXPERIMENTALS
5. EXPERIMENTAL

5.1 Chemical materials and methods:
All chemicals used were special (specific) grade unless otherwise specified, were purchased from commercial source. Water was prepared by filtering deionized water on a Milli-Q Simplicity 185 filtration system from Millipore (Bedford, MA, USA). Discovery DSC-18 SPE tubes for sample clean up were purchased from Supelco (Bellefonte, PA, USA).
Solvents were dried using standard methods and distilled before use. The progress of all reactions was monitored by thin-layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60 F254) using UV light, 50% sulphuric acid, anisaldehyde/H$_2$SO$_4$/EtOH solution or 0.2% ninhydrin in ethanol and heat as developing agent. All flash chromatography was performed with normal phase silica gel (E. Merck 230-400 mesh silica gel), following the general protocol of Still[1].
Melting points were measured on a SMP3 mp apparatus (Stuart Scientific, USA) and are not corrected. NMR spectra were recorded at 25°C on a Bruker AM-500 spectrometer operating at 500.13 MHz for $^1$H and 125.76 MHz for $^{13}$C. The chemical shifts are reported in ppm and coupling constant are given in Hz, relative to CD$_3$OD signal fixed at 3.31 ppm for $^1$H spectra and to CD$_3$OD signal fixed at 49.05 ppm for $^{13}$C spectra, to (DMSO)-$d_6$ signal fixed at 2.50 ppm for $^1$H spectra and to (DMSO)-$d_6$ signal fixed at 39.52 ppm for $^{13}$C spectra, relative to CDCl$_3$ signal fixed at 7.26 ppm for $^1$H spectra and to CDCl$_3$ signal fixed at 77.00, and to internal (CH$_3$)$_3$COH 1.24 ppm and 30.29 ppm for solutions in D$_2$O.
Proton and carbon assignments were established, if necessary, with $^1$H-$^1$H and $^1$H-$^{13}$C correlated NMR experiments. Data for $^1$H NMR are recovered as follows: chemical shift (ppm), multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br , broad), coupling constant(s) in Hz, number of protons, assignment of proton(s).
Optical rotations were taken on a Perkin-Elmer 241 polarimeter equipped with a 1 dm tube; [a]D values are given in 10$^{-1}$deg cm$^2$ g$^{-1}$ and the concentration are given in g/100 mL. Mass spectrometry was performed using Finnigan LCQDeca quadrupole ion-trap mass spectrometer equipped with an ESI ion source (Finnigan ThermoQuest, San Jose, CA, USA). The spectra were collected in continuous flow mode by connecting the infusion pump directly to the ESI source. Solutions of compounds were infused at a flow rate of 5 mL/min.
The spray voltage was set at 5.0 kV in the positive and at 4.5 kV in the negative ion mode with a capillary temperature of 220 °C. Full-scan mass spectra were recorded by scanning a m/z range of 100-2000. All described compounds showed a purity >98%, as determined by HPLC (UV and MS
detectors). LC-UV/MS data were collected with an Agilent 1100 HPLC connected to a Bruker Esquire 3000+ ion trap mass spectrometer through an ES interface.

5.2 Biological materials and methods:

**Inhibition activity assay on commercial α₂,3-sialyltransferase.** Commercial chemicals were of analytical grade or the highest purity available. α-2,3-Sialyltransferase from *Pasteurella multocida*, CMP-sialic acid, N-acetyl-D-lactosamine were purchased from commercial sources. Malachite Green Phosphate Detection Kit was from R&D Systems. Solvents were distilled before use. The water routinely used was freshly distilled on a glass apparatus. ST activity was measured with a microplate reader (Victor 3, PerkinElmer) set at 620 nm wavelength.

**Inhibition activity assay on GM3 synthase.** Commercial chemicals were of analytical grade or the highest purity available. Human Embryonic Kidney, HEK293A (Invitrogen). Protein concentration was determined by a JASCO V-530 spectrophotometer. The cell pellet was homogenized with a Dounce homogenizer.

[3-3H(sphingosine)]LacCer and other radioactive lipid, used as reference standards was from Prof. Bruno Venerando group that collaborated to my thesis work.

High performance silica gel-precoated thin-layer plates (HPTLC Kieselgel 60) were purchased from Merck (Darmstadt, Germany). Radioactive lipids were visualized with a Beta-Imager 2000 (Biospace, Paris, France) and identified by comparison with radiolabeled standards.

**Sphingolipid pattern evaluation by metabolic labeling with [3-3H] sphingosine ([3-3H] SPH) in HEK cells treated with inhibitors.**

Commercial chemicals were of analytical grade or the highest purity available. Human Embryonic Kidney, HEK293A (Invitrogen). Protein concentration was determined by a JASCO V-530 spectrophotometer. [3-3H]sphingosine (19.8 Ci/mmol) was provided by PerkinElmer (Waltham, MA). The radioactive lipid , used as reference standards was from Prof. Bruno Venerando group that collaborated to my thesis work.

High performance silica gel-precoated thin-layer plates (HPTLC Kieselgel 60) were purchased from Merck (Darmstadt, Germany).

Radioactive lipids were visualized with a Beta-Imager 2000 (Biospace, Paris, France) and identified by comparison with radiolabeled standards.
5.3 Chemistry

5.3.1 Synthetic procedure affording alcohol 9.

Aldehyde 6 (1.20 g, 3.71 mmol) was dissolved in CH$_2$Cl$_2$ (60 mL), added by (triphenylphosphoranylidene) acetaldehyde (1.36 g, 4.46 mmol) and stirred overnight at 23 °C. Then, the orange solution was concentrated in vacuo to give crude aldehyde 7. Then, the crude mixture of aldehyde 6, dissolved in MeOH(100 mL), was directly hydrogenated in the presence of Pd on carbon 10% (160 mg) for 2 h. At this time, the catalyst was filtered, washed with MeOH (60 mL) and the solvent was then evaporated under reduced pressure to afford the crude compound 8. This intermediate was dissolved in MeOH (20 mL) and the reaction was cooled at -20 °C. At this time, NaBH$_4$ (158 mg, 2.05 mmol) was added and after 15 minutes the reaction was stopped with the addition of acetone (4 mL), then neutralized with Amberlite resin IRC-50 (H$^+$). The resin was filtered, washed with MeOH (30 mL) and the solvent was then evaporated under reduced pressure to afford, after purified by chromatography on silica gel (eluting with AcOEt/MeOH, 9:1 v/v), the compound 9 (590 mg, 45%), as white powder: m.p. 97–98°C (from CH$_2$Cl$_2$-diisopropyl ether); $[\alpha]_D^{20}$ = +31.2 (c 1.0 in methanol). $^1$H NMR (CDCl$_3$): $\delta$ = 9.71 (s, 1H; NH), 7.72 (d, $J_{6,5} = 7.5$ Hz; 1H, H-6), 7.42 (d, $J_{5,6} = 7.5$ Hz, 1H, H-5), 5.65 (d, $J_{1',2'} = 1.6$ Hz, 1H; H-1’), 4.97 (dd, $J_{2',3'} = 6.6$, $J_{2',1'} = 1.6$ Hz, 1H; H-2’), 4.62 (1H, br d, $J_{3',2'} = 6.6$, $J_{3',4'} = 4.6$ Hz, 1H; H-3’), 4.19–4.16 (m, 1H; H-4’), 3.67–3.64 (overlapping, 2H; H-7a’ and H-7b’), 2.25 (s, 3H; NHCOCH$_3$ at C-4), 1.87–1.78 (overlapping, 2H; H-5a’ and H-5b’), 1.73–1.67 (overlapping, 2H; H-6a’ and H-6b’), 1.56 (s, 3H; C(CH$_3$)$_2$), 1.33 ppm (s, 3H; C(CH$_3$)$_2$). MS (ESI positive)$m/z$: 376.2 [M+Na]$^+$, 729.1 [2M+Na]$^+$. 11.89. Other physico-chemical properties were identical to those reported in literature.$^1$
The characterization of intermediate aldehydes 6, 7 and 8 purified only in small quantities.

1H NMR (CD$_3$)$_2$SO: $\delta = 9.75$, (s, 1H; NHAc), 9.37 (s, 1H; CHO), 7.68 (d, $J_{6,5} = 7.6$ Hz, 1H; H-6), 7.55 (d, $J_{5,6} = 7.6$ Hz, 1H; H-5), 5.60 (s, 1H; H-1'), 5.30 (br d, $J_{2',3'} = 6.2$ Hz, 1H; H-2'), 5.16 (br d, $J_{3',4'} = 6.2$ Hz, 1H; H-3'), 4.58 (s, 1H; H-4'), 2.30 (s, 3H; OCOCH$_3$ at C-4), 1.55 (s, 3H; C(CH$_3$)$_2$), 1.38 (s, 3H; C(CH$_3$)$_2$); MS (ESI positive) $m/z$: 323.9. [M+H]$^+$. Other physico-chemical properties were identical to those reported in literature.$^{60}$

Aldehyde 7 showed: m.p. 183°C (from CH$_2$Cl$_2$-diisopropyl ether); $\left[\alpha\right]_D^{20} = +24.7$ ($c = 1$ in CHCl$_3$). 1H NMR (CDCl$_3$): $\delta = 9.56$ (d, $J_{7,6'} = 7.8$ Hz, 1H; H-7'), 8.95 (s, 1H; NH), 7.60 (d, $J_{6,5} = 7.5$ Hz, 1H; H-6), 7.43 (d, $J_{5,6} = 7.5$ Hz, 1H; H-5), 7.00 (dd, $J_{5',6'} = 6.2$ $J_{6',6'} = 15.8$, 1H; H-5'), 6.23 (dd, $J_{6',7'} = 7.8$ $J_{6',5'} = 15.8$, 1H; H-6') 5.57 (br s, 1H; H-1'), 5.25 (br d, $J_{2',3'} = 6.3$, 1H; H-2'), 5.07 (dd, $J_{3',4'} = 3.9$, $J_{3',2'} = 6.3$ Hz, 1H; H-3'), 4.87–4.83 (m, 1H; H-4'), 2.25 (s, 3H; NHCOCH$_3$ at C-4), 1.58 (s, 3H; C(CH$_3$)$_2$), 1.36 ppm (s, 3H; C(CH$_3$)$_2$); 13C NMR (CDCl$_3$): $\delta = 193.2$ (C-7'), 170.1 (NHCOCH$_3$), 163.3 (C-4), 154.6 (C-2), 153.0 (C-5'), 147.9 (C-6), 132.3 (C-6'), 114.3 (C(CH$_3$)$_2$), 99.0 (C-1'), 96.8 (C-5), 89.0 (C-4'), 85.2 (C-3'), 85.0 (C-2'), 27.0 (C(CH$_3$)$_2$), 25.2(C(CH$_3$)$_2$), 25.0 (NHCOCH$_3$). MS (ESI positive)$m/z$: 372.1 [M+Na]$^+$. Elemental analysis calcd (%) for C$_{16}$H$_{19}$N$_3$O$_6$: C 55.01, H 5.48, N 12.03; found C 54.86, H 5.34, N 11.92.
Aldehyde 8 showed: m.p. 185°C (from CH₂Cl₂-diisopropyl ether); [α]D<sup>20</sup> = +23.4 (c = 1 in CHCl₃).

<sup>1</sup>H NMR (CDCl₃): δ = 9.75 (br s, 1H; H-7’), 9.23 (s, 1H; NH), 7.63 (d, J₆,₅ = 7.4 Hz, 1H; H-6), 7.42 (d, J₅,₆ = 7.4 Hz, 1H; H-5), 5.60 (br s, 1H; H-1’), 5.04 (br d, J₂,₃’ = 6.5; 1H, H-2’), 4.71-4.67 (m, 1H; H-3’), 4.14-4.0 8 (m, 1H; H-4’), 2.62-2.58 (overlapping, 2H; H-6a’ and H-6b’), 2.26 (s, 3H; NHCOCH₃ at C-4), 2.19-2.03 (overlapping, 2H; H-5a’ and H-5b’), 1.55 (s, 3H; C(CH₃)₂), 1.33 ppm (s, 3H; C(CH₃)₂); <sup>13</sup>C NMR (CDCl₃): δ = 201.2 (C-7’), 170.4 (NHCOCH₃), 163.1 (C-4), 154.5 (C-2), 146.6 (C-6), 114.5 (C(CH₃)₂), 96.7 (C-5), 96.2, (C-1’), 87.2 (C-4’), 84.9(C-2’), 83.8 (C-3’), 40.0 (C-6’), 27.2 (C(CH₃)₂), 25.5 (C-5’), 25.3 (C(CH₃)₂), 25.0 (NHCOCH₃) ppm. MS (ESI positive)m/z: 374.6 [M+Na]<sup>+</sup>. Elemental analysis calcd (%) for C₁₆H₂₁N₃O₆: C 54.69, H 6.02, N 11.96; found C 54.35, H 5.87, N 12.06.
5.3.2 General procedure to synthesize chloro derivatives 11a-c.

To a solution of selected compound 10a-c (0.30 mmol), dissolved in acetyl chloride (35 mL) and cooled at -10°C under argon atmosphere, anhydrous methanol (5 mL) was added. The solution was stirred at room temperature overnight then the mixture was evaporated in vacuo to give a syrup, which was crystallized from hexane-ethyl acetate to affording the desired derivatives 11a-c.

Synthesis of compound 11a.

Starting from the peracetylated Neu5Ac methyl ester 10a (160 mg, 0.3 mmol) the 2-chloro peracetylated Neu5Ac methyl ester 11a (117 mg, 77%) was obtained as only stereoisomer as a white solid. $^1$H NMR (CDCl$_3$): $\delta$ = 5.47 (dd, $J_{7.6} = 2.4$, $J_{7.8} = 7.0$ Hz, 1H; H-7), 5.44 (d, $J_{NH.5} = 10.2$ Hz, 1H; NH), 5.39 (ddd, $J_{4.3a} = 4.8$, $J_{4.5} = 10.4$, $J_{4.3b} = 11.0$ Hz, 1H; H-4), 5.17 (ddd, $J_{8.9a} = 2.7$, $J_{8.9b} = 5.8$, $J_{8.7} = 7.0$ Hz, 1H; H-8), 4.42 (dd, $J_{9a.8} = 2.7$, $J_{9a.9b} = 12.5$ Hz, 1H; H-9a), 4.35 (dd, $J_{6.7} = 2.4$, $J_{6.5} = 11.3$ Hz, 1H; H-6), 4.20 (ddd, $J_{5.NH} = 10.2$, $J_{5.4} = 10.4$, $J_{5.6} = 11.3$ Hz, 1H; H-5), 4.06 (dd, $J_{9b.8} = 5.8$, $J_{9b.9a} = 12.5$ Hz, 1H; H-9b), 3.87 (s, 3H; COOCH$_3$), 2.78 (dd, $J_{3a.4} = 4.8$, $J_{3a.3b} = 13.9$ Hz, 1H; H-3a), 2.28 (dd, $J_{3b.3a} = 11.2$, $J_{3a.3b} = 13.9$ Hz, 1H; H-3b), 2.12 (s, 3H; OCOCH$_3$), 2.05 (s, 3H; OCOCH$_3$), 2.04 (s, 3H; OCOCH$_3$), 2.03 (s, 3H; OCOCH$_3$), 1.91 ppm (s, 3H; NHCOCH$_3$).MS (ESI positive) $m/z$: 532.9 [M+Na]$^+$. Elemental analysis calcld (%) forC$_{20}$H$_{28}$ClNO$_{12}$: C 47.11, H 5.53, N 2.75; found C 47.18, H 5.61, N 2.81. Other physico-chemical properties were identical to those reported in literature.$^{69}$

Synthesis of compound 11b.
Starting from the N-trifluoroacetamido peracetylated Neu5Ac methyl ester 10b $^{59}$ (176 mg, 0.30 mmol) and the 2-chloro derivative 11b (137 mg, 81%) was obtained as only stereoisomer as a white solid: m.p. 132°C; [α]D $^{20}$ = +251.6 (c = 1 in CHCl3). $^1$H NMR (CDCl3): δ = 6.53 (d, J$_{NH,5}$ = 10.1 Hz, 1H; NH), 5.53 (ddd, J$_{4,3a}$ = 4.9, J$_{4,5}$ = 10.2, J$_{4,3b}$ = 11.3Hz, 1H; H-4), 5.44 (dd, J$_{7,6}$= 2.3, J$_{5,8}$ = 7.2 Hz, 1H; H-7), 5.19 (ddd, J$_{8,9a}$ = 2.6, J$_{8,9b}$ = 5.3, J$_{8,7}$ = 7.2Hz, 1H; H-8), 4.51 (dd, J$_{6,7}$= 2.3, J$_{6,5}$= 10.7 Hz, 1H; H-6), 4.40 (dd, J$_{9a,8}$ = 2.6, J$_{9a,9b}$ = 12.6 Hz, 1H; H-9a), 4.13 (dd, J$_{5,NH}$ = 10.1, J$_{5,4}$ = 10.2, J$_{5,6}$= 10.7 Hz, 1H; H-5), 4.08 (dd, J$_{9b,8}$ = 5.3, J$_{9b,9a}$ = 12.6 Hz, 1H; H-9b), 3.89 (s, 3H; COOCH3), 2.83 (dd, J$_{3a-4}$ = 4.9, J$_{3a,3b}$ = 14.0 Hz, 1H; H-3a), 2.31 (dd, J$_{3b,3a}$= 11.3, J$_{5a,3b}$= 14.0 Hz, 1H; H-3b), 2.13 (s, 3H; OCOCH3), 2.09 (s, 3H; OCOCH3), 2.05 ppm (s, 6H; 2 X OCOCH3). $^{13}$C NMR (CDCl3): δ = 170.8, 170.6, 170.4, 169.5 (4C, OCOCH3), 156.5 (C-1), 157.6 (1C, J$_{CF}$ = 38.0 Hz, COCF3), 115.3 (C, J$_{CF}$ = 287.9 Hz, COCF3), 96.0 (C-2), 73.1 (C-6), 70.7 (C-8), 68.1 (C-4), 66.9 (C-7), 62.0 (C-9), 53.7 (COOCH3), 49.0 (C-5), 40.4 (C-3), 20.7, 20.5, 20.4 (4C, OCOCH3) ppm. MS (ESI positive) m/z: 586.2[M+Na]$^+$. Elemental analysis calcd (%) for C$_{20}$H$_{25}$ClF$_{3}$NO$_{12}$: C 42.60, H 4.47, N 2.48; found C 42.52, H 4.48, N 2.63.

**Synthesis of compound 11c**

Starting from the N-trifluoroacetamido peracetylated Neu5Ac methyl ester 10c $^{59}$ (206 mg, 0.30 mmol) and the 2-chloro derivative 11c (160mg, 80%) was obtained as only stereoisomer as a white solid: m.p. 128°C; [α]D $^{20}$ = +125.6 (c = 1 in CHCl3). $^1$H NMR (CDCl3): δ = 7.32 (d, J$_{NH,5}$ = 9.7 Hz, 1H; NH), 5.55 (ddd, J$_{4,3a}$ = 4.9, J$_{4,5}$ = 10.3, J$_{4,3b}$ = 11.2Hz, 1H; H-4), 5.43 (dd, J$_{7,6}$= 2.2, J$_{5,8}$ = 5.7 Hz, 1H; H-7), 5.15 (ddd, J$_{8,9a}$ = 2.5, J$_{8,7}$ = 5.7, J$_{8,9b}$ = 6.4 Hz, 1H; H-8), 4.57 (dd, J$_{6,7}$= 2.2, J$_{6,5}$= 10.7 Hz, 1H; H-6), 4.50 (dd, J$_{9a,8}$ = 2.5, J$_{9a,9b}$ = 12.5 Hz, 1H; H-9a), 4.18 (ddd, J$_{5,NH}$= 9.7, J$_{5,4}$ = 10.3, J$_{5,6}$ = 10.7 Hz, 1H; H-5), 4.10 (dd, J$_{9b,8}$ = 6.4, J$_{9b,9a}$ = 12.5 Hz, 1H; H-9b), 3.88 (s, 3H; COOCH3), 2.83 (dd, J$_{3a-4}$ = 4.9, J$_{3a,3b}$ = 13.9 Hz, 1H; H-3a), 2.24 (dd, J$_{3b,3a}$= 11.2, J$_{3a,3b}$= 13.9 Hz, 1H; H-3b), 2.13 (s, 3H; OCOCH3), 2.10 (s, 3H; OCOCH3), 2.04 (s, 3H; OCOCH3), 2.03 ppm (s, 3H; OCOCH3); $^{13}$C NMR (MeOD): δ = 170.6 (4C, OCOCH3), 169.7(C-1), 158.1 (1C, J$_{CF}$ = 26 Hz, COCF2CF2CF3), 124.0-110.0 (3C, COCF2CF2CF3), 95.9 (C-2), 73.1 (C-6), 70.8 (C-4), 67.8 (C-8), 67.0 (C-7), 62.0 (C-9), 53.9 (COOCH3), 49.5 (C-5), 40.6 (C-3), 20.8, 20.6, 20.5, 20.4 (4C,
CH₃COO) ppm. MS (ESI positive) m/z: 686.3[M+Na]⁺. Elemental analysis calcd (%) for C₂₂H₂₅ClF₇NO₁₂: C 39.80, H 3.80, N 2.11; found C 39.72, H 3.78, N 2.15.
5.3.3 General procedure to synthesize dibromo derivatives 14a-c.

To a solution of appropriate compound 15a-c (0.60 mmol) in dichloromethane (5mL) was added bromine (0.80 mmol) at 0°C under argon atmosphere. After stirring for 30 minutes at room temperature the mixture was evaporated in vacuo to give a syrup, which was crystallized from hexane-ethyl acetate to afford the analougs 2,3-dibromo derivative 14a-c.

Synthesis of compound 14a.

Starting from 15 (284 mg, 0.60 mmol), the 2,3-dibromo derivative 14a (383 mg, 93%) was obtained as a white needles showing: \(^1\)H NMR (CDCl\(_3\)): \(\delta = 5.86\) (d, \(J_{NH,5} = 9.6\) Hz, 1H; NH), 5.69 (dd, \(J_{4,3} = 3.5, J_{4,5} = 10.5\)Hz, 1H; H-4), 5.41 (dd, \(J_{7,6} = 2.1, J_{7,8} = 6.8\) Hz, 1H; H-7), 5.20 (ddd, \(J_{8,9a} = 2.3, J_{8,9b} = 5.8, J_{8,7} = 6.8\) Hz, 1H; H-8), 5.02 (d, \(J_{3,4} = 3.4\) Hz, 1H; H-3), 4.58 (dd, \(J_{5,NH} = 9.6, J_{5,4} = J_{5,6} = 10.5\)Hz, 1H; H-5), 4.47-4.40 (overlapping, 2H; H-6 and H-9a), 4.15-4.07 (m, 1H; H-9b), 3.88 (s, 3H; COOCH\(_3\)), 2.14 (s, 3H; OCOCH\(_3\)), 2.09 (s, 3H; OCOCH\(_3\)), 2.06 (s, 3H; OCOCH\(_3\)), 2.03 (s, 3H; OCOCH\(_3\)), 1.93 ppm (s, 3H, NHCOCH\(_3\)). All other physicochemical properties practically superimposable to those previously reported.\(^{70}\)

Synthesis of compound 14b.

Starting from 15b (316 mg, 0.60 mmol), the 2,3-dibromo derivative 14b (392 mg, 95%) was as a white needles, showing: \([\alpha]_D^{20} = -30.1\) (c = 1 CHCl\(_3\)).\(^1\)H NMR (CDCl\(_3\)): \(\delta = 7.00\) (d, \(J_{NH,5} = 9.1\) Hz, 1H; NH), 5.84 (dd, \(J_{4,3} = 3.2, J_{4,5} = 10.4\)Hz, 1H; H-4), 5.40 (d app, \(J_{7,8} = 6.6\) Hz, 1H; H-7), 5.27-5.23 (m, 1H; H-8), 5.06 (d, \(J_{3,4} = 3.2\) Hz; 1H; H-3), 4.59 (d app, \(J_{6,5} = 10.8\) Hz; 1H, H-6), 4.52-4.45 (overlapping, 2H; H-5 and H-9a), 4.19 (dd, \(J_{9b,8} = 5.5, J_{9b,9a} = 12.6\) Hz, 1H; H-9b), 3.92 (s, 3H; COOCH\(_3\)), 2.17 (s, 3H; OCOCH\(_3\)), 2.11 (s, 3H, OCOCH\(_3\)), 2.10 (s, 3H; OCOCH\(_3\)), 2.05 ppm (s, 3H; OCOCH\(_3\)); \(^{13}\)C NMR (CDCl\(_3\)): \(\delta = 170.7, 170.6, 170.3, 170.1\) (4C, OCOCH\(_3\)), 163.8 (C-1),
157.6 (1C, $J_{C,F} = 38.0$ Hz, COCF$_3$), 115.3 (C, $J_{C,F} = 287.9$ Hz, COCF$_3$), 91.0 (C-2), 75.3 (C-6), 70.6 (C-8), 68.1 (C-4), 66.9 (C-7), 61.9 (C-9), 54.4 (COOCH$_3$), 52.6 (C-3), 46.1 (C-5), 20.9, 20.6, 20.4 (4C, OCOCH$_3$) ppm.; MS (ESI positive) M+Na$^+$ $m/z$: 710.3 [M+Na]$^+$. Elemental analysis calcd (%) for C$_{20}$H$_{24}$Br$_2$F$_3$NO$_{12}$: C 34.96, H 3.52, N 2.04; found C 34.85, H 3.48, N 2.09.

**Synthesis of compound 14c.**

Starting from 14c $^{71}$ (376 mg, 0.60 mmol), the 2,3-dibromo derivative 13c (439 mg, 93%) was as a white needles, showing: $[\alpha]_D^{20} = -18.5$ ($c = 1$ in CHCl$_3$). $^1$H NMR (CDCl$_3$): $\delta = 7.29$ (d, $J_{NH,5} = 8.8$ Hz, 1H; NH), 5.89 (dd, $J_{4,3} = 3.5$, $J_{4,5} = 10.6$Hz, 1H; H-4), 5.37 (dd, $J_{7,6} = 1.8$, $J_{7,8} = 6.3$ Hz; 1H, H-7), 5.25 (ddd, $J_{8,9a} = 2.3$, $J_{8,9b} = 5.7$, $J_{8,7} = 6.3$ Hz, 1H; H-8), 5.08 (d, $J_{3,4} = 3.5$ Hz; 1H, H-3), 4.64 (dd, $J_{6,7} = 1.8$, $J_{6,5} = 10.8$ Hz, 1H, H-6), 4.52 (dd, $J_{9a,8} = 2.3$, $J_{9b,9a} = 12.6$ Hz, 1H, H-9a), 4.43 (ddd, $J_{5,NH} = 8.8$, $J_{5,4} = 10.6$, $J_{5,6} = 10.8$ Hz, 1H; H-5), 4.22 (dd, $J_{9b,8} = 5.7$, $J_{9b,9a} = 12.6$ Hz, 1H; H-9b), 3.91 (s, 3H; COOCH$_3$), 2.18 (s, 3H; OCOCH$_3$), 2.09 (s, 6H; 2 X OCOCH$_3$), 2.04 ppm (s, 3H; OCOCH$_3$). $^{13}$C NMR (MeOD): $\delta = 170.6$, 170.5, 170.4, 169.8 (4C, OCOCH$_3$), 163.8(C-1), 158.0 (1C, $J_{C,F} = 38.0$ Hz, COCF$_3$), 125.0-110.0 (3C, COCF$_2$CF$_2$CF$_3$), 90.6 (C-2), 74.7 (C-6), 70.4 (C-8), 67.7 (C-4), 67.0 (C-7), 61.8 (C-9), 54.0 (COOCH$_3$), 52.8 (C-3), 46.1 (C-5), 20.8, 20.6, 20.5, 20.3 ppm (4C, OCOCH$_3$). MS (ESI positive) $m/z$: 810.3 [M+Na]$^+$. Elemental analysis calcd (%) for C$_{22}$H$_{24}$Br$_2$F$_7$N$_4$O$_{12}$: C 33.57, H 3.07, N 1.78; found C 33.49, H 2.98, N 1.79.
5.3.4 Synthetic glycosilation procedures affording to derivatives 12a-c and 13b-c

**Procedure A**: general glycosilation procedure via chloro derivatives

![Chemical structure of 11a-c](image1)

To a stirred solution of the appropriate chlorine derivative 11a-c (0.95 mmol) and of alcohol 8 (265 mg, 0.75 mmol) in anhydrous mixture of toluene (4.0 mL) and nitromethane (4.0 mL), containing molecular sieves 4Å, was added a solution of AgOTf (283 mg, 1.10 mmol) dissolved in toluene (2.0 mL) and nitromethane (2.0 mL) at room temperature under argon. The crude was stirred for 3h at room temperature and filtered through celite bed and solid was washed with AcOEt/MeOH mixture. The combined filtrates were evaporated in vacuo to a residue, that was purified by chromatography on silica gel to afford the desired compounds 12a-c and 13a-c.

**Procedure B**: General glycosilation procedure via dibromo derivatives (step 1) and reductive dibromination (step 2)

![Chemical structure of 14a-c and 16a-c](image2)

a: R=CH₃  
b: R=CF₃  
c: R=C₃F₇

![Chemical structure of 12a-c](image3)
(Step 1) To a stirred solution of the appropriate dibromide 14a-c (1.10 mmol) and of alcohol 8 (265 mg, 0.75 mmol), in anhydrous mixture of toluene (4.0 mL) and nitromethane (4.0 mL), containing molecular sieves 4Å, was added a solution of AgOTf (308 mg, 1.20 mmol) dissolved in toluene (2.0 mL) and nitromethane (2.0 mL) at room temperature under argon. The crude was stirred for 3h at room temperature and filtered through celite bed and solid was washed with AcOEt/MeOH mixture. The combined filtrates and washing were evaporated in vacuo to a residue, that was purified by chromatography on silica gel, to afford the desired compounds 16a-c.

(Step 2) To a solution of compound 16a-c (0.10 mmol) and in THF (5 mL) was added tri-n-butyltin hydride (0.06 mL, 0.20 mmol), and the reaction was refluxed for 3h. Then the reaction was concentrated and the residue was triturated with diisopropyl ether and hexane to afford the appropriate compounds 12a-c.
Synthesis of compounds 12a and 13a

i) Starting from compound 11a (484 mg; 0.95 mmol) and operating according to procedure A, the compound 12a was obtained as a first eluate, after rapid chromatography (AcOEt/MeOH, 98:2 v/v to AcOEt/MeOH, 80:20 v/v), as a white solid (241 mg, 39%) together with trace of its α-anomer 13a as a second white solid eluate (mg, <1%).

Compound 12a showed: [α]D^20 = -7.2 (c = 1 MeOH).\(^1\)H NMR (CDCl₃): δ = 9.20 (s, 1H, NH at C-4), 7.90 (d, J_{NH,5″} = 9.9 Hz, 1H; NH at C-5″), 7.61 (d, J_{6,5} = 7.4 Hz, 1H; H-6), 7.43 (d, J_{5,6} = 7.4 Hz, 1H; 5-H), 5.89 (dd, J_{5″,8″} = 1.6, J_{7″,6″} = 3.0 Hz, 1H; H-7″), 5.68 (dd, J_{2,1} = 1.2, J_{2,3} = 6.3, 1H; H-2″), 5.43 (br s, 1H; H-1″), 5.35 (dd, J_{4″,3a″} = 4.9, J_{4″,5″} = 10.5, J_{4″,3b″} = 11.1 Hz, 1H; H-4″), 5.11 (dd, J_{8″,7″} = 1.6, J_{8″,9a″} = 2.0, J_{8″,9b″} = 9.5 Hz, 1H; H-8″), 5.00-4.95 (overlapping, 2H; H-3′ and H-9a′″), 4.54 (dd, J_{6″,5″} = 10.6, J_{6″,7″} = 3.0 Hz, 1H; H-6″), 4.28-4.19 (overlapping; 2H, H-4″ and H-5″), 4.12 (dd, J_{9b″,9a″} = 12.0, J_{9b″,9a″} = 9.5 Hz, 1H; H-9b″), 3.80 (s, 3H; COOCH₃), 3.58-3.54 (overlapping, 2H; H-7a″ and H-7b″), 2.60 (dd, J_{3a″,3b″} = 12.7, J_{3″,4″} = 4.9 Hz, 1H; H-3a″), 2.26 (s, 3H; NHOCH₃ at C-4), 2.23 (s, 3H; COOCH₃), 2.06 (s, 3H; OCOCH₃), 1.97 (s, 3H; OCOCH₃), 1.92 (s, 3H; OCOCH₃), 1.81 (s, 3H; NHOCH₃ at C-5″), 1.76-1.59 (overlapping, 4H, H-5a″, H-5b″, H-6a″ and H-6b″), 1.57 (s, 3H; C(CH₃)₂), 1.41 ppm (s, 3H; C(CH₃)₂).

\(^1\)H NMR (MeOD): δ = 8.05 (d, J_{6,5} = 7.5 Hz, 1H, H-6), 7.42 (br d, J_{5,6} = 7.5 Hz, 1H, H-5), 5.83 (d, J_{1″,2″} = 0.9 Hz, 1H, H-1″), 5.48 (dd, J_{7″,6″} = 2.2, J_{7″,8″} = 4.2 Hz, 1H, H-7″), 5.31-5.24 (overlapping, 2H, H-4″ and H-8″), 5.14-5.08 (m, 1H, H-2″), 4.60-4.73 (overlapping, 2H, H-3′ and H-9a′″), 4.18 (dd, J_{6″,5″} = 10.5, J_{6″,7″} = 2.2 Hz, 1H, H-6″), 4.15-4.08 (overlapping, 2H, H-9b′″ and H-4″), 4.00 (t app, J_{5″,6″} = J_{5″,4″} = 10.5, 1H, H-5″), 3.81 (s, 3H, COOCH₃), 3.64-3.55 (m, 1H, H-7a″), 3.46-3.40 (m, 1H, H-7a″), 2.49 (dd, J_{3a″,3b″} = 12.9, J_{3″,4″} = 4.9 Hz, 1H, H-3a″), 2.21 (s, 3H, NHOCH₃ at C-4), 2.14 (s, 3H, OCOCH₃), 2.02 (s, 3H, OCOCH₃), 2.01 (s, 3H, OCOCH₃), 2.00 (s, 3H; OCOCH₃), 1.96-1.86 (overlapping, 5H, H-5a′″, H-5b′″ and NHOCH₃ at C-5″), 1.8-1.65 (overlapping, 1H, H-6a′″ and H-b″″), 1.57 (s, 3H, C(CH₃)₂), 1.38 ppm (s, 3H, C(CH₃)₂); \(^1\)C NMR (MeOD): δ = 173.5 (1C, NHOCH₃ at C-5″), 172.9 (1C, NHOCH₃ at C-4), 172.5 (1C, CH₃COO
at C-9''), 172.0 (2C, CH$_3$COO at C-4'' and C-8''), 171.9 (1C, CH$_3$COO at C-7'''), 169.2(C-1'''), 164.7 (C-4), 157.7 (C-2), 148.5 (C-6), 115.4 (C(CH$_3$)$_2$), 100.0 (C-2'''), 98.2 (C-5), 96.7 (C-1''), 88.8 (C-4'), 86.3 (C-2''), 85.6 (C-3''), 72.8 (C-8''), 72.3 (C-6''), 70.7 (C-4''), 70.3 (C-7'''), 64.7 (C-7''), 63.7 (C-9''), 53.2 (COOCH$_3$), 50.3 (C-5''), 38.5 (C-3''), 31.5 (C-5''), 27.6 (C(CH$_3$)$_2$), 26.8 (C-6''), 25.6 (C(CH$_3$)$_2$), 24.6 (NHCOC$_3$H$_3$ at C-4) 22.9 (1C, NHCOC$_3$H$_3$ at C-5''), 21.0, 20.8, 20.7, 20.7 (4C, CH$_3$COO) ppm. MS (ESI positive) m/z 827.0[M+H]$^+$, 849.3 [M+Na]$^+$; elemental analysis calcd (%) forC$_{36}$H$_{50}$N$_4$O$_{18}$: C 52.30; H 6.10; N 6.10; found C 52.41; H 6.18; N 6.09.

**Compound 13a (α-anomer) MS (ESI positive) m/z 827.3[M+H]$^+$, 849.0 [M+Na]$^+$.

**ii) Starting from the dibromide 14a (697 mg, 1.10 mmol) and operating according to procedure B**

**step 1** intermediate 16a was obtained after rapid chromatography (eluting with AcOEt/MeOH, 98:2, v/v), as a white powder (346mg, 51%), showing: m.p. 154-156°C dec. (from CH$_2$Cl$_2$/diisopropyl ether); [α]$_D^{20}$ = -20.2 (c = 0.5 in chloroform); [α]$_D^{20}$ = +65.0 (c = 1.0 in methanol). $^1$H NMR (CDCl$_3$): δ = 9.23 (s, 1H; NH at C-4), 7.89 (d, J$_{NH,5''}$ = 9.9 Hz, 1H; NH at C-5''), 7.59 (d, J$_{6,5} = 7.3$ Hz, 1H; H-6), 7.42 (d, J$_{5,6} = 7.3$ Hz, 1H; H-5), 5.87 (dd, J$_{7'',8''} = 1.6$, J$_{7'',6''} = 2.8$ Hz, 1H; H-7'''), 5.63 (d, J$_{2,3'} = 6.3$ Hz, 1H; H-2'), 5.41 (br s, 1H; H-1'), 5.25 (dd, J$_{4'',3''} = 3.7$, J$_{4'',5''} = 10.4$ Hz, 1H; H-4''), 5.12 (ddd, J$_{3'',7''} = 1.6$, J$_{8''',9a''} = 2.1$, J$_{8''',9b''} = 9.5$ Hz, 1H; H-8'''), 5.05 (dd, J$_{9a''',8''} = 2.1$, J$_{9a''',9b''} = 12.1$ Hz, 1H; H-9'''), 4.91 (dd, J$_{3',4'} = 1.8$, J$_{3',5'} = 6.3$ Hz, 1H; H-3''), 4.71 (d, J$_{3',4'} = 3.7$ Hz, 1H; H-3'''), 4.67 (ddd, J$_{5',NH} = 9.9$, J$_{5'',4''} = J_{5'',6''} = 10.3$ Hz, 1H; H-5'''), (dd, J$_{6',5''} = 10.3$, J$_{6'',7''} = 2.8$ Hz, 1H; H-6''), 4.25–4.40 (m, 1H; H-4'), 4.13 (dd, J$_{9b''',8''} = 9.5$, J$_{9b''',9a''} = 12.1$ Hz, 1H; H-9b''), 3.80 (s, 3H; COOCH$_3$), 3.63–3.53 (overlapping, 2H; H-7a' and H-7b'), 2.22 (s, 6H; NHCOC$_3$H$_3$ at C-4 and OCOCH$_3$), 2.09 (s, 3H; OCOCH$_3$), 1.94 (s, 3H; OCOCH$_3$), 1.89 (s, 3H; OCOCH$_3$), 1.79 (s, 3H; NHCOC$_3$H$_3$ at C-5''), 1.70–1.58 (overlapping, 4H; H-5a', H-5b', H-6a' and H-6b'), 1.54 (s, 3H; C(CH$_3$)$_2$), 1.39 ppm (3H; C(CH$_3$)$_2$). $^1$H NMR (CD$_3$OD): δ = 8.05 (d, J$_{6,5} = 7.4$ Hz, 1H; H-6), 7.44 (d, J$_{5,6} = 7.4$ Hz, 1H; H-5), 5.81 (d, J$_{1',2'} = 1.6$ Hz, 1H; H-1''), 5.47 (dd, J$_{7'',6''} = 2.1$, J$_{7'',8''} = 4.4$ Hz, 1H; H-7'''), 5.38–5.31 (overlapping, 2H; H-8'' and H-4''), 5.14 (dd, J$_{2',1} = 1.6$, J$_{2',3'} = 6.4$ Hz, 1H; H-2'), 4.83 (dd, J$_{9a'',8''} = 1.5$ Hz, J$_{9a'',9b''} = 12.7$ Hz, 1H; H-9a''), 4.70 (t, J$_{3',4'} = 4.2$, J$_{3',5'} = 6.4$ Hz, 1H; H-3'), 4.71 (d, J$_{3',4'} = 3.6$ Hz, 1H; H-3''), 4.51 (t, J$_{5',4''} = J_{5'',6''} = 10.5$ Hz, 1H; H-5''), 4.24 (dd, J$_{5',5''} = 10.5$, J$_{6'',7''} = 2.1$ Hz, 1H; H-6''), 4.18–4.09 (overlapping, 2H; H-4' and H-9''), 3.84 (s, 3H; COOCH$_3$), 3.68–3.61 (m, 1H; H-7a'), 3.39–3.33 (m, 1H; H-7b'), 2.21 (s, 3H; NHCOC$_3$H$_3$ at C-4), 2.18 (s, 3H; OCOCH$_3$), 2.06 (s, 3H; OCOCH$_3$), 2.02 (s, 3H; OCOCH$_3$), 2.01 (s, 3H; OCOCH$_3$), 1.96–1.86 (overlapping, 5H; H-5a', H-5b' and NHCOC$_3$H$_3$ at C-5''), 1.83–1.76 (m, 1H; H-6a'), 1.72–1.65 (m, 1H; H-6b'), 1.57 (s, 3H; C(CH$_3$)$_2$), 1.37 ppm (s, 3H; C(CH$_3$)$_2$). $^{13}$C NMR (CD$_3$OD): δ = 173.5 (1C, NHCOC$_3$H$_3$ at C-5''), 172.9 (1C, NHCOC$_3$H$_3$ at C-4), 172.5,
172.0, 171.9, 171.6 (4C, OCOCH$_3$), 167.2 (C-1’’’), 164.7 (C-4), 157.7 (C-2), 148.9 (C-6), 115.3 (C(CH$_3$)$_2$), 101.5 (C-2’’’), 98.2 (C-5), 97.2 (C-1’’), 88.8 (C-4’’), 86.2 (C-2’’), 85.7 (C-3’’), 72.7 and 72.6 (C-6’’’ and C-8’’’), 70.6 (C-4’’’), 70.1 (C-7’’’), 66.3 (C-7’’), 63.8 (C-9’’’), 53.4 (COOCH$_3$), 52.9 (C-3’’’), 46.4 (C-5’’’), 31.3 (C-5’’), 27.6 (C(CH$_3$)$_2$), 26.6 (C-6’’), 25.6 (C(CH$_3$)$_2$), 24.6 (NHCOCH$_3$ at C-4), 22.9 (1C, NHCOCH$_3$ at C-5’’’), 21.0, 20.8, 20.7, 20.6 (4C, OCOCH$_3$) ppm. MS (ESI positive) $m/z$: 927.1 [100%; $^{79}$Br-M+Na]$^+$, 929.1 [94%; $^{81}$Br-M+Na]$^+$.

Elemental analysis calcd (%) for C$_{36}$H$_{49}$BrN$_4$O$_{18}$: C 47.74, H 5.45, N 6.19; found C 47.66, H 5.56, N 6.09.

Purified intermediate 16a (0.10 mmol 90.4 mg) was reacted according to procedure B step 2 to afford the title compound 12a (67 mg 81%). MS (ESI positive) $m/z$: 827.4 [M+H]$^+$, 849.2 [M+Na]$^+$. Elemental analysis calcd (%) for C$_{36}$H$_{50}$N$_4$O$_{18}$: calcd C 52.30; H 6.10; N 6.10; found C 52.37; H 6.15; N 6.02. Other physico-chemical properties were identical to those previously reported.
Synthesis of compounds 12b and 13b

i) Starting from compound 11b (564mg, 0.95 mmol) and operating according to procedure A; the compound 12b was obtained as a first eluate, after rapid chromatography (hexan/AcOEt, 70: 30v/v to AcOEt/MeOH 95:5 v/v), as a white solid (238 mg, 36%) toghether with its α-anomer 13b as a second white solid eluate (134 mg, 20%).

Compound 12b showed: m.p. 134°; [α]D 20 = -44.5 (c = 1 in CHCl3), [α]D 20 = -12.2 (c = 1 in MeOH).

1H NMR (CDCl3): δ = 9.16 (d, JNH,5'' = 9.8 Hz, 1H; NH at C-5''), 9.02 (s, 1H; NH at C-4), 7.58 (d, J6,5 = 7.4 Hz, 1H; H-6), 7.42 (d, J5,6 = 7.4 Hz, 1H; H-5), 5.90 (dd, J7'',8'' = 1.9, J7'',6'' = 2.9 Hz, 1H; H-7''), 5.67 (d, J1',2' = 1.5 Hz, 1H; H-1'), 5.43-5.36 (overlapping, 2H; H -3' and H-4''), 5.09 (dd, J9a'',8'' = 2.2, J9a'',9b'' = 9.9 Hz, 1H; H-9a'',9b''), 4.95 (dd, J9a'',8'' = 2.2, J9a'',9b'' = 12.1 Hz, 1H; H-9a''), 4.92 (dd, J2',1' = 1.5, J2',3' = 6.3 Hz, 1H; H-2'), 4.71 (dd, J6'',7'' = 2.9, J6'',5'' = 10.3 Hz, 1H; H-6''), 4.28-4.21 (overlapping, 2H; H- 4' and H-5''), 4.08 (dd, J9b'',8'' = 9.9 Hz, J9b'',9a'' = 12.1 Hz, 1H; H-9b''), 3.79 (s, 3H; COOCH3), 3.60–3.53 (overlapping, 2H; H-7a' and H-7b'), 2.65 (dd, J3a'',4'' = 5.1, J3a'',3b'' = 12.8 Hz, 1H; H-3a''), 2.21 (s, 6H; NHCOCH3 at C-4 and OCOCH3), 2.01 (s, 3H; OCOCH3), 1.95 (s, 3H; OCOCH3), 1.78 (s, 3H; OCOCH3), 1.77-1.69 (m, 1H ; H-3b''), 1.61-1.53 (overlapping, 4H, H-5a', H-5b', H-6a' and H-6b'), 1.55 (s, 3H; C(CH3)2), 1.40 ppm (s, 3H; C(CH3)2).

1H NMR (CD2OD): δ = 8.04 (d, J6,5 = 7.5 Hz, 1H; H-6), 7.35 ( d, J5,6 = 7.5 Hz, 1H; H-5),5.83 (d, J1',2' = 1.1 Hz, 1H; H-1'),5.43 (dd, J7'',6'' = 1.9, J7'',8'' = 4.4 Hz, 1H; H-7''), 5.34(ddd, J4'',3a'' = 4.9,J4'',5'' = 10.5, J4'',3b'' = 10.9Hz, 1H; H-4''), 5.27(ddd, J8'',9a'' = 2.3, J8'',7'' = 4.4, J8'',9b''=6.9 Hz, 1H; H-8''),5.04 (dd, J2'',1'' = 1.1, J2'',3'' = 6.2 Hz, 1H; H-2''),4.74 (dd, J9a'',8'' = 2.3, J9a'',9b'' = 12.4 Hz, 1H; H-9a''),4.70 (dd, J3'',4'' = 4.5, J3'',2'' = 6.2 Hz, 1H; H-3''), 4.31 (dd, J6'',7'' = 1.9, J6'',5'' = 10.5 Hz, 1H; H-6''), 4.15-4.01 (overlapping, 2H; H-4' and H-9b''), 4.00 (t app=J5'',4'' = J5'',6'' = 10.5, 1H; H-5''),3.80 (s, 3H; COOCH3),3.60–3.53 (m, 1H; H-7a''), 2.50 (dd, J3a'',4'' = 4.9, J3a'',3b'' = 13.0 Hz, 1H; H-3a''), 2.19 (s, 3H; NHCOCH3 at C-4),2.12 (s, 3H; OCOCH3),2.01 (s, 3H; OCOCH3),2.00 (s, 3H; OCOCH3),1.97 (s, 3H; OCOCH3),1.90-1.66 (overlapping, 5H; H-5a', H-5b', H-6a', H-6b' and H-3b''), 1.55 (s, 3H; C(CH3)2), 1.35 ppm (s, 3H; C(CH3)2); 13C NMR (MeOD): δ = 172.9 (1C, NHCOCH3 at C-4), 172.5, 172.0, 171.8, 171.7. (4C,
OCOCH₃), 169.0(C-1’’), 164.5 (C-4), 159.3(1C, J_{C,F} = 37 Hz, COCF₃), 157.7 (C-2), 149.1 (C-6), 121.7 (1C, J_{C,F} = 318 Hz COCF₃), 115.5 (C(CH₃)₂), 100.0 (C-2’’), 98.2 (C-5), 95.7 (C-1’), 88.3 (C-4’), 86.4 (C-2’), 85.2 (C-3’), 72.5 (C-8’’), 71.4 (C-6’’), 70.0 (2C C-4’’, and C-7’’), 64.8 (C-7’), 63.5 (C-9’’), 53.3 (COOCH₃), 51.0 (C-5’’), 38.4 (C-3’’), 31.3 (C-5’ or C-6’), 27.5 (C(CH₃)₂), 26.8 (C-6’ or C-5’), 25.6 (C(CH₃)₂), 24.6 (NHCOCH₃ at C-4), 21.0, 20.7, 20.6 (4C, 4 X OCOCH₃). MS (ESI positive) m/z: 903.1 [M+Na]⁺. Elemental analysis calcd (%) for C₃₆H₄₇F₃N₄O₁₈: C, 49.09; H, 5.38; N, 6.36; found C, 49.17; H, 5.35; N, 6.32.

Compound 13b (α-anomer): m.p. 120°C; [α]D$^{20}$ = -4.70 (c = 1 in CHCl₃); $^1$H NMR (CDCl₃): δ = 9.30 (s, 1H; NH at C-4), .772 (d, J₆₅= 7.5 Hz, 1H; H-6), 7.42 (d, J₅₆= 7.5 Hz, 1H; H-5), 6.92 (d, J_{NH,5a’}= 9.7 Hz, 1H; NH at C-5’’), 5.66 (br s, 1H; H-1’’), 5.41 (dd, J₈₇,7= 8.7, J₈₉,9a’’= 2.9, J₈₉,9b’’= 4.6, Hz, 1H; H-8’’), 5.33 (dd, J₇₆,8= 8.7, J₇₆,9= 1.9, Hz, 1H; H-7’’), 5.09 (dd, J₄₃,3a’’= 4.6, J₄₅,5= 10.7, J₄₅,3b’’= 12.0Hz, 1H; H-4’’), 5.03 (d app, J₂,3= 6.1 Hz, 1H; H-2’), 4.64 (dd, J₃₄,4’= 4.7, J₃₅,2= 6.1 Hz, 1H; H-3’), 4.28 (dd, J₆₅,7= 1.9, J₆₅,8= 10.7 Hz, 1H; H-6’’), 4.23-4.19 (overlapping, 2H; H-9a’’ and H-9b’’), 4.16-4.04 (m, 1H; H-4’), 4.00 (dd app, J₅₆,8= 9.7, J₅₆,4’= 10.7, 1H; H-5’’), 3.83-3.77 (m, 1H; H-7a’), 3.75 (3H; COOCH₃), 3.31-3.24 (m, 1H; H-7b’), 2.65 (dd, J₃₄,5,3b’’= 12.9 Hz, 1H; H-3a’’), 2.24 (s, 3H; NHCOCH₃ at C-4), 2.13 (s, 3H; OCOCH₃), 2.11 (s, 3H; OCOCH₃), 2.04 (s, 3H; OCOCH₃), 2.02 (s, 3H; OCOCH₃), 1.93 (dd, J₃₄,5,3b’’= 12.1, J₃₄,3b’’= 12.9 Hz, H-3b’’), 1.83-1.76 (overlapping, 2H, H-5a’ and H-5b’), 1.71-1.58 (overlapping, 2H H-6a’ and H-6b’), 1.56 (s, 3H; C(CH₃)₂), 1.34 ppm (s, 3H; C(CH₃)₂); $^1$H NMR (CD$_3$OD): δ = 8.05 (d, J₆₅= 7.5 Hz, 1H; H-6), 7.42 (d, J₅₆= 7.5 Hz, 1H; H-5), 5.85 (d, J₁,₂= 2.0 Hz, 1H; H-1’), 5.40 (dd, J₈₉,9a’’= 2.9, J₈₉,9b’’= 5.2, J₈₉,7= 9.0Hz, 1H; H-7’’), 5.30 (dd, J₇₆,9= 9.0 Hz, 1H; H-7’’), 4.97 (dd, J₂,3= 2.0, J₂,3= 6.4 Hz, 1H; H-2’), 4.91 (dd, J₄₃,3a’’= 4.6, J₄₅,5= 10.4, J₄₅,3b’’= 12.1Hz, 1H; H-4’’), 4.65 (dd, J₃₄,4’= 4.4, J₃₅,2= 6.4 Hz, 1H; H-3’), 4.32 (dd, J₆₇,8= 2.2, J₆₇,9= 10.5 Hz, 1H; H-6’’), 4.27 (dd, J₉₈,9a’’= 2.6 Hz, J₉₈,9b’’= 12.5 Hz, 1H; H-9a’’), 4.18-4.13 (m, 1H; H-4’), 4.09 (dd, J₉₉,9b’’= 5.2 Hz, J₉₉,9a’’= 12.5 Hz, 1H; H-9b’’), 3.97 (dd, J₅₆,8= 10.5, J₅₆,4’= 10.4, 1H, H-5’’), 3.83-3.77 (overlapping, 4H; COOCH₃ and H-7a’), 3.39–3.33 (m, 1H; H-7b’), 2.67 (dd, J₃₄,5,3b’’= 4.7, J₃₄,3b’’= 12.7 Hz, 1H; H-3a’’), 2.18 (s, 3H; NHCOCH₃ at C-4), 2.14 (s, 3H; OCOCH₃), 2.10 (s, 3H; OCOCH₃), 1.99 (s, 3H; OCOCH₃), 1.97 (s, 3H; OCOCH₃), 1.88-1.63 (overlapping, 5H; H-5a’’, H-5b’’, H-6a’’, H-6b’’ and H-3b’’), 1.56 (s, 3H; C(CH₃)₂), 1.35 ppm (s, 3H; C(CH₃)₂); $^{13}$C NMR (MeOD): δ = 173.0 (1C, NHCOCH₃ at C-4), 172.5, 171.8,171.6 (4C, OCOCH₃), 169.6(C-1’’), 164.7 (C-4), 159.4 (1C, J_{C,F} = 37 Hz, COCF₃), 157.7 (C-2), 147.6 (C-6), 117.1 (1C, COCF₃), 115.4 (C(CH₃)₂), 100.1 (C-2’’), 98.1 (C-5), 95.5 (C-1’), 88.6 (C-4’’), 88.5 (C-2’’), 85.3 (C-3’’), 72.5 (C-6’’), 70.2 (C-4’’), 69.4 (C-8’’), 68.4 (C-7’’), 65.6
ii) Starting from the dibromide 14b (866 mg, 1.10 mmol) and operating according to procedure B step 1 intermediate 16b was obtained after rapid chromatography eluting with (hexane /AcOEt, 70: 30v/v to AcOEt/MeOH 95:5 v/v) as a white powder (34.6 mg, 48%), showing: m.p. 134-136 °C dec.

(C-7'), 63.4 (C-9'''), 53.4 (COOCH3), 50.8 (C-5'''), 39.1 (C-3'''), 31.1 (C-5'), 27.6 (C(CH3)2), 27.1 (C-6'), 25.7 (C(CH3)2), 24.6 (NHCOCH3 at C-4), 21.3, 20.8, 20.7, 20.6 (4C, CH3COO) ppm. MS (ESI positive) m/z:903.4[M+Na]+. Elemental analysis calcld (%) for C36H47F3N4O18: C, 49.09; H, 5.38; N, 6.36; found C, 49.15; H, 5.41; N, 6.30.
\[ ^{79}\text{Br-M+Na}]^+ \text{, } 983.1 \text{ [94\%]; } ^{81}\text{Br-M+Na}]^+. \text{ Elemental analysis calcd (\%)} \text{ for C}_{36}\text{H}_{46}\text{BrF}_3\text{N}_4\text{O}_{18}: \text{ C} 45.06, \text{ H} 4.83, \text{ N} 5.84; \text{ found C} 45.06, \text{ H} 4.83, \text{ N} 5.94.

Purified intermediate \textbf{16b} (96 mg 0.10 mmol) was reacted according to \textbf{procedure B step 2} to afford the title compound \textbf{12b} (73 mg 83\%). \text{ MS (ESI positive) } m/z: 903.4[M+Na]^+. \text{ C}_{36}\text{H}_{47}\text{F}_3\text{N}_4\text{O}_{18}: \text{ calcd C, 49.09; H, 5.38; N, 6.36; found C, 49.02; H, 5.45; N, 6.40.}
Synthesis of compounds 12c and 13c

\[ \text{i) Starting from compound 11c (536 mg; 0.95 mmol) and operating according to procedure A, the compound 12c was obtained as a first eluate, after rapid chromatography (hexane/AcOEt 70:30 v/v to AcOEt/MeOH 95:5 v/v), as a white solid (199 mg, 27%) togheder with its α-anomer 13c as a second white solid eluate (177 mg, 24%).} \]

Compound 12c showed: m.p. 128-130 °C; [α]_D^20 = +3.9 (c = 1.0 in methanol). ^1H NMR (CDCl₃): δ = 9.19 (d, J_NH,5' = 9.7 Hz, 1H; NH at C-5’’), 9.08 (s, 1H; NH at C-4), 7.58 (d, J_6,5 = 7.4 Hz, 1H; H-6), 7.42 (d, J_5,6 = 7.4 Hz, 1H; H-5), 5.96 (dd, J_7',8' = 1.8, J_7',6' = 3.2, Hz, 1H; H-7’’), 5.68 (d, J_1',2' = 1.6 Hz, 1H; H-1’), 5.39 (dd, J_3',4' = 4.4, J_3',2' = 6.3 Hz, 1H; H-3’’), 5.36 (ddd, J_4',3a'' = 5.0, J_4',5'' = 10.4, J_4',3b'' = 11.0Hz, 1H; H-4’’), 5.11 (ddd, J_8',7' = 1.8, J_8'',9a'' = 2.4, J_8'',9b'' = 9.6 Hz, 1H; H-8’’), 4.95 (dd, J_9a'',8'' = 2.4, J_9a'',9b'' = 12.1 Hz, 1H; H-9a’’), 4.92 (dd, J_2'',1' = 1.6, J_2'',3' = 6.3 Hz, 1H; H-2’’), 4.73 (dd, J_6'',7'' = 3.2, J_6'',5'' = 10.7 Hz, 1H; H-6’’), (4.32 ddd, J_NH,5'' = 9.7, J_5'',4'' = 10.4, J_5'',6'' = 10.7 Hz, 1H; H-5’’), 4.27-4.23 (m, 1H; H-4’’), 4.07 (dd, J_9b'',8'' = 9.6, J_9b'',9a'' = 12.1 Hz, 1H; H-9b’’), 3.79 (3H; COOCH₃), 3.58–3.54 (overlapping, 2H; H-7a’ and H-7b’), 2.68 (dd, J_3a'',4'' = 5.1, J_3a'',3b'' = 12.8 Hz, 1H; H-3a’’), 2.22 (s, 3H; NHCOCH₃ at C-4), 2.21 (s, 3H; OCOCH₃), 1.99 (s, 3H; OCOCH₃), 1.95 (s, 3H; OCOCH₃), 1.79 (s, 3H; OCOCH₃), 1.76-1.73 (m, 1H; H-3b’’), 1.70-1.58 (overlapping, 4H, H-5a’, H-5b’, H-6a’ and H-6b’), 1.55 (s, 3H; C(CH₃)₂), 1.40 ppm (s, 3H; C(CH₃)₂); ^1H NMR (CD₃OD): δ = 8.05 (d, J_6,5 = 7.5 Hz, 1H; H-6), 7.36 (d, J_5,6 = 7.5 Hz, 1H; H-5), 5.87 (d, J_1,2' = 1.6 Hz, 1H; H-1’), 5.41 (dd, J_7',8' = 1.9, J_7',8' = 4.6 Hz, 1H; H-7’’), 5.37 (ddd, J_4',3a'' = 5.0, J_4',5'' = 10.6, J_4',3b'' = 11.7Hz, 1H; H-4’’), 5.29 (br ddd, J_8'',9a'' = 2.5, J_8'',7'' = 4.6, J_8'',9b'' = 7.1 Hz, 1H; H-8’’), 5.03 (dd, J_2'',1' = 1.6, J_2'',3' = 6.5 Hz, 1H; H-2’’), 4.79-4.70 (overlapping, 2H; H-3’ and H-9a’’), 4.36 (dd, J_6'',7'' = 1.9, J_6'',5'' = 10.3 Hz, 1H; H-6’’), 4.17-4.10 (overlapping, 2H; H-4’ and H-9b’’), 4.07 (dd, J_5'',6'' = 10.3, J_5'',4'' = 10.6 Hz, 1H; H-5’’), 3.82 (s, 3H; COOCH₃), 3.61–3.57 (m, 1H; H-7a’), 3.45-3.41 (m, 1H; H-7b’), 2.55 (dd, J_3a'',4'' = 5.0, J_3a'',3b'' = 13.0 Hz, 1H; H-3a’’), 2.22 (s, 3H; NHCOCH₃ at C-4), 2.14 (s, 3H; OCOCH₃), 2.05 (s, 3H; OCOCH₃), 2.03 (s, 3H; OCOCH₃), 1.96 (s, 3H; OCOCH₃), 1.92-1.72 (overlapping, 5H; H-5a’, H-5b’, H-6a’, H-6b’ and H-3b’’), 1.57 (s, 3H; C(CH₃)₂), 1.37 ppm (s, 3H; C(CH₃)₂); ^13C NMR (MeOD): δ = 172.9 (1C,
Compound 13c (α-anomer): m.p. 132-136°C; [α]D 25 = -26.1 (c = 1 in CH3OH); 1H NMR (CDCl3): δ = 9.30 (s, 1H; NH at C-4), 7.72 (d, J_6,5 = 7.5 Hz, 1H; H-6), 7.42 (d, J_5,6 = 7.5 Hz, 1H; H-5), 6.93 (d, J_NH,5'' = 10.0 Hz, 1H; NH at C-5''), 5.66 (br s, 1H; H-1'), 5.41 (dd, J_8'',9a = 2.7, J_8'',9b = 4.6, 8.7 Hz, 1H; H-8''), 5.33 (dd, J_7'',6'' = 1.8, J_7'',8'' = 8.7 Hz , 1H; H-7''), 5.09 (ddd, J_4'',3a'' = 4.6, J_4'',5'' = J_4'',3b'' = 10.5Hz, 1H; H-4''), 5.05-5.01 (m;1H; H-2'), 4.64 (dd, J_3,y,4' = 4.6, J_3,y,2 = 6.1 Hz, 1H; H-3'), 4.28 (dd, J_6,y,7'' = 1.8, J_6,y,5'' = 10.7 Hz, 1H; H-6''), 4.26-4.19 (overlapping, 2H; H-9a'' and H-9b''), 4.16-4.09 (m, 1H; H-4''), 4.00 (dd app, J_5,4'' = 10.0, J_5,6'' = 10.7, 1H; H-5''), 3.83-3.77 (m, 1H; H-7a''), 3.75 (3H; COOCH3), 3.31-3.25 (m, 1H; H-7b''), 2.65 (dd, J_3,y,4'' = 4.6, J_3,a'',3b'' = 12.9 Hz, 1H; H-3a''), 2.34 (s, 3H; NHOCH3 at C-4), 2.13 (s, 3H; COOCH3), 2.11 (s, 3H; COOCH3 ), 2.04 (s, 3H; COOCH3), 2.02 (s, 3H; COOCH3), 1.91 (dd, J_3,y,4'' = 10.5, J_3,a'',3b'' = 12.9 Hz,1H ; H-3b''), 1.83-1.75 (overlapping, 2H, H-5a' and H-5b'), 1.72-1.60 (overlapping, 2H, H-6a' and H-6b''), 1.56 (s, 3H; C(CH3)2), 1.34 ppm (s, 3H; C(CH3)2); 1H NMR (CD2OD): δ = 8.05 (d, J_6,5 = 7.5 Hz, 1H; H-6), 7.42 (br d, J_5,6 = 7.5 Hz, 1H; H-5), 5.85 (d, J_1,y,2 = 2.0 Hz, 1H; H-1'), 5.39 (ddd, J_8,y,9a = 2.6, J_8,y,9b = 5.1, J_8,y,7'' = 8.9Hz, 1H; H-8''), 4.97 (dd, J_7,y,6'' = 2.0, J_7,y,8'' = 8.9 Hz, 1H; H-7''), 4.97 (dd, J_2,y,1 = 2.0, J_2,y,3 = 6.4 Hz, 1H; H-2''), 4.92 (ddd, J_4,y,3a'' = 4.7, J_4,y,5'' = 10.5, J_4,y,3b'' = 12.0Hz, 1H; H-4''), 4.65 (dd, J_3,y,4'' = 4.4, J_3,y,2 = 6.4 Hz, 1H; H-3'), 4.34 (dd, J_6,y,7'' = 2.0, J_6,y,5'' = 10.5 Hz, 1H; H-6''), 4.27 (dd, J_9a'',8'' = 2.6, J_9a'',8'' = 12.5 Hz, 1H; H-9a''), 4.19-4.13 (m, 1H; H-4'), 4.09 (dd, J_9b'',9a = 5.1,J_9b'',9a = 12.5 Hz, 1H; H-9b''), 4.03 (t app, J_5,6''=J_5,4'' = 10.5 Hz, 1H; H-5''), 3.85-3.76 (overlapping, 4H; COOCH3 and H-7a''), 3.39-3.33 (m, 1H; H-7b''), 2.69 (dd, J_3,y,4'' = 4.7, J_3,a'',3b'' = 12.7 Hz, 1H; H-3a''), 2.18 (s, 3H; NHCOCH3 at C-4), 2.15 (s, 3H; COOCH3), 2.11 (s, 3H; COOCH3), 1.99 (s, 3H; COOCH3), 1.96 (s, 3H; COOCH3), 1.88-1.61 (overlapping, 5H; H-5a', H-5b', H-6a', H-6b' and H-3b''), 1.56 (s, 3H; C(CH3)2), 1.35 ppm (s, 3H; C(CH3)2); 13C NMR (MeOD): δ = 173.0 (1C, NHCOCH3 at C-4), 172.5, 171.8, 171.4 (4C, COOCH3), 169.5(C-1''), 164.7 (C-4), 159.6 (1C, J_CF = 27 Hz, COCF2CF2CF3), 157.7 (C-2), 147.6 (C-6), 124.0-110.0 (3C, COCF2CF2CF3), 115.4 (C(CH3)2), 107.0 (C(CH3)2), 105.7 (C(CH3)2), 105.3 (C(CH3)2), 104.1 (55%)[M+Na]+. Elemental analysis calcd (%) for C_{38}H_{47}F_{7}N_{4}O_{18}: C 46.53, H 4.83, N 5.71; found C 46.42, H 4.78, N 5.89.
ii) Starting from the dibromide 14c (866 mg, 1.10 mmol) and operating according to procedure B step 1 intermediate 16c was obtained after rapid chromatography (hexane/AcOEt 70:30 v/v to AcOEt/MeOH 80:20 v/v, as a white powder (254 mg, 32%), showing: m.p. 138-140°C; [α]D²⁰ = +8.5 (c = 1 CH₃OH);¹H NMR (CDCl₃): δ = 9.23 (d, J₉H₅'' = 9.0 Hz, 1H; NH at C-5''), 9.04 (s, 1H; NH at C-4), 7.58 (d, J₉₅,= 7.4 Hz, 1H; H-6), 7.43 (d, J₅₆ = 7.4 Hz, 1H; H-5), 5.91 (br, s, 1H; H-7'''), 5.63 (dd, J₂''',₁'' = 1.1 Hz, J₂'''',₃''' = 6.3 Hz, 1H; H-2''), 5.39 (d, J₁''',₂'' = 1.1Hz,1H; H-2'') 5.32(dd, J₄''',₃'' = 3.6, J₄''',₅'' = 9.9 Hz, 1H; H-4''), 5.12 (dd, J₄''',₇'' = 1.7, J₄''',₉a'' = 2.4, J₄''',₉b'' = 9.4, Hz, 1H; H-8''), 5.04 (dd, J₉a''',₈'' = 2.4, J₉a''',₉b'' = 12.1 Hz, 1H; H-9a''), 4.89 (dd, J₁',₄' = 1.7, J₃',₂' = 6.3 Hz, 1H; H-3''), 4.76 (d, J₃',₄' = 3.7 Hz, 1H; H-3''), 4.73-4.67 (overlapping, 2H; H-5''; H-6''), 4.26-4.22 (overlapping, 2H; H-7a' and H-7b'), 2.23 (s, 3H; NHCOCH₃), 1.77-1.51 (overlapping, 4H, H-5a', H-5b', H-6a' and H-6b'), 1.51 (s, 3H; C(CH₃)₂), 1.39 ppm (s, 3H; C(CH₃)₂);¹H NMR (CD₂OD): δ = 8.02 (d, J₆₅,₅ = 7.5 Hz, 1H; H-6), 7.43 (d, J₅₆,₅ = 7.5 Hz, 1H; H-5), 5.80 (d, J₁',₂' = 1.8 Hz, 1H; H-1'), 5.46 (dd, J₇',₆'' = 2.0, J₇',₅'' = 4.6 Hz, 1H; H-7''), 5.40(dd, J₄''',₃'' = 3.6, J₄''',₅'' = 10.5, Hz, 1H; H-4'''), 5.29 (ddd, J₈'',₉a'' = 2.6, J₈'',₇'' = 4.6, J₈'',₉b'' = 7.3, Hz, 1H; H-8''), 5.09 (dd, J₂''',₁'' = 1.8, J₂''',₃'' = 6.4 Hz, 1H; H-2''), 4.81(dd, J₉a''',₈'' = 4.6, J₉a''',₉b'' = 12.4 Hz, 1H; H-9a''), 4.74-4.69. (overlapping, 2H; H-3' and H-3''), 4.50 (dd, J₅'',₆'' = 10.6, J₅'',₄'' = 10.5 Hz, 1H; H-5''), 4.38 (dd, J₆'',₇'' = 1.9, J₆'',₅'' = 10.6 Hz, 1H; H-6''), 4.17-4.07(overlapping, 2H; H-4' and H-9b''), 3.83 (s, 3H; COOCH₃), 3.69-3.63 (m, 1H; H-7a'), 3.47-3.41 (m, 1H; H-7b'), 2.19 (s, 3H; NHCOCH₃ at C-4), 2.15 (s, 3H; COOCH₃), 2.03 (s, 3H; COOCH₃), 2.00-1.99 (overlapping, 6H; 2X COOCH₃), 1.92-1.66 (overlapping, 4H; H-5a', H-5b', H-6a' and H-6b'), 1.54 (s, 3H; C(CH₃)₂), 1.35 ppm (s, 3H; C(CH₃)₂).¹³C NMR (MeOD): δ = 173.0 (1C, NHCOCH₃ at C-4), 172.5, 172.0, 171.6, 171.2 (4C, COOCH₃), 167.0 (C-1'''), 164.7 (C-4), 159.6 (1C, J₉,F = 26 Hz, COCF₂CF₂CF₃), 157.7 (C-2), 148.3 (C-6), 124.0-110.0 (3C, COCF₂CF₂CF₃), 115.5 (C(CH₃)₂), 101.5 (C-2'''), 98.2 (C-5), 96.2 (C-1''), 88.3 (C-4''), 86.3 (C-2''), 85.4 (C-3''), 72.5 (C-8'''), 71.8 (C-6'''), 69.9(C-4''), 69.6 (C-7'''), 66.4 (C-7'), 63.5 (C-9''), 53.6 (COOCH₃), 52.6 (C-3'''), 47.1 (C-5'''), 31.1 (C-5'or C-6'), 27.6 (C(CH₃)₂), 26.7 (C-6' or C-5'), 25.6 (C(CH₃)₂), 24.6 (NHCOCH₃ at C-4), 21.0, 20.7, 20.7, 20.5 (4C, 4X OCOCH₃) ppm. MS (ESI positive) m/z:1003.1[M+Na]⁺Elemental analysis calcd (%) for C₃₈H₄₇F₇N₃O₁₈: C 46.53, H 4.83, N 5.71; found C 46.42, H 4.78, N 5.89.
positive) m/z:1082.6 [M+Na]^+. Elemental analysis calcd (%) for C_{38}H_{46}BrF_{7}N_{4}O_{18}: C 43.07, H 4.38, N 5.29; found C 43.02, H 4.38, N 5.35.

Purified intermediate 16c (105 mg, 0.10 mmol) was reacted according to procedure B step 2 to afford the title compound 12c (85.3 mg 87%). MS (ESI positive) m/z:1003.3[M+Na]^+; elemental analysis calcd (%) for C_{38}H_{47}F_{7}N_{4}O_{18}: C 46.53, H 4.83, N 5.71; found C 46.50, H 4.76, N 5.79.
5.3.5 Synthetic deprotection procedures affording derivatives 3a-e

**Step 1: General procedure of actonide deprotection.**
To a solution of appropriate 12a-c or 13b-c (0.40 mmol) in dichloromethane (5mL) moist TFA (0.6 mmol) was added and the mixture was stirred for 30 minutes or 1 hour at reflux. Then the mixture, treated with weak basic resin IRA 67, was filtered and evaporated in vacuo to give a syrup, which was purified by chromatography on silica gel, to afford the desired de-acetonide intermediate.

**Step 2: General procedure of deacetylation.**
To a solution of de-acetonide intermediate (0.20 mmol) in dry methanol a 0.5 M solution of sodium methoxide in dry methanol was added. After stirring for 1 h the solution was neutralized by addition of Amberlite weakly acid CG50, filtered, and evaporated. The residue was purified by flash chromatography to afford the methyl esters of 3a-e.

**Step 3: General procedures methyl ester deprotection.**
The residue (0.10 mmol) was reacted according one of these procedures.

a) In aqueous methanol (1.0 mL, 2:1 v/v) saturated with K$_2$CO$_3$, was stirred for 12 h at 23 °C. Then the solution was treated with an acidic resin Amberlite weakly acid CG50. The solution was filtered and the solvent was removed under reduced pressure to afford the free glycoside.

b) The appropriate protected sialoside (0.10 mmol) dissolved in methanol–water (1.5 mL, 2:1 v/v) was treated with Et$_3$N (0.90 mL) under stirring for 12 h at 23 °C. Then the solvent was removed under reduced pressure and the residue was recovered with water and lyophilized many times until complete elimination of Et$_3$N.

c) The residue (0.18 mmol) dissolved in MeOH and was treated with NaOH aq solution for 40 min. under stirring at 23°C. Then the reaction is neutralized with Amberlite weakly acid CG50, filtered and the solvent was removed under reduced pressure.

**Step 4: General procedure of purification by HPLC-RP chromatography**
All final compounds 3a-e were purified by HPLC-RP chromatography using the C$_{18}$ reverse phase column (Atlantis C-18-Preper T3 ODB, 5um, 19X10 mm HPLC column) and starting from 100% of aqueous 0.1% (v/v) formic acid to 100% CH3CN as eluent.
Synthesis of compound 3a via 3 step deprotection and preparative HPLC purification.

Step 1: Starting from 12a, (331 mg, 0.40 mmol) according to the general deprotection procedure step 1 the de-acetonide compound was obtained, after flash chromatography (eluting with AcOEt/MeOH 9:1) as white powder (230 mg 75%) showing: m.p. 118-121 °C; \([\alpha]_D^{25} = +14.2 (c = 1 \text{ in methanol})\).

H NMR (MeOD): \(\delta = 8.03 (d, J_{6,5} = 7.5 \text{ Hz}, 1\text{H}; H-6), 7.40 (d, J_{5,6} = 7.5 \text{ Hz}, 1\text{H}; H-5), 5.83 (d, J_{1,2} = 2.5, 1\text{H}; H-1'), 5.41 (dd, J_{7',6'} = 2.2, J_{7',8'} = 4.1 \text{ Hz}, 1\text{H}; H-7''), 5.32–5.28 (m, 1\text{H}; H-8''), 5.26–5.22 (m, 1\text{H}; H-4''), 4.80 (dd, J_{9a',8'} = 2.5 \text{ Hz}, J_{9a',8'} = 12.4 \text{ Hz}, 1\text{H}; H-9a''), 4.20 (dd, J_{2',1'} = 2.5, J_{2',3} = 5.2 \text{ Hz}, 1\text{H}; H-2'), 4.18–4.07 (overlapping, 2\text{H}; H-6'' and H-9b''), 4.06–4.02 (m, 1\text{H}; H-4''), 3.95 (t, J_{5'',4''}=J_{5'',6''}= 10.41 \text{ Hz}, 1\text{H}; H-5''), 3.86 (dd, J_{3',2'} = 5.2, J_{3',4'} = 7.1 \text{ Hz}, 1\text{H}; H-3''), 3.80 (s, 3\text{H}; COOCH_3) 3.66–3.59 (m, 1\text{H}; H-7a''), 3.47–3.41 (m, 1\text{H}; H-7b''), 2.47 (dd, J_{3a'',4''} = 5.0, J_{3a'',3b''} = 13.0 \text{ Hz}, 1\text{H}; H-3a''), 2.19 (s, 3\text{H}; NHCOCH_3 at C-4), 2.11 (s, 3\text{H}; OCOCH_3), 2.04–1.96 (overlapping, 11\text{H}; 3 X OCOCH_3, H-5a' and H-5b''), 1.90–1.81 (overlapping, 5\text{H}; NHCOCH_3 at C-5'', H-6a' and H-6b''), 1.79 ppm (dd, J_{3b'',4''} = 11.5, J_{3b'',3a''} = 13.0 \text{ Hz}, 1\text{H}; H-3b''); \(^{13}\text{C} \text{ NMR (MeOD)}: \delta = 173.6 (1\text{C}, \text{ NHCOCH}_3 \text{ at C-5''}), 173.6 (1\text{C}, \text{ NHCOCH}_3 \text{ at C-4}), 172.7, 172.3, 172.1, 171.8 (4\text{C}, \text{ OCOCH}_3), 169.2(\text{C-1'''}, 164.2 (\text{C-4}), 158.1 (\text{C-2}), 146.2 (\text{C-6}), 100.0 (\text{C-2''}), 98.4 (\text{C-5}), 94.0 (\text{C-1''}), 84.5 (\text{C-4''}), 76.3 (\text{C-2''}), 75.0 (\text{C-3''}), 73.0 (\text{C-8''}), 72.3 (\text{C-6''}), 70.5 (\text{C-4'''}, 70.1 (\text{C-7'''}, 64.9 (\text{C-7}), 64.9 (\text{C-9'''}, 53.3 (\text{COOCH}_3), 50.3 (\text{C-5''}), 38.5 (\text{C-3'''}, 31.2 (\text{C-5}), 24.6 (\text{C}, \text{ NHCOCH}_3 \text{ at C-4}), 22.8 (1\text{C}, \text{ NHCOCH}_3 \text{ at C-5''}), 21.0, 20.9, 20.8, 20.8 ppm (4\text{C}, \text{ OCOCH}_3); MS (ESI positive) \text{ m/z:809.3[M+Na]^+}. \text{ Elemental analysis calcd (\%)} \text{ for C}_{33}\text{H}_{46}\text{N}_{4}\text{O}_{18}: \text{ C 50.38, H 5.89, N 7.12; found C 50.46, H 5.80, N 7.03.}
Step 2: This intermediate (157 mg, 0.20 mmol) was deacetylated according to general deprotection procedure step 2 to afford, after flash chromatography (eluting with AcOEt/MeOH 8:2), the intermediate methyl ester of 3a as white powder (104 mg 90%) showing: m.p. 118-120 °C; $[\alpha]_D^{25} = +15.1$ (c = 1 in H$_2$O); $^1$H NMR (D$_2$O): $\delta = 7.67$ (d, $J_{6,5} = 7.6$ Hz, 1H; H-6), 6.03 (d, $J_{5,6} = 7.6$ Hz, 1H; H-5), 5.84 (d, $J_{1',2'} = 3.9$ Hz, 1H; H-1'), 4.33 (dd, $J_{2',1'} = 3.9$, $J_{2',3'} = 4.9$ Hz, 1H; H-2'), 4.14-4.00 (overlapping, 3H; H-4', H-4'' and H-3'), 3.96-3.88 (overlapping, 2H; H-5'' and H-6''), 3.88-3.81 (overlapping, 5H; COOCH$_3$, H-8'' and H-9a''), 3.76-3.74 (overlapping, 2H; H-7a''), 3.40-3.33 (m, 1H; H-7b''), 2.43 (dd, $J_{3a'',4''} = 4.7$, $J_{3a'',3b''} = 13.2$ Hz, 1H; H-3a''), 2.05 (s, 3H; NHCOC$H_3$), 1.95-1.70 ppm (overlapping, 5H; H-5a', H-5b', H-6a', H-6b' and H-3b'')); $^{13}$C NMR (D$_2$O): $\delta = 176.2$ (1C, NHCOC$H_3$), 171.9 (C-1''), 166.8 (C-4'), 157.6 (C-2'), 143.4 (C-6), 100.0 (C-2''), 97.5 (C-5), 92.0 (C-1'), 84.5 (C-4'), 75.0 (C-2'), 74.3 (C-3'), 71.9 (C-6''), 71.2 (C-8''), 69.3 (C-7''), 67.7 (C-4''), 64.7 (C-9''), 64.6 (C-7'), 54.8 (COOCH$_3$), 53.2 (C-5''), 40.7 (C-3''), 30.5 (C-5'), 26.3 (C-6'), 23.4 ppm (1C, NHCOC$H_3$); MS (ESI positive) m/z: 599.3[M+Na]$^+$. Elemental analysis calcd (%) for C$_{23}$H$_{36}$N$_4$O$_{13}$: C 47.91, H 6.29, N 9.72; found C 47.87, H 6.09, N 9.83.

![3a](image)

Step 3 Finally, methyl ester of 3a (104 mg, 0.10 mmol) was deprotected according to general deprotection procedure step 3(C) to afford free acid 3a (93 mg 92%), after purification by HPLC-RP chromatography according to general deprotection procedure step 4. The compound 3a was a white solid that showed: $[\alpha]_D^{25} = +13.9$ (c = 1 in H$_2$O); $^1$H NMR (D$_2$O): $\delta = 7.64$ (d, $J_{6,5} = 7.5$ Hz, 1H; H-6), 6.00 (d, $J_{5,6} = 7.5$ Hz, 1H; H-5), 5.87 (d, $J_{1',2'} = 3.9$ Hz; 1H; H-1'), 4.31 (t app, $J_{2',1'} = 3.9$, $J_{2',3'} = 5.4$ Hz, 1H; H-2'), 4.11-4.06 (overlapping, 2H; H-4' and H-4''), 4.02 (t app, $J_{3',2'} = 5.4$ Hz, 1H; H-3'), 3.97-3.84 (overlapping, 4H; H-5'', H-6'', H-8'' and H-9a''), 3.71 (dd, $J_{9b'',8''} = 5.3$, $J_{9a'',9b''} = 11.7$ Hz, 1H; H-9b''), 3.62-3.56 (m, 1H; H-7a'), 3.53 (d, $J_{7'',8''} = 9.4$ Hz, 1H; H-7''), 3.38-3.31 (m, 1H; H-7b'), 2.37 (dd, $J_{3a'',4''} = 4.8$, $J_{3a'',3b''} = 12.9$ Hz, 1H; H-3a''), 2.05 (s, 3H; NHCOC$H_3$), 1.94-1.86 (m, 1H; H-5a'), 1.82-1.69 (overlapping, 3H; H-5b', H-6a' and H-6b'), 1.63
ppm (t app, $J_{3\beta''}, 3\alpha''$ = 12.9 Hz, 1H; H-3b’’); $^{13}$C NMR (D$_2$O): $\delta$ = 176.3 (C-1’’), 175.6 (1C, NHCOCH$_3$), 167.0 (C-4), 158.4 (C-2), 142.4 (C-6), 100.7 (C-2’’), 97.1 (C-5), 91.2 (C-1’), 84.1 (C-4’), 74.7 (C-2’), 73.8 (C-3’), 70.9 (C-6’’), 70.8 (C-8’’), 69.2 (C-7’’), 67.9 (C-4’’), 64.4 (C-9’’), 63.3 (C-7’), 52.9 (C-5’’), 40.8 (C-3’’), 30.3 (C-5’), 25.9 (C-6’), 22.9 ppm (1C, NHCOCH$_3$); MS (ESI negative) m/z: 561.3[M-H$^-$], 583.3[M-2H+Na$^-$]. Elemental analysis calcd (%) for C$_{22}$H$_{34}$N$_{4}$O$_{13}$: C 46.97, H 6.09, N 9.96; found C 46.87, H 6.00, N 9.85.
Synthesis of compound 3b via 3 step deprotection and preparative HPLC purification.

**Step 1:** Starting from **12b**, (352 mg, 0.40 mmol) according to the general deprotection procedure step 1 the intermediate de acetonide was obtained, after flash chromatography (eluting with AcOEt/MeOH 9:1 as white powder (259 mg 77%) showing m.p. 128-130 °C; [α]D-20 = +21.5 (c = 1 MeOH); 1H NMR (CD3OD): δ= 8.02 (d, J6,5= 7.5 Hz, 1H; H-6), 7.47 (d, J5,6 = 7.5 Hz, 1H; H-5), 5.81 (d, J1',2' = 2.0 Hz, 1H; H-1'), 5.39 (dd, J7',6'= 2.0, J7',9'= 4.2 Hz, 1H; H-7'''), 5.38-5.29 (overlapping, 2H; H-4'' and H-8''), 4.78 (dd, J9a'',8''= 2.1,J9a'',9b'' = 12.4 Hz 1H; H-9a''), 4.28 (dd, J6'',7''= 2.0, J6'',5'' = 9.5 Hz, 1H; H-6''), 4.18 (dd, J2',4' = 2.0, J2',3' = 4.9 Hz, 1H; H-2'), 4.12 (dd, J9b'',8''= 7.4,J9a'',9b'' = 12.4 Hz 1H; H-9b''), 0.45-3.96 (overlapping, 2H; H-4' and H-5''), 3.73-3.77 (overlapping, 4H; H-3' and COOCH3), 3.67-3.59 (m, 1H; H-7'a''), 3.48-3.42 (m, 1H; H-7'b''), 2.51 (dd, J3a'',4''= 4.9, J3a'',3b'' = 12.9 Hz, 1H; H-3a''), 2.18 (s, 3H; NHCOCH3 at C-4), 2.12 (s, 3H; OCOCH3), 2.03 (s, 3H; OCOCH3), 2.02 (s, 3H; OCOCH3), 1.97 (s, 3H; OCOCH3), 1.92-1.77 (overlapping, 5H; H-5a', H-5b', H-6a', H-6b' and H-3b''). 13C NMR (MeOD): δ = 173.0 (1C, NHCOCH3 at C-4), 172.5, 172.2, 171.7, 171.5 (4C, 4 X OCOCH3), 169.0(C-1''), 164.4 (C-4), 159.3 (1C, Jc,F = 38 Hz, COCF3), 158.0 (C-2), 146.1 (C-6), 117.1 (1C, Jc,F = 287 Hz COCF3), 100.0 (C-2''), 98.3 (C-5), 94.1(C-1''), 84.4 (C-4''), 76.2 (C-2''), 75.0 (C-3''), 72.7 (C-8''), 71.6 (C-6''), 70.0 (C-4''), 69.9 (C-7''), 65.0 (C-7''), 63.5 (C-9''), 53.3 (COOH3), 51.0 (C-5''), 39.5 (C-3''), 31.1 (C-5' or C-6''), 27.2 (C-6' or C-5''), 24.6 (NHCOCH3 at C-4), 21.0, 20.7, 20.6 ppm (4C, 4 X OCOCH3); MS (ESI positive) m/z:863.1[M+Na]+. Elemental analysis calcd (%) for C35H43F3N4O16: C 47.15, H 5.16, N 6.66; found C 47.22, H 5.28, N 6.50.
Step 2: This intermediate (168 mg, 0.20 mmol) was deacetylated according to the general deprotection procedure step 2 to afford, after flash chromatography (eluting with AcOEt/MeOH 8:2), the intermediate methyl ester of 3b (115 mg 91%) as white powder showing: m.p. 120°C; $[\alpha]_D^{20} = +18.1$ (c = 1 in CH$_2$OH). $^1$H NMR (CD$_3$OD): $\delta$ = 7.60 (d, $J_{6,5}$ = 7.4 Hz, 1H; H-6), 5.93 (d, $J_{5,6}$ = 7.4 Hz, 1H; H-5), 5.77 (d, $J_{1',2'}$ = 3.2 Hz, 1H; H-1'), 4.20-4.09 (overlapping, 3H; H-2', H-4'', and H-6''), 4.00 (t app, $J_{5'',4''} = J_{5'',6''}$ = 10.3, 1H; H-5''), 3.96-3.90 (m, 1H; H-4''), 3.85-3.76 (overlapping, 7H; H-3', H-7a', H-8'', H-9a'' and COOCH$_3$), 3.67 (dd, $J_{9b'',8''} = 5.2, J_{9a'',9b''} = 11.3$ Hz, 1H; H-9b''), 3.46 (d app, $J_{7'',8''} = 9.4, 1H$; H-7''). 13C NMR (MeOD): $\delta$ = 171.5 (C-1''), 167.7 (C-4), 159.8 (1C, $J_{C,F} = 37$ Hz, COCF$_3$), 158.5 (C-2), 142.8 (C-6), 117.6 (1C, $J_{C,F} = 287$ Hz, COC$_3$F), 100.1 (C-2''), 96.3 (C-5), 93.1 (C-1'), 84.3 (C-4''), 75.8 (C-2''), 75.0 (C-3''), 71.8 (C-8''), 71.4 (C-6''), 70.1 (C-7''), 67.3 (C-4''), 65.2 (C-9''), 64.4 (C-7''), 54.3 (C-5''), 53.4 (COOCH$_3$), 41.9 (C-3''), 31.1 (C-5' or C-6'), 27.0 (C-6' or C-5') ppm; (ESI positive) m/z:653.0[M+Na]$^+$, MS (ESI negative) m/z:629.1[M-H]$^-$.

Elemental analysis calcd (%) for C$_{23}$H$_{33}$F$_3$N$_4$O$_{13}$: C 43.81, H 5.28, N 9.04; found C 43.62, H 5.10, N 9.30.

Step 3: Finally methyl ester of 3b (113.5 mg, 0.10 mmol) was deprotected according to the general deprotection procedure step 3 to afford, by HPLC/preparative according to step 4 of general deprotection the compounds 3b (97 mg 88%) as white powder showing: m.p. 129-131°C; $[\alpha]_D^{20} = +18.9$ (c = 1 in H$_2$O). $^1$H NMR (D$_2$O): $\delta$ = 7.86 (d, $J_{6,5}$ = 7.9 Hz, 1H; H-6), 6.23 (d, $J_{5,6}$ = 7.9 Hz, 1H; H-5), 5.83 (d, $J_{1',2'}$ = 3.8 Hz, 1H; H-1'), 4.35 (dd, $J_{2',1'} = 3.8, J_{2',3'} = 5.1$ Hz, 1H; H-2'), 4.23 (t app, $J_{4',3a''} = 4.9, J_{4',5'} = J_{4',3b''} = 11.1$Hz, 1H; H-4''), 4.13-3.99 (overlapping, 4H; H-3', H-4', H-5'' and H-6''), 3.89-3.82 (overlapping, 2H; H-8'' and H-9a''), 3.69-3.62 (overlapping, 2H; H-7a' and H-9b''), 3.53 (d app, $J_{7'',8''} = 9.7, 1H$; H-7''). 13C NMR (D$_2$O): $\delta$ = 174.4 (C-1''), 160.6 (C-4), 159.8 (1C, $J_{C,F} = 39$ Hz, COCF$_3$), 149.0 (C-2), 144.9 (C-6), 116.4 (1C, $J_{C,F} = 286$ Hz, COCF$_3$), 100.0 (C-2''), 95.8 (C-5), 91.0 ppm; (ESI positive) m/z:653.0[M+Na]$^+$, MS (ESI negative) m/z:629.1[M-H]$^-$. Elemental analysis calcd (%) for C$_{23}$H$_{33}$F$_3$N$_4$O$_{13}$: C 43.81, H 5.28, N 9.04; found C 43.62, H 5.10, N 9.30.
91.4 (C-1’), 86.4 (C-4’), 74.4 (C-2’), 73.5 (C-3’), 70.7 (C-8’’), 70.1 (C-6’’), 68.8 (C-7’’), 66.6 (C-4’’), 64.1 (C-9’’), 63.6 (C-7’), 53.5 (C-5’’), 40.5 (C-3’’), 30.0 (C-5’ or C-6’), 25.8 (C-6’ or C-5’) ppm; MS (ESI negative) m/z 615.2[M-H]. Elemental analysis calcd (%) for C_{22}H_{31}F_{3}N_{4}O_{13}: C 42.86, H 5.07, N 9.09; found C 43.01, H 5.0, N 9.12.
Synthesis of compound 3c via 3 step deprotection and preparative HPLC purification.

Step 1: Starting from 12c, (392 mg, 0.40 mmol) according to the general deprotection procedure step 1, the de-acetonide intermediate was obtained, after flash chromatography (eluting with AcOEt/MeOH 9:1) as white powder (297 mg 79%) showing: m.p. 130-132°C, [α]D20 = +19.4 (c = 1.0 CH3OH); 1H NMR (CD3OD): δ = 8.04 (d, J6.5 = 7.4 Hz, 1H; H-6), 7.37 (d, J5.6 = 7.4 Hz, 1H; H-5), 5.83 (d, J1',2' = 2.6 Hz, 1H; H-1'), 5.41-5.27 (overlapping, 3H; H-7', H-4'' and H-8''), 4.76 (dd, J9a',8' = 2.3, J9a',9' = 12.4 Hz, 1H; H-9a''), 4.32 (d, J6''-5'' = 10.4 Hz, 1H; H-6''), 4.19 (dd, J2',1' = 2.6, J2',3' = 5.2 Hz, 1H; H-2'), 4.15-4.00 (overlapping, 3H; H-4', H-5'' and H-9b''), 3.87 (dd, J3',2' = 5.2, J3',4' = 6.5 Hz, 1H; H-3'), 3.80 (s, 3H; COOCH3), 3.67-3.59 (m, 1H; H-7a'), 3.48-3.41 (m, 1H; H-7b'), 2.53 (dd, J3a',4' = 4.7, J3a',3b' = 12.9 Hz, 1H; H-3a''), 2.19 (s, 3H; NHCOCH3 at C-4), 2.12 (s, 3H; OCOCH3), 2.04 (s, 3H; OCOCH3), 2.01 (s, 3H; OCOCH3), 1.96 (s, 3H; OCOCH3), 1.92-1.76 ppm (overlapping, 5H; H-5a', H-5b', H-6a', H-6b' and H-3b''); 13C NMR (MeOD): δ = 173.0 (1C, NHCOCH3 at C-4), 172.5, 172.2, 171.6, 171.4 (4C, 4 X OCOCH3), 169.0(C-1''), 164.2 (C-4), 159.6 (1C, JCF = 26 Hz, COCF2CF2CF3), 158.1 (C-2), 146.1 (C-6), 121.0-106.0 (3C, COCF2CF2CF3), 100.0 (C-2''), 98.4 (C-5), 93.8 (C-1''), 84.6 (C-4''), 76.3 (C-2''), 74.9 (C-3''), 72.7 (C-8''), 71.5 (C-6''), 69.8 (2C, C-4'' and C-7''), 65.0 (C-7''), 63.5 (C-9''), 53.3 (COOCH3), 51.0 (C-5''), 38.5 (C-3''), 31.1 (C-5''), 27.2 (C-6''), 24.6 (NHCOCH3 at C-4), 21.0, 20.7, 20.6 (4C, 4 X OCOCH3) ppm;MS (ESI positive) m/z:963.1 [M+Na]+. Elemental analysis calcd (%) for C33H43F7N4O16: C 44.69, H 4.61, N 5.96; found C 44.52, H 4.70, N 5.93.

Step 2: This intermediate (188 mg, 0.20 mmol) was deacetylated according to general deprotection procedure step 2 to afford, after flash chromatography (eluting with AcOEt/MeOH 8:2), the intermediate methyl ester of 3c (130 mg 89%) as white powered showing: m.p. 130-
132°C; [α]D 20 = +5.0 (c = 1 in CH3OH). 1H NMR (CD3OD): δ = 7.60 (d, J6,5 = 7.5 Hz, 1H; H-6), 5.93 (d, J5,6 = 7.5 Hz, 1H; H-5), 5.77 (d, J1,2 = 3.4 Hz, 1H; H-1'), 4.20-4.02 (overlapping, 4H; H-2', H-4', H-5' and H-6''), 3.96-3.89 (m, 1H; H-4'), 3.86-3.74 (overlapping, 7H; H-3', H-7a', H-8'', H-9a'' and COOCH3), 3.63 (dd, J9b'',8'' = 5.6, J9a'',9b'' = 11.4 Hz, 1H; H-9b''), 3.44 (d app, J7'',9'' = 9.4, 1H; H-7''), 3.29–3.24 (m, 1H; H-7b'), 2.39 (dd, J3a'',4'' = 4.8, J3a'',3b'' = 12.9 Hz, 1H; H-3a''), 1.93-1.69 (overlapping, 4H; H-5a', H-5b', H-6a' and H-6b'), 1.66 ppm (dd, J3b'',4'' = 11.2, J3a'',3b'' = 12.9 Hz, 1H; H-3b''). 13C NMR (MeOD): δ = 171.6 (C-1''), 167.7 (C-4), 159.9 (1C, JCF = 25.7 Hz, COCF2CF2CF3), 158.4 (C-2), 142.8 (C-6), 120.9-107.10 (3C, COCF2CF2CF3), 100.1 (C-2''), 96.3 (C-5), 93.1 (C-1'), 84.2 (C-4'), 75.8 (C-2'), 75.0 (C-3'), 71.7 (C-8''), 71.4 (C-6''), 70.2 (C-7''), 67.3 (C-4''), 65.3 (C-9''), 64.4 (C-7'), 54.4 (C-5''), 53.5 (COOCH3), 42.0 (C-3''), 31.0 (C-5' or C-6'), 27.0 (C-6' or C-5'). ppm; MS (ESI positive) m/z: 753.1[M+Na]+, (ESI negative) m/z: 729.1[M-H]−. Elemental analysis calc % for C23H33F7N4O13: C 41.10, H 4.55, N 7.67; found C 41.30, H 4.40, N 7.82.

Step 3: Finally, methyl ester of 3b (130 mg, 0.10 mmol) was deprotected according to general deprotection procedure step 3(a) to afford 3c as white powder (119 mg 92%), after purification by HPLC-RP chromatography according to step 4 of general deprotection procedure. The compound 3c showed: m.p. 125-126°C; [α]D 20 = +22.1 (c = 1 in H2O). 1H NMR (D2O): δ = 7.64 (d, J6,5 = 7.6 Hz, 1H; H-6), 6.05 (d, J5,6 = 7.6 Hz, 1H; H-5), 5.86 (d, J1,2 = 4.0 Hz, 1H; H-1'), 4.30 (dd, J2',3' = 4.0, J2',5' = 4.6 Hz, 1H; H-2'), 4.20 (ddd, J4',3a'' = 4.9, J4',5'' = 10.2, J4',3b'' = 11.2 Hz, 1H; H-4''), 4.09-4.00 (overlapping, 4H; H-3', H-4', H-5'' and H-6''), 3.91-3.82 (overlapping, 2H; H-8'' and H-9a''), 3.63-3.57 (overlapping, 2H; H-7a and H-9b''), 3.47 (d app, J7'',8'' = 9.4, 1H; H-7''), 3.37-3.31 (m, 1H; H-7b'), 2.40 (dd, J3a'',4'' = 4.9, J3a'',3b'' = 13.2 Hz, 1H; H-3a''), 1.95-1.69 (overlapping, 4H; H-5a', H-5b', H-6a' and H-6b'), 1.66 ppm (dd, J3b'',4'' = 11.2, J3a'',3b'' = 13.2 Hz, 1H; H-3b''). 13C NMR (D2O): δ = 176.1 (C-1''), 166.9 (C-4), 160.4 (1C, JCF = 26.6 Hz, COCF2CF2CF3), 158.3 (C-2), 142.2 (C-6), 120.9-107.0 (3C, COCF2CF2CF3), 100.6 (C-2''), 97.1 (C-5), 91.0 (C-1''), 84.0 (C-4''), 74.6 (C-2''), 73.6 (C-3'), 70.8 (C-8''), 70.0 (C-6''), 69.1 (C-7''), 67.0 (C-4''), 64.3 (C-9''), 63.2 (C-7'), 53.8 (C-5'') 41.0 (C-3''), 30.1 (C-5' or C-6'), 25.9 (C-6' or C-5').
ppm; MS (ESI negative) $m/z$ 715.2[M-H], 737.4[M-2H+Na]. Elemental analysis calcd (%) for C$_{24}$H$_{31}$F$_7$N$_4$O$_{13}$: C 40.23, H 4.36, N 7.82; found C 40.60, H 4.13, N 7.30.
Synthesis of compound 3d via 3 step deprotection and preparative HPLC purification.

**Step 1:** Starting from 13b, (352 mg, 0.40 mmol) according to the general deprotection procedure step 1, the de-acetonide compound of 13b (255 mg 76%) was obtained, after flash chromatography (eluting with AcOEt/MeOH 85:15), as a white solid and showing: m.p. 132-134°C; [α]D 20 = -24.1 (c = 1 in MeOH); 1H NMR (CD3OD): δ 8.08 (d, J6,5 = 7.4 Hz, 1H; H-6), 7.49 (d, J5,6 = 7.4 Hz, 1H; H-5), 5.85 (d, J1',2' = 1.4, 1H; H-1'), 5.47-5.41 (m, 1H; H-8'), 5.33 (d, J7',8' = 8.7 Hz, 1H; H-7'), 4.96-4.84 (m, 1H; H4''), 4.36-4.27 (overlapping, 2H; H-6'' and H-9a''), 4.18-4.15 (m, 1H; H-2'), 4.14-4.03 (overlapping, 3H; H-3', H-4' and H-9b''), 4.02 (t app, J5'',4'' = J5'',6'' = 10.7 Hz, 1H; H-5'') 3.90-3.80 (overlapping, 4H; H-7a' and COOCH3), 3.44-3.38 (m, 1H; H-7b'), 2.71 (dd, J3a'',4'' = 4.6, J3b'',4b'' = 12.5 Hz, 1H; H-3a''), 2.20 (s, 3H; NHCOCOCH3), 2.17 (s, 3H; CH3COO), 2.13 (s, 3H; CH3COO), 2.01 (s, 3H; CH3COO), 1.99 (s, 3H; CH3COO), 1.94-1.72 ppm (overlapping, 5H; H-5a', H-5b', H-6a', H-6b' and H-3b'''). MS (ESI positive) m/z:863.6[M+Na]+. Elemental analysis calcd (%) for C33H43F3N4O18: C 47.15, H 5.16, N 6.66, found C 47.66, H 5.20, N 6.40.

**Step 2:** This intermediate (164 mg, 0.20 mmol) was deacetylated according to general deprotection procedure step 2 to afford, after flash chromatography, (eluting with AcOEt/MeOH 8:2), the intermediate methyl ester of 3d (106 mg 84%) as white powdered showing: m.p. 132-134°C; [α]D 20 = -16.2 (c = 1 in MeOH); 1H NMR (CD3OD): δ 7.61 (d, J6,5 = 7.3 Hz, 1H; H-6), 5.95 (d, J5,6 = 7.3 Hz, 1H; H-5), 5.79 (br d, J1,2 = 1.7Hz 1H; H-1'), 4.09 (br s, 1H; H-2'), 3.99-3.91 (overlapping, 2H; H-4' and H-5''), 3.90-3.80 (overlapping, 8H; H-3', H-6'', H-7a', H-8'', H-9a''' and COOCH3), 3.71 (dd, J4'',3a'' = 4.6, J4'',5'' = 10.2, J4'',3b''' = 12.4 Hz, 1H; H-4''), 3.64 (dd, J9b'',8'' = 5.9, J9a'',9b'' = 5.9, J9a'',9b'' = 5.9, J9a'',9b'' = 5.9, J9a'',9b'')}
11.9 Hz, 1H; H-9b’’), 3.50-3.42 (overlapping, 2H; H-7a’ and H-7b’), 2.68 (dd, \(J_{3\text{a}'',4''} = 4.6, J_{3\text{a}'',3\text{b}''} = 12.8\) Hz, 1H; H-3a’’), 1.91-1.63 ppm (overlapping, 5H; H-5a’, H-5b’, H-6a’, H-6b’ and H-3b’’).

MS (ESI negative) \(m/z: 629.2\) [M-H]. Elemental analysis calcd (%) for \(\text{C}_{23}\text{H}_{33}\text{F}_3\text{N}_4\text{O}_{13}\): C 43.81, H 5.28, N 8.89, found C 43.94, H 5.40, N 8.72.

**Step 3:** Finally, methyl ester of 3d (114 mg, 0.10 mmol) was deprotected according to **general deprotection procedure step 3(B)** to afford the compound 3d (96 mg 86%) as white powder after purification by HPLC-RP chromatography showing: m.p. 125-128°C; \([\alpha]_D^{25} = -20.5\) (c = 1 H2O); \(^1\)H NMR (D2O): \(\delta = 8.12\) (d, \(J_{6,5} = 7.7\) Hz, 1H; H-6), 6.18 (d, \(J_{5,6} = 7.7\) Hz, 1H; H-5), 5.88 (d, \(J_{1',2'} = 3.9\) Hz, 1H; H-1’), 4.34–4.29 (m, 1H; H-2’), 4.12–3.99 (overlapping, 3H; H-3’, H-4’ and H-5’’), 3.96–3.84 (overlapping, 4H; H-6’, H-7a’, H-8’’ and H-9a’’), 3.85–3.68 (m, 1H; H-4’’), 3.64 (dd, \(J_{9\text{a}'',8''} = 6.2, J_{9\text{a}'',9\text{b}''} = 11.5\) Hz, 1H; H-9b’’), 3.59-3.50 (overlapping, 2H; H-7b’ and H-7’’), 2.81 (dd, \(J_{3\text{a}'',4''} = 4.6, J_{3\text{a}'',3\text{b}''} = 12.6\) Hz, 1H; H-3a’’), 1.88–1.67 ppm (overlapping, 5H; H-5a’, H-5b’, H-6a’, H-6b’ and H-3b’’); MS (ESI negative) \(m/z: 629.1\) [M-H]. Elemental analysis calcd (%) for \(\text{C}_{23}\text{H}_{33}\text{F}_3\text{N}_4\text{O}_{13}\): C 43.81, H 5.28, N 8.89; found: C 45.76, H 5.10, N 8.96.
Synthesis of compound 3e via 3 step deprotection and preparative HPLC purification.

Step 1: Starting from 13c, (392 mg, 0.40 mmol) according to the general deprotection procedure step 1, the de-acetonide compound (271 mg 72%) was obtained, as white powder, after flash chromatography (eluting with AcOEt/MeOH 85:15), showing: m.p. 122-124 °C; [α]D20 = -14.9 (c = 1 in MeOH); 1H NMR (CD3OD): δ 8.06 (d, J6,5 = 7.5 Hz, 1H; H-6), 7.46 (d, J5,6 = 7.5 Hz, 1H; H-5), 5.83 (d, J1,2 = 2.5, 1H; H-1’), 5.41 (ddd, J8,9a = 2.7, J8,9b = 5.2, J8,7 = 8.9 Hz, 1H; H-8”), 5.29 (dd, J7,6 = 2.1, J7,8 = 8.9 Hz, 1H; H-7”), 4.96-4.88 (m, 1H; H4”), 4.34 (dd, J6,7 = 1.9 Hz, J6,5 = 10.7 Hz, 1H; H-6a”), 4.29 (dd, J9a,8 = 2.7 Hz, J9a,8 = 12.5 Hz, 1H; H-9a”), 4.15 (dd, J2,1 = 2.5, J2,3 = 5.2 Hz, 1H; H-2’), 4.09-4.00 (overlapping, 3H; H-4’, H-5’ and H-9b”), 3.88-3.78 (overlapping, 5H; H-3’, H-7a’ and COOCH3), 3.42-3.36 (m, 1H; H-7b’), 2.70 (dd, J3a,4 = 4.7, J3a,3b = 12.7 Hz, 1H; H-3a”), 2.18 (s, 3H; NHCOCH3), 2.15 (s, 3H; CH3COO), 2.11 (s, 3H; CH3COO), 1.99 (s, 3H; CH3COO), 1.96 (s, 3H; CH3COO), 1.91-1.69 ppm (overlapping, 5H; H-5a’, H-5b’, H-6a’, H-6b’ and H-3b’); 13C NMR (MeOD): δ = 173.0 (1C, NHCOCH3 at C-4), 172.5, 171.9, 171.5, 171.4 (4C, CH3COO), 169.5(C-1”), 164.2 (C-4), 159.6 (1C, JCF = 27 Hz, COCF2,CF2,CF3), 158.1 (C-2), 146.1 (C-6), 124.0-110.0 (3C, COCF2,CF2,CF3), 100.1 (C-2”), 98.2 (C-5), 93.8 (C-1”), 84.7 (C-4”), 76.4 (C-2”), 75.0 (C-3”), 72.4 (C-6”), 72.3 (C-4”), 70.5 (C-8”), 68.6 (C-7”), 65.7 (C-7”), 63.5 (C-9”), 53.3 (COOCH3), 50.7 (C-5”), 39.2 (C-3”), 30.7 (C-5”), 27.5 (C-6”), 24.6 (1C, NHCOCH3 at C-4), 21.3, 20.8, 20.7, 20.6 ppm (4C, CH3COO). MS (ESI positive) m/z:963.1[M+Na]⁺. Elemental analysis calcd (%) for C_{35}H_{43}F_{7}N_{4}O_{18}: C 44.69, H 4.61, N 5.96, found C 44.66, H 4.73, N 6.03.
**Step 2:** This intermediate (188 mg, 0.20 mmol) was deacetylated according to general deprotection procedure step 2 to afford, after flash chromatography (eluting with AcOEt/MeOH 8:2), the intermediate methyl ester of 3e (126 mg 86%) as white powered showing: m.p. 125-127 °C; [α]_{D}^{20} = -11.1 (c = 1 in MeOH); ¹H NMR (CD_{3}OD): δ 7.61 (d, J_{6,5} = 7.3 Hz, 1H; H-6), 5.94 (br d, J_{5,6} = 7.3 Hz, 1H; H-5), 5.78 (br s, 1H; H-1’), 4.09 (br s, 1H; H-2’), 4.03 (dd, J_{5''',6'''} = J_{5''',4'''} = 10.2, 1H; H-5’’’), 3.96-3.91 (m, 1H; H-4’’’), 3.90-3.79 (overlapping, 8H; H-3’, H-6’, H-7a’, H-8’’, H-9a’’ and COOCH{subscript}3), 3.81-3.72 (m, 1H; H-4’’’), 3.69 (dd, J_{9b''',8} = 6.3, J_{9a''',9b'''} = 11.6 Hz, 1H; H-9b’’’), 3.49-3.42 (overlapping, 2H; H-7b’ and H-7’’’), 2.68 (dd, J_{3a''',4'''} = 4.5, J_{3a''',3b'''} = 12.7 Hz, 1H; H-3a’’’), 1.92-1.65 ppm (overlapping, 5H; H-5a’, H-5b’, H-6’, H-6b and H-3b’’’); ¹³C NMR (CD_{3}OD): δ = 170.9 (C-1’’’), 167.7 (C-4), 160.0 (1C, J_{CF} = 26 Hz, COCF_{2}CF_{2}CF_{3}), 158.4 (C-2), 142.6 (C-6, 124.0-110.0 (3C, COCF_{2}CF_{2}CF_{3}), 100.3 (C-2’’’), 96.3 (C-5), 92.8 (C-1’’), 84.1 (C-4’’’), 75.9 (C-2’’), 75.0 (C-3’’), 73.7 (C-6’’’ or C-8’’’), 72.8 (C-6’’’ or C-8’’’), 70.3 (C-7’’’), 68.3 (C-4’’’), 64.8 (C-7’ or C-9’’’), 64.7 (C-7’ or C-9’’’), 54.1 (C-5’’’), 53.3 (COOCH_{3}), 41.9 (C-3’’’), 30.7 (C-5’’), 27.2 ppm (C-6’’’); MS (ESI positive) m/z: 753.6 [M+Na]⁺. Elemental analysis calcd (%) for C_{25}H_{37}F_{7}N_{3}O_{13}: C 41.10, H 4.55, N 7.67; found C 41.19, H 4.61, N 7.59.

[Diagram of compound 3e]

**Step 3:** Finally methyl ester of 3e (130 mg, 0.10 mmol) was deprotected according to general deprotection procedure step 3(A) to afford 3e (108 mg, 84%) as white powder after purification with HPLC-RP chromatography in accordance with general procedure step 4. The compound 3a showed: [α]_{D}^{25} = -11.3 (c = 1 H_{2}O); ¹H NMR (D_{2}O): δ = 8.05 (d, J_{6,5} = 7.5 Hz, 1H; H-6), 6.09 (br d, J_{5,6} = 7.5 Hz, 1H; H-5), 5.90 (d, J_{1',2'} = 3.8 Hz, 1H; H-1’), 4.36-4.26 (m, 1H; H-2’), 4.12–3.99 (overlapping, 3H; H-3’, H-4’ and H-5’’’), 3.97–3.82 (overlapping, 4H; H-6’’, H-7a’, H-8’’’ and H-9a’’’), 3.81–3.72 (m, 1H; H-4’’’), 3.64 (dd, J_{9b''',8} = 6.3, J_{9a''',9b'''} = 11.5 Hz, 1H; H-9b’’’), 3.59–3.50 (overlapping, 2H; H-7b’ and H-7’’’), 2.79 (dd, J_{3a''',4'''} = 4.8, J_{3a''',3b'''} = 12.6 Hz, 1H; H-3a’’’), 1.92–1.67 ppm (overlapping, 5H; H-5a’, H-5b’, H-6a’, H-6b’ and H-3b’’’); ¹³C NMR (D_{2}O): δ = 176.6 (C-1’’’), 166.3 (C-4), 159.8 (1C, J_{CF} = 26 Hz, COCF_{2}CF_{2}CF_{3}), 157.7 (C-2), 141.5 (C-6), 120.0-110.0 (3C, COCF_{2}CF_{2}CF_{3}), 100.8 (C-2’’’), 96.4 (C-5), 90.2 (C-1’’), 83.4 (C-4’’’), 74.0 (C-2’’), 73.0 (C-3’’), 72.1 (C-6’’’ or C-8’’’), 71.9 (C-6’’’ or C-8’’’), 68.4 (C-7’’’), 68.1 (C-4’’’), 64.5 (C-7’’’), 62.6 (C-9’’’), 53.0 (C-5’’’), 40.6 (C-3’’’), 29.3 (C-5’’), 25.5 ppm (C-6’’’); MS (ESI negative) m/z: 715.3 [M-H]⁻;
elemental analysis calcd (%) for C$_{34}$H$_{31}$F$_7$N$_4$O$_{13}$: C 40.23, H 4.36, N 7.82; found: C 40.39, H 4.24, N 7.85.
5.3.6 General procedure of 1,7 lactonization reaction

Triethylamine (1.24 mmol) was added to a stirred solution of starting material 3b-c or 17 (0.10 mmol) in THF-DMF mixture (1.5 mL and 1 mL), stirring the solution at 0 °C, for 5 min. Then, CbzCl (0.14 ml, 0.98 mmol), dissolved in THF (1 mL), was added dropwise and the mixture was stirred at 23 °C, for 1 h. Then, the reaction MeOH (1.5 mL) was added and the stirring was continued for 15 min. After evaporation of the solvent under high vacuum (0.1 mmHg), a crude residue was obtained which, after purification by flash chromatography afforded the pure lactone 18 and 4b-c.
Synthesis of compound 4a.

The compound 12a (165 mg) was treated with the general deprotection procedure step 2 and step 3(C) to afford, the compound 17 (95 mg 79%) as white solid showing: [α]D20 = +39.1.1 (c = 1, H2O). 1H NMR (D2O): δ = 7.64 (d, J6,5 = 7.5 Hz, 1H; H-6), 6.02 (d, J5,6 = 7.5 Hz, 1H; H-5), 5.81 (d, J1',2' = 2.1 Hz; 1H; H-1’), 5.09 (dd, J2',1' = 2.1, J2',3' = 6.5 Hz, 1H; H-2’), 4.73-4.64 (m, 1H; H-3’), 4.16-4.11 (m, 1H; H-4’), 4.07 (dd, J4''',3a'''' = 4.9, J4''',5'''' = 10.4 J4'',3b'''' = 10.8, Hz, 1H; H-4''') 3.91-3.77 (overlapping,4H; H-5'', H-6'', H-8'' and H-9a’’), 3.66 (dd, J9b',8'' = 5.6, J9a',9b'' = 12.1 Hz, 1H; H-9b’’), 3.56-3.50 (overlapping, 2H; H-7a’ and H-7’’), 3.36-3.29 (m, 1H; H-7b’), 2.37 (dd, J3a'',4'''' = 4.8, J3a'',3b'''' = 13.0 Hz, 1H; H-3a’’), 2.04 (s, 3H; NHCOCH3), 1.84-1.77 (overlapping, 2H; H-5a’’and H-5b’’), 1.72-1.61 ppm (overlapping, 3H; H-6a’, H-6b’ and H-3b’’). 13C NMR (D2O): δ = 178.1(C-1’’), 175.4 (1C, NHCOCH3), 167.1 (C-4), 157.8 (C-2), 143.7 (C-6), 115.6 (C(CH3)2), 100.5 (C-1’’), 96.7 (C-5), 91.2 (C-1’’), 86.9 (C-4’’), 85.0 (C-2’’), 83.7 (C-3’’), 70.8 (C-6’’), 70.7 (C-8’’), 69.0 (C-7’’), 67.8 (C-4’’), 64.2 (C-9’’), 63.0 (C-7’’), 52.8 (C-5’’), 40.7 (C-3’’), 30.2 (C-5’’), 26.6 (C-6’’), 25.7 (C(CH3)2 ), 25.1 (C(CH3)2), 22.8 (1C, NHCO CH3); MS (ESI negative) m/z:601.4[M-H]. Elemental analysis calcd (%) forC25H38N6O13: C 49.83, H 6.36, N 9.30; found C 49.89, H 6.14, N 9.10.
The intermediate compound 17 (60 mg, 0.10 mmol), according to general procedure of 1,7 lactonization, is transformed to pure lactone 18 (41 mg 70%) after purification with flash chromatography (eluting with AcOEt/MeOH 80:20 v/v). Compound 18 showed: [α]_D = +23.1 (c = 1, CH3OH). 1H NMR (CD3OD): δ = 7.63 (d, J_6,5 = 7.5 Hz, 1H, H-6), 5.91 (d, J_5,6 = 7.5 Hz, 1H, H-5), 5.77 (d, J_1,2 = 2.3 Hz, 1H, H-1'), 4.95 (dd, J_2',1' = 2.3, J_2',3' = 6.5 Hz, 1H, H-2'), 4.63 (dd, J_3',4' = 4.8, J_3',2' = 6.5 Hz, 1H, H-3'), 4.58 (br s, 1H, H-6''), 4.46 (dd, J_2',6'' = 1.0, J_1',2' = 7.8 Hz, 1H, H-7''), 4.07-4.04 (m, 1H, H-4''), 4.04-3.99 (m, 1H, H-4'), 3.97 (br d, J_5',4' = 1.2 Hz, 1H, H-5''), 3.82-3.69 (overlapping, 4H, H-7a'', H-8'', H-9a'' and H-9b''), 3.48-3.42 (m, 1H, H-7b'), 2.12 (dd, J_3a',4' = 3.3, J_3a',3b' = 14.1 Hz, 1H, H-3a''), 2.06 (dd, J_3b',4' = 2.1, J_3b',3a' = 14.1 Hz, 1H, H-3b''), 2.02 (s, 3H, NHCOCH3), 1.84-1.78 (overlapping, 2H, H-5a' and H-5b'), 1.74-1.66 (overlapping, 2H, H-6a' and H-6b'), 1.55 (s, 3H, C(CH3)2), 1.35 ppm (s, 3H, C(CH3)3).

The compound 18 (50 mg 0.09 mmol) was dissolve in 200 µL of water and it was treated with a solution of CF3COOH aq at 95% v/v (8 µL 0.10 mmol) under stirred, for 30 minute at room temperature. Then the reaction is neutralized by the added of Amberlite IRA-67, filtered and evaporated in vacuo. The crude reaction was purified by HPLC-RP according to the general procedure of deprotection step 4, to afford as first eluate the pure compound 4a (30 mg 65%) and 3a as second eluate (5 mg 10%). Compound 4a showed: [α]_D = +31.0 (c = 1 in MeOH). 1H NMR (CD3OD): δ = 7.60 (d, J_5,6 = 7.5 Hz, 1H, H-6), 5.91 (d, J_5,6 = 7.5 Hz, 1H, H-5), 5.77 (d, J_1,2 = 3.3 Hz, 1H, H-1'), 4.56 (br s, 1H, H-6''), 4.44 (d app, J_7',8'' = 7.8, 1H, H-7''), 4.11-4.08 (m, 1H, H-2'), 4.05-4.02 (m, 1H, H-4''), 3.96-3.94 (m, 1H, H-4'), 3.94-3.84 (m, 1H, H-5''), 3.84-3.68 (overlapping, 5H, H-3', H-7a', H-8'', H-9a'' and H-9b''), 3.50-3.43 (m, 1H, H-7b'), 2.10 (dd, J_5a',4' = 3.4, J_3a',3b' = 14.1 Hz, 1H, H-3a''), 2.05 (dd, J_3b',4' = 2.1, J_3b',3a' = 14.2 Hz, 1H, H-3b''), 1.88-1.69 (overlapping, 4H, H-5a', H-5b', H-6a' and H-6b'). MS (ESI positive) m/z: 567.1 [M+Na]⁺. Elemental analysis calcd (%) for C25H36N4O12: C 51.37, H 6.21, N 9.58; found C 51.28, H 6.29, N 9.61.
Compound 3a showed: $\delta = 7.62$ (d, $J_{6,5} = 7.5$ Hz, 1H; H-6), 6.01 (d, $J_{5,6} = 7.5$ Hz, 1H; H-5), 5.87 (d, $J_{1',2'} = 3.9$ Hz; 1H; H-1'), 4.31 (t app, $J_{2',1'} = 3.9$, $J_{2',3'} = 5.4$ Hz, 1H; H-2'), 4.11-4.06 (overlapping, 2H; H-4' and H-4''), 4.02 (t app, $J_{3',2'} = 5.4$ Hz, 1H; H-3'), 3.97-3.82 (overlapping, 4H; H-5'', H-6'', H-8'' and H-9a''), 3.71 (dd, $J_{9b'',8''} = 5.4$, $J_{9a'',9b''} = 11.7$ Hz, 1H; H-9b''), 3.62-3.56 (m, 1H; H-7a'), 3.53 (d, $J_{7'',8''} = 9.4$ Hz, 1H; H-7''), 3.38-3.30 (m, 1H; H-7b'), 2.37 (dd, $J_{3a'',4''} = 4.8$, $J_{3a'',3b''} = 12.9$ Hz, 1H; H-3a''), 2.05 (s, 3H; NHCOCH$_3$), 1.94-1.86 (m, 1H; H-5a'), 1.82-1.69 (overlapping, 3H; H-5b', H-6a' and H-6b'), 1.63 ppm (t app, $J_{3b'',3a''} = 12.9$ Hz, 1H; H-3b''). MS (ESI negative) $m/z$:561.1[M-H]. All other physicochemical properties practically are superimposable to those previously reported for the same compound.
Synthesis of compound 4b.

Starting from compound 3b (61 mg, 0.10) and operating according to 1,7 lactonization procedure, the compound 4b (42 mg 70%) was obtained in pure form, after flash chromatography (eluting with: AcOEt/MeOH 85:15). The compound 4b showed: m.p. 123-124 °C; [α]D 20 = +19.1 (c = 1 in MeOH). 1H NMR (CD3OD): δ = 7.62 (d, J6,5 = 7.5 Hz, 1H; H-6), 5.96 (d, J5,6 = 7.5 Hz, 1H; H-5), 5.80 (d, J1',2' = 3.5 Hz, 1H; H-1’), 4.67 (br s, 1H; H-6’’), 4.47 (d app, J7'',8'' = 7.9, 1H; H-7’’), 4.14-4.08 (overlapping, 2H; H-2’ and H-4’’), 4.00 (br s, 1H; H-5’’), 3.95-3.90 (m, 1H; H-4’’), 3.86-3.75 (overlapping, 4H; H-3’, H-7a’, H-8’’ and H-9a’’), 3.75-3.69 (m, 1H; H-9b’’), 3.49-3.42 (m, 1H; H-7b’’), 2.16 (dd, J3a'',d'' = 3.5, J3a'',3b'' = 14.2 Hz, 1H; H-3a’’), 2.07 (dd, J3b'',d'' = 1.5, J3b'',3a'' = 14.2 Hz, 1H; H-3b’’), 1.89-1.61 (overlapping, 4H; H-5a’, H-5b’, H-6a’ and H-6b’’). 13C NMR (MeOD): δ = 170.1 (C-1’’), 167.6 (C-4), 159.0 (C-1C, JCF = 38 Hz, COCF3), 158.6 (C-2), 142.5 (C-6), 117.3 (1C, COCF3), 96.5 (C-5), 96.1 (C-2’’), 92.5 (C-1’), 84.4 (C-4’’), 79.7 (C-7’’), 76.0 (C-2’), 74.9 (C-3’), 73.0 (C-8’’), 71.3 (C-6’’), 67.0 (C-4’’), 65.0 (C-7’), 63.5 (C-9’’), 53.8 (C-5’’), 38.0 (C-3’’), 30.9 (C-5’ or C-6’), 27.2 (C-6’ or C-5’). MS (ESI positive) m/z: 621.2 [M+Na]+. Elemental analysis calcd (%) for C22H29F3N4O12: C 44.15, H 4.89, N 9.36; found C 44.71, H 4.65, N 9.10.
Synthesis of compound 4c.

Starting from compound 3c (72 mg, 0.10 mmol) and operating according to 1,7 lactonization procedure, the compound 4c (50 mg, 71%) was obtained in pure form, after flash chromatography (eluting with: AcOEt/MeOH 85:15). The compound 4c showed: $[\alpha]_D^{20} = -42.9$ (c = 1 in MeOH).$^1$H NMR (CD$_3$OD): $\delta = 7.60$ (d, $J_{6,5} = 7.5$ Hz, 1H; H-6), 5.92 (d, $J_{5,6} = 7.5$ Hz, 1H; H-5), 5.77 (d, $J_{1',2'} = 3.3$ Hz, 1H; H-1’), 4.68 (br s, 1H; H-6’’), 4.46 (d app, $J_{7'',8''} = 8.0$, 1H; H-7’’), 4.13-4.07 (overlapping, 2H; H-2’ and H-4’’), 4.04 (br s, 1H; H-5’’), 3.94-3.88 (m, 1H; H-4’’), 3.84-3.75 (overlapping, 4H; H-3’, H-7a’, H-8’’ and H-9a’’), 3.74-3.70 (m, 1H; H-9b’’), 3.48-3.43 (m, 1H; H-7b’’) 2.15 (dd, $J_{3a'',4''} = 3.4$, $J_{3a'',3b''} = 14.2$ Hz, 1H; H-3a’’), 2.07 (dd, $J_{3b'',4''} = 2.3$, $J_{3b'',3a''} = 14.2$ Hz, 1H; H-3b’’), 1.89-1.68 (overlapping, 4H; H-5a’, H-5b’, H-6a’ and H-6b’’).$^{13}$C NMR (MeOD): $\delta = 170.0$ (C-1’’), 167.6 (C-4), 159.4 (C-1, $J_{C,F} = 27$ Hz, COCF$_2$CF$_2$CF$_3$), 158.5 (C-2), 142.6 (C-6), 120.1-109.3 (3C, COCF$_2$CF$_2$CF$_3$), 96.4 (C-5), 96.1 (C-2’’), 92.6 (C-1’), 84.3 (C-4’), 79.7 (C-7’’), 76.0 (C-2’), 74.9 (C-3’), 73.0 (C-8’’), 71.3 (C-6’’), 66.9 (C-4’’), 65.0 (C-7’), 63.5 (C-9’’), 54.1 (C-5’’), 38.0 (C-3’’), 30.9 (C-5’ or C-6’), 27.2 (C-6’ or C-5’); MS (ESI positive) $m/z$: 721.1 [M+Na]$^+$. Elemental analysis calcd (%) for C$_{24}$H$_{29}$F$_7$N$_4$O$_{12}$: C 41.27, H 4.18, N 8.02; found C 41.11, H 4.39, N 8.13.
5.3.7 General procedure to synthesize peracetylated compounds 5a-c and 5e.

**Step 1: General procedure of actonide deprotection**
To a solution of appropriate 12a-c and 13c (0.4 mmol) in dichloromethane (5mL) moist TFA (0.6 mmol) was added and the mixture was stirred for 30 minutes or 1 hour at reflux. Then the mixture, treated with weak basic resin IRA 67, was filtered and evaporated in vacuo to give a syrup, which was purified by chromatography on silica gel, to afford the desired deprotected intermediate.

**Step 2: General procedure of acetylation**
The de-acetonide compounds of 12a-c or 13c (0.2 mmol) was dissolved in pyridine (2 ml) and, to this solution, was added acetic anhydride (1.6 mmol). Then the reaction was stirred for 3h at 23°C. At this time, the reaction mixture was diluted with AcOEt, washed with aqueous HCl (1 M) and worked-up, to afford, after purification by flash chromatography the desiderated peracetylated compounds.
Synthesis of compound 5a

**Step 1:** Starting from 12a, (331 mg, 0.4 mmol) according to the general **procedure to obtain peracetylated compound step 1**, the de-acetonide compound of 12a was obtained, after flash chromatography (eluting with AcOEt/MeOH 9:1) as white powder (242 mg 77%). This compound showed: $^1$H NMR (MeOD): $\delta = 8.02$ (d, $J_{6,5} = 7.4$ Hz, 1H; H-6), 7.40 (d, $J_{5,6} = 7.4$ Hz, 1H; H-5), 5.83 (d, $J_{1',2'} = 2.5$, 1H; H-1’), 5.41 (dd, $J_{7',6'} = 2.2, J_{7',8'} = 4.1$ Hz, 1H; H-7’), 5.30–5.25 (m, 1H; H-8’), 5.26–5.22 (m, 1H; H-4’), 4.80 (dd, $J_{9a',8'} = 2.4$ Hz, $J_{9a',3'} = 12.4$ Hz, 1H; H-9a’), 4.20 (dd, $J_{2',1'} = 2.5, J_{2',3} = 5.2$ Hz, 1H; H-2’), 4.18–4.07 (overlapping, 2H; H-6’ and H-9b’), 4.06–4.02 (m, 1H; H-4’), 3.95 (t, $J_{3',4'} = J_{5',6'} = 10.3$ Hz, 1H; H-5’), 3.86 (dd, $J_{3',2'} = 5.2, J_{3',4} = 7.1$ Hz, 1H; H-3’), 3.80 (s, 3H; COOCH$_3$) 3.66–3.59 (m, 1H; H-7a’), 3.47—3.41 (m, 1H; H-7b’), 2.47 (dd, $J_{3a',4'} = 5.0, J_{3a',3b'} = 13.0$ Hz, 1H; H-3a’), 2.19 (s, 3H; NHCOCH$_3$ at C-4), 2.11 (s, 3H; OCOCH$_3$), 2.04–1.96 (overlapping, 11H; 3X OCOCH$_3$, H-5a’ and H-5b’), 1.90–1.81 (overlapping, 5H; NHCOCH$_3$ at C-5’, H-6a’ and H-6b’), 1.79 ppm (dd, $J_{3b',4'} = 11.5, J_{3b',3a'} = 13.0$ Hz, 1H; H-3b’). MS (ESI positive) $m/z$:809.1[M+Na]$^+$. All other physicochemical properties are practically superimposable to those previously reported for the same compound.

**Step 2:** The de-acetonide compound of 12a (157 mg, 0.2 mmol) was treated, according to the general **procedure to obtain peracetylated compound step 2**, for affording the compound 5a (159 mg, 91%) after flash chromatography (eluting with AcOEt/MeOH 99:1) as white solid showing: $[\alpha]_D^{20} = +32.1$ (c = 1 CH$_3$OH). $^1$H NMR(CD$_3$OD) $\delta = 8.02$ (d, $J_{6,5} = 7.5$ Hz, 1H; H-6), 7.47 (d, $J_{5,6} = 7.5$ Hz, 1H; H-5), 5.91 (d, $J_{1',2'} = 3.8$ Hz, 1H; H-1’), 5.57 (dd, $J_{2',1'} = 3.8, J_{2',3} = 5.9$
Hz, 1H; H-2’), 5.41 (dd, \(J_{7''},6'' = 2.0, J_{7''},8'' = 4.0\) Hz, 1H; H-7’’), 5.30-5.24 (overlapping 2H; H-3’
and H-8’’), 5.21 (ddd, \(J_{4''},3a'' = 4.9, J_{4''},5'' = 10.5, J_{4''},3b'' = 11.4\) Hz, 1H; H-4’’), 4.80 (dd, \(J_{9a''},8'' = 2.3, J_{9a''},9b'' = 12.3\) Hz, 1H; H-9a’’), 4.21-4.15 (m, 1H; H-4’’), 4.12-4.06 (overlapping, 2H; H-6’’ and H-9b’’), 3.97 (dd, \(J_{5''},4'' = 10.5, J_{5''},6'' = 10.4\) Hz, 1H; H-5’’), 3.80 (s, 3H; COOCH₃), 3.64–3.57 (m, 1H; H-7a’’), 3.49-3.39 (m, 1H; H-7b’’), 2.45 (dd, \(J_{3a''},4'' = 4.9, J_{3a''},3b'' = 12.9\) Hz, 1H; H-3a’’), 2.19 (s, 3H; NHCOCH₃ at C-4), 2.11 (s, 6H; 2X OCOCH₃), 2.09 (s, 3H; OCOCH₃) 2.02 (s, 6H; 2X OCOCH₃), 1.98 (s, 3H; OCOCH₃), 1.96-1.87 (overlapping, 2H; H-5a’’ and H-5b’’), 1.85 (s, 3H; OCOCH₃), 1.83-1.57 ppm (overlapping, 3H; H-6a’, H-6b’ and H-3b’’). MS (ESI positive) \(m/z\): 893.5[M+Na]+. Elemental analysis calcd (%) for C₃₇H₅₀F₇N₄O₂₀: C 51.03, H 5.79, N 6.43; found C 51.23, H 5.56, N 6.32.
Synthesis of compound 5b

**Step 1:** Starting from 12b, (352 mg, 0.40 mmol) according to the **general procedure to obtain peracetylated compound step 1**, the de-acetonide compound of 12b was obtained, after flash chromatography (eluting with AcOEt/Exane 9:1) as white powder (266 mg 79%). This compounds showed: $^1$H NMR (CD$_3$OD): $\delta$= 8.02 (d, $J_{6,5}$= 7.5 Hz, 1H; H-6),7.47 (d, $J_{5,6}$ = 7.5 Hz, 1H; H-5),5.83 (d, $J_{1',2'}$ = 2.0 Hz, 1H; H-1’), 5.39 (dd, $J_{7',6'}$= 2.0, $J_{7',8'}$= 4.2 Hz, 1H; H-7’’), 5.38-5.29 (overlapping, 2H; H-4’’ and H-8’’), 4.78 (dd, $J_{9a',8'}$= 2.1,$J_{9a',9b'}$ = 12.2 Hz 1H; H-9a’’), 4.28 (dd, $J_{6',7'}$= 2.0, $J_{6',5'}$= 9.5 Hz, 1H; H-6’’), 4.18 (dd, $J_{2',3'}$ = 2.0, $J_{2',3}$ = 4.9 Hz, 1H; H-2’), 4.12 (dd, $J_{9b',8'}$= 7.4,$J_{9a',9b'}$ = 12.2 Hz 1H; H-9b’’),4.05-3.96 (overlapping, 2H; H-4’ and H-5’’), 3.87-3.77 (overlapping, 4H; H-3’ and COOCH$_3$),3.67–3.59 (m, 1H; H-7a’),3.48-3.42 (m, 1H; H-7b’),2.51 (dd, $J_{3a',4'}$= 4.9, $J_{3a',3b'}$ = 12.9 Hz, 1H; H-3a’’),2.16 (s, 3H; NHCOCH$_3$ at C-4),2.12 (s, 3H; OCOCH$_3$),2.03 (s, 3H; OCOCH$_3$),2.02 (s, 3H; OCOCH$_3$),1.97 (s, 3H; OCOCH$_3$),1.92-1.75 (overlapping, 5H; H-5a’, H-5b’, H-6a’, H-6b’ and H-3b’’). MS (ESI positive) $m/z$:863.3[M+Na]$^+$. All other physicochemical properties are practically superimposable to those previously reported for the same compound.

**Step 2:** The de-acetonide compound of 12b (168 mg, 0.20) was treated, according to the **general procedure to obtain peracetylated compounds step 2**, for affording the compound 5b (164.5 mg 89 %), after flash chromatography (eluting with AcOEt/hexane 9:1) as white solid showing: m.p. 130-131 °C; $[a]_D^{20}$ = +21.3 (c = 1 CH$_3$OH); $^1$H NMR(CD$_3$OD) $\delta$= 8.02 (d, $J_{6,5}$= 7.5 Hz, 1H; H-6), 7.47 (d, $J_{1',2'}$ = 2.0 Hz, 1H; H-1’), 5.39 (dd, $J_{7',6'}$= 2.0, $J_{7',8'}$= 4.2 Hz, 1H; H-7’’), 5.38-5.29 (overlapping, 2H; H-4’’ and H-8’’), 4.78 (dd, $J_{9a',8'}$= 2.1,$J_{9a',9b'}$ = 12.2 Hz 1H; H-9a’’), 4.28 (dd, $J_{6',7'}$= 2.0, $J_{6',5'}$= 9.5 Hz, 1H; H-6’’), 4.18 (dd, $J_{2',3'}$ = 2.0, $J_{2',3}$ = 4.9 Hz, 1H; H-2’), 4.12 (dd, $J_{9b',8'}$= 7.4,$J_{9a',9b'}$ = 12.2 Hz 1H; H-9b’’),4.05-3.96 (overlapping, 2H; H-4’ and H-5’’), 3.87-3.77 (overlapping, 4H; H-3’ and COOCH$_3$),3.67–3.59 (m, 1H; H-7a’),3.48-3.42 (m, 1H; H-7b’),2.51 (dd, $J_{3a',4'}$= 4.9, $J_{3a',3b'}$ = 12.9 Hz, 1H; H-3a’’),2.16 (s, 3H; NHCOCH$_3$ at C-4),2.12 (s, 3H; OCOCH$_3$),2.03 (s, 3H; OCOCH$_3$),2.02 (s, 3H; OCOCH$_3$),1.97 (s, 3H; OCOCH$_3$),1.92-1.75 (overlapping, 5H; H-5a’, H-5b’, H-6a’, H-6b’ and H-3b’’). MS (ESI positive) $m/z$:863.3[M+Na]$^+$. All other physicochemical properties are practically superimposable to those previously reported for the same compound.
d, $J_{5,6} = 7.5$ Hz, 1H; H-5), 5.91 (d, $J_{1',2'} = 3.8$ Hz, 1H; H-1’), 5.51 (dd, $J_{2',1'} = 3.8$, $J_{2',3'} = 6.0$ Hz, 1H; H-2’), 5.38 (dd, $J_{7',6''} = 2.1$, $J_{7',8''} = 4.5$ Hz, 1H; H-7’’), 5.35-5.27 (overlapping 2H; H-4’’ and H-8’’), 5.25 (t app, $J_{3',2'} = J_{4'',5''} = 6.0$ Hz, 1H; H-3’’), 4.77 (dd, $J_{9a'',8''} = 2.5$, $J_{9a'',9b''} = 12.4$ Hz, 1H; H-9a’’), 4.27 (dd, $J_{6'',7''} = 1.9$, $J_{6'',5''} = 10.4$ Hz, 1H; H-6’’), 4.21-4.15 (m, 1H; H-4’’), 4.14-4.06 (m, 1H; H-9b’’), 3.99 (dd, $J_{5'',4''} = 10.3$, $J_{5'',6''} = 10.4$, 1H; H-5’’), 3.81 (s, 3H; COOCH$_3$), 3.64–3.57 (m, 1H; H-7a’), 3.47-3.40 (m, 1H; H-7b’), 2.49 (dd, $J_{3a'',4''} = 5.0$, $J_{3a'',3b''} = 13.0$ Hz, 1H; H-3a’’), 2.18 (s, 3H; NHCOCH$_3$ at C-4), 2.12 (s, 3H; OCOCH$_3$), 2.10 (s, 3H; OCOCH$_3$), 2.09 (s, 3H; OCOCH$_3$) 2.03 (s, 3H; OCOCH$_3$), 2.01 (s, 3H; OCOCH$_3$), 1.96 (s, 3H; OCOCH$_3$), 1.96-1.72 (overlapping, 5H; H-5a’, H-5b’, H-6a’, H-6b’ and H-3b’’). MS (ESI positive) $m/z$:947.6 [M+Na]$^+$. Elemental analysis calcd (%) for C$_{37}$H$_{47}$F$_3$N$_4$O$_{20}$: C 48.05, H 5.12, N 6.06; found C 48.18, H 5.40, N 6.38
Synthesis of compound 5c

**Step 1:** Starting from 12c, (392 mg, 0.40 mmol) according to the general procedure to obtain peracylated compound step 1, the de-acetonide compound of 12c (297 mg 79%) was obtained, after flash chromatography (eluting with AcOEt/MeOH 9:1) as white powder. This compounds showed: $^1$H NMR (CD$_3$OD); δ= 8.04 (d, $J_{6,5}$ = 7.4 Hz, 1H; H-6), 7.37 (d, $J_{5,6}$ = 7.4 Hz, 1H; H-5), 5.83 (d, $J_{1',2'}$ = 2.6 Hz, 1H; H-1’), 5.41-5.27 (overlapping, 3H; H-7’, H-4’’ and H-8’’), 4.76 (dd, $J_{9a',8'}$= 2.3,$J_{9a',5'}$= 12.4 Hz, 1H; H-9a’’), 4.30 (d app, $J_{6',5'}$= 10.4 Hz, 1H; H-6’’), 4.19 (dd, $J_{2',1'}$ = 2.6, $J_{2',3'}$ = 5.0 Hz, 1H; H-2’’), 4.14-4.00 (overlapping, 3H; H-4’, H-5’’ and H-9b’’), 3.87 (dd, $J_{3',2'}$= 5.0, $J_{3',4'}$ = 6.5 Hz, 1H; H-3’), 3.80 (s, 3H; COOCH$_3$), 3.67-3.59 (m, 1H; H-7a’), 3.48-3.41 (m, 1H; H-7b’), 2.53 (dd, $J_{3a',4'}$= 4.7, $J_{3a',3b'}$= 12.9 Hz, 1H; H-3a’’), 2.19 (s, 3H; NHCOCH$_3$ at C-4), 2.12 (s, 3H; OCOCH$_3$), 2.04 (s, 3H; OCOCH$_3$), 2.01 (s, 3H; OCOCH$_3$), 1.96 (s, 3H; OCOCH$_3$), 1.92-1.76 ppm (overlapping, 5H; H-5a’, H-5b’, H-6a’, H-6b’ and H-3b’’); MS (ESI positive) m/z: 963.4[M+Na]$^+$. All other physicochemical properties are practically superimposable to those previously reported for the same compound.

**Step 2:** The de-acetonide compound of 12c (188 mg, 0.20 mmol) was treated, according to the general procedure to obtain peracylated compound step 2, affording the compound 5c (182.4 mg 89 %), after flash chromatography (eluting with AcOEt/hexane 9:1) as white solid showing: [α]$_D^{20}$ = +21.3 (c = 1 CH$_3$OH). $^1$H NMR(CD$_3$OD) δ= 8.02 (d, $J_{6,5}$= 7.5 Hz, 1H; H-6), 7.47 ( d, $J_{5,6}$ = 7.5 Hz, 1H; H-5), 5.91 (d, $J_{1',2'}$ = 3.8 Hz, 1H; H-1’), 5.54 (dd, $J_{2',1'}$ = 3.8, $J_{2',3'}$ = 6.0 Hz, 1H; H-2’), 5.36 (dd, $J_{7',6'}$= 2.0, $J_{7',8'}$= 4.5 Hz, 1H; H-7’’), 5.35-5.27 (overlapping 2H; H-4’’ and H-8’’), 5.25 (t app, $J_{3',2'}$ = 4.5 Hz, 1H; H-3’’), 4.76 (dd, $J_{9a',8'}$= 2.5,$J_{9a',9b'}$= 12.4 Hz, 1H; H-9a’’),
4.29 (dd, $J_{6',7'} = 1.1, J_{6',5'} = 10.6$ Hz, 1H; H-6’), 4.22-4.15 (m, 1H; H-4’), 4.11 (dd, $J_{9b',8'} = 7.3, J_{9b',9a'} = 12.4$ Hz, 1H; H-9a’ 1H; H-9b’), 4.05 (dd, $J_{5',4'} = 10.3, J_{5',6'} = 10.6$, 1H; H-5’), 3.80 (s, 3H; COOCH$_3$), 3.64–3.57 (m, 1H; H-7a’), 3.46–3.40 (m, 1H; H-7b’), 2.51 (dd, $J_{3a',4'} = 5.0, J_{3a',3b'} = 13.0$ Hz, 1H; H-3a’), 2.18 (s, 3H; NHCOCH$_3$ at C-4), 2.12 (s, 3H; OCOCH$_3$), 2.10 (s, 3H; OCOCH$_3$), 2.09 (s, 3H; OCOCH$_3$) 2.03 (s, 3H; OCOCH$_3$), 2.02 (s, 3H; OCOCH$_3$), 1.95 (s, 3H; OCOCH$_3$), 1.93-1.73 ppm (overlapping, 5H; H-5a’, H-5b’, H-6a’, H-6b’ and H-3b’). MS (ESI positive) $m/z$: 1047.2[M+Na]$^+$. Elemental analysis calcld (%) for C$_{39}$H$_{47}$F$_7$N$_4$O$_20$: C 45.71, H 4.62, N 5.47; found C 45.11, H 4.75, N 5.23.
Synthesis of compound 5e

Step 1: Starting from 13c, (392 mg, 0.40mmol) according to the general procedure to obtain peracylated compound step 1, the de-acetonide compound of 13c (275 mg 74%) was obtained, after flash chromatography (eluting with AcOEt/MeOH 9:1) as white powder. This compounds showed: \(^{1}\)H NMR (CD\(_{3}\)OD): \(\delta\) 8.06 (d, \(J_{6,5}\) = 7.5 Hz, 1H; H-6), 7.46 (d, \(J_{5,6}\) = 7.5 Hz, 1H; H-5), 5.82 (d, \(J_{1',2'}\) = 2.5, 1H; H-1’), 5.41 (dd, \(J_{8''},9a''\) = 2.7, \(J_{8''},9b''\) = 5.2, \(J_{8''},7''\) = 8.9Hz, 1H; H-8’’), 5.29 (dd, \(J_{7''},8''\) = 1.8, \(J_{7''},6''\) = 8.9 Hz, 1H; H-7’’), 4.96-4.88 (m, 1H; H4’’), 4.34 (dd, \(J_{6''},7''\) = 1.8 Hz, \(J_{6''},5''\) = 10.7 Hz, 1H; H-6a’’), 4.29 (dd, \(J_{9a''},8''\) = 2.7 Hz, \(J_{9a''},8''\) = 12.5 Hz, 1H; H-9a’’), 4.15 (dd, \(J_{2',1'}\) = 2.5, \(J_{2',3'}\) = 5.2 Hz, 1H; H-2’’), 4.08-4.00 (overlapping, 3H; H-4’, H-5’’ and H-9b’’), 3.88-3.78 (overlapping, 5H; H-3’, H-7a’ and COOCH\(_3\)), 3.42-3.36 (m, 1H; H-7b’), 2.70 (dd, \(J_{3a''},4''\) = 4.6, \(J_{3a''},3b''\) = 12.7 Hz, 1H; H-3a’’), 2.18 (s, 3H; NHCOC\(_3\)H\(_3\)), 2.15 (s, 3H; CH\(_3\)COO), 2.11 (s, 3H; CH\(_3\)COO), 1.99 (s, 3H; CH\(_3\)COO), 1.96 (s, 3H; CH\(_3\)COO), 1.91-1.69 ppm (overlapping, 5H; H-5a’, H-5b’, H-6a’, H-6b’ and H-3b’’). MS (ESI positive) \(m/z\):963.2[M+Na]+. All other physicochemical properties are practically superimposable to those previously reported for the same compound.

Step 2: The de-acetonide compound of 13c (188 mg, 0.20 mmol) was treated, according to the general procedure to obtain peracylated compound step2, for affording the compound 5e (180.4 mg 88 %), after flash chromatography (eluting with AcOEt/hexane 9:1) as white solid, showing:m.p. 132-129 °C; \(\alpha\)\(D\)\(_{20}\) = -18.3 (c = 1 in MeOH); \(^{1}\)H NMR (CD\(_{3}\)OD): \(\delta\) 8.05 (d, \(J_{6,5}\) = 7.5 Hz, 1H; H-6), 7.47 (d, \(J_{5,6}\) = 7.5 Hz, 1H; H-5), 5.93 (d, \(J_{1',2'}\) = 3.8, 1H; H-1’), 5.51 (dd, \(J_{2',1'}\)=3.8,\(J_{2',3'}\)= 6.0 Hz,1H; H-2’), 5.40 (dd, \(J_{8''},9a''\) = 2.6, \(J_{8''},9b''\) = 5.4, \(J_{8''},7''\) = 8.6Hz, 1H; H-8’’),
5.28 (dd, $J_{7''}', 6''' = 2.1, J_{7''}', 8''' = 8.6$ Hz, 1H; H-7’’’), 5.22 (t app, $J_{3''}, 2'''' = J_{3''}, 4'''' = 6.0$ Hz, 1H; H-3’), 4.92 (dd, $J_{4''''}', 3a'''' = 4.6, J_{4''''}', 5'''' = 11.1, J_{4''''}', 3b'''' = 11.9$ Hz, 1H; H4’’’), 4.34 (dd, $J_{6''''}', 7'''' = 2.1$ Hz, $J_{6''''}', 5'''' = 10.7$ Hz, 1H; H-6’’’), 4.30 (dd, $J_{9a''''}', 8'''' = 2.6$ Hz, $J_{9a''''}', 9b'''' = 12.5$ Hz, 1H; H-9a’’’), 4.21-4.16 (m, 1H; H-4’), 4.13-4.01 (overlapping, 2H; H-5’’’ and H-9b’’’), 3.84-3.77 (overlapping, 4H; H-7a’’ and COOCH3), 3.42-3.35 (m, 1H; H-7b’), 2.69 (dd, $J_{3a'''', 4'''''} = 4.6, J_{3a'''', 3b'''''} = 12.7$ Hz, 1H; H-3a’’’), 2.18 (s, 3H; NHCOC3), 2.14 (s, 3H; CH3COO), 2.11 (s, 3H; CH3COO), 2.10-2.09 (overlapping, 6H; 2XCH3COO), 1.99 (s, 3H; CH3COO), 1.96 (s, 3H; CH3COO), 1.90-1.63 ppm (overlapping, 5H; H-5a’, H-5b’, H-6a’, H-6b’ and H-3b’’’); MS (ESI positive) $m/z$: 1047.1 [M+Na]+. Elemental analysis calcd (%) for C39H47F7N4O20: C 45.71, H 4.62, N 5.47; found C 45.13, H 4.70, N 5.31.
5.4 Biological

5.4.1 Inhibition activity assay on commercial α 2,3-sialyltransferase

**ST activity and inhibition assay**

Inhibition of α 2,3-sialyltransferase activity is performed essentially according to using a commercial sialyltransferase activity kit\(^6\). This kit takes advantage of a 5’-nucleotidase to remove inorganic phosphate from the leaving nucleotide cytidine 5’-monophosphate (CMP) of sialyltransferase reactions and malachite green phosphate detection reagents that turn inorganic phosphate to a green colored complex. The amount of inorganic phosphate released by the 5’-nucleotidase is equal to the CMP-sialic acid consumed or the sialyl-conjugate produced; therefore, the rate of inorganic phosphate produced reflects the kinetics of a sialyltransferase reaction.

Briefly, the ST reaction was carried out in 50 µL of reaction buffer (25 mM Tris, 150 mM NaCl, 5 mM MgCl\(_2\) and 5 mM MnCl\(_2\), pH 7.5) in a 96-well plate at room temperature for 20 min. To determine the kinetic parameters of ST, multiple reactions with varied amounts of either the enzyme or substrates were carried out simultaneously in the presence of fixed amounts of all other components, including a coupling phosphatase. Particularly, specific activity against donor substrate CMP-NeuAc in the presence of 1 mM acceptor N-acetyl-D-lactosamine; specific activity vs. acceptor substrate N-acetyl-D-lactosamine in the presence of 0.2 mM CMP-NeuAc and activity vs. enzyme dose in the presence of 1 mM CMP-NeuAc and 4 mM N-acetyl-D-lactosamine. One well containing all components except for the enzyme was used as a blank control. The reactions were initiated by adding the substrates and phosphatase to the enzyme and terminated by the addition of 30µL of Malachite reagent A and 100µL of water to each well. The color was developed by the addition of 30µL of Malachite reagent B to each well followed by gentle mixing and incubation at room temperature for 20 min. Following color development, the plate was read at 620 nm with the multiwell plate reader. A phosphate standard curve was also performed to determine the conversion factor between the absorbance and the inorganic phosphate contents.

For \(K_M\) and \(V_{\text{max}}\) determination, the results were plotted against substrate concentrations and fitted to the Michaelis–Menten equation using the KaleidaGraph 4 program (www.synergy.com).

In order to test our inhibitors, the incubation mixture (final volume of 50µL) contained 0.1 mU of α-2,3-Sialyltransferase from *Pasteurella Multocida*, various amounts of inhibitors (0-500 mM), 1 mM CMP-NeuAc and 4 mM N-acetyl-D-lactosamine, buffer (25 mM Tris, 150 mM NaCl, 5 mM MgCl\(_2\) and 5 mM MnCl\(_2\), pH 7.5). After incubation at 37°C for 20 min, the reactions are stopped by the
addition of 30 µL of Malachite reagent A and 100 µL of water to each well. The color was developed by the addition of 30 µL of Malachite reagent B to each well followed by gentle mixing and incubation at room temperature for 20 min and the ST activity is determined by multiwell plate reader at 620 nm. The inhibition values are obtained by linear regression (using Microsoft Excel) of different concentrations of inhibitors from 0 to 500 mM (five concentrations of each inhibitor are used) with a fixed concentration of substrates. Typical concentration–response plots are obtained from the average values of triplicate assay results.
**5.4.2 Inhibition activity assay on GM3 synthase**

**Cell culture**

The Human Embryonic Kidney, HEK293A, were grown at 37°C in the presence of 5% CO₂, in DMEM medium supplemented with 10% (v/v) FBS, 2mM glutamine and 1mM Penicillin/Streptomycin.

The cryopreservation of these cells is commonly performed in liquid nitrogen and involves the use of freezing medium, containing a mixture of 90% FBS and 10% DMSO.

**Protein quantification: Bradford method**

A standard calibration curve with BSA (bovine serum albumin), at known concentrations, was prepared to determine the protein concentration of the samples.

1 ml of blue dye Coomassie diluted in water 1:4 was added to each sample; then, absorbance was detected with a JASCO spectrophotometer, at 595 nm.

**Inhibiton assay on GM3 synthase**

Cells cultured in 100-mm dishes were harvested using a plastic scraper and washed two times with phosphate-buffered saline. Cells were resuspended in 150 mM sodium cacodylate-HCl buffer, pH 6.6 (20 mg of cell protein/ml) with protease inhibitors (2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.0016 mM aprotinin, 0.044 mM leupeptin, 0.08 mM bestatin, 0.03 mM pepstatin A, 0.028 mM E-64) and homogenized with a Dounce homogenizer (10 strokes, tight). In each reaction tube, 10 µl of Triton CF-54 1.5% (v/v) in chloroform/methanol (2:1) were mixed with [3-³H(sphingosine)]LacCer, corresponding to 45 nCi, from a stock solution in chloroform/methanol (2:1) and dried under N₂. To this mixture, 8 µl of 750 mM sodium cacodylate-HCl buffer, pH 6.6, 4 µl of 125 mM MgCl₂, 4 µl of 125 mM 2-mercaptoethanol, 10 µl of 5 mM CMP-NeuAc, and 10 µl of cell homogenate (containing 200 µg of protein) were added in a total reaction volume of 50 µl.

Briefly, the GM3 activity assay was performed using as positive control the cell homogenate incubated with the radioactive LacCer and in absence of GM3 synthase inhibitors; the negative control was performed using heat-inactivated cell homogenates (100 °C for 3 min) in the presence of 3-³H(sphingosine)] LacCer; the GM3 inhibitors were tested using a final concentration of 1mM or 10µM incubated with the cell homogenate and the radioactive LacCer. All the incubations were performed at 37 °C for 3 h with continuous shaking. The reactions were stopped by adding 1.5 ml of chloroform/methanol (2:1). The reaction mixture (68dpm) was analyzed by HPTLC using the
solvent system chloroform/methanol/water (55:20:3 v/v). Radioactive lipids were detected by analysis with the Beta-Imager 2000 (Biospace, Paris, France) and quantified by densitometric analysis using M3 Vision software (Biospace, Paris, France).

Data are means ±SD of three different experiments, statistical differences were determined by 1-way Anova.
5.4.3 Sphingolipid pattern evaluation by metabolic labeling with [3-^3H] sphingosine ([3-^3H] SPH) in HEK cells treated with inhibitors.

Metabolic assay
The sphingolipid pattern of HEK293 cells, treated with GM3 synthase inhibitors, was determined by metabolic labeling with [3-^3H] sphingosine ([3-^3H] SPH). [3-^3H] SPH is a natural precursor of sphingolipids. When cells are placed in culture medium supplemented with radiolabeled sphingosine, [3-^3H] SPH is absorbed and used into the biosynthetic pathways of sphingolipids. The metabolic products are radioactive, and after extraction and separation by HPTLC, it is possible to evaluate the cellular sphingolipid content in terms of both quality and quantity.

Cells were incubated with 10 µM GM3 synthase inhibitors 2 hours before the addition of [3-^3H] SPH. During the entire assay cells were maintained in the presence of inhibitors. (0.4 µCi) [3-^3H] SPH was administered to the cells after being dissolved in culture medium at a final concentration of 3x10^{-8}M. Subsequently, the lipids were extracted and separated by HPTLC and the chromatographic profile was obtained by Beta-Imager equipment (Biospace).

Data are means ±SD of three different experiments, statistical differences were determined by 1-way Anova.

1. **[3-^3H] SPHINGOSINE PREPARATION:**

   [3-^3H] SPH was dissolved in sterile conditions in DMEM medium with 10% FBS, 2 mM Glutamine.

   The solution was sonicated for 2 min and vortexed for 1 min. This procedure was repeated 3 times. The degree of solubilization (> 70%) was verified by counting the radioactivity by β-counter (Perkin Elmer).

2. **PULSE:**

   The day before, 2,5x10^5 cells were plated.

   The culture medium was replaced with 5ml of medium containing [3-^3H] SPH.

   The cells were then incubated for 2 hours at 37 °C, with 5% CO₂.

   In this phase the cells absorbed the [3-^3H] SPH present in the medium.

   After two hours, the medium was taken from plates and stored for the quantification of [3-^3H] SPH absorbed by cells, by counting the radioactivity with a β-counter (Perkin Elmer).

3. **CHASE**
The cells were grown in 8ml of growth medium without [3-\(^3\)H] SPH, for 48 hours, at 37 ° C with 5% CO\(_2\).

4. **CELL HARVESTING**

At the end of the chase, medium was collected from plates and kept for counting by β-Counter (Perkin Elmer). The cells were washed three times with PBS and harvested.

After being harvested by centrifugation (300xg, 10 min, 4 °C), the cells were freeze and lyophilized.

5. **EXTRACTION OF TOTAL LIPIDS FROM CELL PELLET**

The lyophilized cells were resuspended in 25µl of water, sonicated in ultrasonic bath and vortexed. In order to obtain a good solubilization, two lipid extractions were made.

**FIRST EXTRACTION**

10 volumes of methanol were added to the aqueous solution of cells. The resulting mixture was sonicated in ultrasonic bath for 2 min and vortexed for 1 min.

Then, 20 volumes of chloroform were added. Sonication in ultrasonic bath for 2 minutes and agitation were repeated.

The samples were shaken on an Eppendorf shaker for 10 min and centrifuged at 10,000xg, for 10 min at room temperature.

The supernatant containing the lipids was transferred to a new eppendorf.

**SECOND EXTRACTION**

10 volumes of a mixture of chloroform / methanol 2:1 were added to pellet.

The samples were sonicated in ultrasonic bath, vortexed and shaken for 10 min; then, they were centrifuged at 10,000xg, for 10 min, at room temperature. The lipid supernatant was collected and combined with that collected after the first extraction.

The protein pellets, after the evaporation of solvent, was digested overnight at room temperature in 50µl of 1N NaOH and then, subsequently, increased to 1 ml with water.

Proteins were then measured by the method of Lowry.

The lipid radioactivity was evaluated to determine the percentage yield of the lipid extraction.
PARTITION OF LIPIDS

This procedure allows to divide the extracted lipid in an aqueous phase (FA), containing gangliosides, and an organic phase (FO), containing neutral glycolipids.

The partition was divided into two phases:

FIRST PARTITION

In order to separate the aqueous from the organic phase, a volume of water equal to 20% of the total solution was added to lipid extracts. Samples were vortexed and mixed on Eppendorf mixer for 15 min and centrifuged at 3,500xg for 5 min. The aqueous phase was collected and transferred to a new eppendorf.

SECOND PARTITION

A mixture of methanol / water 1: 1, equal to 40% of the initial volume, was added to the organic phase. The separation of the aqueous phase from the organic phase was obtained by vortexing and agitation on an Eppendorf shaker for 15 min and, finally, centrifugation at 3,500xg for 5 min.

The aqueous phase was collected and added to that obtained in the first separation.

The two separated phases were dried under nitrogen and suspended in a mixture of chloroform/methanol 2:1. The aqueous phase was resuspended in 100 µl of solvent, while the organic phase was resuspended in 200 µl.

Radioactivity assays of the two phases were performed to determine the percentage yield of the partition. The total lipids, neutral glycolipids and gangliosides were then separated by HPTLC and the content of the component detected was expressed as dpm / mg total protein.

In order to separate total lipids and gangliosides was used a solvent consisting of chloroform/methanol/0.2% CaCl₂ (60:40:9) and to separate neutral lipids in the organic phase, a solvent consisting chloroform/methanol/water (110:40:6).
6. REFERENCES
6. REFERENCES


60. J.C.S. Perkin I **1982**.


