Selective inhibitors of human neuraminidase 1 (NEU1)

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ABSTRACT: Inhibitors of human neuraminidase enzymes (NEU) are recognized as important tools for the study of the biological functions of NEU and will be potent tools for elucidating the role of these enzymes in regulating the repertoire of cellular glycans. Here we report the discovery of selective inhibitors of the human neuraminidase 1 (NEU1) and neuraminidase 2 (NEU2) enzymes with exceptional potency. A library of modified 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA) analogs, with variability in the C5- or C9-positions, were synthesized and evaluated against four human neuraminidase isoenyzmes (NEU1–4). Hydrophobic groups with an amide linker at the C5 and C9 positions were well accommodated by NEU1, and a hexanamido group was found to give the best potency at both positions. While the C5-hexanamido-C9-hexanamido-DANA analog did not show synergistic improvements for combined modification, an extended alkylamide at an individual position combined with a smaller group at the second gave increased potency. The best NEU1 inhibitor identified was a C5-hexanamido-C9-acetamido DANA that had a K_i of 53 ± 5 nM and 340-fold selectivity over other isoenzymes. Additionally, we demonstrated that C5-modifications combined with a C4-guandino group provided the most potent NEU2 inhibitor reported, with a K_i of 1.3 ± 0.2 μM and 7-fold selectivity over other NEU isoenzymes.

Introduction

The structure of glycoconjugates influences their physical properties and regulates intermolecular interactions that are crucial to cell function.[1, 2] The addition and removal of sialic acids from the termini of complex glycans is controlled by two major classes of enzymes: sialyltransferases (SiaT) and sialidases (also called neuraminidases, NEU).[3] Thus, both enzyme families are implicated in extracellular glycan remodeling, a process thought to modify the function of glycans through small structural changes.[4, 5] This process provides a rapid mechanism to alter biosynthetically complex glycoconjugates, and sialic acid's placement at the periphery of the glycan and its unique properties (such as negative charge) makes it an ideal participant in such a process.[6] In particular, regulation of the sialylation state of glycans for cell surface glycoproteins and gangliosides by NEU is important in disease and in homeostasis.[7, 8] The effects of bulk desialylation have often been studied in human cells using bacterial sialidases or unspecific inhibitors such as 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA); however, the precise roles of human homologues continues to be an active area of investigation.[9-12]

Four isoenzymes of human NEU (hNEU), have been reported: NEU1, NEU2, NEU3, and NEU4.[7] Since the discovery of human NEU genes almost 20 years ago, investigations of their function have implicated these enzymes in diseases including disorders of metabolism,[13] inflammation,[14] cancer,[15] diabetes,[16-19] and atherosclerosis.[20-23] Although these four isoenzymes vary in their tissue expression, subcellular location, and substrate specificity; their precise biochemical roles remain to be clearly elucidated.[7, 8] Our group has continued to pursue the

development of small molecule inhibitors with selectivity for individual isoenzymes in the hNEU family to be used as research tools to reveal the specific roles of these enzymes.[24]

Several selective inhibitors of human neuraminidases have been previously reported (**Figure 1**).[24] Current examples are based on the DANA (**1**, **Figure 1**) scaffold, which in recent years has yielded a number of potent inhibitors. Inhibitors designed for viral and bacterial enzymes, such as zanamivir **2**, have typically shown lower activity against hNEU.[25, 26] The first selective inhibitor of hNEU reported was a C9-amido analog of DANA **8**,[27] which was found to have micromolar IC_{50} against NEU1 over the other isoenzymes.[28] Several analogues of DANA with C9-triazolyl groups have shown nanomolar activity for NEU3 and NEU4. The most potent inhibitor reported for NEU4 is C9-4-hydroxymethyltriazolyl DANA (C9-4HMT-DANA **3**, K_i = 30 ± 19 nM, 500-fold selective).[29] Modification of DANA with a C9-biphenyltriazolyl generates a bifunctional NEU3/NEU4 inhibitor (C9-4BPT-DANA **4**, K_i = 280 ± 40 nM for NEU3, K_i = 260 ± 40 nM for NEU4); combining this C9 modification with a C4 guanidino group resulted in a NEU3-selective inhibitor (**5**, K_i =320 ± 40 nM, 10-fold selectivity).[26, 30, 31] These examples all illustrate that modifications of the DANA scaffold can lead to potent and selective inhibitors of hNEU.

Despite continued efforts, there have been few reports of selective inhibitors for the NEU1 or NEU2 isoenzymes with good potency. Based on substrate specificity studies of NEU2, Chen and coworkers found that Neu5Ac$N_3$2en **6** was twice as potent as DANA for this isoenzyme.[31-33] Selectivity for NEU2 was shown for a DANA analog where the C7-C9 glycerol side chain of DANA was replaced by an amino-linked benzyl group (**7**), but these modifications resulted in lower potency than the parent DANA.[34] To date, C9-BA-DANA is the most selective inhibitor of

NEU1 reported (IC_{50} 10 μM),[28] and the compound has been used in biological studies to block NEU1 activity in lung cells.[35] Anti-tumor activities have been proposed for oseltamivir **9** due to activity against NEU1 in vivo;[36] however, in vitro inhibition assays find low activity for this compound against hNEU isoenzymes.[25, 26]

In this work, we report on the discovery of the first nanomolar potency NEU1 inhibitors with high selectivity. These compounds were identified from a library of C5-, C9-, and C5, C9-modified DANA analogues. Within the library we also identified inhibitors of NEU2 with single-digit micromolar activity and good selectivity. These findings offer a significant expansion of the tool box for the functional study of human neuraminidases with small molecule inhibitors. Furthermore, the data provide new insights into the topology of the NEU1 active site for which little structural data is available.

Results

Inhibitor Design and Synthesis

Among the four hNEU isoenzymes, NEU1 shows the least sequence identity with NEU2/3/4,[37] and efforts at molecular modeling of NEU1 have been challenging.[25, 38] Several factors contribute to the lack of structural information regarding NEU1, including its occurrence in a membrane-associated complex with carboxypeptidase protective protein/cathepsin A (PPCA) and a β-galactosidase,[39] and the inherent instability of the protein. In the absence of validated structural models of NEU1, we based our design on the limited structure-activity relationship (SAR) studies reported for the target. Magesh et al. screened a panel of amide-linked C9-modified DANA analogues in vitro and found that NEU1 was able to accommodate hydrophobic groups at the C9

position. The ability of NEU1 to accommodate C9-modifications may be the result of a shorter loop between the seventh and eighth strands of the β-propeller fold (the β7–β8 loop) in NEU1 compared with NEU2/3/4.[35] Interestingly, multiple groups have found that small modifications at C5 have maintained or improved the activity of DANA analogs with NEU2 and NEU3.[31-33] From alignments of NEU1/2/3/4, it can be proposed that the C5 binding pocket of all the four human neuraminidase isoenzymes consist of primarily hydrophobic amino acid residues. Based on the above information we set out to explore the C9- and C5-binding pockets of hNEU with hydrophobic groups.

Synthesis of C5-modified DANA analogues began with a previously reported C5-amino-DANA derivative with O-acetyl protection (**10**, **Scheme 1**).[33] Amides were generated using the corresponding acyl chloride or anhydride, with subsequent alkaline deprotection of the O-acyl and C1-methyl ester to provide the final compounds (**11a-l**, **Scheme 1**). We also developed compounds with a C5-aryltriazolyl group after coupling **10** with the N-hydroxysuccinimidyl-2-azidoacetate to provide C5-azidoacetyl-DANA derivative (**12, Scheme 2**).[33, 40] A subsequent CuAAC reaction and deprotection was performed to construct C5-aryltriazolyl DANA analogs **13a-h** (**Scheme 2**).[33] We also applied a previous strategy to generate libraries of C9-amido analogs of DANA **15a-l** (Scheme **3**) using Staudinger reaction of **14** with activated esters, followed by deprotection of C1-methyl ester (**Scheme 3**).[28]

We next developed a route to doubly-modified DANA analogs containing C5, C9- and C4, C5-fragments (**Schemes 4 & 5**, respectively). The synthesis of C5, C9-modified compounds was accomplished using compound **16**, which was synthesized as previously described.[32] The C9-azido

group was converted to a C9-amide moiety using the strategy above. The C5 amide was introduced using the corresponding acyl chlorides or anhydrides after deprotection of *tert*-butyloxycarbonyl group (Boc) with trifluoroacetic acid. The final C5, C9-products **17a-f** were obtained after hydrolysis of the C1-methyl ester. Previous work had suggested that C4 modification of DANA could result in improved activity for NEU2 and NEU3.[26, 30, 31] Thus, we developed a route to compounds which could combine C5-amide modifications with a C4-guanidino group in our library to be tested for selectivity among the hNEU isoenzymes. The C5, C4-doubly modified DANA analogue was synthesized from a globally protected C4-azido-DANA derivative **18** (**Scheme 5**).[41] The C5-acetamido group was first protected with di-*tert*-butyloxydicarbonate (Boc_2O), and then selectively deacylated under mild conditions to generate compound **19**.[33] After protection of the hydroxy groups, Boc was removed. We introduced a C5-amide group that had increased potency for NEU2 in our screens of C5-modified compounds (*vide infra*), the 4-methylbutanamido group (**11h**, **Table 1**), using the 4-methylbutanoyl chloride, yielding compound **20**. The C4-azido group was then reduced by triphenylphosphine and transformed to a guanidino using *N*, *N'*-di-Boc-1*H*-pyrazole-1-carboxamidine to form compound **21**. The final product **22** was obtained after deprotection of the Boc group and hydrolysis of the acyl groups and methyl ester.

Inhibition Assays

The inhibitory activity of DANA analogues were evaluated using the fluorogenic neuraminidase substrate, 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4MU-NANA) and samples of individual hNEU isoenzymes.[42, 43] The IC_{50} assay results for each of the four hNEU enzymes are summarized in **Tables 1-3**, and are grouped by the site of modification on the DANA scaffold: C5

(**11a-11l**, **13a-13i**; **Table 1**), C9 (**15a-15m**; **Table 2**) and C5, C9 (**17a-17h**, **22**; **Table 3**). Substituents were linked to DANA analogs through either an amide (**11a-11l**, **15a-15l**, **17a-17h**) or an azidoacetyl group that was converted to a triazole (**13a-13i**). We observed the largest differences in potency for this library against NEU1; however, selective inhibitors of NEU2 and SAR for NEU3 and NEU4 was apparent from these data, as discussed in detail below.

C5-amides with linear and branched alkanes improve potency for NEU1

The C5 pocket of NEU1 was sensitive to the length and branching of alkyl groups. Extension of the C5 chain from one methyl (DANA) to as long as a hexyl chain (**11a-11e**) spanned a more than 100-fold range of potency for NEU1 IC_{50} from 49 ± 8 μM (**DANA 1**) to 0.42 ± 0.06 μM (**11d**). Branched alkanes gave mixed results against NEU1. For example, an α-methyl branch in **11f** (5.3 ± 0.8 μM) had approximately 3-fold improved potency as compared with propylamide **11a** (18 ± 1 μM). In contrast, the β-methyl group in **11g** (32 ± 5 μM) caused a 4-fold loss in potency for NEU1 as compared with butylamide **11b** (8.4 ± 0.5 μM). Introduction of a γ-methyl group in **11h** (1.7 ± 0.1 μM) may lead to a small decrease in NEU1 potency as compared with the pentylamide **11c** (0.99 ± 0.07 μM). Small cycloalkanes, such as cyclopropyl (**11i**, 6.9 ± 0.4 μM) and cyclobutyl (**11j**, 12 ± 2 μM) groups had similar potency to propyl (**11b**, 8.4 ± 0.5 μM) and ethyl (**11a**, 18 ± 1 μM) substituents with NEU1. We observed that a benzylamide was also accommodated at the C5 pocket of NEU1 (**11k**, 11 ± 1 μM), showing a 4-fold increase in potency as compared to DANA, and similar to the cyclobutylamide of **11j**. The polarity of the C5 amide was critical for potency with NEU1. Inclusion of a terminal amine caused a significant loss of activity for NEU1 (**11l**, 480 ± 260 μM) and NEU2 (>500 μM).

Virtually all large groups at the C5 position resulted in low potency for NEU3 and NEU4. The only exception observed was the C5-butylamide-DANA **11b** which had modest potency against NEU3 (15 ± 2 μM) and NEU4 (8.4 ± 0.4 μM). These observations confirm that the C5 pocket of NEU3 and NEU4 is relatively restricted.

C5-triazolyl alkyl and aromatic groups improved selectivity for NEU2

We included a series of compounds containing a triazolyl linker at C5, and observed that this modification abolished activity against NEU1 (**13a-i**, **Table 1**). A similar trend was observed for C9 modifications, where analogues containing a triazolyl linker had greatly decreased potency (**15m**, **Table 2**).[30]

C5-modifications were well accommodated by NEU2, in agreement with reports that DANA and Neu5Ac$N_3$2en **6** has improved potency for this isoenzyme.[31] In contrast with NEU1, NEU2 showed a preference for the γ-branched alkyl chain of **11h** (7.3 ± 1.2 μM for NEU2, **Table 1**) and aryltriazolyl groups (**13a-h**, **Table 1**). The NEU2 active site was able to accommodate C5-aryltriazolyl DANA analogues with neutral (**13d**, 3.3 ± 0.3 μM) or basic (**13c**, 4.4 ± 0.3 μM) substitutions on the benzene ring, while acidic substitution (**13h**, 180 ± 30 μM) was not well tolerated. Compound **11h** (7.3 ± 1.2 μM) had the greatest potency among the alkyl series, while **13c** (4.4 ± 0.3 μM), **13d** (3.3 ± 0.3 μM), and **13f** (4.5 ± 0.3 μM) were the best among the aryltriazolyl series. Previous reports have found that a guanidino group at the C4 position could improve activity for NEU2,[26, 30, 31] thus we decided to generate compounds that combined modifications at C5 and C4. Compound **22**, containing a C4-guanidino and a γ-branched pentylamide, demonstrated a 3-fold increase in potency against NEU2 (2.1 ± 0.1 μM) as compared

to the C5-single modified analogue (**11h,** 7.3 ± 1.2 μM) or the C4-guanidino-containing zanamivir **2** (7.8 ± 2.0 μM).[25] Although **22** had the greatest potency for NEU2, its selectivity for the isoenzyme was inferior when compared with **13c** and **13d**, as **22** also had moderate potency for NEU1. We measured the K_i values of **13c** and **13d** for NEU2 to be 2.1 ± 0.6 μM and 2.7 ± 0.6 μM, respectively (**Table 4**). These values indicate both compounds to be 30-fold selective for NEU2, identifying them as the most selective NEU2 inhibitors reported. Compound **22,** with a K_i of 1.3 ± 0.2 μM, is the most potent NEU2 inhibitor reported, but lacks selectivity for NEU2 over NEU1.

C9-amide groups improved selectivity and potency for NEU1

Testing the activity of a series of compounds containing C9-modifications revealed important features of the NEU1 C9 pocket (**Table 2**). First, while alkylamido groups at this position provide improved potency over DANA **1** (e.g. **8**, 3.4 ± 0.2 μM), branching at the α-position of the amide was detrimental to activity (**15d**, 250 ± 60 μM), while β- or γ-methyl groups (**15e**, 2.5 ± 0.3 μM; **15f**, 3.2 ± 0.3 μM) provided similar to the activity of C9-butanamido-DANA **8** and C9-pentanamido-DANA (**15a**, 4.0 ± 0.5 μM).[28] These results indicate that the C9 binding pocket of NEU1 may have a narrow opening, but can accommodate larger groups distal to the C9-amide. The hydrophobic nature of the C9 binding pocket has been previously suggested,[28] and we sought to explore this further with larger groups. A series of phenylamides (**15g**-**15k**) suggest that there are specific contacts that can be gained from the C9 pocket. Compound **15g** (2.5 ± 0.3 μM) had similar potency to **8** and **15b** (2.9 ± 0.2 μM). Introduction of a *p*-amino group on the phenyl ring (**15i**, 29 ± 5 μM) decreased potency 11-fold as compared to **15g** (2.5 ± 0.3 μM). Conversion of the *p*-amino group to a less polar *p*-acetamido group (**15h**, 1.9 ± 0.4 μM) maintained similar potency

to **15g**. However, modifications at the meta position of the phenyl group to an amino (**15j**, 7.5 ± 1 μM) or acetamido (**15k**, 6.5 ± 0.7 μM) showed a similar, but minor, decrease in potency against NEU1. Extension of a linear alkyl chain from the C9 position with a terminal *p*-acetamidophenyl group (**15l**, 240 ± 70 μM) resulted in substantial loss of potency. Finally, we investigated the role of the C9-linker. While the C9-hexanamido derivative **15b** showed improved potency from DANA against NEU1, a 4-hexanyl-triazole (**15m**, 77 ± 30 μM) showed a significant decrease in potency; confirming that the C9-amide linker was partly responsible for improved potency against NEU1. This series of compounds allowed us to conclude that the NEU1 C9 pocket has distinct steric requirements, such as exclusion of α-branching and specific orientation of polar groups. Additionally, we note that none of the above modifications improved potency over the C5-hexanamido-DANA **11d**.

The C9-amide-DANA series contained generally poor inhibitors of NEU3 and NEU4. Medium sized amides were tolerated (e.g. **15c**, 39 ± 8 μM; and **15g**, 34 ± 5 μM for NEU3) but had modest potency. Benzamides **15h** and **15j** had single-digit IC_{50} values for NEU3 (~7 μM); and this activity was sensitive to the placement of the amide group (e.g. **15i**, 31 ± 6 μM vs. **15j**, 7.1 ± 0.7 μM). Comparison of the amide linker with the triazole linker for a pentyl chain (**15b**, 83 ± 9 μM vs **15m**, 6.7 ± 1.2 μM) showed a 12-fold preference for the triazole, suggesting that the linker contributes substantially to potency in NEU3.

Combined C5, C9-alkylamide modifications produce high potency NEU1 inhibitors

Based on the singly-modified series discussed above, we set out to generate combinations of C5- and C9-modifications that could provide increased potency for NEU1. First, we combined the

alkyl groups at C5 (**11d**, 0.42 ± 0.06 μM) and C9 (**15b**, 2.9 ± 0.2 μM) which had produced the best activity, and synthesized C5-hexanamido, C9-hexanamido-DANA **17a** (**Table 3**). Unfortunately, **17a** (4.3 ± 0.8 μM) was 10-fold less potent than the singly-modified **11d**, and 1.4-fold less potent than **15b**. Combination of two shorter chains in the C5-pentanamido, C9-pentanamido-DANA **17b** (1.5 ± 0.2 μM) was still 1.5-fold less potent than its singly-modified counterparts **11c** (0.99 ± 0.07 μM), but did show a modest improvement in potency over the C9-modified analogue **15b** (2.9 ± 0.2 μM). We hypothesized that these results could support a bridged model where the C5- and C9-pockets of NEU1 share occupancy. Following on this hypothesis, we tested compounds which combined a long and short alkyl chain modification at these two positions (**17c-h**). Placing the longer chain at the C9 position (**17c**, 1.6 ± 0.2 μM; **17d**, 1.4 ± 0.2 μM) showed modest improvement over **8** (3.4 ± 0.2 μM), **15b**, and **11a** (18 ± 1 μM). The largest improvements were observed when a longer chain was introduced at C5, and a shorter chain was included on C9 (**17e-17h**). The C5-pentanamido, C9-acetamido-DANA **17e** (0.35 ± 0.03 μM) and C5-hexanamido, C9-acetamido DANA **17f** (0.14 ± 0.01 μM) were approximately 3-fold more potent than their C5 singly-modified DANA analogues **11c** and **11d**. The addition of the C9-acetamido group increased inhibitor selectivity for NEU1, while potency for NEU2, NEU3, and NEU4 for these compounds was decreased. Compounds with longer C9-chains, such as C5-hexnanamido, C9-propionamido-DANA **17g** (0.40 ± 0.10 μM) and C5-heptanamido, C9-butanamido-DANA **17h** (1.2 ± 0.1 μM) had a modest decrease in potency. Measurement of the inhibitory constant for the best compounds from this series confirmed that the C5-hexanamido, C9-acetamido-DANA **17f** (K_i = 53 ± 5 nM, Table 4) was the most potent inhibitor reported for NEU1 to date. Additionally, the C9-

hexanamido-DANA **11d** (K_i = 180 ± 20 nM), which is more synthetically accessible, had good potency in the nanomolar range.

Activity of inhibitors against murine NEU orthologues

After identifying selective human NEU1 inhibitors through in vitro screening, we proceeded to test their selectivity and potency against mouse orthologues. The availability of NEU knock-out (KO) animals allowed us to isolate tissue lacking expression of individual isoenzymes. These tissues then provide a confirmation of in vitro assays using recombinant enzymes.[30] We assayed the inhibition of neuraminidase activity by **17f** in the brain homogenate of wild type mice (**Figure 3A**), NEU1 knockout (*Neu1*$^{-/-}$) mice (**Figure 3B**), and a double NEU3/NEU4 knockout (*Neu3*$^{-/-}$ *Neu4*$^{-/-}$) mice (**Figure 3C**).[44] In the brain tissues from wild-type mice (C57B16), up to 40% of total neuraminidase activity was blocked by **17f**. In contrast, the NEU1 KO tissue showed no significant inhibition when treated with **17f**, supporting that the compound did not have significant activity against other NEU isoenzymes expressed in the brain (NEU2, NEU3, and NEU4). In the brain tissues of NEU3/NEU4 double knockout mice, the majority of neuraminidase activity was blocked by **17f** at high concentration. The residual NEU activity in these samples may be the result of NEU2 expression, as small amounts of NEU2 were detected in the WT mice brain,[45] with higher levels in the adult brain.[46] To further probe the selectivity of **17f**, we tested its effects on NEU activity from kidney homogenates of wild type mice (**Figure 3D**), which is known to exclusively express NEU1.[44, 47] Compound **17f** showed 90% of inhibition of total neuraminidase activity from kidney tissue, confirming its selectivity for NEU1 over other NEU isoenzymes. Differences in the

apparent potency of **17f** as compared to IC_{50} determinations are likely due to differences in the substrate concentration and unknown concentration of active NEU isoenzymes.

Discussion

Human neuraminidase enzymes are responsible for the cleavage of sialic acid residues from glycoproteins and glycolipids. These enzymes have the potential to rapidly remodel glycan structures and may therefore influence cellular events mediated by glycans. NEU1 has the highest expression level among all the four hNEU isoenzymes.[26] Deficiency of lysosomal NEU1 can lead to lysosomal storage disorders such as sialidosis and galactosialidosis.[7] NEU1 is also found on the plasma membrane and likely plays a role in many signaling pathways in health and disease.[8, 20, 22, 48, 49] One of the major challenges of studying the role of individual hNEU isoenzymes is the existence of multiple isoenzymes and their overlapping biological activity and specificity. For example, both NEU1 and NEU3 are reported to regulate cell signaling pathways mediated by EGFR[50, 51] and IR,[17, 18] as well as lipid metabolism.[22]

Inhibitors of NEU have become essential research tools to address the issue of multiple isoenzymes. However, no commercially available inhibitors have been designed for specificity against hNEU isoenzymes. NEU inhibitors developed for bacterial or viral targets typically have broad or weak activity against hNEU.[25, 26] The role of sialidase activity was investigated with DANA and zanamivir in skin senescence, where sialidases down regulated myofibroblast differentiation via desialylation of CD44.[50] DANA and oseltamivir have been used as hNEU inhibitors for the study of apoptosis and phagocytosis of platelets.[52] Testing in vitro suggested that

zanamivir had modest potency for NEU3, but little activity for NEU1,[30] while oseltamivir has very low activity against any hNEU isoenzyme.[25, 26] A bi-specific inhibitor of NEU3 and NEU4, C9-4BPT-DANA **4**,[30] was used to interrogate the role of NEU1 activity in the tissues of mice exposed to high-fat diet as a model of obesity-induced type 2 diabetes.[53] These examples highlight that there remains a need for potent and selective hNEU inhibitors to facilitate biological studies of the role of human NEU isoenzymes.

In this work, we generated inhibitors based on the DANA scaffold containing modifications at the C5- and C9-positions to obtain potent and selective inhibitors of NEU1. In an earlier report from Magesh et al., C9-amide derivatives of DANA were found to act as selective inhibitors of NEU1.[28] We generated a panel of C9-amido derivatives of DANA with various alkyl chains and found that many of these were accommodated by NEU1. Interestingly, the C9-amide linker was found to be critical to the activity of these compounds as C9-triazolyl derivatives had reduced potency for NEU1 (despite their improved activity for NEU3 and NEU4).[29, 30] The most potent NEU1 inhibitors from the C9-series contained a C9-hexanamido chain, while longer chains had reduced activity. We extended this strategy to the C5 position of DANA. We were surprised to observe that the C5-binding pocket of NEU1 was able to accommodate linear, branched, and cycloalkane groups. Among these DANA analogues, compounds with C5-pentanamido or C5-hexanamido groups gave sub-micromolar IC_{50} values. The C5 pocket of NEU1 did not accommodate alkyl- or aryl-triazolyl groups and was sensitive to branching of the alkyl chain.

Combined modification of C5 and C9 positions allowed us to generate inhibitors of NEU1 with exceptional potency. Initial combinations of the best alkylamido groups from the singly-modified

series did not show additive potency. Instead, we found that a single pentanamido or hexanamido group at C5, when combined with a short amido group (e.g. acetamido, ethylamido), had potency as low as 53 nM K_i for NEU1. Furthermore, these compounds show unprecedented selectivity with compounds **17e**, **17f**, and **17g** ranging from 200-450–fold preference for NEU1. The selectivity and potency of **17f** against NEU1 was also confirmed in murine brain homogenates (**Figure 3**), supporting consistency between assays. Based on these results, we propose a model for the active site of NEU1 (**Figure 2B**). The co-crystal structure of DANA in the active site of NEU2 provides a map of the key residues used by that enzyme to complex the inhibitor. Previous work from our group has suggested that the C9 pocket of NEU2 is exquisitely sensitive to increased steric bulk at this position,[54] and the inhibitors tested here support that conclusion. However, the C5-, C9-, and C5, C9-series suggest a unified C5 and C9 pocket in NEU1. Furthermore, our data suggest that this pocket prefers hydrophobic groups linked through an amide, rather than a triazole. This should be contrasted with the C5 pocket of NEU3 and NEU4, which appears to be small and prefers more polar groups.[33] The C9 pocket of both NEU3 and NEU4 accommodates large hydrophobic groups when linked through a triazole, but the linker provides an additional element of discrimination between the isoenzymes.[30]

The panels of compounds tested here also identified selective inhibitors for NEU2 with low micromolar potency. The C5 pocket of NEU2 was able to accommodate large aryltriazolyl groups, yielding inhibitors with 30-fold selectivity (**13c**, **13d**). The presence of a C4-guanidino group in compounds such as zanamivir has been previously found to increase potency for NEU2. We

synthesized compound **22**, bearing a C5-(4-methylpentanamido) together with a C4-guaindino group, which had the most potent K_i reported against NEU2 (**22**, $K_i = 1.3 \pm 0.2$ μM).

Conclusions

Our results establish a rational strategy for the design of improved inhibitors of human NEU isoenzymes NEU1 and NEU2. Using a panel of compounds with C4, C5, C9, and combined modifications we were able to identify the most potent and selective inhibitors of both isoenzymes reported to date. The best inhibitor of NEU1 tested was **17f**, with a K_i of 53 ± 5 nM and 330-fold selectivity. The more synthetically accessible compound, **11d** (K_i = 0.18 ± 0.02 μM), had comparable potency but reduced selectivity of 35-fold. The most potent inhibitor of NEU2 tested was **22**, with a K_i of 1.3 ± 0.2 μM and 7-fold selectivity; while the most selective inhibitors of NEU2 tested were **13c** (4.4 ± 0.3 μM) and **13d** (3.3 ± 0.3 μM), each with 30-fold selectivity for the isoenzyme. Moreover, **17f** selectively inhibited NEU1 in mouse orthologs from brain and kidney homogenates, demonstrating that these inhibitors will be important new tools for investigation of the role of hNEU enzymes in biological systems.

Experimental Section

General Synthetic Procedures. All reagents and solvents were purchased from Sigma-Aldrich unless otherwise noted and used without further purification. Reactions were monitored with TLC (Merck TLC Silica gel 60 F_{254}) and spots were visualized under UV light (254 nm) or by charring with 0.5 % H_2SO_4/EtOH. Compounds were purified by flash column chromatography with silica gel (SiliCycle SiliaFlash® F60, 40-63 μm particle size) or recrystallization. Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on Varian 500 (500 MHz for ^{1}H; 125 MHz for ^{13}C) or Varian 700 (700 MHz for ^{1}H; 175 MHz for ^{13}C). High-resolution mass spectrometry (HR-MS) analysis was performed on Agilent Technologies 6220 TOF spectrometer. Purity of all final products used for inhibitor assays and pharmacokinetic studies was determined to be ≥95% by HPLC (see Supporting Information for details).

5-Acetamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (DANA, 1) was synthesized as previously reported.[33] ^{1}H NMR (500 MHz, CD_3OD) δ 5.67 (d, *J* = 2.3 Hz, 1H, H-3), 4.36 (dd, *J* = 8.6, 2.3 Hz, 1H, H-4), 4.10 (dd, *J* = 10.9, 1.1 Hz, 1H, H-6), 3.99 (dd, *J* = 10.9, 8.6 Hz, 1H, H-5), 3.87 (ddd, *J* = 9.1, 5.4, 3.1 Hz, 1H, H-8), 3.80 (dd, *J* = 11.4, 3.1 Hz, 1H, H-9), 3.65 (dd, *J* = 11.4, 5.4 Hz, 1H, H-9'), 3.52 (dd, *J* = 9.1, 1.1 Hz, 1H, H-7), 2.02 (s, 3H, $COCH_3$). ^{13}C NMR (125 MHz, CD_3OD) δ 174.68, 170.02 (2 × C=O), 149.95 (C-2), 108.34 (C-3), 77.24 (C-6), 71.29 (C-8), 70.22 (C-7), 68.70 (C-4), 64.94 (C-9), 51.96 (C-5), 22.82 ($COCH_3$). HR-MS (ESI) calcd. for $C_{11}H_{16}NO_8$ $[M-H]^-$, 290.0876; found 290.0879

5-(*N*-2-azidoacetyl)-2, 3, 5 -trideoxy-D-glycero-D-galacto-2-nonulopyranosonic acid (6) Compound **6** was synthesized as previously reported.[33] ^{1}H NMR (500 MHz, CD_3OD) δ 5.86 (d, *J*

= 1.5 Hz, 1H, H-3), 4.46 (dd, *J* = 8.7, 1.5 Hz, 1H, H-4), 4.24 (d, *J* = 10.8 Hz, 1H, H-6), 4.07 (dd, *J* = 10.8, 8.7 Hz, 1H, H-5), 3.97 (q, *J* = 16.1 Hz, 2H, N-CH_2-CO), 3.88 (br, 1H, H-8), 3.81 (dd, *J* = 11.4, 2.6 Hz, 1H, H-9), 3.66 (dd, *J* = 11.4, 5.2 Hz, 1H, H-9'), 3.57 (d, *J* = 9.0 Hz, 1H, H-7). ^{13}C NMR (126 MHz, CD_3OD) δ 171.55 (C=O), 111.68 (C-3), 77.43 (C-6), 71.41 (C-8), 70.04 (C-7), 68.23 (C-4), 64.86 (C-9), 53.01 (CH_2N_3), 51.93 (C-5). HRMS (ESI) calcd. for $C_{11}H_{15}N_4O_8$ [M-H]$^-$, 331.0890; found 331.0894

5-Acetamido-9-pentanamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (8) Compound **8** was synthesized as previously reported.[28] ^{1}H NMR (500 MHz, CD_3OD) δ 5.93 (d, *J* = 1.7 Hz, 1H, H-3), 4.43 (dd, *J* = 8.6, 1.7 Hz, 1H, H-4), 4.18 (d, *J* = 10.8 Hz, 1H, H-6), 4.02 – 3.95 (m, 1H, H-5), 3.95 – 3.87 (m, 1H, H-8), 3.59 (dd, *J* = 13.9, 2.9 Hz, 1H, H-9), 3.43 (d, *J* = 9.0 Hz, 1H, H-7), 3.32 – 3.27 (m, 1H, H-9'), 2.22 (t, *J* = 7.6 Hz, 2H, α-CH_2), 2.03 (s, 3H, $COCH_3$), 1.64 – 1.54 (m, 2H, β-CH_2), 1.35 (dd, *J* = 15.0, 7.5 Hz, 2H, γ-CH_2), 0.92 (t, *J* = 7.4 Hz, 3H, δ-CH_2). ^{13}C NMR (126 MHz, CD_3OD) δ 177.26, 174.88 (N-C=O), 165.81 (C-1), 145.81 (C-2), 113.17 (C-3), 77.79 (C-6), 71.50 (C-7), 70.24 (C-4), 68.03 (C-8), 51.97 (C-5), 44.40 (C-9), 36.87 (C-α), 29.28 (C-β), 23.44 (γ), 22.86 (CO*C*H_3), 14.22 (C-δ). HRMS (ESI) calcd. for $C_{16}H_{25}N_2O_8$ [M-H]$^-$, 373.1616; found 373.1614.

General Procedure for the synthesis of compounds 11a-l. A solution of compound **10** in anhydrous dichloromethane and triethylamine (4 eq) was cooled down to 0 °C and anhydride or acyl chloride (1.5 eq) were added dropwise. The solution was allowed to warm to room temperature and kept stirring overnight. After completion, the reaction was quenched with water, concentrated and purified by flash chromatography to give the protected product. The protected

product was then dissolved in methanol and 0.5 N NaOH was added. The solution was stirred under room temperature until completion. After completion, the solution was neutralized by Amberlite IR 120 (H+). The suspension was then filtered, and the filtrate was concentrated and purified by flash chromatography or precipitated in a mixture of methanol and ethyl acetate to give the desired product.

5-Propionamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (11a) Compound **11a** was synthesized from compound **10** using propionic anhydride. 70 mg (65% (86% × 76%), over two steps) ^{1}H NMR (500 MHz, CD_3OD) δ 5.90 (d, *J* = 2.4 Hz, 1H, H-3), 4.43 (dd, *J* = 8.7, 2.4 Hz, 1H, H-4), 4.15 (dd, *J* = 10.8, 0.9 Hz, 1H, H-6), 4.01 – 3.95 (m, 1H, H-5), 3.91 – 3.86 (m, 1H, H-8), 3.81 (dd, *J* = 11.4, 3.0 Hz, 1H, H-9), 3.65 (dd, *J* = 11.4, 5.4 Hz, 1H, H-9'), 3.55 (dd, *J* = 9.2, 0.9 Hz, 1H, H-7), 2.31 (q, *J* = 7.6 Hz, 2H, CH_2), 1.15 (t, *J* = 7.6 Hz, 3H, CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 178.80 (N-C=O), 166.46 (C-1), 112.52 (C-3), 77.98 (C-6), 71.17 (C-8), 70.17 (C-7), 68.10 (C-4), 64.93 (C-9), 51.80 (C-5), 30.20 (C-α), 10.33 (C-β). HRMS (ESI) calcd. for $C_{12}H_{18}NO_8$ $[M-H]^-$, 304.1032; found 304.1039.

5-Butyramido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (11b) Compound **11b** was synthesized from compound **10** using butyric anhydride. 21 mg (70% (79% × 89%), over two steps) ^{1}H NMR (500 MHz, CD_3OD) δ 5.89 (d, *J* = 2.4 Hz, 1H, H-3), 4.42 (dd, *J* = 8.7, 2.4 Hz, 1H, H-4), 4.15 (d, *J* = 10.8 Hz, 1H, H-6), 3.99 (dd, *J* = 10.8, 8.7 Hz, 1H, H-5), 3.89 (ddd, *J* = 9.0, 5.3, 3.0 Hz, 1H, H-8), 3.81 (dd, *J* = 11.4, 3.0 Hz, 1H, H-9), 3.63 (dd, *J* = 11.4, 5.3 Hz, 1H, H-9'), 3.56 (d, *J* = 9.0 Hz, 1H, H-7), 2.29 – 2.23 (m, 2H, CH_2), 1.66 (dt, *J* = 13.4, 7.0 Hz, 2H, CH_2), 0.97 (t, *J* = 7.0 Hz, 3H, CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 177.98 (N-C=O), 166.50

(C-1), 112.47 (C-3), 78.00 (C-6), 71.17 (C-7), 70.26 (C-4), 68.09 (C-8), 64.99 (C-9), 51.85 (C-5), 39.04 (C-α), 20.31 (C-β), 14.11 (C-γ). HRMS (ESI) calcd. for $C_{13}H_{20}NO_8$ [M-H]$^-$, 318.1189; found 318.1196

5-Pentanamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (11c). Compound **11c** was synthesized from compound **10** using valeric anhydride. 100 mg (56% (64% × 87%), over two steps) ^{1}H NMR (500 MHz, CD_3OD) δ 5.88 (d, *J* = 2.1 Hz, 1H, H-3), 4.44 (dd, *J* = 8.8, 2.1 Hz, 1H, H-4), 4.15 (d, *J* = 10.8 Hz, 1H, H-6), 3.99 (dd, *J* = 10.8, 8.8 Hz, 1H, H-5), 3.89 (m, 1H, H-8), 3.81 (dd, *J* = 11.4, 2.9 Hz, 1H, H-9), 3.64 (dd, *J* = 11.4, 5.3 Hz, 1H, H-9'), 3.57 (d, *J* = 9.0 Hz, 1H, H-7), 2.29 (t, *J* = 7.6 Hz, 2H, α-CH_2), 1.62 (m, 2H, β-CH_2), 1.37 (dq, *J* = 14.8, 7.4 Hz, 2H, γ-CH_2), 0.93 (t, *J* = 7.4 Hz, 3H, CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 178.13 (N-C=O), 166.89 (C-1), 146.72 (C-2), 112.33 (C-3), 77.91 (C-6), 71.30 (C-7), 70.18 (C-4), 68.09 (C-8), 64.90 (C-9), 51.76 (C-5), 36.92 (C-α), 29.08 (C-β), 23.46 (C-γ), 14.21 (C-δ). HRMS (ESI) calcd. for $C_{14}H_{22}NO_8$ [M-H]$^-$, 332.1345; found 332.1348

5-Hexanamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (11d) Compound **11d** was synthesized from compound **10** using hexanoic anhydride. 42 mg (60% (85% × 71%), over two steps). ^{1}H NMR (700 MHz, CD_3OD) δ 5.95 (d, *J* = 2.3 Hz, 1H, H-3), 4.43 (dd, *J* = 8.7, 2.3 Hz, 1H, H-4), 4.16 (dd, *J* = 10.8, 0.8 Hz, 1H, H-6), 3.99 (dd, *J* = 10.8, 8.7 Hz, 1H, H-5), 3.91 (brs, 1H, H-8), 3.83 (dd, *J* = 11.4, 2.9 Hz, 1H, H-9), 3.63 (dd, *J* = 11.4, 5.5 Hz, 1H, H-9'), 3.56 (dd, *J* = 9.3, 0.7 Hz, 1H, H-7), 2.29 (t, *J* = 7.5 Hz, 2H, α-CH_2), 1.69 – 1.62 (m, 2H, β-CH_2), 1.35 (m, 2 × 2H, γ-CH_2, δ-CH_2), 0.93 (t, J = 7.0 Hz, 3H, CH_3). ^{13}C NMR (176 MHz, CD_3OD) δ 178.20 (N-C=O), 113.34 (C-3), 78.14 (C-6), 71.06 (C-7), 70.27 (C-4), 67.95 (C-8), 65.03 (C-9),

51.81(C-5), 37.06 (C-α), 32.56 (C-β), 26.59 (C-γ), 23.44 (C-δ), 14.27(C-ε). HRMS (ESI) calcd. for $C_{15}H_{25}NO_8$ [M-H]$^-$, 346.1507; found 346.1506

5-Heptanamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (11e). Compound **11e** was synthesized from compound **10** using heptanoyl chloride. 28 mg (46% (65% × 70%), over two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.93 (d, *J* = 2.1 Hz, 1H, H-3), 4.40 (dd, *J* = 8.7, 2.1 Hz, 1H, H-4), 4.13 (d, *J* = 10.8 Hz, 1H, H-6), 3.98 (dd, *J* = 10.8, 8.7 Hz, 1H, H-5), 3.89 (brs, 1H, H-8), 3.81 (dd, *J* = 11.4, 2.7 Hz, 1H, H-9), 3.61 (dd, *J* = 11.4, 5.5 Hz, 1H, H-9'), 3.53 (d, *J* = 8.8 Hz, 1H, H-7), 2.27 (t, *J* = 7.5 Hz, 2H, α-CH_2), 1.69 – 1.55 (m, 2H, β-CH_2), 1.42 – 1.16 (m, 3 × 2H, γ-CH_2, δ-CH_2, ε-CH_2), 0.89 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, CD_3OD) δ 178.23 (N-C=O), 113.38 (C-3), 78.18 (C-6), 71.07 (C-7), 70.32 (C-4), 67.98 (C-8), 65.06 (C-9), 51.84 (C-5), 37.12 (C-α), 32.72 (C-β), 30.06 (C-γ), 26.89 (C-δ), 23.58 (C-ε), 14.40 (C-ζ). HRMS (ESI) calcd. for $C_{16}H_{26}NO_8$ [M-H]$^-$, 360.1664; found 360.1665

5-Isobutyramido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (11f). Compound **11f** was synthesized from compound **10** using isobutyryl chloride. 25 mg (38% (54% × 70%), over two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.93 (d, *J* = 2.5 Hz, 1H, H-3), 4.43 (dd, *J* = 8.7, 2.5 Hz, 1H, H-4), 4.15 (dd, *J* = 10.8, 1.1 Hz, 1H, H-6), 3.95 (dd, *J* = 10.8, 8.7 Hz, 1H, H-5), 3.88 (s, 1H, H-8), 3.80 (dd, *J* = 11.4, 2.9 Hz, 1H, H-9), 3.62 (dd, *J* = 11.4, 5.4 Hz, 1H, H-9'), 3.52 (dd, *J* = 9.2, 1.1 Hz, 1H, H-7), 2.57 – 2.46 (m, 1H, α-CH), 1.14 (dd, *J* = 6.9, 2.4 Hz, 6H, 2 × β-CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 182.10 (N-C=O), 165.49 (C-1), 113.48 (C-3), 78.20 (C-6), 71.05 (C-7), 70.21 (C-4), 67.89 (C-8), 64.98 (C-9), 51.68 (C-5), 36.41 (C-α), 20.10, 19.70 (2 ×C-β). HRMS (ESI) calcd. for $C_{13}H_{21}NO_8$ [M-H]$^-$, 318.1194; found 318.1193.

5-(3-Methylbutanamido)-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (11g). Compound **11g** was synthesized from compound **10** using isovaleryl chloride. 30 mg (43% (62% × 69%), over two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.93 (d, *J* = 2.5 Hz, 1H, H-3), 4.40 (dd, *J* = 8.7, 2.5 Hz, 1H, H-4), 4.14 (dd, *J* = 10.8, 1.1 Hz, 1H, H-6), 3.98 (dd, *J* = 10.8, 8.7 Hz, 1H, H-5), 3.89 (s, 1H, H-8), 3.80 (dd, *J* = 11.4, 2.9 Hz, 1H, H-9), 3.61 (dd, *J* = 11.4, 5.5 Hz, 1H, H-9'), 3.57 (dd, *J* = 9.3, 1.1 Hz, 1H, H-7), 2.17 – 2.04 (m, 3H, α-CH_2, β-CH_2), 0.96 (dd, *J* = 6.5, 3.8 Hz, 6H, 2×γ-CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 177.53 (N-C=O), 113.50 (C-3), 78.18 (C-6), 71.05 (C-7), 70.34 (C-4), 67.95 (C-8), 65.04 (C-9), 51.87 (C-5), 46.33 (C-α), 27.43 (C-β), 22.89, 22.83 (2 ×C- γ). HRMS (ESI) calcd. for $C_{14}H_{22}NO_8$ $[M-H]^-$, 332.1351; found 332.1348.

5-(4-Methylpentanamido)-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (11h). Compound **11h** was synthesized from compound **10** using 4-methylpentanoyl chloride. 30 mg (41% (56% × 74%), over two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.93 (d, *J* = 2.3 Hz, 1H, H-3), 4.42 (dd, *J* = 8.7, 2.3 Hz, 1H, H-4), 4.14 (d, *J* = 10.8 Hz, 1H, H-6), 3.96 (dd, *J* = 10.8, 8.8 Hz, 1H, H-5), 3.89 (s, 1H, H-8), 3.81 (dd, *J* = 11.4, 2.8 Hz, 1H, H-9), 3.62 (dd, *J* = 11.4, 5.4 Hz, 1H, H-9'), 3.54 (d, *J* = 8.7 Hz, 1H, H-7), 2.32 – 2.25 (m, 2H, α-CH_2), 1.62 – 1.47 (m, 3H, β-CH_2, γ-CH), 0.91 (dd, *J* = 6.4, 1.1 Hz, 6H, 2 × δ-CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 178.46 (N-C=O), 113.46 (C-3), 78.14 (C-6), 71.07 (C-7), 70.22 (C-4), 67.97 (C-8), 64.99 (C-9), 51.81 (C-5), 35.86 (C-α), 35.19 (C-β), 29.00 (C-γ), 22.75, 22.67 (2 × C-δ). HRMS (ESI) calcd. for $C_{15}H_{24}NO_8$ $[M-H]^-$, 346.1507; found 346.1496.

5-Cyclopropanecarboxamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (11i). Compound **11i** was synthesized from compound **10** using cyclopropanecarbonyl

chloride. 30 mg (13% (17% × 77%), over two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 8.26 (d, *J* = 8.4 Hz, 1H, NH), 5.88 (d, *J* = 1.5 Hz, 1H, H-3), 4.47 (d, *J* = 8.7 Hz, 1H, H-4), 4.16 (d, *J* = 10.8 Hz, 1H, H-6), 4.01 (m, 1H, H-5), 3.93 – 3.85 (m, 1H, H-8), 3.80 (dd, *J* = 11.4, 2.6 Hz, 1H, H-9), 3.69 – 3.62 (m, 1H, H-9), 3.56 (d, *J* = 9.0 Hz, 1H, H-7), 1.69-1.66 (m, 1H, CH), 0.92-0.88 (m, 2H, CH_2), 0.81-0.78 (m, 2H, CH_2). ^{13}C NMR (126 MHz, CD_3OD) δ 178.35 (N-C=O), 166.87 (C-1), 112.31 (C-3), 78.06 (C-6), 71.28 (C-7), 70.10 (C-4), 68.19 (C-8), 64.84 (C-9), 51.96 (C-5), 15.10 (C-α), 8.10 (C-β), 7.75 (C-β'). HRMS (ESI) calcd. for $C_{13}H_{18}NO_8$ $[M-H]^-$, 316.1032; found 316.1030

5-Cyclobutanecarboxamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (11j). Compound **11j** was synthesized from compound **10** using cyclobutanecarbonyl chloride. 35 mg (16% (18% × 88%), over two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.89 (d, *J* = 1.7 Hz, 1H, H-3), 4.44 (dd, *J* = 8.9, 1.7 Hz, 1H, H-4), 4.15 (d, *J* = 10.6 Hz, 1H, H-6), 3.99 (dd, *J* = 10.6, 8.9 Hz, 1H, H-5), 3.88 (m, 1H, H-8), 3.80 (dd, *J* = 11.4, 2.7 Hz, 1H, H-9), 3.65 (dd, *J* = 11.4, 5.2 Hz, 1H, H-9'), 3.53 (d, *J* = 9.0 Hz, 1H, H-7), 3.19 (p, *J* = 8.5 Hz, 1H, CH), 2.30-2.22 (m, 2H, CH_2), 2.20 – 2.10 (m, 2H, CH_2), 1.89-1.82 (m, 2H, CH_2). ^{13}C NMR (126 MHz, CD_3OD) δ 179.57 (N-C=O), 166.68 (C-1), 112.52 (C-3), 77.96 (C-6), 71.26 (C-7), 70.10 (C-4), 68.04 (C-8), 64.87 (C-9), 51.69 (C-5), 40.83 (C-α), 26.42 (C-β), 26.09 (C-β'), 19.08 (C-γ). HRMS (ESI) calcd. for $C_{14}H_{20}NO_8$ $[M-H]^-$, 330.1189; found 330.1195

5-Benzamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (11k) Compound **11k** was synthesized from compound **10** using benzyl chloride. 21 mg (28% (44% × 64%), two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 7.88 (dd, *J* = 8.3, 1.2 Hz, 2H, Ar-H), 7.57 – 7.50

(m, 1H, Ar-H), 7.49 – 7.41 (m, 2H, Ar-H), 5.98 (d, *J* = 2.3 Hz, 1H, H-3), 4.64 (dd, *J* = 8.7, 2.3 Hz, 1H, H-4), 4.32 (d, *J* = 11.0 Hz, 1H, H-6), 4.24 (dd, *J* = 11.0, 8.7 Hz, 1H, H-5), 3.92 (s, 1H, H-8), 3.80 (dd, *J* = 11.5, 2.8 Hz, 1H, H-9), 3.68 – 3.59 (m, 2H, H-9', H-7). ^{13}C NMR (126 MHz, CD_3OD) δ 171.74 (N-C=O), 135.08, 133.05, 129.55, 128.67 (Ar-C), 113.62 (C-3), 78.17 (C-6), 71.16 (C-7), 70.17 (C-4), 68.06 (C-8), 64.86 (C-9), 52.47 (C-5). HRMS (ESI) calcd. for $C_{16}H_{18}NO_8$ $[M-H]^-$, 352.1038; found 352.1035

5-(5-amino)pentanamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (11l) Compound **11l** was synthesized from compound **10** using 5-((tert-butoxycarbonyl)amino)pentanoic anhydride. 20 mg (20% (87% × 23% yield for two deprotection steps), over three steps). ^{1}H NMR (700 MHz, D_2O) δ 5.71 (d, *J* = 2.2 Hz, 1H, H-3), 4.49 (dd, *J* = 8.8, 2.2 Hz, 1H, H-4), 4.25 (d, *J* = 10.8 Hz, 1H, H-6), 4.09 (dd, *J* = 10.8, 8.8 Hz, 1H, H-5), 3.99 – 3.93 (m, 1H, H-8), 3.89 (dd, *J* = 12.0, 2.6 Hz, 1H, H-9), 3.68 (dd, *J* = 12.0, 5.9 Hz, 1H, H-9'), 3.61 (d, *J* = 9.4 Hz, 1H, H-7), 2.98 (t, *J* = 6.8 Hz, 2H, δ-CH_2), 2.40 (t, *J* = 6.9 Hz, 2H, α- CH_2), 1.73-1.67 (m, 4H, γ-CH_2, β-CH_2). ^{13}C NMR (126 MHz, D_2O) δ 177.71 (C=O), 170.62 (C-1), 148.86 (C-2), 108.65 (C-3), 76.24 (C-6), 70.65 (C-8), 69.22 (C-7), 68.47 (C-4), 63.98 (C-9), 50.78 (C-5), 40.15 (δ-C), 36.15 (α-C), 27.49 (γ-C), 23.12 (β-C). HRMS (ESI) calcd. for $C_{12}H_{23}N_2O_8$ $[M-H]^-$, 347.1460; found 347.1458

5-(N-2-azido)acetyl-4,7,8,9-tetra-O-acetyl-2,3,5-trideoxy-D-glycero-D-galacto-2-nonulopyranosonate (12) Compound **12** was synthesized using *N*-hydroxysuccinimidyl-2-azidoacetate as reported.[33] 600 mg (62%) ^{1}H NMR (500 MHz, CD_3OD) δ 5.93 (t, *J* = 2.7 Hz, 1H, H-3), 5.59 (dd, *J* = 8.7, 2.7 Hz, 1H, H-4), 5.48 (dd, *J* = 6.5, 2.4 Hz, 1H, H-7), 5.34 (td, *J* = 6.2, 2.8

Hz, 1H, H-8), 4.55 (dd, *J* = 12.5, 2.8 Hz, 1H, H-9), 4.50 (dd, *J* = 10.5, 2.4 Hz, 1H, H-6), 4.29 (dd, *J* = 10.5, 8.7 Hz, 1H, H-5), 4.15 (dd, *J* = 12.5, 6.2 Hz, 1H, H-9'), 3.86 – 3.73 (m, 5H, N-CH_2-CO, $COOCH_3$), 2.09, 2.03, 2.01(3 × s, 12H, 4 × $COOCH_3$). ^{13}C NMR (126 MHz, CD_3OD) δ 172.41, 172.24, 171.72, 171.45, 170.47 (C=O), 163.11 (C-1), 146.75 (C-2), 109.78 (C-3), 77.56 (C-6), 71.51 (C-8), 70.57 (C-7), 68.68 (C-4), 63.12 (C-9), 53.10, 53.07 ($COOCH_3$, CH_2N_3), 47.84 (C-5), 20.91, 20.85, 20.82, 20.74 ($COCH_3$). HRMS (ESI) calcd. for $C_{20}H_{26}N_4NaO_{12}$ $[M+Na]^+$, 537.1445; found 537.1436.

General Procedure of CuAAC Reaction and Hydrolyzation of Methyl Ester for Synthesis of Compounds 13a-h. To a solution of methyl 5-acetamido-9-azido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonate (**12**)[33] (1 eq) and the corresponding alkyne (1.5 eq) in THF-H_2O (2:1), sodium L-ascorbate (0.5 eq) and copper (II) sulfate (0.5 eq) were added sequentially. The reaction mixture was kept stirring at room temperature and monitored by TLC until no azide remained. Silica gel was then added to the reaction mixture and the solvent was removed under reduced pressure. The residue was separated by flash chromatography to provide the desired products with yields of 42%-88%. To hydrolyze the C1-methyl ester, the product was dissolved in MeOH, and 0.5 M NaOH was added. The mixture was kept stirring at room temperature. After completion, the pH of the solution was adjusted to 2 with Amberlite IR-120 (H^+). The solution was filtered and purified by flash chromatography to provide the desired products with yields of 45%-88%.

5-(2-(4-(4-(dimethylamino)phenyl)-1H-1,2,3-triazol-1-yl))acetamido-2,3,5-trideoxy-D-glycero-D-galacto-2-nonulopyranosonic acid (13a). Compound **13a** was synthesized from

compound **12** using 4-dimethylaminophenylacetylene. 13 mg (20% (50% × 41%), two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 8.18 (s, 1H, triazole-H), 7.57 (d, *J* = 8.6 Hz, 2H, Ar-H), 6.81 (d, *J* = 8.5 Hz, 2H, Ar-H), 5.70 (d, *J* = 2.2 Hz, 1H, H-3), 5.28 (s, 2H, N-CH_2-CO), 4.47 (dd, *J* = 8.7, 2.2 Hz, 1H, H-4), 4.25 (d, *J* = 10.8 Hz, 1H, H-6), 4.09 (dd, *J* = 10.8, 8.7 Hz, 1H, H-5), 3.93 – 3.86 (m, 1H, H-8), 3.83 (dd, *J* = 11.6, 2.8 Hz, 1H, H-9), 3.66 (dd, *J* = 11.6, 5.7 Hz, 1H, H-9'), 3.60 (d, *J* = 9.3 Hz, 1H, H-7). ^{13}C NMR (126 MHz, CD_3OD) δ 169.93 (CH_3*C*O), 169.04 (C-1), 149.58, 122.78 (Triazole-C), 148.93 (C-2), 127.94, 121.52, 117.07 (Ar-C), 108.57 (C-3), 76.73 (C-6), 71.42 (C-8), 70.02 (C-7), 68.81 (C-4), 64.87 (C-9), 53.35 (N-*C*H_2-CO), 52.19 (C-5). HRMS (ESI) calcd. for $C_{19}H_{22}N_5O_8$ [M-H]$^-$, 448.1468; found 448.1481.

5-(2-(4-(4-acetamido)phenyl)-1H-1,2,3-triazol-1-yl))acetamido-2, 3, 5 -trideoxy- D-glycero-D-galacto-2-nonulopyranosonic acid (13b). Compound **13b** was synthesized from compound **12** using 4-acetamidophenylacetylene. 35 mg (46% (59% × 79%), two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 8.30 (s, 1H, triazole-H), 7.75 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.62 (d, *J* = 8.6 Hz, 2H, Ar-H), 5.69 (d, *J* = 2.2 Hz, 1H, H-3), 5.26 (s, 2H, N-CH_2-CO), 4.43 (dd, *J* = 8.6, 2.2 Hz, 1H, H-4), 4.23 (d, *J* = 10.8 Hz, 1H, H-6), 4.10 (dd, *J* = 10.8, 8.6 Hz, 1H, H-5), 3.90 – 3.84 (m, 1H, H-8), 3.81 (dd, *J* = 11.4, 2.9 Hz, 1H, H-9), 3.67 (dd, *J* = 11.4, 5.3 Hz, 1H, H-9'), 3.57 (d, *J* = 9.2 Hz, 1H, H-7), 2.13 (s, 3H, NAc).^{13}C NMR (126 MHz, CD_3OD) δ 171.70 (CH_3*C*O), 169.98 (C-1), 168.75 (C=O), 150.14 (C-1), 148.58, 123.59 (Triazole-C), 140.08, 127.37, 127.17, 121.36 (Ar-C), 108.11 (C-3), 76.80 (C-6), 71.44 (C-8), 70.17 (C-7), 68.78 (C-4), 64.92 (C-9), 53.30 (N-*C*H_2-CO), 52.23 (C-5), 23.90 (CH_3). HRMS (ESI) calcd. for $C_{21}H_{25}N_5O_9$ [M-H]$^-$, 490.1574; found 490.1584.

5-(2-(4-(4-aminophenyl)-1H-1,2,3-triazol-1-yl)) acetamido-2, 3, 5 -trideoxy-D-glycero-D-galacto-2-nonulopyranosonic acid (13c). Compound **13c** was synthesized from compound **12** using 4-aminophenylacetylene. 27 mg (31% (63% × 50%), two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 8.18 (s, 1H, triazole-H), 7.57 (d, *J* = 8.6 Hz, 2H, Ar-H), 6.81 (d, *J* = 8.5 Hz, 2H, Ar-H), 5.70 (d, *J* = 2.2 Hz, 1H, H-3), 5.28 (s, 2H, N-CH_2-CO), 4.47 (dd, *J* = 8.7, 2.2 Hz, 1H, H-4), 4.25 (d, *J* = 10.8 Hz, 1H, H-6), 4.09 (dd, *J* = 10.8, 8.7 Hz, 1H, H-5), 3.93 – 3.86 (m, 1H, H-8), 3.83 (dd, *J* = 11.6, 2.8 Hz, 1H, H-9), 3.66 (dd, *J* = 11.6, 5.7 Hz, 1H, H-9'), 3.60 (d, *J* = 9.3 Hz, 1H, H-7). ^{13}C NMR (126 MHz, CD_3OD) δ 169.93 (CH_3*C*O), 169.04 (C-1), 149.58, 122.78 (Triazole-C), 148.93 (C-2), 127.94, 121.52, 117.07 (Ar-C), 108.57 (C-3), 76.73 (C-6), 71.42 (C-8), 70.02 (C-7), 68.81 (C-4), 64.87 (C-9), 53.35 (N-*C*H_2-CO), 52.19 (C-5). HRMS (ESI) calcd. for $C_{19}H_{22}N_5O_8$ $[M-H]^-$, 448.1468; found 448.1481.

5-(2-(4-(4-*p*-tolyl)-1H-1,2,3-triazol-1-yl)) acetamido-2, 3, 5 -trideoxy-D-glycero-D-galacto-2-nonulopyranosonic acid (13d). Compound **13d** was synthesized from compound **12** using 4-methylphenylacetylene. 35 mg (52% (74% × 70%), two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 8.31 (s, 1H, triazole-H), 7.70 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.24 (d, *J* = 8.0 Hz, 2H), 5.87 (d, *J* = 2.2 Hz, 1H, H-3), 5.29 (s, 2H, N-CH_2-CO), 4.49 (dd, *J* = 8.7, 2.2 Hz, 1H, H-4), 4.29 (d, *J* = 10.8 Hz, 1H, H-6), 4.10 (dd, *J* = 10.7, 8.7 Hz, 1H, H-5), 3.91 – 3.84 (m, 1H, H-8), 3.81 (dd, *J* = 11.4, 2.9 Hz, 1H, H-9), 3.67 (dd, *J* = 11.4, 5.3 Hz, 1H, H-9'), 3.64 (d, *J* = 9.1 Hz, 1H, H-7), 2.35 (s, 3H, $PhCH_3$). ^{13}C NMR (126 MHz, CD_3OD/DMSO-d_6) δ 168.97 (CH_3*C*O), 167.13 (C-1), 148.95, 123.68 (Triazole-C), 146.97 (C-2), 139.45, 130.64, 128.89, 126.71 (Ar-C), 111.62 (C-3), 77.42

(C-6), 71.51 (C-8), 70.06 (C-7), 68.30 (C-4), 64.86 (C-9), 53.29 (N-*C*H_2-CO), 52.18 (C-5), 21.37 (Ph*C*H_3). HRMS (ESI) calcd. for $C_{20}H_{23}N_4O_8$ $[M-H]^-$, 447.1516; found 447.1520.

5-(2-(4-(4-methoxyphenyl)-1H-1,2,3-triazol-1-yl))acetamido-2, 3, 5 -trideoxy- D-glycero-D-galacto-2-nonulopyranosonic acid (13e). Compound **13e** was synthesized from compound **12** using 4-methoxylphenylacetylene. 40 mg (51% (82% × 62%), two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 8.27 (s, 1H, triazole-H), 7.74 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.01 (d, *J* = 8.7 Hz, 2H, Ar-H), 5.89 (d, *J* = 1.8 Hz, 1H, H-3), 5.31 (s, 2H, N-CH_2-CO), 4.56 – 4.45 (m, 1H, H-4), 4.33 (d, *J* = 10.8 Hz, 1H, H-6), 4.11 (dd, *J* = 10.5, 8.9 Hz, 1H, H-5), 3.92 – 3.86 (m, 1H, H-8), 3.85-3.83 (m, 4H, H-9, OCH_3), 3.70 – 3.63 (m, 2H, H-7, H-9'). ^{13}C NMR (126 MHz, CD_3OD/DMSO-d_6) δ 169.15 (CH_3*C*O), 167.27 (C-1), 148.92, 123.53 (Triazole-C), 146.62 (C-2), 161.19, 128.28, 124.06 115.61 (Ar-C), 112.08 (C-3), 77.26 (C-6), 71.38 (C-8), 69.81 (C-7), 68.41 (C-4), 64.77 (C-9), 56.20 (PhO*C*H_3), 53.38 (N-*C*H_2-CO), 52.02 (C-5). HRMS (ESI) calcd. for $C_{20}H_{23}N_4O_9$ $[M-H]^-$, 463.1465; found 463.1471

5-(2-(4-(4-fluorophenyl)-1H-1,2,3-triazol-1-yl))acetamido-2,3,5-trideoxy-D-glycero-D-galacto-2-nonulopyranosonic acid (13f). Compound **13f** was synthesized from compound **12** using 4-fluorophenylacetylene. 25 mg (46% (79 % × 59%), two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 8.33 (s, 1H, triazole-H), 7.81 (dd, *J* = 8.6, 5.4 Hz, 2H, Ar-H), 7.14 (t, *J* = 8.7 Hz, 2H, Ar-H), 5.80 (s, 1H, H-3), 5.29 (s, 2H, N-CH_2-CO), 4.48 (d, *J* = 8.2 Hz, 1H, H-4), 4.27 (d, *J* = 10.7 Hz, 1H, H-6), 4.14 – 4.07 (m, 1H, H-5), 3.88 (m, 1H, H-8), 3.81 (d, *J* = 11.0 Hz, 1H, H-9), 3.73 – 3.60 (m, 2H, H-9', H-7). ^{13}C NMR (126 MHz, CD_3OD) δ 168.94 (CH_3*C*O), 168.64 (C-1), 165.11, 163.15 (d, *J* = 246.3 Hz), 148.28 (C-2), 147.97, 123.91 (Triazole-C), 128.70 (d, *J* = 8.2 Hz, Ar-C),

128.11 (d, *J* = 3.2 Hz, Ar-C), 116.78 (d, *J* = 22.0 Hz, Ar-C), 110.10 (C-3), 77.12 (C-6), 71.64 (C-8), 69.99 (C-7), 68.50 (C-4), 64.75 (C-9), 53.32 (N-*C*H_2-CO), 52.18 (C-5). HRMS (ESI) calcd. for $C_{19}H_{20}N_4O_8$ [M-H]$^-$, 451.1265; found 451.1271.

5-(2-(4-(4-(trifluoromethyl)phenyl)-1H-1,2,3-triazol-1-yl))acetamido-2, 3, 5 -trideoxy-D-glycero-D-galacto-2-nonulopyranosonic acid (13g). Compound **13g** was synthesized from compound **12** using 4-trifluoromethylphenylacetylene. 25 mg (32% (78% × 41%), two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 8.47 (s, 1H, Triazole-H), 7.97 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.68 (d, *J* = 8.3 Hz, 2H, Ar-H), 5.76 (s, 1H, H-3), 5.32 (s, 2H, N-CH_2-CO), 4.47 (dd, *J* = 8.6, 1.5 Hz, 1H, H-4), 4.27 (d, *J* = 10.8 Hz, 1H, H-6), 4.12 (dd, *J* = 10.8, 8.6 Hz , 1H, H-5), 3.89 (br, 1H, H-8), 3.84 – 3.76 (m, 1H, H-9), 3.69 (dd, *J* = 11.4, 5.1 Hz, 1H, H-9'), 3.64 (d, *J* = 8.9 Hz, 1H, H-7). ^{13}C NMR (126 MHz, CD_3OD) δ 168.79 (CH_3*C*O), 147.36, 125.07 (Triazole-C), 135.56 (Ar-C), 130.94 (q, *J* = 32.3 Hz, Ar-C), 127.05 (Ar-C), 127.87 (q, *J* = 3.7 Hz, Ar-C), 109.19 (C-3), 76.99 (C-6), 71.60 (C-8), 70.06 (C-7), 68.68 (C-4), 64.80 (9), 53.36 (N-*C*H_2-CO), 52.24 (C-5). HRMS (ESI) calcd. for $C_{20}H_{20}F_3N_4O_8$ [M-H]$^-$, 501.1233; found 501.1243.

5-(2-(4-(4-carboxyphenyl)-1H-1,2,3-triazol-1-yl)) acetamido-2, 3, 5 -trideoxy-D-glycero-D -galacto-2-nonulopyranosonic acid (13h). Compound **13h** was synthesized from compound **12** using 4-carboxylphenylacetylene. 30 mg (52% (86% × 60%), two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 8.48 (s, 1H, Triazole-H), 8.10 – 8.04 (m, 2H, Ar-H), 7.95 – 7.90 (m, 2H, Ar-H), 5.88 (d, *J* = 2.4 Hz, 1H, H-3), 5.32 (s, 2H, N-CH_2-CO), 4.49 (dd, *J* = 8.7, 2.4 Hz, 1H, H-4), 4.30 (dd, *J* = 10.8, 0.6 Hz, 1H, H-6), 4.11 (dd, *J* = 10.8, 8.7 Hz, 1H, H-5), 3.91 – 3.86 (m, 1H, H-8), 3.82 (dd, *J* = 11.4, 3.0 Hz, 1H, H-9), 3.68 (dd, *J* = 11.5, 5.4 Hz, 1H, H-9'), 3.64 (dd, *J* = 9.2, 0.6 Hz, 1H, H-

7). ^{13}C NMR (126 MHz, CD_3OD/DMSO-d_6) δ 169.44, 168.85 (C=O), 167.00 (C-1), 147.83, 125.04 (Triazole-C), 146.85 (C-2), 136.10, 131.60, 131.48, 126.53 (Ar-C), 111.73 (C-3), 77.44 (C-6), 71.45 (C-8), 70.09 (C-7), 68.32 (C-4), 64.88 (C-9), 53.32 (N-*C*H_2-CO), 52.21 (C-5). HRMS (ESI) calcd. for $C_{20}H_{21}N_4O_{10}$ $[M-H]^-$, 477.1258; found 477.1268.

5-(2-(4-pentyl-1H-1,2,3-triazol-1-yl)) acetamido-2, 3, 5 -trideoxy-d-glycero-d -galacto-2-nonulopyranosonic acid (13i). Compound **13i** was synthesized from compound **12** using 1-heptyne. 33 mg (52% (85% × 61%), two steps). ^{1}H NMR (700 MHz, CD_3OD) δ 7.84 (s, 1H, triazole-H5), 5.96 (d, *J* = 2.2 Hz, 1H, H-3), 5.21 (s, 2H, N-CH_2-CO), 4.47 (dd, *J* = 8.7, 2.2 Hz, 1H, H-4), 4.30 (d, *J* = 10.7 Hz, 1H, H-6), 4.08 (dd, *J* = 10.5, 8.7 Hz, 1H, H-5), 3.91 – 3.86 (m, 1H, H-8), 3.82 (dd, *J* = 11.4, 2.7 Hz, 1H, H-9), 3.66 (dd, *J* = 11.4, 5.4 Hz, 1H, H-9'), 3.61 (d, *J* = 9.3 Hz, 1H, H-7), 2.76 – 2.68 (m, 2H, α-CH_2), 1.73 – 1.66 (m, 2H, β-CH_2), 1.37 (dd, *J* = 7.0, 3.6 Hz, 4H, γ-CH_2, δ-CH_2), 0.92 (t, *J* = 6.9 Hz, 3H, ε-CH_3). ^{13}C NMR (176 MHz, CD_3OD) δ 168.93 (CH_3*C*O), 165.52 (C-1), 145.41(Triazole-C4), 125.30 (Triazole-C5), 113.18 (C-3), 77.68 (C-6), 71.26 (C-7), 70.07 (C-4), 68.07 (C-8), 64.89 (C-9), 53.17 (N-*C*H_2-CO), 52.12 (C-5), 32.44 (C-α), 30.09 (C-β), 26.15 (C-γ), 23.41(C-δ), 14.30 (C-ε). HRMS (ESI) calcd. for $C_{18}H_{27}N_4O_8$ $[M-H]^-$, 427.1834; found 427.1837.

General Procedure of Staudinger Reaction for Synthesis of Compounds of 15a-l

The C9-azido DANA methyl ester (**14**) was dissolved in THF-H_2O and cooled to 0 ºC with an ice water bath. Triphenyl phosphine was then added followed by anhydride or acyl chloride. The solution was allowed to warm to room temperature and kept stirring overnight. After completion, the reaction was quenched with water, concentrated, and purified by flash chromatography to give

the desired product. The product was then dissolved in MeOH, and 0.5 M NaOH was added. The mixture was kept stirring at room temperature. After completion, the mixture was neutralized with Amberlite IR-120 (H^+), filtered and purified by flash chromatography to provide the desired products.

5-Acetamido-9-butyramido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (15a). Compound **15a** was synthesized from compound **14** using butyryl anhydride (60% (68% × 89%), over two steps) ^{1}H NMR (500 MHz, CD_3OD) δ 5.93 (d, *J* = 2.5 Hz, 1H, H-3), 4.41 (dd, *J* = 8.7, 2.5 Hz, 1H, H-4), 4.20 – 4.15 (m, 1H, H-6), 3.96 (dd, *J* = 10.7, 8.7 Hz, 1H, H-5), 3.94 – 3.88 (m, 1H, H-8), 3.57 (dd, *J* = 14.0, 3.3 Hz, 1H, H-9), 3.41 (dd, *J* = 9.1, 1.1 Hz, 1H, H-7), 2.22 – 2.15 (m, 2H, α-CH_2), 2.00 (d, *J* = 9.2 Hz, 3H, $COCH_3$), 1.62 (dd, *J* = 14.8, 7.4 Hz, 2H, β-CH_2), 0.93 (t, *J* = 7.4 Hz, 3H, γ-CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 177.13, 174.87 (C=O), 165.51 (C-1), 113.36 (C-3), 77.83 (C-6), 71.49 (C-7), 70.17 (C-4), 67.96 (C-8), 52.01 (C-5), 44.40 (C-9), 38.97 (C-α), 22.73 (CO*C*H_3), 20.45 (C-β), 14.05 (C-γ). HRMS (ESI) calcd. for $C_{16}H_{25}N_2O_8$ [M-H]$^-$, 359.1460; found 359.1453

5-Acetamido-9-hexanamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (15b). Compound **15b** was synthesized from compound **14** using hexanoic anhydride. 66 mg (58% (79% × 72%), over two steps) ^{1}H NMR (500 MHz, CD_3OD) δ 5.92 (d, *J* = 2.5 Hz, 1H, H-3), 4.42 (dd, *J* = 8.7, 2.5 Hz, 1H, H-4), 4.17 (dd, *J* = 10.7, 1.0 Hz, 1H, H-6), 3.97 (dd, *J* = 10.7, 8.7 Hz, 1H, H-5), 3.94 – 3.88 (m, 1H, H-8), 3.59 (dd, *J* = 13.9, 3.3 Hz, 1H, H-9), 3.42 (dd, *J* = 9.0, 1.0 Hz, 1H, H-7), 3.31 – 3.27 (m, 1H, H-9'), 2.23 – 2.16 (m, 2H, α-CH_2), 2.01 (s, 3H, $COCH_3$), 1.59 (dt, *J* = 15.0, 7.6 Hz, 2H, β-CH_2), 1.39 – 1.25 (m, 4H, γ-CH_2, δ-CH_2), 0.89 (t, *J* = 7.1 Hz, 3H, ε-

CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 177.25, 174.87 (C=O), 165.63 (C-1), 113.30 (C-3), 77.82 (C-6), 71.55 (C-7), 70.24 (C-4), 67.98 (C-8), 51.97 (C-5), 44.41 (C-9), 37.09 (C-α), 32.58 (C-β), 26.81 (C-γ), 23.45 (C-δ), 22.81 ($CO\mathit{C}H_3$), 14.32 (C-ε). HRMS (ESI) calcd. for $C_{17}H_{27}N_2O_8$ $[M-H]^-$, 387.1773; found 387.1766.

5-Acetamido-9-heptanamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (15c). Compound **15c** was synthesized from compound **14** using heptanoyl chloride. 18 mg (45% (74% × 61%), over two steps) ^1H NMR (500 MHz, CD_3OD) δ 5.77 (d, *J* = 2.3 Hz, 1H, H-3), 4.37 (dd, *J* = 8.6, 2.3 Hz, 1H, H-4), 4.16 – 4.08 (m, 1H, H-6), 3.97 (dd, *J* = 10.7, 8.7 Hz, 1H, H-5), 3.89 (ddd, *J* = 8.7, 7.1, 3.4 Hz, 1H, H-8), 3.58 (dd, *J* = 13.8, 3.4 Hz, 1H, H-9), 3.40 (d, *J* = 8.7 Hz, 1H, H-7), 3.28 – 3.23 (m, 1H, H-9'), 2.26 – 2.13 (m, 2H, α-CH_2), 2.01 (s, 3H, $COCH_3$), 1.64 – 1.52 (m, 2H, β-CH_2), 1.37 – 1.23 (m, 6H, γ-CH_2, δ-CH_2, ε-CH_3), 0.88 (dd, *J* = 8.8, 5.1 Hz, 3H, ζ-CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 177.04, 174.70 (N-C=O), 110.32 (C-3), 77.35 (C-6), 71.50 (C-7), 70.44 (C-4), 68.36 (C-8), 51.99 (C-5), 44.23 (C-9), 37.15 (C-α), 32.73 (C-β), 30.07 (C-γ), 27.10(C-δ), 23.58 (C-ε), 22.80 (C-ζ), 14.39 ($CO\mathit{C}H_3$). HRMS (ESI) calcd. for $C_{18}H_{29}N_2O_8$ $[M-H]^-$, 401.1929; found 401.1931.

5-Acetamido-9-isobutyramido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (15d). Compound **15d** was synthesized from compound **14** using isobutyryl chloride. 15 mg (21% (40% × 52%), over two steps) ^1H NMR (700 MHz, CD_3OD) δ 5.74 (d, *J* = 2.3 Hz, 1H, H-3), 4.37 (dd, *J* = 8.7, 2.3 Hz, 1H, H-4), 4.14 (dd, *J* = 10.8, 1.0 Hz, 1H, H-6), 3.99 (dd, *J* = 10.8, 8.7 Hz, 1H, H-5), 3.91 (ddd, *J* = 9.1, 6.9, 3.4 Hz, 1H, H-8), 3.58 (dd, *J* = 13.8, 3.4 Hz, 1H, H-9), 3.40 (dd, *J* = 9.1, 1.0 Hz, 1H, H-7), 3.33 (dd, *J* = 13.8, 6.9 Hz, 1H, H-9'), 2.49 (dt, *J* = 13.8, 6.9 Hz, 1H,

α-CH_2), 2.02 (s, 3H, $COCH_3$), 1.12 (dd, *J* = 6.9, 0.5 Hz, 6H, 2 × β-CH_3). ^{13}C NMR (176 MHz, CD_3OD) δ 180.76, 174.56 (N-C=O), 109.51(C-3), 77.30 (C-6), 71.67 (C-7), 70.20 (C-4), 68.50 (C-8), 52.07 (C-5), 44.23 (C-9), 36.28 (C-α), 22.73, 19.96, 19.90 (2 ×C-β, CO*C*H_3). HRMS (ESI) calcd. for $C_{15}H_{23}N_2O_8$ [M-H]$^-$, 359.1460; found 359.1458.

5- Acetamido-9-(3-methylbutanamido)-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (15e). Compound **15e** was synthesized from compound **14** using 3-methylbutanoyl chloride. 18 mg (24% (44% × 55%), over two steps). 1H NMR (700 MHz, CD_3OD) δ 5.74 (d, J = 2.3 Hz, 1H, H-3), 4.37 (dd, J = 8.7, 2.3 Hz, 1H, H-4), 4.13 (dd, J = 10.8, 1.0 Hz, 1H, H-6), 3.99 (dd, J = 10.8, 8.7 Hz, 1H, -5), 3.91 (ddd, J = 9.0, 6.9, 3.4 Hz, 1H, H-8), 3.60 (dd, J = 13.8, 3.4 Hz, 1H, H-9), 3.43 – 3.39 (dd, J = 13.8, 6.9 Hz, 1H, H-9'), 3.33 (m, 1H, H-7), 2.10 – 2.09 (m, 2H, α-CH_2), 2.08-2.03 (m, 1H, β-CH), 2.02 (s, 3H, $COCH_3$), 0.96 (dd, J = 6.4, 1.6 Hz, 6H, 2 × γ-CH_3). ^{13}C NMR (176 MHz, CD_3OD) δ 176.27, 174.60 (N-C=O), 109.43 (C-3), 77.30 (C-6), 71.71 (C-7), 70.26 (C-4), 68.51 (C-8), 52.08 (C-5), 46.32 (C-9), 44.18 (C-α), 27.42 (C-β), 22.77, 22.75, 22.74 (2 ×C- γ, CO*C*H_3). HRMS (ESI) calcd. for $C_{16}H_{25}N_2O_8$ [M-H]$^-$, 373.1616; found 373.1617.

5-Acetamido-9-(4-methylpentanamido)-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (15f). Compound **15f** was synthesized from compound **14** using 4-methylpentanoyl chloride. 18 mg (23% (38% × 61%), over two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.69 (d, *J* = 2.3 Hz, 1H, H-3), 4.36 (dd, *J* = 8.7, 2.3 Hz, 1H, H-4), 4.10 (dd, *J* = 10.8, 0.8 Hz, 1H, H-6), 3.97 (dd, *J* = 10.8, 8.7 Hz, 1H, H-5), 3.92 – 3.85 (m, 1H, H-8), 3.58 (dd, *J* = 13.8, 3.3 Hz, 1H, H-9), 3.38 (dd, *J* = 8.9, 0.8 Hz, 1H, H-7), 3.28 – 3.21 (m, 1H, H-9'), 2.25 – 2.18 (m, 2H, α-CH_2), 2.01 (s, 3H, $COCH_3$), 1.60 – 1.44 (m, 3H, β-CH_2, γ-CH), 0.90 (d, *J* = 6.5 Hz, 6H,

2 × δ-CH_3). 13C NMR (126 MHz, CD_3OD) δ 177.19, 174.68 (N-C=O), 108.93 (C-3), 77.12 (C-6), 71.55 (C-7), 70.35 (C-4), 68.59 (C-8), 52.00 (C-5), 44.22 (C-9), 36.12 (C-α), 35.23 (C-β), 29.04 (C-γ), 22.86, 22.74 (2 × C-δ, CO*C*H_3). HRMS (ESI) calcd. for $C_{17}H_{27}N_2O_8$ $[M-H]^-$, 387.1773; found 387.1765.

5-Acetamido-9-benzamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (15g). Compound **15g** was synthesized from compound **14** using benzyl chloride. 20 mg (25% (37% × 68%), over two steps). ^1H NMR (500 MHz, CD_3OD) δ 7.85 – 7.79 (m, 2H, Ar-H), 7.54 – 7.48 (m, 1H, Ar-H), 7.43 (dd, *J* = 10.3, 4.7 Hz, 2H, Ar-H), 5.84 (d, *J* = 2.3 Hz, 1H, H-3), 4.40 (dd, *J* = 8.7, 2.3 Hz, 1H, H-4), 4.18 (d, *J* = 10.8 Hz, 1H, H-6), 4.07 – 3.96 (m, 2H, H-8, H-5), 3.77 (dd, *J* = 13.8, 3.4 Hz, 1H, H-9), 3.55 (dd, *J* = 13.8, 6.8 Hz, 1H, H-9'), 3.48 (d, *J* = 8.9 Hz, 1H, H-7), 1.96 (s, 3H, $COCH_3$). ^{13}C NMR (126 MHz, CD_3OD) δ 174.79, 170.91 (2 × N-C=O), 135.63, 132.71, 129.57, 128.36 (Ar-C), 111.64 (C-3), 77.53 (C-6), 71.60 (C-7), 70.35 (C-4), 68.20 (C-8), 51.92 (C-5), 45.02 (C-9), 22.74 (CO*C*H_3). HRMS (ESI) calcd. for $C_{18}H_{21}N_2O_8$ $[M-H]^-$, 393.1303; found 393.1302

5-Acetamido-9-(4-acetamido)benzamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (15h). Compound **15h** was synthesized from compound **14** using 4-acetamidobenzyl chloride. 45 mg (28% (36 % × 77%), over two steps). ^1H NMR (500 MHz, D_2O) δ 7.80 (d, *J* = 8.2 Hz, 2H, Ar-H), 7.58 (d, *J* = 8.2 Hz, 2H, Ar-H), 5.95 (s, 1H, H-3), 4.54 (d, *J* = 7.8 Hz, 1H, H-4), 4.33 (d, *J* = 10.9 Hz, 1H, H-6), 4.14 (t, *J* = 9.5 Hz, 2H, H-5, H-8), 3.86 – 3.80 (m, 1H, H-9), 3.68 – 3.58 (m, 2H, H-7, H-9'), 2.22 (s, 3H, $COCH_3$), 2.07 (s, 3H, $COCH_3$). ^{13}C NMR (126 MHz, D_2O) δ 175.64, 173.90, 171.46 (3 × N-C=O), 168.07 (C-1), 141.49, 130.56, 129.13,

121.72 (Ar-C), 111.40 (C-3), 76.57 (C-6), 70.37 (C-7), 69.56 (C-4), 68.16 (C-8), 50.72 (C-5), 44.12 (C-9), 24.04, 23.02 (2 × CO*C*H$_3$). HRMS (ESI) calcd. for $C_{18}H_{21}N_2O_8$ [M-H]$^-$, 450.1518; found 450.1525

5-Acetamido-9-(4-amino)benzamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (15i). Compound **15i** was synthesized from compound **14** using *N*-hydroxysuccinimidyl-4-((tert-butoxycarbonyl) amino) benzoate. 30 mg (20% (45 % × 45%, yields for two steps of deprotection), over three steps). ^{1}H NMR (500 MHz, CD$_3$OD) δ 7.91 (d, *J* = 7.3 Hz, 2H), 7.33 (d, *J* = 7.3 Hz, 2H), 5.94 (s, 1H, H-3), 4.46 (d, *J* = 8.0 Hz, 1H, H-4), 4.21 (d, *J* = 10.6 Hz, 1H, H-6), 4.08 – 3.96 (m, 2H, H-5, H-8), 3.77 (d, *J* = 12.9 Hz, 1H, H-9), 3.51 (m, 2H, H-9', H-7), 1.97 (s, 3H, COCH$_3$). ^{13}C NMR (126 MHz, CD$_3$OD) δ 165.66 (C-1), 145.21, 130.38, 122.41 (Ar-C), 113.68 (C-3), 77.75 (C-6), 71.40 (C-7), 70.28 (C-4), 67.93 (C-8), 51.73 (C-5), 45.05 (C-9), 22.92 (CO*C*H$_3$). HRMS (ESI) calcd. for $C_{18}H_{21}N_2O_8$ [M-H]$^-$, 408.1412; found 408.1415

5-Acetamido-9-(3-acetamido)benzamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (15j). Compound **15j** was synthesized from compound **14** using 3-acetamidobenzyl chloride. 40 mg (30% (36 % × 82%), over two steps). ^{1}H NMR (500 MHz, CD$_3$OD) δ 7.97 (t, *J* = 1.5 Hz, 1H, Ar-H), 7.69 (dd, *J* = 7.9, 1.5 Hz, 1H, Ar-H), 7.52 (d, *J* = 7.9 Hz, 1H, Ar-H), 7.37 (t, *J* = 7.9 Hz, 1H, Ar-H), 5.78 (d, *J* = 2.0 Hz, 1H, H-3), 4.38 (dd, *J* = 8.6, 2.0 Hz, 1H, H-4), 4.16 (d, *J* = 10.8 Hz, 1H, H-6), 4.00 (m, 2H, H-5, H-8), 3.76 (dd, *J* = 13.8, 3.3 Hz, 1H, H-9), 3.53 (dd, *J* = 13.8, 6.8 Hz, 1H, H-9'), 3.47 (d, *J* = 8.8 Hz, 1H, H-7), 2.12 (s, 3H, COCH$_3$), 1.97 (s, 3H, COCH$_3$). ^{13}C NMR (126 MHz, CD$_3$OD) δ 174.74, 171.81, 170.56 (3 × N-C=O),

140.21, 136.51, 130.03, 124.15, 123.74, 120.14 (Ar-C), 110.38 (C-3), 77.38 (C-6), 71.78 (C-7), 70.21 (C-4), 68.40 (C-8), 51.96 (C-5), 45.07 (C-9), 23.86, 22.77 (2 × CO*C*H$_3$). HRMS (ESI) calcd. for $C_{18}H_{21}N_2O_8$ [M-H]$^-$, 450.1518; found 450.1515

5-Acetamido-9-(3-amino)benzamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (15k). Compound **15k** was synthesized from compound **14** using 3-amidobenzyl chloride. ^{1}H NMR (500 MHz, CD$_3$OD) 30 mg (18% (46 % × 40%, over two steps). δ 7.94 (d, *J* = 7.8 Hz, 1H, Ar-H), 7.89 (s, 1H, Ar-H), 7.63 (t, *J* = 7.8 Hz, 1H, Ar-H), 7.60 – 7.56 (m, 1H, Ar-H), 5.95 (d, *J* = 2.4 Hz, 1H, H-3), 4.45 (dd, *J* = 8.7, 2.4 Hz, 1H, H-4), 4.21 (d, *J* = 10.9 Hz, 1H, H-6), 4.08 – 3.97 (m, 2H, H-5, H-8), 3.84 – 3.79 (m, 1H, H-9), 3.57 – 3.48 (m, 2H, H-7, H-9'), 1.99 (s, 3H, CO*C*H$_3$). ^{13}C NMR (126 MHz, CD$_3$OD) δ 175.00, 168.86 (2 × N-C=O), 165.60 (C-1), 145.27 (C-2), 137.83, 132.62, 131.55, 128.67, 127.16, 123.51 (Ar-C), 113.61(C-3), 77.79 (C-6), 71.72 (C-7), 70.03 (C-4), 67.90 (C-8), 51.81 (C-5), 45.29 (C-9), 22.80 (CO*C*H$_3$). HRMS (ESI) calcd. for $C_{18}H_{21}N_2O_8$ [M-H]$^-$, 408.1412; found 408.1411

5-Acetamido-9-(5-(4-acetamidobenzamido))pentanamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (15l). Compound **15l** was synthesized from compound **14** using *N*-hydroxysuccinimidyl-5-(4-acetamidobenzamido) pentanoate. 30 mg (22% (42% × 53%, over two steps). ^{1}H NMR (500 MHz, CD$_3$OD) δ 7.77 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.64 (d, *J* = 8.7 Hz, 2H, Ar-H), 5.90 (d, *J* = 2.3 Hz, 1H, H-3), 4.42 (dd, *J* = 8.7, 2.3 Hz, 1H, H-4), 4.19 – 4.13 (d, *J* = 10.7, 1H, H-6), 3.98 (dd, *J* = 10.7, 8.7 Hz, 1H, H-5), 3.93 – 3.86 (m, 1H, H-8), 3.59 (dd, *J* = 13.8, 3.1 Hz, 1H, H-9), 3.42 (d, *J* = 8.9 Hz, 1H, H-7), 3.37 (t, *J* = 6.0 Hz, 2H, δ-CH$_2$), 2.27 (t, *J* = 7.1 Hz, 2H, α-CH$_2$), 2.13, 2.01 (2 × s, 2 × 3H, 2 × COCH$_3$), 1.73 – 1.56 (m, 4H, β-CH$_2$, γ-CH$_2$).

^{13}C NMR (126 MHz, CD_3OD) δ 176.77, 174.82, 171.90, 169.57 (4 × N-C=O), 143.07 (C-2), 130.74, 129.15, 120.26 (Ar-C), 112.90 (C-3), 77.69 (C-6), 71.35(C-8), 70.24 (C-7), 51.81(C-4), 49.88(C-5), 44.28(C-9), 40.53(δ-CH_2), 36.59(α-CH_2), 30.04 (γ-CH_2), 24.42(α-CH_2), 24.03, 22.86(2 × CO*C*H_3). HRMS (ESI) calcd. for $C_{25}H_{33}N_4O_{10}$ $[M-H]^-$, 549.2202; found 549.2207.

5-Acetamido-9-(4-pentyltriazolyl)-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (15m). To a solution of methyl 5-acetamido-9-azido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonate (**14**) (50 mg, 1 eq) and 1-heptyne (30mg, 2 eq) in THF-H_2O (2:1), sodium L-ascorbate (5 mg, 0.3 eq) and copper (II) sulfate (3 mg, 0.3 eq) were added. The reaction mixture was kept stirring at room temperature and monitored by TLC until compound **14** was consumed. Solvents were then removed, and the residue was separated by flash chromatography to provide the desired C1-methyl ester product 59 mg (92%). The product was dissolved in 2 mL MeOH, and 0.5 M NaOH was added. The mixture was kept stirring at room temperature. After completion, the pH of the solution was adjusted to 2 with Amberlite IR-120 (H^+). The solution was filtered, concentrated and dissolved in a minimum amount of methanol. Ethyl acetate was added to allow the product precipitate. The solid was collected by filtration and dried in vacuum. 40 mg (70%). ^{1}H NMR (500 MHz, CD_3OD) δ 7.71 (s, 1H, Triazole-H), 5.91 (d, *J* = 2.3 Hz, 1H, H-3), 4.77 (dd, *J* = 14.0, 2.5 Hz, 1H, H-9), 4.44 – 4.33 (m, 2H, H-4, H-6), 4.24 – 4.16 (m, 1H, H-8), 4.14-4.12 (m, 1H, H-9'), 3.98 (dd, *J* = 10.7, 8.7 Hz, 1H, H-5), 3.39 (d, *J* = 9.1 Hz, 1H, H-7), 2.66 (t, *J* = 7.7 Hz, 2H, α-CH_2), 2.01 (s, 3H, $COCH_3$), 1.71 – 1.59 (m, β-2H), 1.39 – 1.27 (m, 4H, γ-CH_2, δ-CH_2), 0.89 (dd, *J* = 9.7, 4.3 Hz, 3H, ε-CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 175.10 (*C*OCH_3), 166.11 (C-1), 145.91 (Triazole-C4), 124.31 (Triazole-C5), 112.97 (C-3), 77.69 (C-6),

71.34 (C-7), 69.86 (C-4), 67.93 (C-8), 55.00 (C-9), 51.92 (C-5), 32.51 (C-α), 30.36 (C-β), 26.29 (C-γ), 23.46 (C-δ), 22.72 ($COCH_3$), 14.35 (C-ε). HRMS (ESI) calcd. for $C_{18}H_{27}N_4O_7$ $[M-H]^-$, 411.1885; found 411.1889

General procedure for synthesis of C5, C9 double modified DANA analogue 17a-f. Compound **16** was dissolved in THF-H_2O and cooled to 0 ºC with an ice water bath. Triphenyl phosphine was then added followed by activated esters. The solution was allowed to warm to room temperature and kept stirring overnight. After completion, the reaction was quenched with water, concentrated and purified by flash chromatography to give the desired C9-modified product. The product was then dissolved in anhydrous TFA-DCM (10%) and the solution was stirred for 2-4 hours at ambient temperature. Solvents were removed under vacuum and the residue was dissolved in anhydrous DCM and TEA was added. The solution was cooled to 0 ºC and the corresponding activated esters for C5 modification were added dropwise. The solution was allowed to warm to room temperature and kept stirring overnight. After completion, the reaction was quenched with water, concentrated and purified by flash chromatography to give the desired C5-modified product. The product was then dissolved in MeOH, and 0.5 N NaOH was added. The mixture was kept stirring at room temperature. After completion, the mixture was neutralized with Amberlite IR-120 (H^+), filtered and purified by flash chromatography to provide the final C5, C9- double modified compounds.

5-Hexanamido-9-hexanamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (17a). Compound **17a** was synthesized from compound **16** using hexanoic anhydride. 20 mg. (22% (77% × 54% × 52%), over three steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.78 (s, 1H, H-3),

4.38 (d, J = 8.5 Hz, 1H, H-4), 4.13 (d, J = 10.6 Hz, 1H, H-6), 4.02 – 3.86 (m, 2H, H-5, H-8), 3.56 (d, J = 13.2 Hz, 1H, H-9), 3.42 (d, J = 7.9 Hz, 1H, H-7), 2.31 – 2.12 (m, 4H, α-CH_2, α'-CH_2), 1.68 – 1.53 (m, 4H, β-CH_2, β'-CH_2), 1.40 – 1.23 (m, 8H, γ-CH_2, γ'-CH_2, δ-CH_2, δ'-CH_2), 0.90 (q, J = 6.9 Hz, 6H, ε-CH_3, ε'-CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 177.85, 177.06 (N-C=O), 110.69 (C-3), 77.49 (C-6), 71.39 (C-7), 70.66 (C-4), 68.30 (C-8), 51.88 (C-5), 44.25 (C-9), 37.18, 37.11(C-α, C-α'), 32.64, 32.61 (C-β, C-β'), 26.78, 26.66 (C-γ, C-γ'), 23.46 (C-δ, C-δ'), 14.32, 14.30 (C-ε, C-ε'). HRMS (ESI) calcd. for $C_{21}H_{35}N_2O_8$ [M-H]$^-$, 443.2399; found 443.2396.

5-Pentanamido-9-pentanamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (17b). Compound **17b** was synthesized from compound **16** using valeric anhydride. 50 mg. (36% (90% × 72% × 55%), over three steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.74 (d, J = 1.9 Hz, 1H, H-3), 4.38 (dd, J = 8.7, 1.9 Hz, 1H, H-4), 4.11 (d, J = 10.8 Hz, 1H, H-6), 3.97 (dd, J = 10.7, 8.8 Hz, 1H, H-5), 3.93 – 3.85 (m, 1H, H-8), 3.57 (dd, J = 13.4, 3.1 Hz, 1H, H-9), 3.38 (d, J = 8.7 Hz, 1H, H-7), 3.26 (dd, J = 13.4, 6.0 Hz, 1H, H-9'), 2.30 – 2.23 (m, 2H, α-CH_2), 2.23 – 2.15 (m, 2H, α'-CH_2), 1.59 (tdd, J = 15.3, 11.2, 7.5 Hz, 4H, β-CH_2, β'-CH_2), 1.34 (dq, J = 22.0, 7.4 Hz, 4H, γ-CH_2, γ'-CH_2), 0.92 (q, J = 7.4 Hz, 6H, δ-CH_3, δ'-CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 177.78, 176.97 (N-C=O), 109.98 (C-3), 77.36 (C-6), 71.51 (C-7), 70.36 (C-4), 68.40 (C-8), 51.87 (C-5), 44.27(C-9), 36.96, 36.89 (C-α, C-α'), 29.24, 29.11 (C-β, C-β'), 23.49, 23.45 (C-γ, C-γ'), 14.19 (C-δ, C-δ'). HRMS (ESI) calcd. for $C_{19}H_{31}N_2O_8$ [M-H]$^-$, 415.2086; found 415.2081.

5-Propionamido-9-pentanamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (17c). Compound **17c** was synthesized from compound **16** using valeric anhydride and propionic anhydride. 15 mg. (18% (60% × 53% × 58%), over three steps). ^{1}H NMR (500 MHz,

CD_3OD) δ 5.90 (d, *J* = 2.4 Hz, 1H, H-3), 4.41 (dd, *J* = 8.7, 2.3 Hz, 1H, H-4), 4.16 (d, *J* = 10.8 Hz, 1H, H-6), 4.01 – 3.87 (m, 2H, H-5, H-8), 3.61 – 3.53 (m, 1H, H-9), 3.41 – 3.35 (m, 1H, H-7), 2.28 (q, *J* = 7.6 Hz, 2H, α-CH_2), 2.24 – 2.17 (m, 2H, α-CH_2'), 1.57 (dt, *J* = 13.0, 7.5 Hz, 2H, β'-CH_2), 1.33 (dq, *J* = 14.7, 7.4 Hz, 2H, γ'-CH_2), 1.14 (t, *J* = 7.6 Hz, 3H, β-CH_3), 0.91 (t, *J* = 7.4 Hz, 3H, δ'-CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 178.61, 177.24 (N-C=O), 112.84 (C-3), 77.83 (C-6), 71.51 (C-7), 70.19 (C-4), 68.00 (C-8), 51.87 (C-5), 44.40 (C-9), 36.82 (C-α'), 30.20 (C-β'), 29.23 (C-γ'), 23.42 (C-α), 14.13 (C-δ'), 10.37 (C-β). HRMS (ESI) calcd. for $C_{17}H_{27}N_2O_8$ $[M-H]^-$, 387.1773; found 387.1770.

5-Propionamido-9-hexanamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (17d). Compound **17d** was synthesized from compound **16** using hexanoic anhydride and propionic anhydride. 13 mg. (17% (63% × 46% × 60%), over three steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.89 (s, 1H, H-3), 4.40 (d, *J* = 8.7 Hz, 1H, H-4), 4.15 (d, *J* = 10.7 Hz, 1H, H-6), 4.01 – 3.86 (m, 2H, H-5, H-8), 3.57 (d, *J* = 11.9 Hz, 1H, H-9), 3.39 (d, *J* = 8.6 Hz, 1H, H-7), 2.28 (q, *J* = 7.5 Hz, 2H, α-CH_2), 2.24 – 2.14 (m, 2H, α'-CH_2), 1.59 (dt, *J* = 14.8, 7.5 Hz, 2H, β'-CH_2), 1.38 – 1.25 (m, 4H, , γ'-CH_2, δ'-CH_3), 1.14 (t, *J* = 7.6 Hz, 3H, β-CH_3), 0.89 (t, *J* = 7.0 Hz, 3H, ε-CH_3'). ^{13}C NMR (126 MHz, CD_3OD) δ 178.60, 177.22 (2 × N-C=O), 112.68 (C-3), 77.89 (C-6), 71.56 (C-7), 70.25 (C-4), 68.10 (C-8), 51.87 (C-6), 44.40 (C-9), 37.07 (C-α'), 32.58 (C-β'), 30.21 (C-γ'), 26.77 (C-δ'), 23.44 (C-α), 14.27(C-ε'), 10.37 (C-β). HRMS (ESI) calcd. for $C_{18}H_{29}N_2O_8$ $[M-H]^-$, 401.1929; found 401.1926.

5-Pentanamido-9-acetamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (17e). Compound **17e** was synthesized from compound **16** using acetic anhydride and valeric

anhydride. 20 mg. (24% (74% × 50% × 65%), over three steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.86 (d, *J* = 2.4 Hz, 1H, H-3), 4.40 (dd, *J* = 8.7, 2.4 Hz, 1H, H-4), 4.14 (d, *J* = 10.7 Hz, 1H, H-6), 3.96 (dd, *J* = 10.7, 8.7 Hz, 1H, H-5), 3.93 – 3.87 (m, 1H, H-8), 3.58 (dd, *J* = 13.9, 3.2 Hz, 1H, H-9), 3.38 (dd, *J* = 9.0, 0.7 Hz, 1H, H-7), 3.27 – 3.23 (m, 1H, H-9'), 2.27 (t, 2H, J=7.5 Hz, α-CH_2), 1.95 (s, 3H, $COCH_3$), 1.61 (dt, *J* = 13.1, 7.5 Hz, 2H, β-CH_2), 1.37 (dt, *J* = 15.0, 7.4 Hz, 2H, γ-CH_2), 0.93 (t, *J* = 7.4 Hz, 3H, δ-CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 177.96, 174.02 (2 × N-C=O), 166.67 (C-1), 112.19 (C-3), 77.73 (C-6), 71.69 (C-7), 70.00 (C-4), 68.08 (C-8), 51.87 (C-5), 44.58 (C-9), 36.90 (C-α), 29.13 (C-β), 23.45 (C-γ), 22.58 (CO*C*H_3), 14.16 (C-δ). HRMS (ESI) calcd. for $C_{16}H_{25}N_2O_8$ $[M-H]^-$, 373.1616; found 373.1615.

5-Hexanamido-9-acetamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (17f). Compound **17f** was synthesized from compound **16** using acetic anhydride and hexanoic anhydride. 24 mg (30% (74% × 55% × 74%), over three steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.74 (d, *J* = 2.3 Hz, 1H, H-3), 4.39 (dd, *J* = 8.7, 2.3 Hz, 1H, H-4), 4.11 (d, *J* = 10.8 Hz, 1H, H-6), 3.96 (dd, *J* = 10.8, 8.7 Hz, 1H, H-5), 3.93 – 3.87 (m, 1H, H-8), 3.60 (dd, *J* = 13.8, 3.2 Hz, 1H, H-9), 3.40 – 3.35 (m, 1H, H-7), 3.23 (dd, *J* = 13.8, 7.2 Hz, 1H, H-9'), 2.29 – 2.23 (m, 2H, α-CH_2), 1.95 (s, 3H, $COCH_3$), 1.66 – 1.59 (m, 2H, β-CH_2), 1.37 – 1.28 (m, 4H, γ-CH_2, δ-CH_3), 0.90 (t, *J* = 7.0 Hz, 3H, ε-CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 178.01, 174.03 (2 × N-C=O), 168.67 (C-1), 110.16 (C-3), 77.34 (C-6), 71.71 (C-7), 70.06 (C-4), 68.37 (C-8), 51.82 (C-5), 44.53 (C-9), 37.25 (C-α), 32.61(C-β), 26.70 (C-γ), 23.45 (C-δ), 22.69 (CO*C*H_3), 14.37 (C-ε). HRMS (ESI) calcd. for $C_{16}H_{25}N_2O_8$ $[M-H]^-$, 387.1773; found 387.1774

5-Hexanamido-9-propionamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (17g). Compound **17g** was synthesized from compound **16** using propionic anhydride and hexanoic anhydride. 20 mg (30% (96% × 41% × 75%), over three steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.73 (d, *J* = 2.3 Hz, 1H, H-3), 4.36 (dd, *J* = 8.6, 2.3 Hz, 1H, H-4), 4.10 (dd, *J* = 10.8, 0.8 Hz, 1H, H-6), 3.97 (dd, *J* = 10.8, 8.7 Hz, 1H, H-5), 3.89 (ddd, *J* = 9.2, 5.2, 2.7 Hz, 1H, H-8), 3.59 (dd, *J* = 13.8, 3.2 Hz, 1H, H-9), 3.36 (dd, *J* = 9.0, 0.7 Hz, 1H, H-7), 3.25 (dd, *J* = 13.8, 7.1 Hz, 1H, H-9'), 2.29 – 2.15 (m, 4H, α-CH_2, α'-CH_2), 1.63 (dt, *J* = 14.8, 7.6 Hz, 2H, β-CH_2), 1.38 – 1.28 (m, 4H, γ-CH_2, δ-CH_2), 1.11 (t, *J* = 7.6 Hz, 3H, β'-CH_3), 0.91 (t, *J* = 7.0 Hz, 3H, ε-CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 177.78, 177.58 (2 × N-C=O), 109.74 (C-3), 77.38 (C-6), 71.81 (C-7), 70.10 (C-4), 68.43 (C-8), 51.87 (C-5), 44.43 (C-9), 37.17 (C-α), 32.63 (C-β), 30.18 (C-α'), 26.67 (C-γ), 23.45 (C-δ), 14.32 (C-ε), 10.55 (C-β'). HRMS (ESI) calcd. for $C_{18}H_{29}N_2O_8$ $[M-H]^-$, 401.1929; found 401.1929

5-Hexanamido-9-butanamido-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid (17h). Compound **17h** was synthesized from compound **16** using butyric anhydride and hexanoic anhydride. 22 mg (34% (96% × 46% × 78%), over three steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.70 (d, *J* = 2.2 Hz, 1H, H-3), 4.36 (dd, *J* = 8.6, 2.3 Hz, 1H, H-4), 4.09 (dd, *J* = 10.8, 0.8 Hz, 1H, H-6), 3.97 (dd, *J* = 10.8, 8.7 Hz, 1H, H-5), 3.92 – 3.85 (m, 1H, H-8), 3.59 (dd, *J* = 13.8, 3.2 Hz, 1H, H-9), 3.36 (d, *J* = 8.9 Hz, 1H, H-7), 3.25 (dd, *J* = 13.8, 7.0 Hz, 1H, H-9'), 2.29 – 2.21 (m, 2H, α-CH_2), 2.20 – 2.11 (m, 2H, α'-CH_2), 1.62 (ddd, *J* = 14.9, 7.5, 2.6 Hz, 4H, β-CH_2, β'-CH_2), 1.34-1.32 (m, 4H, γ-CH_2, δ-CH_2), 0.94-0.89 (m, 6H, ε-CH_3, γ'-CH_3). ^{13}C NMR (126 MHz, CD_3OD) δ 177.75, 176.70 (2 × N-C=O), 109.28 (C-3), 77.31 (C-4), 71.74 (C-7), 70.22 (C-

6), 68.50 (C-8), 51.89 (C-5), 44.32 (C-9), 39.04 (C-α), 37.18 (C-α'), 32.63 (C-β), 26.66 (C-γ), 23.46 (C-β'), 20.44 (C-δ), 14.33 (C-ε), 14.08 (C-γ'). HRMS (ESI) calcd. for $C_{19}H_{32}N_2O_8$ [M-H]$^-$, 415.2086; found 415.2088

Methyl 5-(*tert*-butoxycarbonyl)amino-4-azido-2,6-anhydro-3,4,5-trideoxy-dideoxy-D-*glycero*-D-*galacto*-non-2-enonate (19). Compound **18** (200 mg, 1 eq), di-tert-butyl dicarbonate (241 mg, 2.7 eq) and 4-dimethylaminopyridine (76 mg, 1.6 eq) were dissolved in 60 mL anhydrous THF. The solution was then refluxed for 2 h. After completion, solvents were removed under reduced pressure and the residue was separated by flash chromatography, providing crude product (240 mg). The crude product was dissolved in 10 mL MeOH. After the solution was cooled down to 0 ºC, NaOMe (20 mg, 1eq) was added slowly. The mixture was stirred at 0 ºC for 1 h. After completion, Amberlite IR 120 (H+) was added to adjust the pH of the solution to 2. After filtration, solvent was removed under reduced pressure and the residue was purified by flash chromatography, providing the desired product 140 mg (82%, over two steps). ^{1}H NMR (500 MHz, CD_3OD) δ 5.86 (d, *J* = 2.4 Hz, 1H, H-3), 4.31 (dd, *J* = 9.4, 2.4 Hz, 1H, H-4), 4.21 (d, *J* = 10.9 Hz, 1H, H-6), 3.88 – 3.79 (m, 3H, H-5, H-8, H-9), 3.77 (s, 3H, $COOCH_3$), 3.71 – 3.61 (m, 2H, H-7, H-9'), 1.45 (s, 9H, ^{t}Boc). ^{13}C NMR (126 MHz, CD_3OD) δ 169.53, 163.95 (C-1), 158.44 (^{t}Boc−OCO), 146.55 (C-2), 108.74 (C-3), 81.25 (^{t}Boc-*C*CH_3), 78.40 (C-6), 71.28 (C-8), 69.75 (C-7), 64.87 (C-9), 60.08 (C-4), 53.00 (C-5), 50.53 (COO*C*H_3), 28.57 (^{t}Boc-CH_3). HRMS (ESI) calcd. for $C_{15}H_{24}N_4NaO_8$ [M-H]$^-$, 411.1486; found 411.1487.

Methyl 5-(4-methylpentanamido)-4-azido-7,8,9-tri-*O*-acetyl-2,6-anhydro-3,4,5-trideoxy-D-*glycero*-D-*galacto*-non-2-enonate (20). A solution of compound **19** (140 mg, 1 eq) in anhydrous

pyridine was cooled to 0 °C and acetic anhydride (400 µL, 10 eq) was added dropwise. The mixture was then allowed to warm to ambient temperature and kept stirring overnight. After completion, the reaction was quenched with methanol and solvents were removed under reduced pressure. The residue was dissolved in 200 mL ethyl acetate and carefully washed with 0.1 M HCl, water, brine and dried over Na_2SO_4. The solution was concentrated to give 160 mg as a yellow oil, which was dissolved in 20 mL anhydrous DCM and 2 mL TFA was added slowly. The solution was then stirred at room temperature for 2 hours. After completion, DCM and TFA were removed under reduced pressure. The residue was dissolved in 10 mL anhydrous DCM and TEA (124 µL, 3 eq) was added. The mixture was then cooled to 0 °C and 4-methylpentanoyl chloride (75 mg, 1.2 eq) was added. The solution was allowed to warm to room temperature and kept stirring overnight. After completion, the reaction was quenched with water, concentrated and purified by flash chromatography to give the desired product. 105 mg (52%, over three steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.05 (d, *J* = 8.3 Hz, 1H, NH), 5.94 (d, *J* = 2.1 Hz, 1H, H-3), 5.41 (d, *J* = 5.2 Hz, 1H, H-7), 5.29 (td, *J* = 6.5, 2.7 Hz, 1H, H-8), 4.59 (dd, *J* = 12.4, 2.6 Hz, 1H, H-9), 4.54-4.50 (m, H5, H-4), 4.16 (dd, *J* = 12.4, 6.6 Hz, 1H, H-9'), 3.78 (s, 3H, $COOCH_3$), 2.17 (t, *J* = 7.8 Hz, 2H, α-CH_2), 2.11, 2.03, 2.02 (3 × s, 9H, 3 × $COCH_3$), 1.62 – 1.43 (m, 3H, β-CH_2, γ-CH), 0.88 (d, *J* = 6.3 Hz, 6H, 2 × δ-CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.00, 170.69, 170.27, 170.08 (4 × C=O), 161.55 (C-1), 145.05 (C-2), 107.66 (C-3), 75.58 (C-6), 70.66 (C-8), 67.74 (C-7), 62.00 (C-9), 57.51 (C-4), 52.56 (COO*C*H_3), 48.74 (C-5), 34.71 (C-β), 34.00(C- γ), 27.72, 22.24, 22.20 (3 × CO*C*H_3), 20.82, 20.72 (2 × C-δ). HRMS (ESI) calcd. for $C_{22}H_{32}N_4NaO_8$ $[M-H]^-$, 535.2011; found 535.2003.

Methyl 5-(4-methylpentanamido)-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-[2,3-bis(tert-butoxycarbonyl)guanidino]- 3,4,5-trideoxy-D-*glycero*-D-*galacto*-non-2-enonate (21). To a solution of compound **20** (50 mg, 1 eq) in THF (2 mL), 1 N HCl (200 μL, 2.2 eq) was added, followed by triphenylphosphine (29 mg, 1.2 eq). The resulting mixture was stirred at room temperature overnight. After completion, solvents were removed under reduced pressure and the residue was purified by flash chromatography, providing crude product (50 mg). The residue was dissolved in 5 mL anhydrous DCM, and TEA (50 μL, 4 eq) was added. The solution was cooled to 0 ºC and *N*, *N*′-Di-Boc-1*H*-pyrazole-1-carboxamidine (600 mg, 2 eq) added. The reaction mixture was allowed to warm up to room temperature and kept stirring overnight. After completion, the reaction was quenched with water, extracted with ethyl acetate. The organic phase was washed with brine, dried over Na_2SO_4, concentrated and purified by flash chromatography to give the desired product. 60 mg (87%, over two steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.46 (d, *J* = 8.9 Hz, 1H, NH), 7.76 (brs, 1H, NH), 6.16 (d, *J* = 9.2 Hz, 1H, NH), 5.88 (d, *J* = 2.4 Hz, 1H, H-3), 5.42 (dd, *J* = 4.9, 1.7 Hz, 1H, H-7), 5.28 (ddd, *J* = 7.4, 4.9, 2.7 Hz, 1H, H-8), 5.19 (td, *J* = 9.7, 2.4 Hz, 1H, H-4), 4.67 (dd, *J* = 12.4, 2.7 Hz, 1H, H-9), 4.31 (dd, *J* = 10.5, 9.7 Hz, 1H, H-5), 4.26 (dd, *J* = 10.5, 1.7 Hz, 1H, H-6), 4.15 (dd, *J* = 12.4, 7.4 Hz, 1H, H-9'), 3.79 (s, 3H, $COOCH_3$), 2.17 – 1.96 (m, 11H, 3 × $COCH_3$, α-CH_2), 1.56 – 1.33 (m, 21H, 2 × ^{t}Boc, β-CH_2, γ-CH), 0.85 (dd, *J* = 6.5, 2.7 Hz, 6H, 2 × δ-CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.01, 170.56, 170.24, 170.07 (4 × C=O), 161.70 (^{t}Boc−OCO), 157.23 (C-1), 152.62 (C=N), 145.07(C-2), 109.71(C-3), 83.87, 79.77 (^{t}Boc−*C*(CH_3)$_3$), 78.11 (C-6), 71.57(C-8), 67.76(C-7), 62.29(C-9), 52.45(COO*C*H_3), 48.88 (C-4), 47.60 (C-5), (CO*C*H_3), 34.67 (C-α), 34.12 (C-β), 28.27, 28.03 (^{t}Boc−C(*C*H_3)$_3$), 27.70 (C-γ), 22.32,

22.13 (C-δ), 20.91, 20.87, 20.79 (CO*C*H_3). HRMS (ESI) calcd. for $C_{33}H_{52}N_4NaO_{14}$ $[M+Na]^+$, 751.3372; found 751.3378.

5-(4-methylpentanamido)-2,6-anhydro-4-guanidino-3, 4, 5-trideoxy-D-glycero-D-galacto-non-2-enonic acid (**22**)**.** To a solution of compound **21** (60 mg) in 5 mL DCM, 500 μL TFA was added. The solution was then stirred at room temperature for 2 h. After completion, DCM and TFA were removed under reduced pressure. The residue was dissolved in methanol and 2 mL 1 N NaOH was added. The solution was stirred at room temperature for 1 h. After completion, the reaction mixture was added with Amberlite IR 120 (H+) to make the pH of the solution 7. The suspension was then filtered, and the filtrate was concentrated to give a light yellow oil. The residue was dissolved in a minimum of methanol and the product was precipitated by ethyl acetate. The product was obtained by filtering as a white solid. 15 mg (48%, over two steps). ^{1}H NMR (700 MHz, CD_3OD) δ 5.50 (s, 1H, H-3), 4.37 (d, *J* = 8.5 Hz, 1H, H-4), 4.33 (d, *J* = 10.0 Hz, 1H, H-6), 4.19 (t, *J* = 9.4 Hz, 1H, H-5), 3.89 – 3.79 (m, 2H, H-8, H-9), 3.65 (dd, *J* = 11.3, 5.4 Hz, 1H, H-9'), 3.58 (d, *J* = 9.2 Hz, 1H, H-7), 2.26 (t, *J* = 7.5 Hz, 2H, α-CH_2), 1.61 – 1.49 (m, 3H, β-CH_2, γ-CH), 0.92 (d, *J* = 6.4 Hz, 6H, 2 × δ-CH_3). ^{13}C NMR (176 MHz, CD_3OD) δ 177.19 (*C*OCH_3), 169.63 (C-1), 158.78 (C=N), 151.58 (C-2), 103.33(C-3), 77.04 (C-6), 71.33 (C-8), 70.31 (C-7), 64.88 (C-9), 52.26 (C-4), 35.85 (C-α), 35.21 (C-β), 28.86 (C-γ), 22.72, 22.63 (2 × C-δ). HRMS (ESI) calcd. for $C_{33}H_{52}N_4NaO_{14}$ $[M+Na]^+$, 387.1885; found 387.1879.

Inhibition Assays. Inhibition assays against 4MU-NANA hydrolysis was performed using protocols reported previously.[34] NEU3 and NEU2 were expressed as N-terminal MBP fusion proteins in *E. coli* and purified as previously reported.[55] NEU4 was expressed as an MBP fusion

protein in *E. coli* and purified.[56] NEU1 was overexpressed as a $(His)_6$ fusion protein in HEK293 cells, and used as a crude preparation from cell lysate.[30, 57] All assays were conducted in 0.1 M sodium acetate buffer at optimum pH for each enzyme (4.5 for NEU1, NEU3 and NEU4; 5.5 for NEU2).[34] To get comparable IC_{50} measurements among the four isoenzymes, similar activity units of each enzyme were used in the assay.

For assays using 4MU-NANA as the substrate, inhibitors over 3-fold serial dilutions of concentration were incubated with enzyme at 0 ºC for 15 min. 4MU-NANA was then added to the mixture, making the final concentration of 4MU-NANA 50 μM and the total volume of the reaction mixture 20 μL. After incubation at 37 ºC for 30 min, the reaction was quenched with 100 μL of 0.2 M sodium glycine buffer (pH 10.2). The reaction mixture was transferred to a 386-well plate and the enzyme activity was determined by measuring fluorescence (λ_{ex} = 365 nm; λ_{em} = 445 nm) using a plate reader (Molecular Devices, Sunnyvale CA). Assays were performed with duplicates for each point and IC_{50} was obtained by plotting the data with Graphpad Prism 7.0. For curves that showed less than a 50% decrease in signal, fits were conducted using maximum inhibition values found for DANA.

***K*i Determinations.** Enzymes were incubated with serial concentrations of inhibitors at 0 °C for 15 min and serial concentrations of 4MU-NANA were added. The reaction mixture was transferred to 386-well plate immediately and the rate of product formation was obtained by measuring fluorescence (λ_{ex} = 315 nm; λ_{em} = 450 nm) every 1 min over 30 min. Lineweaver-Burk plots for some inhibitors indicated noncompetitive inhibition, sometimes called mixed inhibition (Figure S2).[58] Kinetic data were processed with Graphpad Prism 7.0 using a competitive or noncompetitive

inhibition models to obtain K_i and α values.[58, 59] Competitive inhibition dominates most of the inhibitors (with $\alpha > 4$), compounds that had significant noncompetitive character are noted in Table 3, and fit K_i and α values for the noncompetitive model are provided in Table S3. We note that noncompetitive or mixed inhibition has been previously observed for other sialidase enzymes.[60-62]

Neuraminidase Assay in Mouse Tissue Homogenates. Mice with a targeted disruption of the *Neu1* gene (*Neu1*$^{-/-}$) or a combined deficiency of the *Neu3* and *Neu4* genes (*Neu3*$^{-/-}$; *Neu4*$^{-/-}$) have been previously described.[44] Mice were housed in an enriched environment with continuous access to food and water, under constant temperature and humidity, and on a 12 h light:dark cycle. Approval for the animal care and use in experiments was granted by the Animal Care and Use Committee of the Ste-Justine University Hospital Research Center.

At the age of 24 weeks for the *Neu3/4* DKO mice and 8 weeks for the WT and *Neu1* KO mice, the animals were anaesthetized with isoflurane and sacrificed by cervical dislocation and their brains and kidneys extracted, snap-frozen with liquid nitrogen and kept at -80 ºC. For the measurement of neuraminidase activity, 50 mg of frozen brain tissue was homogenized in water in a ratio of 250 µL of water per 50 mg of tissue in 1.5 mL Eppendorf tubes using a Sonic Dismembrator (Artek Systems Corporation). Protein concentration in the homogenate was measured by the Bradford method using the Bio-Rad reagent. Acidic α-neuraminidase activity was assayed at pH 4.6 using fluorogenic 4MU-NANA substrate as previously described.[63] The reaction mixture contained an aliquot of homogenate corresponding to 100 µg of total protein, neuraminidase inhibitor in a concentration of 0-150 µM and substrate in a final concentration of 250 µM. The reaction was carried on at 37 °C for 60 min after which it was terminated by the

addition of 960 μL of 0.4 M glycine buffer, pH 10.4. For blank samples, the reaction mixture contained buffer, inhibitor, and substrate and the same volume of homogenate was added after termination of the reaction.

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Author Contributions

TG synthesized and characterized all compounds, conducted IC_{50} and K_i measurements and wrote the manuscript; RH-R conducted mouse ortholog experiments; RH-R and AVP designed ortholog experiments and wrote the manuscript; CZ and RZ developed and implemented protein purification protocols; CWC designed experiments and wrote the manuscript.

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ABBREVIATIONS

NEU, neuraminidase enzyme; Neu5Ac, 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid; DANA, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid; 4MU-NANA, 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid; SAR, structure-activity relationship; CuAAC, copper-catalyzed azide–alkyne cycloaddition;

ASSOCIATED CONTENT

Supporting Information. Supplementary information including protein expression protocols, HPLC traces for intermediates and final products, IC_{50} curves, K_i determinations, ^{1}H and ^{13}C NMR data, HR-MS data, and Molecular Formula Strings are provided. This material is available free of charge via the Internet at http://pubs.acs.org

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FIGURES

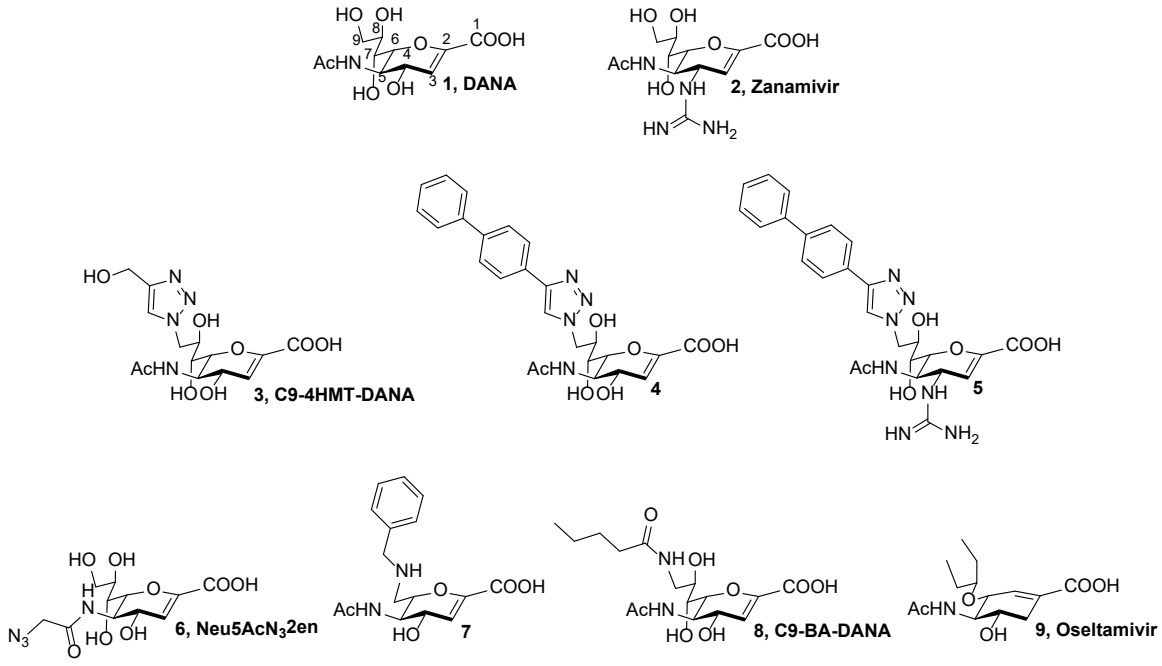


Figure 1. **Reported inhibitors for human neuraminidases.** Compounds previously reported as selective inhibitors of the human neuraminidase enzymes are shown. DANA **1** is a pan-selective inhibitor (with carbon atom numbering for the scaffold shown).[24] The antiviral NEU inhibitor, zanamivir **2**, has moderate selectivity for NEU3 and NEU2.[25] The C9-4HMT-DANA **3** has a 30 nM K_i against NEU4, and is 500-fold selective.[29] The C9-biphenyltriazole compound **4** inhibits both NEU3 and NEU4 at sub-micromolar potency, while compound **5** is selective for NEU3.[30] The azidoacetyl derivative **6** has potency for NEU2.[32, 33] Compound **7** is reported to have selectivity for NEU3.[34] The C9-pentylamido derivative of DANA **8** has been reported to have selective inhibition of NEU1 at micromolar potency.[28] The antiviral NEU inhibitor oseltamivir **9** (shown as the carboxylate form), has poor in vitro potency against human NEU.[25, 26]

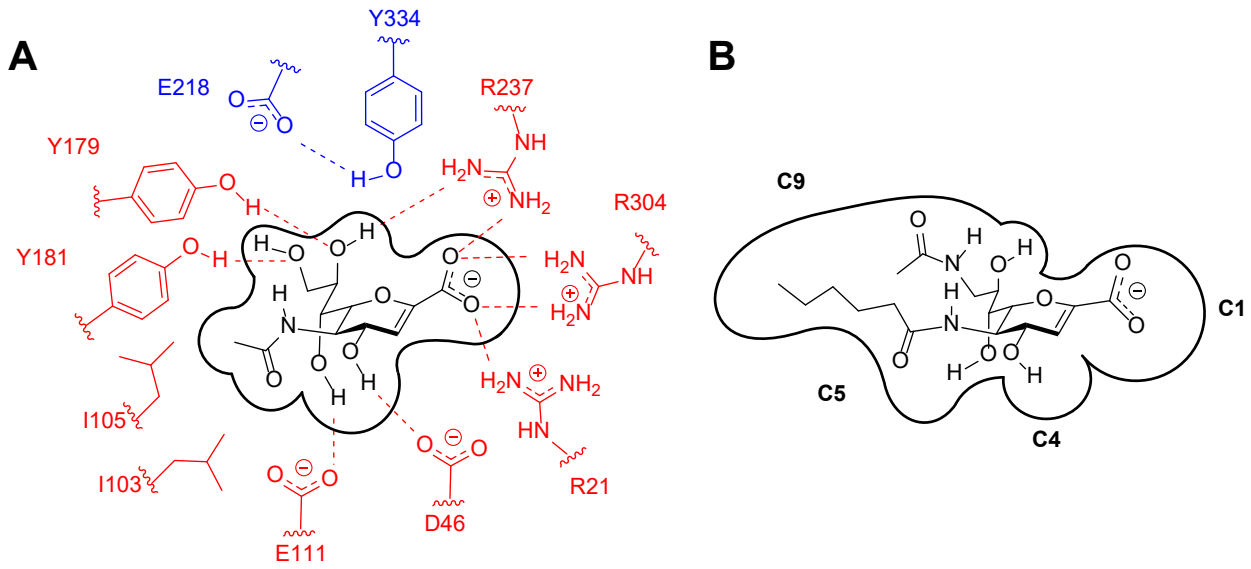


Figure 2. Schematic illustration of the binding sites of hNEU. A. Contacts between DANA and the binding site of NEU2 are summarized, with presumed H bond interactions. Residues shown are based on the co-crystal structure previously reported (PDB ID: 1VCU).[64] B. A model of the NEU1 binding site with **17f**. We propose that the proximity of the C5 and C9 sites allows crossover of large groups attached at either position, and the C5 and C9 pockets act as a single large pocket, in contrast to other hNEU active sites.

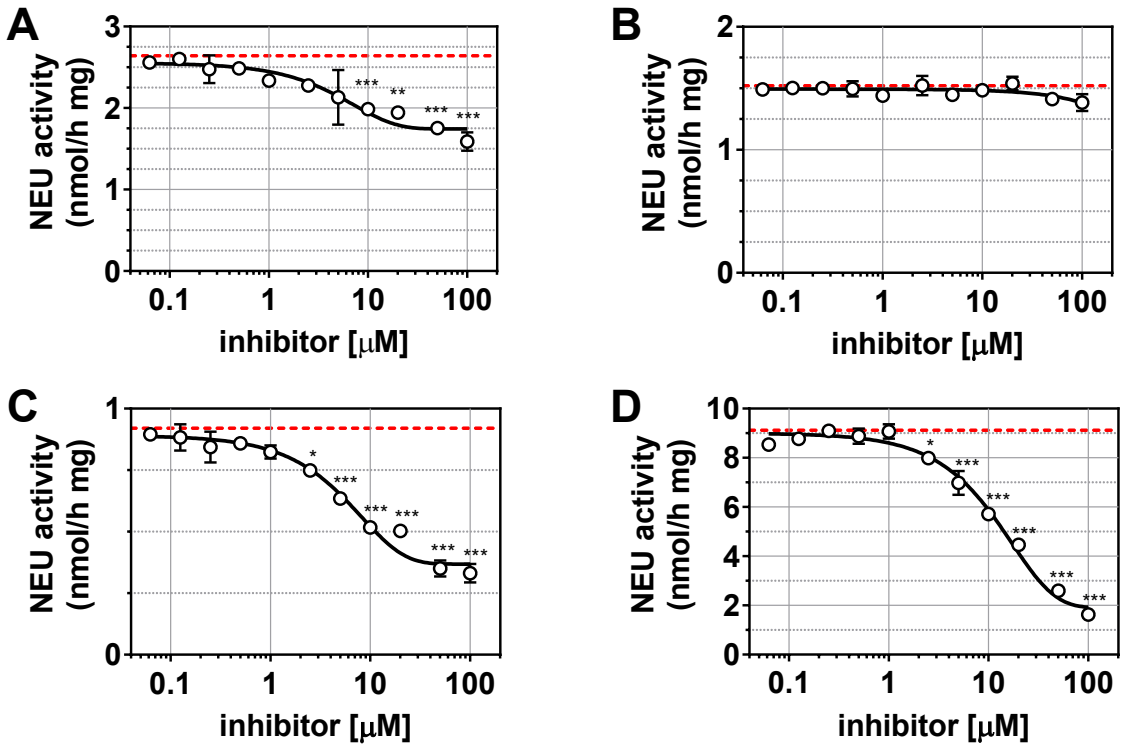
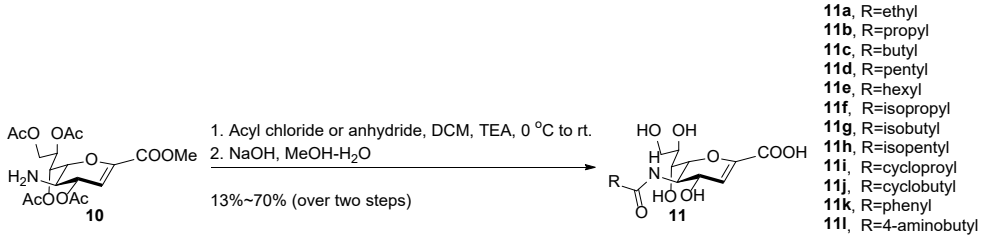


Figure 3. Inhibition of NEU activity in homogenates of murine tissues by 17f. Brains and kidneys were extracted from WT C57Bl6, *Neu1* KO (*Neu1*$^{-/-}$), or *Neu3/4* double KO (DKO) mice (*Neu3*$^{-/-}$, *Neu4*$^{-/-}$). The homogenates were assayed for inhibition of neuraminidase activity by compound **17f** against 4MU-NANA. The WT brain homogenate (**A**) showed partial inhibition (up to 40% at 100 μM) of neuraminidase activity, while the *Neu1* KO brain homogenate (**B**) showed no inhibition. The *Neu3/4* DKO brain homogenate (**C**) and WT kidney homogenate (**D**), which express only *Neu1*, showed dramatic inhibition. All homogenates showed a complete inhibition of neuraminidase activity at 150 μM of DANA (data not shown). Points are labeled as significantly different (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$) from activity in the absence of the inhibitors

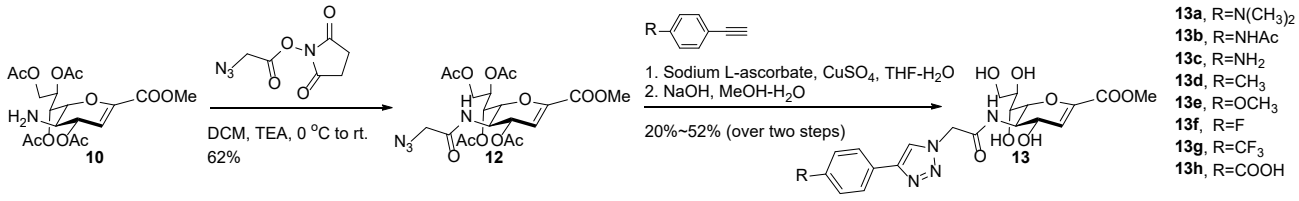
(dotted line) according to a t-test with a Dunlett post-test. Data are shown as mean values ± SD of 4 independent experiments.

SCHEMES

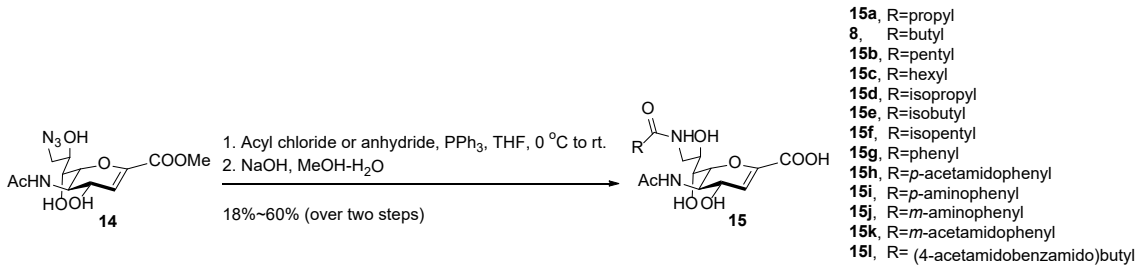
Scheme 1. Synthetic route to C5-modified DANA analogues **11a-l**



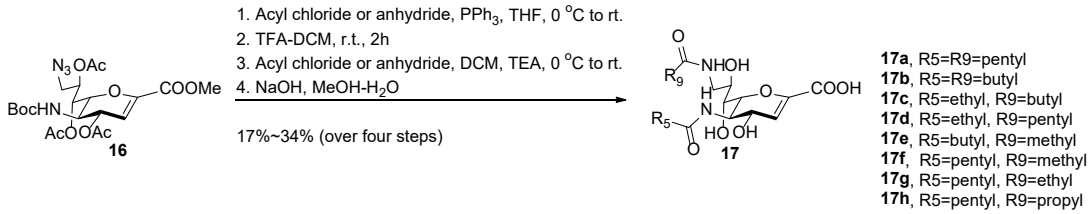
Scheme 2. Synthetic route to C5-modified DANA analogues **13a-h**



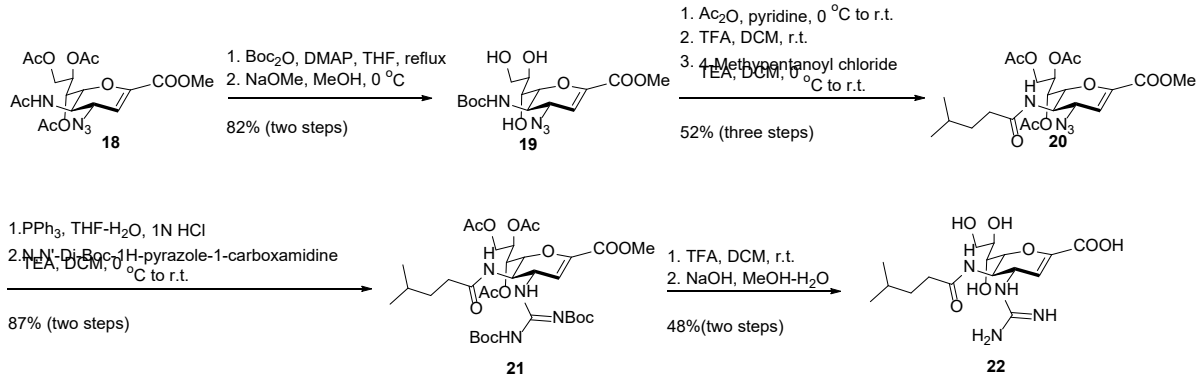
Scheme 3. Synthetic route to C9-modified DANA analogues **15a-l**



Scheme 4. Synthetic route to C5, C9-modified DANA analogues **16a-f**

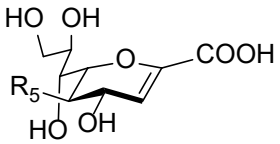


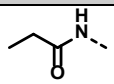
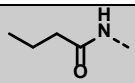
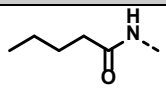
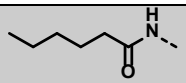
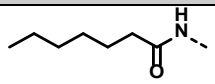
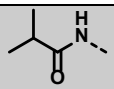
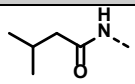
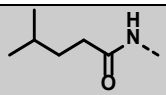
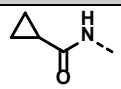
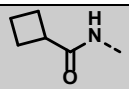
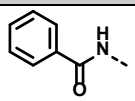
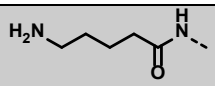
Scheme 5. Synthetic route to C4, C5-modified DANA analogue **22**



TABLES

Table 1. IC_{50} results for C5-modified DANA analogues



Structures		IC_{50} [μM]			
Compd.	R5	NEU1	NEU2	NEU3	NEU4
DANA, 1		49 ± 8	37 ± 6	7.7 ± 0.8	8.3 ± 1.0
11a		18 ± 1	86 ± 4	60 ± 7	87 ± 18
11b		8.4 ± 0.5	40 ± 5	15 ± 2	8.4 ± 0.4
11c		0.99 ± 0.07	33 ± 2	140 ± 10	110 ± 20
11d		0.42 ± 0.06	15 ± 2	210 ± 60	440 ± 150
11e		2.1 ± 0.2	37 ± 6	210 ± 70	470 ± 200
11f		5.3 ± 0.8	170 ± 50	>500	71 ± 17
11g		32 ± 5	39 ± 10	>500	>500
11h		1.7 ± 0.1	7.3 ± 1.2	150 ± 20	370 ± 160
11i		6.9 ± 0.4	220 ± 10	170 ± 40	150 ± 40
11j		12 ± 2	60 ± 3	100 ± 20	81 ± 15
11k		11 ± 1	41 ± 12	480 ± 100	>500
11l		480 ± 260	>500	>500	>500

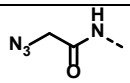
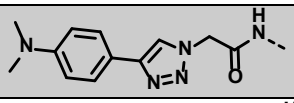
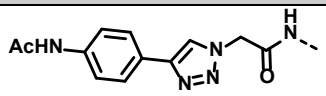
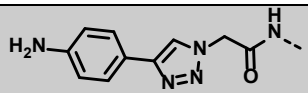
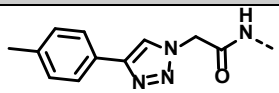
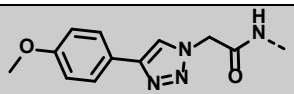
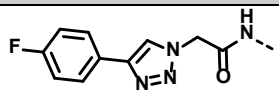
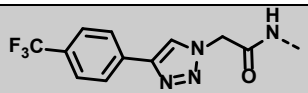
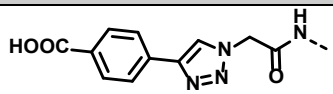
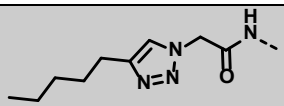
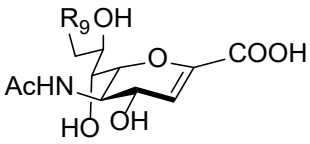
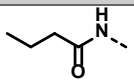
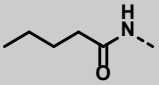
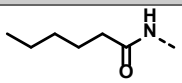
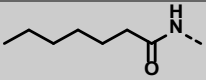
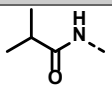
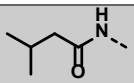
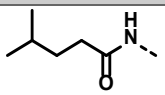
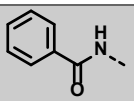
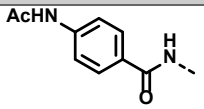
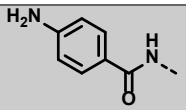
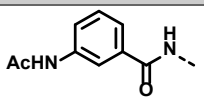
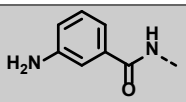
Neu5AcN$_3$2en (6)		24 ± 2	22 ± 3	4.4 ± 0.9	5.6 ± 0.8
13a		110 ± 10	76 ± 6	46 ± 7	36 ± 5
13b		>500	17 ± 3	>500	>500
13c		>500	4.4 ± 0.3	430 ± 100	130 ±20
13d		>500	3.3 ± 0.3	>500	110 ± 20
13e		>500	11 ± 1	>500	87 ± 14
13f		>500	4.5 ± 0.3	240 ± 40	100 ± 20
13g		>500	50 ± 7	>500	300 ± 60
13h		>500	180 ± 30	>500	400 ± 160
13i		>500	78 ± 14	>500	>500

Table 2. IC_{50} results for C9-modified DANA analogues



Structures		IC_{50} [μM]			
Compd.	R9	NEU1	NEU2	NEU3	NEU4
DANA (1)	HO--	49 ± 8	37 ± 6	7.7 ± 0.8	8.3 ± 1.0
15a		4.0 ± 0.5	>500	250 ± 90	74 ± 14
C9-BA-DANA (8)		3.4 ± 0.2	>500	110 ± 40	220 ± 50
15b		2.9 ± 0.2	>500	83 ± 9	290 ± 30
15c		9.9 ± 1.3	410 ± 110	39 ± 8	310 ± 30
15d		250 ± 60	220 ± 30	96 ± 28	230 ± 60
15e		2.5 ± 0.3	120 ± 20	72 ± 20	130 ± 40
15f		3.2 ± 0.3	160 ± 40	54 ± 6	150 ± 50
15g		2.5 ± 0.3	>500	34 ± 5	150 ± 10
15h		1.9 ± 0.4	90 ± 20	7.2 ± 1.2	24 ± 6
15i		29 ± 5	190 ± 70	31 ± 6	210 ± 80
15j		7.5 ± 1	130 ± 10	7.1 ± 0.7	52 ± 9
15k		6.5 ± 0.7	180 ± 40	35 ± 6	150 ± 40

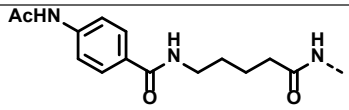
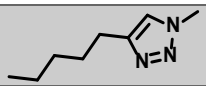
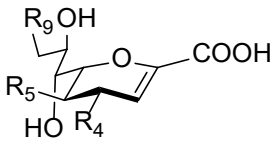
15l		240 ± 70	390 ± 80	31 ± 5	46 ± 13
15m		77 ± 30	450 ± 170	6.7 ± 1.2	2.6 ± 0.6

Table 3. IC_{50} results for DANA analogues with combined modifications



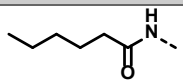
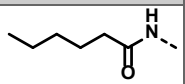
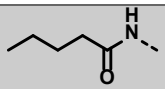
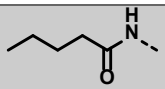
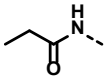
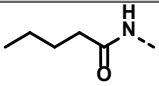
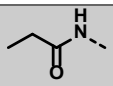
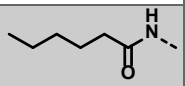
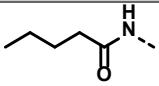
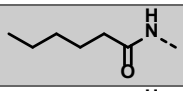
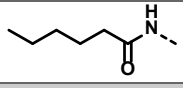
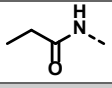
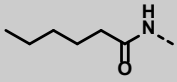
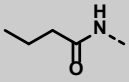
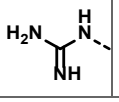
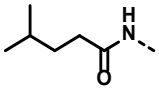
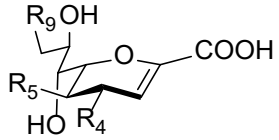
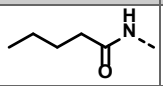
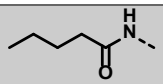
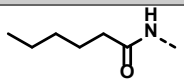
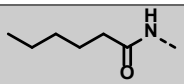
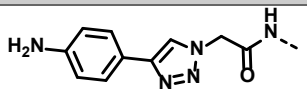
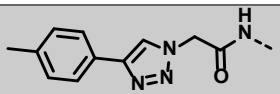
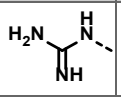
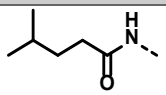
Structures				IC_{50} [μM]			
Compd.	R4	R5	R9	NEU1	NEU2	NEU3	NEU4
DANA	HO--		HO--	49 ± 8	37 ± 6	7.7 ± 0.8	8.3 ± 1.0
17a	HO--			4.3 ± 0.8	26 ± 6	>500	>500
17b	HO--			1.5 ± 0.2	59 ± 26	>500	>500
17c	HO--			1.6 ± 0.2	140 ± 30	>500	190 ± 70
17d	HO--			1.4 ± 0.2	31 ± 8	260 ± 70	210 ± 90
17e	HO--			0.35 ± 0.03	170 ± 70	>500	>500
17f	HO--			0.14 ± 0.01	47 ± 14	>500	170 ± 100
17g	HO--			0.40 ± 0.10	90 ± 10	>500	270 ± 70
17h	HO--			1.2 ± 0.1	32 ± 4	>500	>500
22			HO--	14 ± 2	2.1± 0.1	150 ± 20	47 ± 7

Table 4. K_i of NEU1 and NEU2 inhibitors



Structures				K_i [μM][a]		
Compd.	R4	R5	R9	NEU1	NEU2	Targets
DANA	HO--		HO--	12 ± 1	25 ± 4 [b]	NEU1-4
8	HO--			0.83 ± 0.15 [b]	ND	NEU1
11c	HO--		HO--	0.24 ± 0.03	ND	NEU1
11d	HO--		HO--	0.18 ± 0.02	ND	NEU1
17f	HO--			0.053 ± 0.005	ND	NEU1
13c	HO--		HO--	ND	2.1 ± 0.6 [b]	NEU2
13d	HO--		HO--	ND	2.7 ± 0.6 [b]	NEU2
22			HO--	ND	1.3 ± 0.2	NEU2

[a] Values obtained from analysis using a competitive inhibition model.

[b] Indicated compounds exhibited noncompetitive inhibition with $\alpha \leq 1$, see Table S3.

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