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Targeting GNE myopathy: A dual prodrug approach for the delivery of *N*-acetylmannosamine 6-phosphate.

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Abstract

ProTides comprise an important class of prodrugs currently marketed and developed as antiviral and anticancer therapies. The ProTide technology employs a phosphate masking groups capable of providing more favorable drug-like properties and an intracellular activation mechanism for enzyme-mediated release of a nucleoside monophosphate. Herein we describe the application of phosphoramidate chemistry to 1,3,4-*O*-acetylated *N*-acetylmannosamine (Ac_3ManNAc) to deliver ManNAc-6-phosphate (ManNAc-6-P), a critical intermediate in sialic acid biosynthesis. Sialic acid deficiency is a hallmark of GNE myopathy, a rare congenital disorder of glycosylation (CDG) caused by mutations in *GNE* that limit the production of ManNAc-6-P. Synthetic methods were developed to provide a library of Ac_3ManNAc-6-phosphoramidates that were evaluated in a series of studies for their potential as a treatment for GNE myopathy. Prodrug **12b** showed rapid activation in a carboxylesterase (CPY) enzymatic assay and favorable ADME properties, while also being more effective than ManNAc at increasing sialic acid levels in GNE-deficient cell lines than ManNAc. These

results provide a potential platform to address substrate deficiencies in GNE myopathy and other CDGs.

Introduction

Congenital Disorders of Glycosylation (CDGs), first described as Carbohydrate Deficient Glycoprotein Syndrome by Jaeken et al. in 1980[1], are a group of rare, inherited, metabolic diseases characterized by defects in protein or lipid glycosylation. CDGs primarily affect children and can vary in clinical presentation from disabling to life-threatening conditions. Disease manifestations typically impact multiple organ systems, including liver and neurological involvement. Over 100 unique CDGs have been identified to date and the number continues to increase due to advances in the availability and efficiency of diagnostic techniques, namely genome sequencing.[2, 3]

Among the CDGs, Hereditary Inclusion Body Myopathy (HIBM), also known as GNE myopathy (GNEM), is an autosomal recessive neuromuscular disorder characterized by symptoms that typically appear during the third decade of life, starting with skeletal muscle atrophy and weakness that slowly progresses to a significant physical disability. GNEM is caused by biallelic mutations in the *GNE* gene resulting in loss-of-function of uridine 5'-diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) 2-epimerase and *N*-acetylmannosamine (ManNAc) kinase (GNE),[4] a key enzyme in sialic acid biosynthesis (Figure 1).[5] Sialic acids decorate cell surfaces as terminal component of glycoprotein and glycolipids,[6] where they mediate or modulate a variety of physiological and pathological processes like signal transduction, cell adhesion, system embryogenesis, cancer metastasis or viral and bacterial infections.[7] GNE is a bifunctional enzyme capable of catalyzing epimerization of UDP-GlcNAc at the 2-position of the hexose ring to afford ManNAc and subsequent phosphorylation at the 6-position to afford ManNAc-6P (Figure 1). As the rate-limiting step in sialic acid biosynthesis, GNE deficiency results in decreased sialic acid production and consequent hyposialylation of glycoproteins, particularly in muscle tissue. The prevalence of GNEM is estimated to be roughly 1 to 9 in 1,000,000 people. More than 140 mutations in *GNE* have been

associated with GNE myopathy and can compromise the enzymatic activity of either catalytic domain.[8] Each domain is functional when expressed separately, but evolution of some intramolecular dependency is exemplified by mutations causing reductions in both enzyme activities to different extents in a manner independent of domain localization.[9]

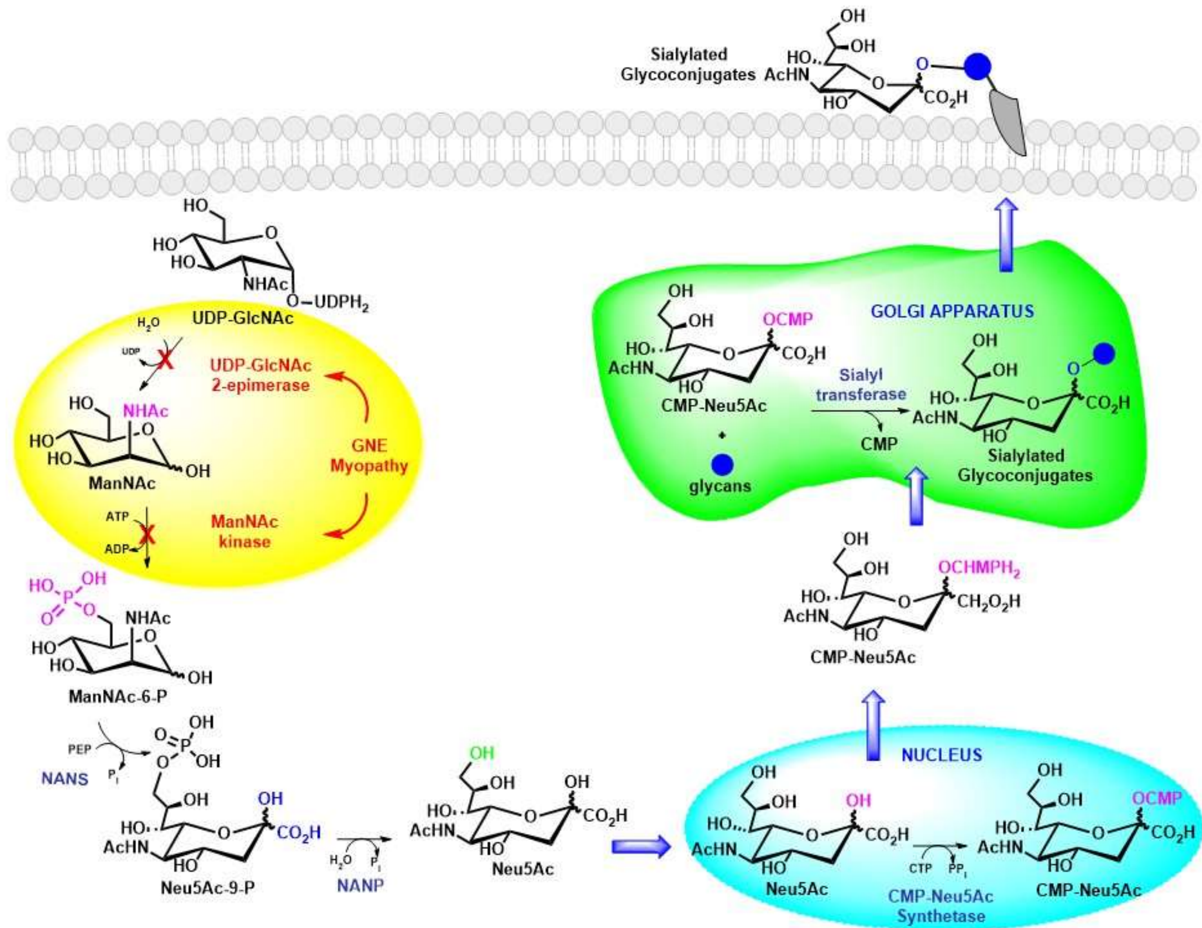


Figure 1. Sialic acid biosynthetic cascade: the point of mutation of UDP-GlcNAc 2-epimerase/ManNAc kinase, responsible for the GNE myopathy, is highlighted in yellow.

To date, there is no approved therapy for GNEM.[10] Palliative treatments, such as physiotherapy, analgesic drugs, mobility devices and psychological support are available to alleviate patient symptoms and improve quality of life. Therapeutic approaches have focused on substrate replacement paradigms seeking to re-establish available pools of intracellular

sialic acid by supplementation of aceneuramic acid (Neu5Ac) or its precursor, ManNAc.[11] Both compounds have proven capable of restoring sialic acid biosynthesis and preventing or arresting disease progression in mouse models of GNEM.[12] ManNAc is currently in late-stage clinical trials to assess its safety and efficacy in patients with GNE myopathy,[13, 14] whereas aceneuramic acid, although safe and well-tolerated, failed to demonstrate efficacy in a Phase 3 double-blind, placebo-controlled trial and was subsequently discontinued.[15] Intravenous administration of immunoglobulin (IVIG) and gene therapy are other therapeutic options. Unfortunately, IVIG, although containing eight micromoles of sialic acid per gram, was not capable of improving muscle sialylation in GNE myopathy patients.[10] Correction of GNE function through liposomal delivery of a wild-type *GNE* gene demonstrated increased production of sialic acid *in vitro* and *in vivo*. However, data supporting the continued stabilization of decline in muscle strength in a single patient are limited and no follow-up studies have been reported thus far.[16]

Although ManNAc presents an intriguing therapeutic approach, poor intracellular transport and feedback inhibition necessitates high daily dosage (from 3g to 12g).[13,17] No dose-response correlation was observed after repeated administration in patients with GNEM. Furthermore, patients with loss-of-function mutations in the ManNAc kinase domain of GNE may not be capable of phosphorylating ManNAc, thereby limiting restoration of sialic acid production.

A study conducted in a GNE myopathy mouse model demonstrated that the lipophilic ManNAc analog *O*-tetra-acetylated *N*-acetylmannosamine (Ac_4ManNAc) was more effective at increasing sialylation and preventing the myopathic phenotype when compared to natural sialic acid metabolites after oral administration.[18] Ac_4ManNAc is a prodrug of ManNAc and can more readily cross cellular membranes via passive diffusion, providing evidence that synthetic sugar analogs may be a practical consideration for the design of therapeutic agents for GNE myopathy. However, Ac_4ManNAc still requires phosphorylation upon release of ManNAc. To

overcome the deficiency in both UDP-GlcNAc-2-epimerase and ManNAc kinase enzyme activity observed in GNE myopathy, administration of ManAc-6-P could be envisaged as an ideal substrate replacement strategy. Due to the high polarity of the phosphate moiety, a prodrug approach would be required to overcome poor cell permeability and mitigate de-phosphorylation by extracellular phosphatases.

Prodrug approaches for delivering phosphate carbohydrate analogs have been reported previously. Meyer *et al* designed *cyclo*saligenyl prodrugs of *O*-tetra-acetylated mannose-1-phosphate[19] whereas Eklund with co-workers synthesized two mannose-1-acetoxymethylated phosphates.[20] Both classes of phosphate prodrugs were designed for the treatment of phosphomannomutase 2 (PMM2) deficiency, characteristic of the PMM2-CDG condition also known as CDG-Ia. Despite encouraging results demonstrating intracellular delivery of the mannose-1-monophosphate capable of correcting the CDG-Ia phenotype *in vitro*, these approaches were not explored further, most likely due to toxicity and stability concerns. To date, no additional studies have been reported.

Our laboratory was previously successful in applying phosphoramidate chemistry to *N*-acetyl glucosamine for the treatment of osteoarthritis[21, 22] and to various analogs of deoxyribose-1-phosphate as potential anti-HIV agents.[23] The *N*-acetylglucosamine phosphoramidate prodrugs generated in these studies showed acceptable biological stability and prodrug activation in chondrocyte cell lysate. In particular, the (*L*)-proline amino acid-containing *O*-3/*O*-4 phosphoramidates demonstrated good activity at non-cytotoxic concentrations in human cartilage *ex vivo*, exceeding that of *N*-acetylglucosamine and emerging as promising candidate for further development for the treatment of osteoarthritis and other musculoskeletal diseases.

Among many existing phosphate prodrug strategies,[24] the ProTide approach has been successfully applied to nucleosides[25, 26] as evidenced by the development and approval of the currently marketed drugs Sofosbuvir[27] and Tenofovir Alafenamide (TAF)[28] (Figure 2). In

addition, in collaboration with our laboratories, NuCana plc is currently pioneering the ProTide technology in oncology, with the clinical candidates NUC-1031 (Acelarin),[29] and NUC-3373,[30] respectively in Phase III and Phase I studies for patients with advanced solid tumors (Figure 2). The phosphoramidate technology more recently was also exploited for the delivery of non-nucleoside derivatives.[31]

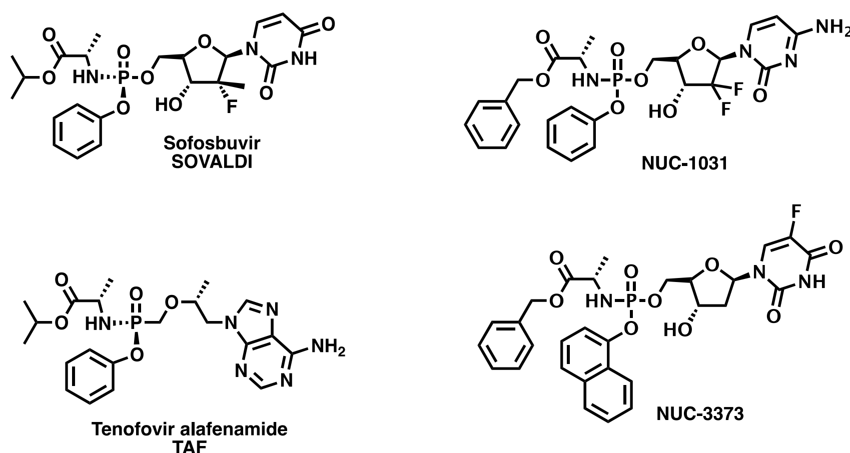


Figure 2. FDA-approved antiviral ProTides, Sofosbuvir and TAF and anticancer clinical candidates ProTides, NUC-1031 and NUC-3373.

Given the importance of phosphoramidate prodrug technology in recent drug discovery, we revived our interest in its application toward the delivery of ManNAc-6-P as a novel approach to the treatment of GNE myopathy. Rationally designed ManNAc phosphoramidate prodrugs could enable the bypass of deficient GNE enzyme observed in GNEM patients by directly providing a source of intracellular ManNAc-6-P. Our approach sought to optimize drug-like properties of ManNAc phosphoramidates to improve upon the low permeability and plasma stability of ManNAc-6-P. In this work, we report the design, synthesis and evaluation of a family of ManNAc-6-P and Ac_4ManNAc-6-P ProTide analogues, including ADME assays, enzymatic activation studies and *in vitro* assessment of sialic acid production in cell-based models of GNE myopathy. These data serve as proof-of-concept that the ProTide platform is a practical approach to substrate replacement therapies for GNE myopathy and potentially for

1 other CDGs characterized by deficiencies in phosphorylated metabolites.

2 RESULTS AND DISCUSSION

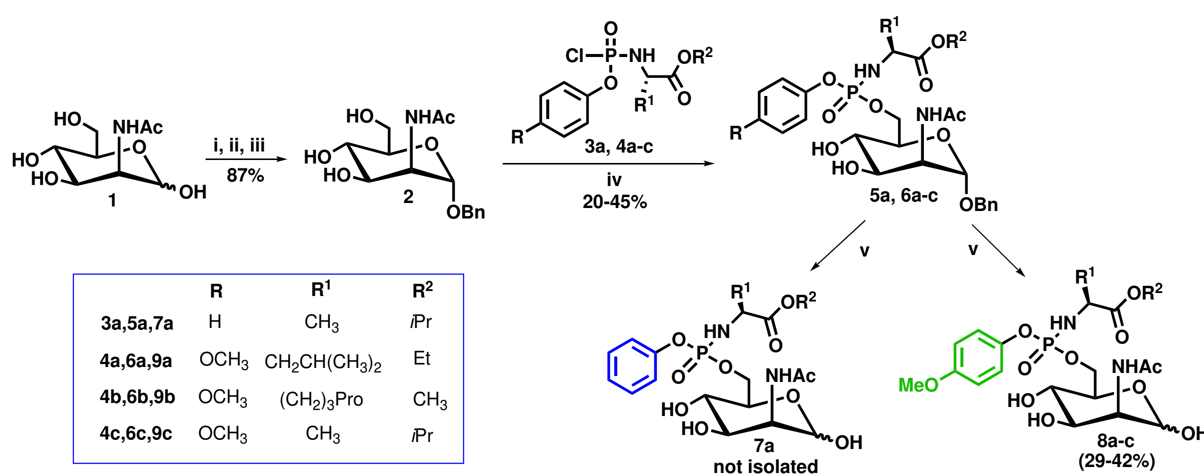
3 Chemistry

4 Our synthetic efforts began with the synthesis of ManNAc-6-phosphoramidates. We initially
5 envisaged the application of a synthetic procedure previously reported by our group for the
6 preparation of glucosamine phosphoroamidates as feasible approach.[21] Surprisingly, when
7 ManNAc (**1**) was coupled with different phosphorochloridates in the presence of *N*-methyl
8 imidazole (NMI),[32] the formation of a complex mixture of by-products was always observed,
9 from which no desired compound could be isolated.

10 We then chose to explore an alternative synthetic route, involving protection of the anomeric
11 hydroxyl prior to attempting the coupling reaction. The benzyl group was selected as the
12 protecting group given its removal via hydrogenation is highly compatible with the
13 phosphoramidate motif, as evidenced by a similar procedure employed by our group in the
14 synthesis of *N*-acetylglucosamine phosphoramidate derivatives.[22]

15 ManNAc (**1**) was fully acetylated (acetic anhydride in pyridine), and selectively benzylated at
16 the anomeric position with benzyl alcohol and a catalytic amount of BF_3OEt_2. Subsequent *O*-
17 deprotection of the acetylated alcohols gave compound **2** as exclusively the α-anomer in 87%
18 yield over three steps (Scheme 1).[33] Pleasingly, the coupling of phosphorochloridates (**3a, 4a-**
19 **c**) with compound **2** was efficient, affording exclusively *O*-6 phosphoramidate derivatives **5a**
20 and **6a-c** (as a mixture of S_p and R_p diastereoisomers) with the same *O*-6 regioselectivity as
21 previously observed for the *N*-acetyl glucosamine series.[22] Finally, hydrogenation (10% Pd/C
22 and H_2) of compound **5a** in methanol proceeded smoothly and after 12 hours both MS and ^1H-
23 NMR analysis of the crude reaction mixture revealed the presence of the desired product with
24 concomitant disappearance of the starting material. Disappointingly, attempts to isolate pure
25 compound **7a** via traditional purification techniques (silica gel chromatography and/or reverse

phase HPLC) were unsuccessful due to partial degradation of the compound. When repeating the synthesis with phosphorochloridates bearing different promoieties, purification following hydrogenation consistently led to the observation of partial degradation and hindered isolation of the desired product. Surprised by these results, we decided to further investigate the degradation pathway via mass spectrometry. LC-MS analysis of **7a** following purification indicated the presence of the desired compound (*m/z* 490) and a side product showing a peak at *m/z* 419 $[M+Na]^+$ consistent with structure **IV** (Figure 3). Lack of UV absorbance in the LC-MS traces for this peak and evidence of phenol release observed in the ^{1}H-NMR spectra of the purified material further supported this hypothesis. LC-MS analysis of other phosphoramidates, whose purification was attempted after hydrogenation, led to similar results that indicated the formation of compound **IV**-like structures (*m/z* values were in agreement with the amino acid ester present in the parent compound). In addition, ^{31}P-NMR of these mixtures showed two peaks at ~9 ppm, indicative of a chiral phosphorodiamidate as in compound **IV** (Figure 3).



	R	R¹	R²
3a,5a,7a	H	CH_3	*i*Pr
4a,6a,9a	OCH_3	$CH_2CH(CH_3)_2$	Et
4b,6b,9b	OCH_3	$(CH_2)_3$Pro	CH_3
4c,6c,9c	OCH_3	CH_3	*i*Pr

Scheme 1. Phosphoramidate synthesis through 1'-*O*-Bn-*N*-acetylmannosamine. [a]Reagents and conditions: (i) Ac_2O, DMAP, Py, rt, 12h; (ii) BnOH, BF_3OEt_2 cat., ACN, 80 °C, 3h; (iii) NaOMe cat. MeOH, rt, 5h; (iv) NMI, THF/pyridine 4/2 v/v, rt, 12h; (v) H_2, 10% Pd/C, MeOH,

1 rt, 12h.

2 A plausible mechanism for the formation of by-product **IV** is proposed in Figure 3.
3 Deprotection of the benzyl group at the anomeric position leads to the formation of both α- (**I**)
4 and β-anomers (**III**) in equilibrium with an open-chain form (**II**). Since the 1-*O*-benzyl
5 phosphoramidates were formed exclusively as α anomers and proved to be stable, we then
6 hypothesized that only the β anomer of **II** could participate in the degradation pathway. The
7 phosphorus atom of the β anomer could be particularly susceptible to intramolecular
8 nucleophilic attack by the nitrogen atom of the sugar, leading to the corresponding cyclic
9 adduct **IV**. Interestingly, this side reaction was not previously observed with any of the *N*-
10 acetyl-glucosamine phosphoroamidate derivatives.[22] The difference in stereochemistry at the
11 2'-position between GlcNAc and ManNAc phosphoramidates may be responsible for the
12 divergence in reactivity toward the intramolecular cyclization process, further supporting the
13 proposed mechanism. This could also justify the difficulty in obtaining the ManNAc
14 phosphoramidate series from *O*-unprotected ManNAc in our earlier attempts. Although it
15 would appear reasonable to assume that the phosphorous atom of the more flexible open chain
16 structure **II** could undergo the same intramolecular nucleophilic attack by the nitrogen atom,
17 we have ruled out this possibility given such degradation was not observed with *N*-acetyl
18 glucosamine derivatives.[22] Further studies to confirm this proposed mechanism are in progress.

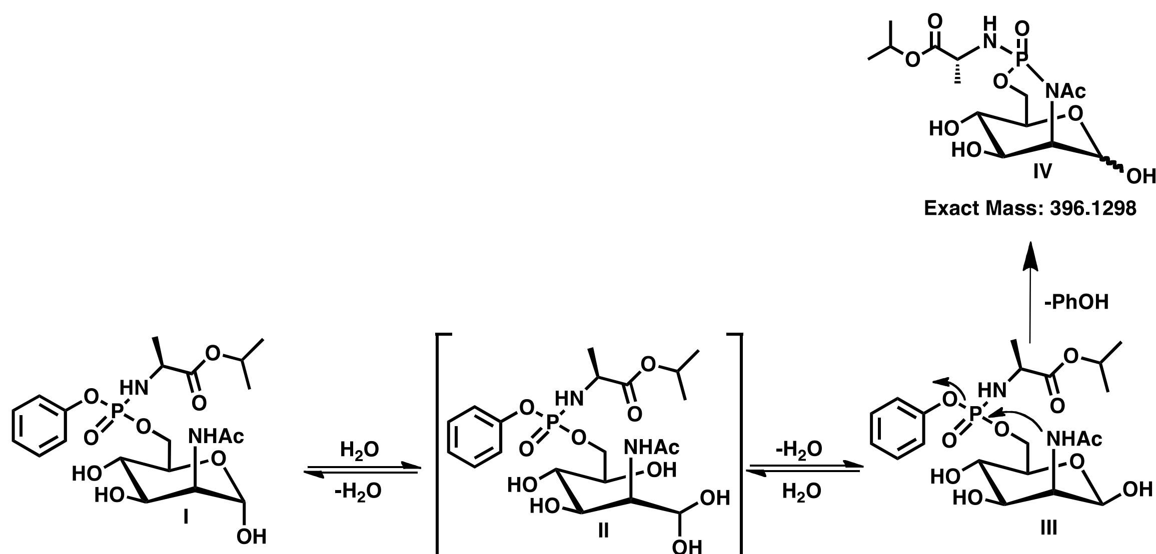
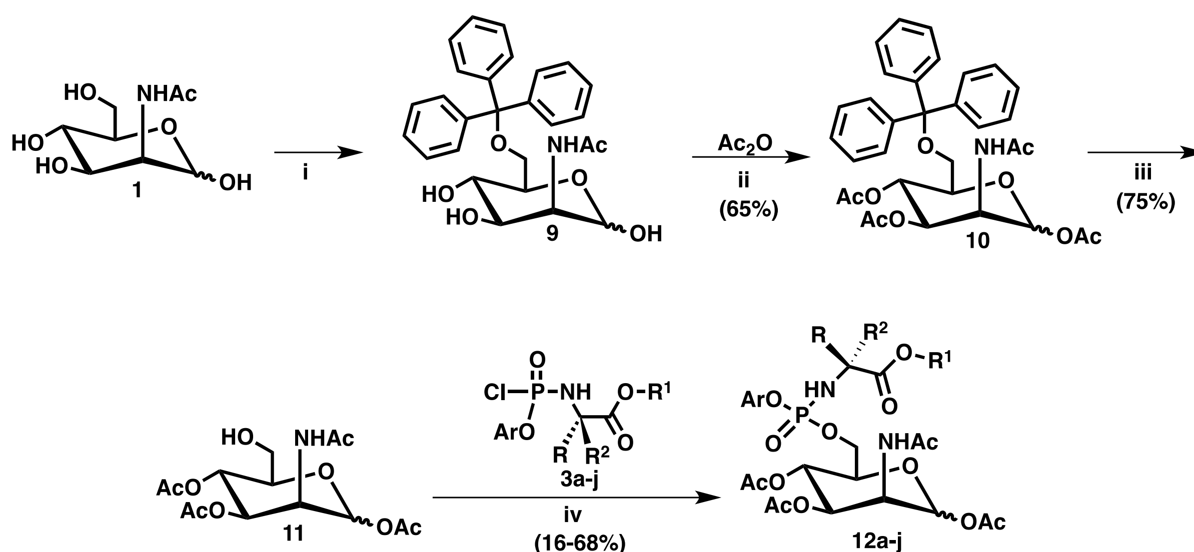


Figure 3. Proposed mechanism for the formation of the side product **IV** during the purification step.

Efforts to block the attack of the nucleophilic nitrogen atom onto the phosphorus center by introducing hindered amino acid esters in conjunction with bulkier aryloxy moieties failed and degradation was still observed. We then sought to stabilize the phosphoroamidate moiety by introducing a *para*-methoxy functionality within the aryl group, thereby decreasing the electrophilicity of the phosphorous atom and making it less susceptible to nucleophilic attack. This resulted in a decreased rate of by-product formation and enabled isolation of the desired products (**8a-c**) (Scheme 1). However, these compounds were not pursued further as possible candidates due to unsatisfactory ADME properties, namely poor cell permeability due to the presence of several free hydroxyl groups.

Given these results, we turned our attention to the preparation of 1,3,4-*O*-triacetylated ManNAc-6-phosphoramidates, which we envisaged as targets with more appropriate drug-like properties. The acetyl protecting groups would further increase the lipophilicity of the compounds while allowing for the possibility of *in vivo* cleavage by plasma esterases following absorption from the gut, thus acting as a dual prodrug. Additionally, 1-*O*-acetylation could stabilize the prodrug structure and prevent the cyclization side-reaction. 1,3,4-*O*-triacetylated

ManNAc (**11**) was prepared according to a literature procedure[34] outlined in Scheme 2. ManNAc (**1**) was tritylated, peracetylated and selectively deprotected at the *O*-6 position to yield compound **11** as a mixture of α and β anomers (1:0.3), which was then coupled with phosphorochloridates **3a-j** in the presence of Grignard reagent[32] to afford compounds **12a-j** as a mixture of S_p and R_p diastereoisomers of α and β anomers. To avoid the formation of regioisomeric phosphoramidates arising from acetyl migration,[35, 36] low reaction temperature was required during phosphoramidate formation.



Scheme 2. Synthetic strategy for the preparation of 1,3,4-*O*-triacetylated ManNAc phosphoramidates. Reagents and conditions: (i) Pyridine, rt, 12h and 60 °C, 1h; (ii) Pyridine, 0 °C to rt, 12h; (iii) glacial acetic acid, 60 °C, 2h; (iv) 1M *t*-BuMgCl, THF, 0 °C to rt, 12h.

A small, rationally designed library of Ac_3ManNAc phosphoramidates was built based on predicted log P values and existing knowledge of prodrug activation kinetics, using different amino acids and ester moieties to sample a diverse set of analogs (Table 1). Furthermore, since our previous report[22] demonstrated that proline-based ProTides of N-acetyl glucosamine were particularly effective in cell-based models of osteoarthritis, we prepared the proline derivative **12c**.

Compound ID	**Ar**	**R**	**R^1**	**R^2**	**log P[a]**	**Yield[b] (%)**
12a	Ph	CH_3	*i*Pr	H	1.32	60
12b	Ph	$CH_2CH(CH_3)_2$	Et	H	1.94	16
12c	Ph	$(CH_2)_3$Pro	Met	H	0.92	19
12d	Naph	H	Met	H	1.30	49
12e	Ph	CH_2Ph	Et	H	2.28	45
12f	Naph	CH_3	Bn	H	2.95	47
12g	Ph	$CH(CH_3)_2$	Bn	H	2.64	39
12h	Ph	$CH_3CH_2CHCH_3$	Et	H	1.95	34
12i	Ph	CH_3	Bn	CH_3	2.38	64
12j	Ph	$CH_2CH(CH_3)_2$	Bn	H	3.04	61

1 **Table 2.** Library of Ac_3ManNAc phosphoramidate prodrugs. [a] Predicted log P calculated using
2 SwissADME webservice.[37] [b] Yield of the isolated compounds.

3 Carboxypeptidase Activation Studies

4 The ProTide technology has proven to be a valuable strategy capable of delivering membrane-
5 permeable nucleotide analogs intracellularly. Once across the cell membrane via passive
6 diffusion, ProTides have been shown to undergo a series of enzymatic reactions leading to the
7 release of the corresponding monophosphate analog. ManNAc phosphoramidates are believed
8 to follow a similar activation sequence as depicted in Figure 4.

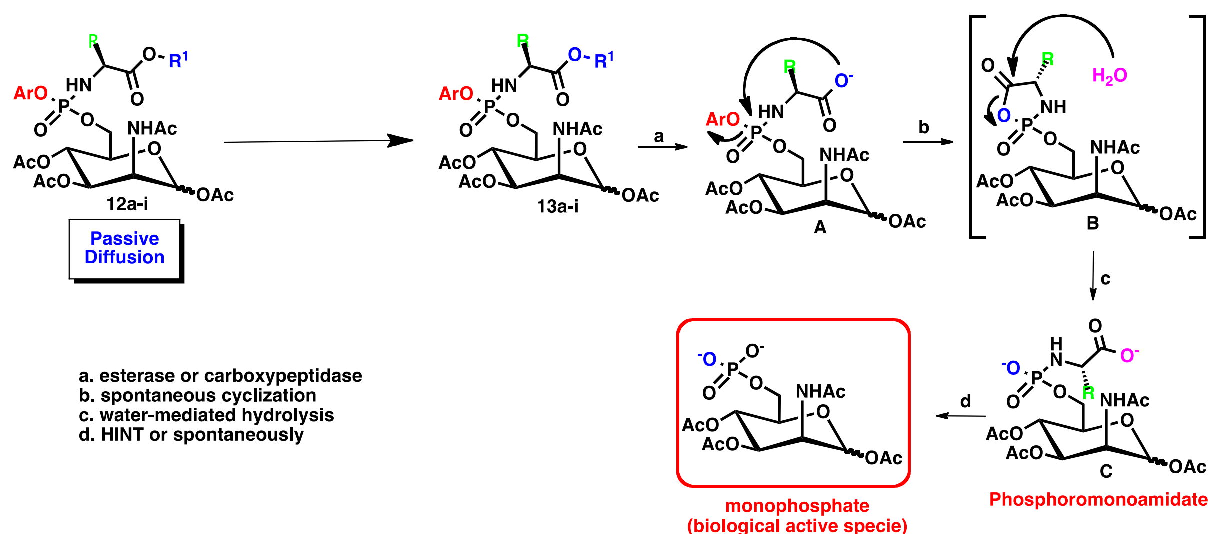


Figure 4. Proposed enzymatic activation mechanism for Ac_3ManNAc phosphoramidates: a) carboxylesterase or carboxypeptidase catalyzed ester hydrolysis to metabolite **A**. Intramolecular nucleophilic displacement of the aryloxy group provide the cyclic anhydride intermediate **B**. c) water-mediated hydrolysis of **B** to phosphoromonoamidate **C**. d) P-N bond cleavage mediated by HINT enzyme or by spontaneous hydrolysis.

To assess whether a carboxypeptidase-mediated activation mechanism to phosphoromonoamidate **C** is also operative with ManNAc phosphoramidates, compounds **12a-i** (Scheme 2 and Table 2) were subjected to enzymatic studies by incubating each compound with carboxypeptidase Y (CPY, from Baker's yeast) in 0.05 M Trizma buffer (pH = 7.6) in deuterated acetone, which allowed monitoring of reaction progress by ^{31}P-NMR and LC-MS analyses. Almost all compounds showed activation of the promoiety with less than 50% of the parent phosphoramidates remaining 15 minutes following the addition of CPY. The only exception was **12c,** which showed no activation (up to 24 hours). Figure 5 displays the ^{31}P-NMR spectra for the enzymatic activation assays of compounds **12b** and **12c**. Although the exact reaction sequence cannot be established from these data alone (and, consequently, the prodrug half-life), we can assert that compound **12b** demonstrates rapid conversion into a species showing two signals at 6.82 and 7.02 ppm. On the other hand, replacement of leucine

with proline (**12b** to **12c**) completely abolishes prodrug activation under identical incubation conditions, as the minor change from ethyl to methyl ester moiety at R^1 is unlikely to cause such a stark contrast in susceptibility to carboxypeptidase-mediated activation.

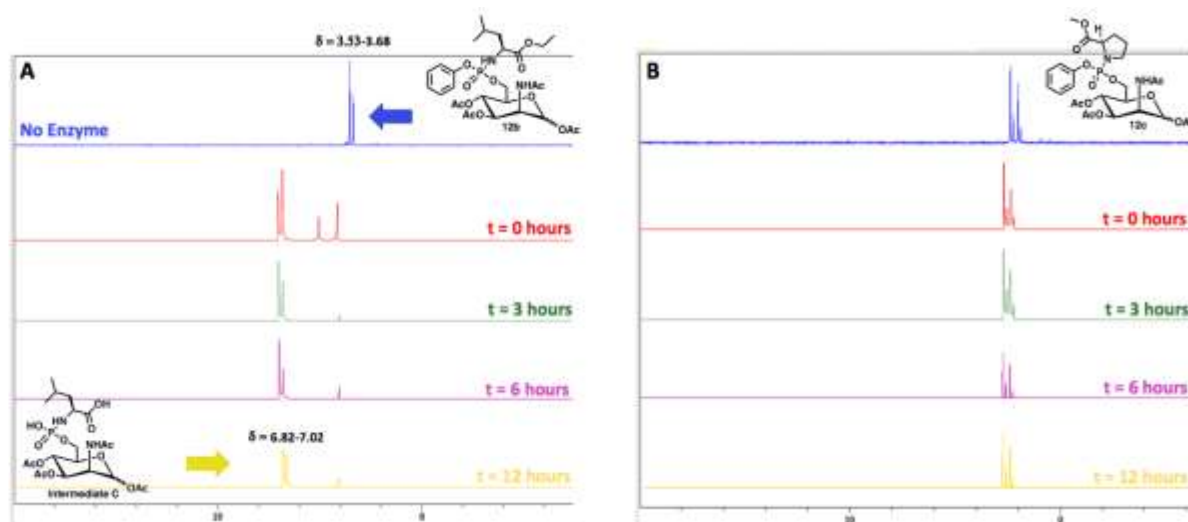
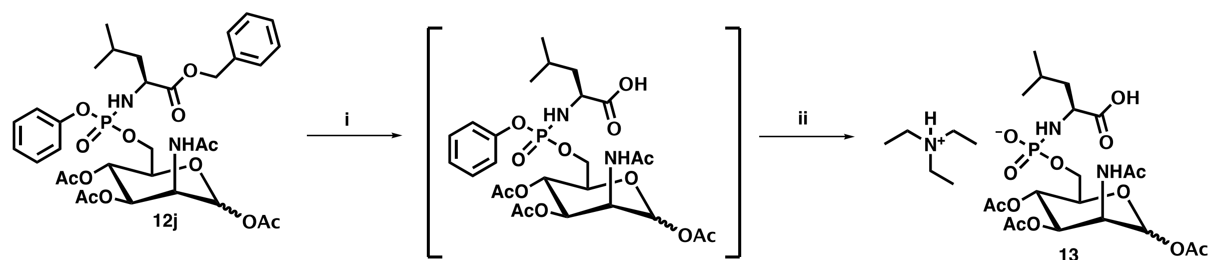


Figure 5. Deconvoluted ^{31}P-NMR spectra of phosphoroamidates **12b** (**A**) and **12c (B)** over time after enzymatic incubation with Carboxypeptidase Y. Conditions: 202MHz, [D_6]acetone, 0.05M Trizma buffer, pH=7.6.

Crude enzymatic reaction mixture of **12b** was further analyzed by LC-MS confirming the presence of intermediate **C** (see Figure 31 in Supporting Information). To independently confirm the real structure of intermediate **C** (two ^{31}P-NMR peaks at 6.82 and 7.02 ppm), a sample of this compound was prepared synthetically. Compound **12j** was debenzylated via catalytic hydrogenation to the corresponding acid, which formed the expected intermediate **C** as a triethyl ammonium salt (**13**) (Scheme 3) after treatment with 0.1 M triethylammonium bicarbonate buffer. Comparison of LC-MS (t_{R} = 2.5, 3.7 min) and ^{31}P-NMR (7.02, 6.97 ppm) data for compound **13** and those obtained from incubation of compound **12b** confirmed the effective activation of **12b** into the corresponding phosphoromonoamidate **C**. This result demonstrates for the first time that carbohydrate phosphoramidates undergo a similar activation pathway observed for nucleoside phosphoramidates. In addition, these studies suggest that

1 even non-nucleoside phosphoramidates are metabolized intracellularly and can serve as a
2 substrate for monophosphate formation via enzymatic (i.e. HINT1) or spontaneous activation,
3 but the selection of the appropriate amino acid side chain is critical towards enabling the
4 activation mechanism.



5

6 **Scheme 3.** Synthesis of compound **13**. Reagents and conditions: (i) H_2, 10% Pd/C,
7 EtOH/EtOAc, 2h at rt; (ii) 0.1 M triethylammonium bicarbonate, 5 min at rt.

8 ADME Evaluation

9 Having established that these compounds can readily undergo enzymatic activation, we
10 decided to evaluate several Ac_3ManNAc phosphoramidates for ADME properties to select lead
11 candidates for cell-based models of GNE myopathy.

12 As previously mentioned, substrate replacement for GNE myopathy with highly polar small
13 molecules were hampered by poor passive diffusion across cellular membranes. While the
14 phosphoramidate promoiety increases cell permeability due to increased lipophilicity, this
15 effect must be balanced with adequate aqueous solubility to ensure proper compound
16 dissolution and absorption. Therefore, compounds **12b-e** were screened for Caco-2 cell
17 permeability, kinetic solubility, human plasma stability and metabolic stability as key
18 parameters that would increase the likelihood of success in cell-based assays. The results of
19 these studies are displayed in Table 3.

Compound ID	P_{app} (B-A/A-B) (10^{-6} cm/s)	Kinetic Solubility (µM, pH 7.4)	Human Plasma Stability (% remaining at 2 h)	HLM ($T_{1/2}$, min)
12b	0.8 / 1.5*	125	20	1.3
12c	0.1 / 0.6*	152	15	1.4
12d	0.3 / 0.6*	129	8.3	1.2
12e	0.3 / 0.4*	124	9.8	0.8

1 **Table 3.** ManNAc phosphoroamidates ADME properties. *with pgp/bcrp inhibitor GF120918
2 (10 µM). Low permeability control was nadolol (P_{app} = 0.09) and high permeability control
3 was metoprolol (P_{app} = 13.6). Efflux control was digoxin, and efflux ratio reduced from 374 to
4 1.69 in the presence of GF120918.

5 Compound **12b** showed improved permeability based on the Caco-2 assay relative to all other
6 compounds. Compound **12c** had comparable permeability to **12d** and **12e** but showed increased
7 plasma stability. Pleasingly, each compound showed acceptable solubility in aqueous
8 environment at physiological pH. Hydrolysis of ester side chains via plasma esterases
9 unsurprisingly causes rapid degradation of the parent compound, and the same phenomenon
10 holds true for metabolic stability when incubated with human liver microsomes (HLMs) as
11 evidenced by short half-lives of each compound. Aside from displaying acceptable ADME
12 properties, **12c** did not demonstrate activation in the aforementioned carboxypeptidase assay,
13 potentially providing a negative control for assessing prodrug efficacy in cell-based assays
14 models of GNE myopathy. Based on the results of the ADME screening, as well as the
15 enzymatic activation data, compounds **12b** and **12c** were selected for further testing.

16 Sialic Acid Quantification in Cell-Based Models of GNE Myopathy

17 The evaluation of prodrugs (**12b** and **12c**) to rescue sialic acid deficiencies *in vitro* was

conducted using two different cell lines: *Lec3* CHO cells and GNEM patient-derived myoblasts. Both cell types present with reduced sialic acid levels due to mutations in *GNE* that mimic the hyposialylation defects observed in patients with GNE myopathy. In particular, *Lec3* mutant CHO cells lack any detectable GNE activity[38], whereas the patient-derived myoblasts contained a D378Y mutation that cause an 80 % reduction in GNE activity.[9] Compounds **12b** and **12c** were initially assessed for cytotoxicity in *GNE* mutant cell lines relative to ManNAc using Trypan Blue staining as an assay for cell viability after 48 hours incubation. At 0.1 mM, **12b** and **12c** induced no observable difference in cell viability relative to controls and at 1 mM, cell viability was comparable to 1 mM ManNAc (see Supporting Info, Figure 32).

Sialic acid quantification was then carried out in duplicate at two concentrations to assess dose-response in GNE-deficient cells after 48-hour incubation with 0.1 mM and 1 mM **12b** or **12c**, 1 and 100 mM for ManNAc, and a negative control with media only. We focused specifically on assessing total and free sialic acid production in membrane protein fractions, as sialic acid availability in cell membranes plays a key role in promoting muscle growth, structure and integrity, a critical issue for GNE myopathy patients. Although clinical studies with therapies like aceneuramic acid and ManNAc supplementation have shown increases in plasma sialic acid levels, it is not clear that these data have correlated with increases in sialic acid levels within tissues.

As shown in Figure 6, **12b** induced dose-dependent increases in total and free sialic acid levels in membrane protein fractions in both *Lec3* CHO cells and GNEM patient-derived myoblasts. Given the lack of activation observed with **12c** in the carboxypeptidase assay, the compound unsurprisingly produced no effects on sialic acid levels in *Lec3* CHO cells. The effects of **12b** were more pronounced than ManNAc, producing >30-fold and >50-fold more sialic acid in *Lec3* CHO cell and GNEM patient-derived myoblasts, respectively, at 1 mM.

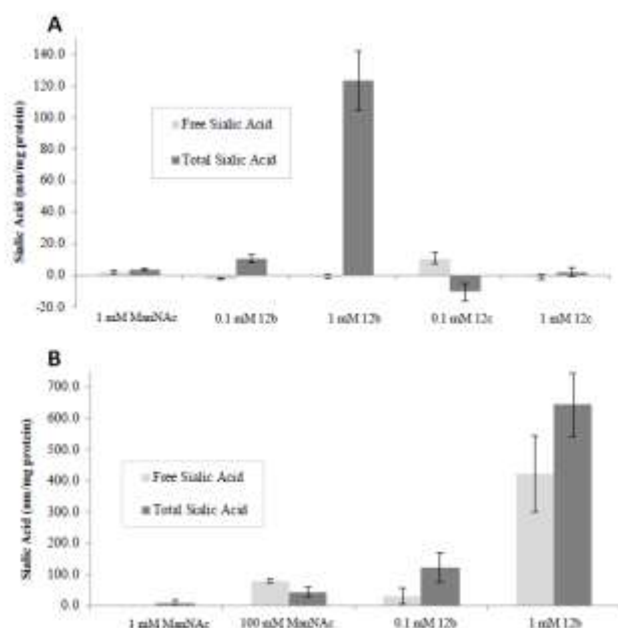


Figure 6. Sialic acid levels in membrane protein fraction. Sialic acid levels were determined using Sialic Acid Assay kit on isolated membrane protein fractions from *Lec3* CHO cells (**A**) and GNEM patient-derived fibroblasts (**B**) incubated with **12b**, **12c**, ManNAc for 48 hours and normalized to control (media only) (n = 2, +/- SEM).

CONCLUSION

A substrate replacement paradigm was applied to the design of novel ProTide prodrugs of ManNAc-6-P as a therapeutic approach for GNE myopathy. A focused library of ManNAc-6-P phosphoramidates was synthesized and evaluated for ADME properties to select lead compounds for profiling in cell-based models of GNE myopathy. Two leads, **12b** and **12c**, displayed the most promising ADME properties, although **12c** did not show activation in a carboxypeptidase assay. The demonstration and independent confirmation of carboxypeptidase-mediated activation of **12b** serves as the first example of a non-nucleoside phosphoramidate activation mechanism. The contrast in prodrug activation between **12b** and **12c** further emphasizes the importance of selecting the appropriate amino acid side chain for the ProTide moiety. Compounds **12b** and **12c** were evaluated in two separate GNE-deficient cell lines for their ability to increase sialic acid production in membrane protein fractions

relative to ManNAc. Compound **12b** produced >30-fold and >50-fold more sialic acid than ManNAc in *Lec3* CHO cell and GNEM patient-derived myoblasts, respectively, at 1 mM. The extension of the ProTide platform to monosaccharide prodrugs presents an opportunity to explore therapeutic options for diseases due to the deficiency of sugar metabolites, such as the CDGs.

Experimental Section

General Experimental Information

All chemicals were purchased from Sigma-Aldrich and Carbosynth and used without any further purification. All ^{1}H-NMR (500MHz), ^{31}P-NMR (202MHz) and ^{13}C-NMR (125MHz) spectra were recorded on a Bruker AVANCE III HD 500 MHz spectrometer at 25 °C. The deuterated solvent used were $CDCl_3$ and CD_3OD and the spectra were calibrated using their residual peaks: $CDCl_3$, ^{1}H: δ=7.26 ppm; ^{13}C: δ=77.36 ppm and CD_3OD, ^{1}H: δ=3.34 ppm; ^{13}C: δ=49.86 ppm. Chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (J) in Hertz. The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), spt (septet), m (multiplet), br (broad), dd (doublet of doublets) and ddd (doublet of doublet of doublets). The signals in the ^{1}H and ^{13}C NMR spectra were assigned based on analysis of coupling constants and additional two-dimensional experiments (COSY and HSQC). Some peaks in all NMR analyses may be overlapped. Reactions followed by thin-layer chromatography (precoated aluminium-backed plates, 60 F254, 0.2 mm thickness, Merck, were visualized under short-wave ultraviolet light (254 nm) and/or via Pancaldi's solution (molybdate ammonium cerium sulfate solution).

Chromatography purifications were performed through ISOLERA™ 3.0 BIOTAGE OS 852M both with the sample being loaded as a concentrated solution in the same eluent and pre-adsorbed on top of silica. Fractions containing the product were identified by TLC, combined,

1 and the solvent was removed in vacuo. HPLC analysis was carried out to confirm the purity of
2 final compounds by using a Varian Pursuit XRs 5 C18 150 mm × 4.6 mm analytical column.
3 All compounds tested had a purity of >95% as determined by HPLC at two different
4 wavelengths (220 and 254 nm).
5 Low-resolution mass and LC-MS analyses were performed on a Bruker Daltonics MicroTof-
6 LC instrument (atmospheric pressure ionization, electrospray mass spectroscopy) in either the
7 positive- or negative-ion mode. MS parameters were optimized to the following: set capillary
8 4500 V, set end plate offset -500 V, set nebulizer 1.0 Bar, set dry heater 200 ºC, set dry gas 8.8
9 l/min, set divert valve source. Full scan mass spectra were recorded over a range of *m/z* 50-
10 3000.
11 **Standard Procedures.** For practical purposes, standard procedures are given. Any variations
12 from these procedures are discussed individually and described in full.
13 **Synthesis of (α)-(2*R*,3*S*,4*R*,5*S*)-5-Acetamido-2-(acetoxymethyl)-6-(benzyloxy)tetrahydro-**
14 **2H-pyran-3,4-diyl diacetate (2):** A solution of *N*-acetyl-D-mannosamine hydrochloride
15 (45.21 mmol, 10.0 g) and DMAP (4.52 mmol, 0.55 g) in pyridine (200 mL) was reacted with
16 acetic anhydride (43 mL) and stirred at rt for 12 h. The reaction mixture was diluted with DCM,
17 washed with 1M HCl, $NaHCO_3$ and brine. The residue was concentrated in vacuo and purified
18 by column chromatography eluting with DCM/CH_3OH (2:10) to afford *tetra*-acetylated-D-
19 mannosamine as a yellowish oil (74 %) which was then dissolved in ACN (40 mL) followed
20 the addition of benzyl alcohol (57.8 mmol, 6 mL) and $BF_3 \cdot Et_2O$ (1.83 mmol, 230 μL). The
21 resulting mixture was heated at 80 °C and stirred for 12 h. Afterwards, the mixture was cooled
22 down to rt, diluted with DCM (400 mL) and washed with $NaHCO_3$. The expected product was
23 obtained after column chromatography purification eluted with Hexane/EtOAc (1:4) in 75 %
24 yield. Final deacetylation was accomplished via treating the latter compound (2.29 mmol, 1.00

g) dissolved in anhydrous MeOH (50 mL) with a solution of NaOMe (0.23 mmol, 0.012 g) in 300 µL of dry CH_3OH under argon atmosphere. The resulting solution was stirred at rt for 5 h and then Amberlite IR-120 hydrogen form was added leaving the mixture under stirring for another 1 h. The mixture was then filtered through celite and the solvent was removed under reduced pressure. The resulting solid was triturated with Et_2O and further purified by column chromatography on silica using EtOAc/MeOH (4:1) to obtain the final product (**2**) in 87 % as two anomers α and β anomers (0.62 g). White solid. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.35-7.28 (m, 5H, $\underline{Ph}$), 4.81 (s, 1H, $\underline{H\text{-}1}$), 4.64 (d, 1H, *J* = 11.7 Hz, $\underline{CH_{2a}}Ph$), 4.47-4.44 (m, 2H, $\underline{CH_{2b}}Ph$ and $\underline{H\text{-}2}$), 4.11-4.09 (m, 1H, $\underline{H\text{-}3}$), 3.95 (d, 1H, *J* = 10.8 Hz, $\underline{H\text{-}4}$), 3.79-3.730 (m, 2H, H-5, $\underline{H\text{-}6}$), 3.61 (d, 1H, *J* = 9.4 Hz, $\underline{H\text{-}6}$), 2.00 (s, 3H, $OCOC\underline{H_3}$) ppm. MS (ES+): *m/z* 334.15 $[M+Na]^+$

Standard Procedure 1. **General Synthesis of Phosphorochloridate (3a-i/4a-c):** A solution of aryl dichlorophosphate (1 equiv) and an appropriate amino acid ester (1 equiv) in anhydrous DCM under argon atmosphere was cooled down to -78 °C. Afterwards, Et_3N (2 equiv) was added dropwise and the resulting mixture was stirred for 15 minutes at -78 °C. Then, the reaction mixture was allowed to reach room temperature and left under stirring for 1 hour. The solvent was removed under reduced pressure, the solid residue was redissolved in diethyl ether and the resulting mixture was stirred under argon atmosphere for 20 min. The solid was filtered off and the solvent was evaporated giving the pure compound as a colorless oil which was used in the next step without further purification.

Isopropyl (chloro(phenoxy)phosphoryl)-L-alaninate (3a): Colorless oil; 99 %, 8.36 g. ^{31}P-NMR (202 MHz, $CDCl_3$, mixture of R_P and S_P diastereoisomers, a:b): δ_P 8.09 (1P), 7.71 (0.9) ppm. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.39-7.36 (m, 2H, $C\underline{H}$-Ph), 7.28-7.22 (m, 3H, $C\underline{H}$-Ph), 5.10-5.05 (m, 1H, $C\underline{H}(CH_3)_2$), 4.16-4.08 (m, 1H, $NHC\underline{H}CO$), 1.50 (dd, 3H, *J* = 2.4, 7.1 Hz,

NHCHC$\underline{H}_3$), 1.30-1.26 (m, 6H, CH(C$\underline{H}_3$)$_2$) ppm.

Ethyl (chloro(phenoxy)phosphoryl)-L-leucinate (3b): Prepared according to *Standard procedure 1* in 98 %, yield as a mixture of two diastereoisomers (*SS*$_P$ and *SR*$_P$). Colorless oil; 4.20 g. ^{31}P-NMR (202 MHz, CDCl$_3$): δ_P 8.51 (1P), 8.27 (1P) ppm. ^{1}H-NMR (500 MHz, CDCl$_3$): δ_H 7.38-7.34 (m, 2H, C$\underline{H}$-Ph), 7.27-7.21 (m, 3H, C$\underline{H}$-Ph), 4.34-4.19 (m, 3H, N$\underline{H}$, OC$\underline{H}_2$CH$_3$), 4.14-4.02 (m, 1H, NHC$\underline{H}$CO), 1.86 (spt, 0.5H, J = 6.6 Hz, C$\underline{H}_a$(CH$_3$)$_2$), 1.79 (spt, 0.5H, J = 6.6 Hz, C$\underline{H}_b$(CH$_3$)$_2$), 1.64-1.59 (m, 2H, CHC$\underline{H}_2$CH), 1.30-1.26 (m, 3 H, OCH$_2$C$\underline{H}_3$), 0.96-0.93 (m, 6H, C$\underline{H}_3$CHC$\underline{H}_3$) ppm.

Methyl (chloro(phenoxy)phosphoryl)-D-prolinate (3c): Prepared according to *Standard procedure 1* in 80 %, yield as a mixture of two diastereoisomers (*SS*$_P$ and *SR*$_P$). Light yellow oil; 1.20 g. ^{31}P-NMR (202 MHz, CDCl$_3$): δ_P 7.95 (1P), 7.76 (0.9P) ppm. ^{1}H-NMR (500 MHz, CDCl$_3$): δ_H 7.38-7.34 (m, 2H, C$\underline{H}$-Ph), 7.30-7.28 (m, 1H, C$\underline{H}$-Ph), 7.24-7.20 (m, 2H, C$\underline{H}$-Ph), 4.50-4.46 (m, 0.5H, NHC$\underline{H}_a$CO), 4.32-4.39 (m, 0.5H, NHC$\underline{H}_b$CO), 3.73 (s, 1.5H, COOC$\underline{H}_3$), 3.72 (s, 1.5H, COOC$\underline{H}_3$), 3.58-3.49 (m, 2H, $\underline{H}$-Pro), 2.28-2.19 (m, 1H, $\underline{H}$-Pro), 2.15-2.08 (m, 1H, $\underline{H}$-Pro), 2.07-1.95 (m, 2H, $\underline{H}$-Pro) ppm.

Methyl (chloro(naphthalen-1-yloxy)phosphoryl)glycinate (3d): Prepared according to *Standard procedure 1* in 78 %, yield as a mixture of two stereoisomers (*SS*$_P$ and *SR*$_P$). Light yellow oil; 2.30 g. ^{31}P-NMR (202 MHz, CDCl$_3$): δ_P 9.08 ppm. ^{1}H-NMR (500 MHz, CDCl$_3$): δ_H 8.06 (d, 1H, J = 8.3 Hz, C$\underline{H}$-Naph), 7.87 (d, 1H, J = 7.7 Hz, C$\underline{H}$-Naph), 7.72 (d, 1H, J = 8.3 Hz, C$\underline{H}$-Naph), 7.60-7.52 (m, 3H, C$\underline{H}$-Naph), 7.43 (t, 1H, J = 8.3 Hz, C$\underline{H}$-Naph), 4.43-4.40 (br, 1H, N$\underline{H}$), 4.00 (dd, 2H, J = 4.2, 9.7 Hz, NHC$\underline{H}_2$CO), 3.80 (s, 3H, COOC$\underline{H}_3$) ppm.

Ethyl (chloro(phenoxy)phosphoryl)-L-phenylalaninate (3e): Prepared according to *Standard procedure 1* in > 99 %, yield as a mixture of two diastereoisomers (*SS*$_P$ and *SR*$_P$). Colorless oil; 6.30 g. ^{31}P-NMR (202 MHz, CDCl$_3$): δ_P 8.03 (1P), 7.96 (1P) ppm. ^{1}H-NMR (500 MHz, CDCl$_3$): δ_H 7.39-7.15 (m, 10H, C$\underline{H}$-Ar), 4.48-4.35 (m, 1H, NHC$\underline{H}$CO), 4.23-4.16 (m,

2H, $OC\underline{H_2}CH_3$), 3.21-3.10 (m, 2H, $C\underline{H_2}Ph$), 1.26-1.21 (m, 3H, $OCH_2C\underline{H_3}$) ppm.

Benzyl (chloro(naphthalen-1-yloxy)phosphoryl)-L-alaninate (3f): Prepared according to *Standard procedure 1* in 96 %, yield as a mixture of two diastereoisomers (*SS*$_P$ and *SR*$_P$). Colorless oil; 7.2 g. ^{31}P-NMR (202 MHz, $CDCl_3$): δ_P 8.17 (0.8P), 7.90 (1P) ppm. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 8.06-8.04 (m, 1H, $C\underline{H}$-Ar), 7.88-7.86 (m, 1H, $C\underline{H}$-Ar), 7.74-7.72 (d, 1H, J = 8.3 Hz, $C\underline{H}$-Ar), 7.60-7.52 (m, 3H, $C\underline{H}$-Ar), 7.44-7.40 (m, 1H, $C\underline{H}$-Ar), 7.39-7.35 (m, 2H, $C\underline{H}$-Ar), 7.34-7.32 (m, 3H, $C\underline{H}$-Ar), 5.23-5.15 (m, 2H, $OC\underline{H_2}Ph$), 4.52-4.39 (m, 1H, $N\underline{H}$), 4.37-4.31 (br, 1H, $NHC\underline{H}CO$), 1.55 (dd, 3H, J = 7.3, 10.2 Hz, $CHC\underline{H_3}$) ppm.

Benzyl (chloro(phenoxy)phosphoryl)-L-valinate (3g): Prepared according to *Standard procedure 1* in 89 %, yield as a mixture of two diastereoisomers (*SS*$_P$ and *SR*$_P$). Light yellow oil; 4.40 g. ^{31}P-NMR (202 MHz, $CDCl_3$): δ_P 9.53 (1P), 9.02 (1P) ppm.^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.38-7.22 (m, 10H, $C\underline{H}$-Ar), 5.32-5.07 (AB system, 2H, J = 7.6 Hz, $OC\underline{H_2}Ph$), 4.32-4.16 (m, 1H, $N\underline{H}$), 4.04-3.93 (m, 1H, $NHC\underline{H}CO$), 2.21-2.13 (m, 1H, 3H, $C\underline{H}(CH_3)_2$), 1.00 (m, 3H, $CH(C\underline{H_3})_2$), 0.90 (m, 3H, $CH(C\underline{H_3})_2$) ppm.

Ethyl (chloro(phenoxy)phosphoryl)-L-isoleucinate (3h): Prepared according to *Standard procedure 1* in 98 %, yield as a mixture of two diastereoisomers (*SS*$_P$ and *SR*$_P$). Light yellow oil; 8.40 g. ^{31}P-NMR (202 MHz, $CDCl_3$): δ_P 9.47 (1P), 8.91 (0.9P) ppm. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.39-7.35 (m, 2H, $C\underline{H}$-Ph), 7.29-7.22 (m, 3H, $C\underline{H}$-Ph), 4.56-4.48 (m, 1H, $N\underline{H}$), 4.28-4.20 (m, 2H, $OC\underline{H_2}CH_3$), 4.03-3.93 (m, 1H, $NHC\underline{H}CO$), 1.91-1.88 (m, 1H, $CH_3C\underline{H}CH_2$), 1.56-1.48 (m, 1H, $CHC\underline{H_{2a}}CH_3$), 1.30 (m, 3H, $OCH_2C\underline{H_3}$), 1.27-1.23 (m, 1H, $CHC\underline{H_{2b}}CH_3$), 1.00-0.92 (m, 6H, $CH(C\underline{H_3})_2$) ppm.

Benzyl 2-((chloro(phenoxy)phosphoryl)amino)-2-methylpropanoate (3i): Prepared according to *Standard procedure 1* in 96 %, yield as a mixture of two stereoisomers (*SS*$_P$ and *SR*$_P$). Light yellow oil; 96 %; 3.84 g. ^{31}P-NMR (202 MHz, $CDCl_3$): δ_P 5.62 ppm. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.28-7.11 (m, 10H, $C\underline{H}$-Ph), 5.12 (s, 2H, $OC\underline{H_2}Ph$), 1.62 (s, 3H, $C(C\underline{H_3})_2$),

1.60 (s, 3H, $C(C\underline{H}_3)_2$) ppm.

Benzyl (chloro(phenoxy)phosphoryl)-L-leucinate (3j): Prepared according to *Standard procedure 1* in > 99 %, yield as a mixture of two diastereoisomers (SS_P and SR_P). Colorless oil; 2.43 g. ^{31}P-NMR (202 MHz, $CDCl_3$): δ_P 8.31 (0.9P), 8.00 (1P) ppm. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.30-7.26 (m, 7H, $C\underline{H}$-Ar), 7.18-7.13 (m, 3H, $C\underline{H}$-Ar), 5.16-5.08 (m, 2H, $OC\underline{H}_2Ph$), 4.14-4.01 (m, 2H, $N\underline{H}$ and $NHC\underline{H}CO$), 1.81-1.67 (m, 1H, $C\underline{H}(CH_3)_2$), 1.61-1.49 (m, 2H, $CHC\underline{H}_2CH$), 0.88-0.85 (m, 6H, $CH(C\underline{H}_3)_2$) ppm.

Ethyl (chloro(4-methoxyphenoxy)phosphoryl)-L-leucinate (4a): Prepared according to *Standard procedure 1* in > 99 %, yield as a mixture of two diastereoisomers (SS_P and SR_P). Colorless oil; 4.58 g. ^{31}P-NMR (202 MHz, $CDCl_3$): δ_P 9.03 (1P), 8.84 (1P) ppm. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.18-7.16 (m, 2H, $C\underline{H}$-Ph), 6.87-6.85 (m, 2H, $C\underline{H}$-Ph), 4.22 (m, 2H, $OC\underline{H}_2CH_3$), 4.15-4.00 (m, 2H, $N\underline{H}$ and $NHC\underline{H}CO$), 3.78 (s, $OC\underline{H}_3$), 1.86 (spt, 0.5H, J = 6.7 Hz, $C\underline{H}_a(CH_3)_2$), 1.79 (p, 0.5H, J = 6.7 Hz, $C\underline{H}_b(CH_3)_2$), 1.66-1.51 (m, 2H, $CHC\underline{H}_2CH$), 1.29 (m, 3 H, $OCH_2C\underline{H}_3$), 0.97-0.94 (m, 6H, $CH(C\underline{H}_3)_2$) ppm.

Methyl (chloro(4-methoxyphenoxy)phosphoryl)-D-prolinate (4b): Prepared according to *Standard procedure 1* in > 99 %, yield as a mixture of two diastereoisomers (SS_P and SR_P). Light yellow oil; 10.00 g. ^{31}P-NMR (202 MHz, $CDCl_3$): δ_P 8.60 (1P), 8.42 (0.9P) ppm. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.20 (dd, 1H, J = 1.8, 9.3 Hz, $C\underline{H}$-Ph), 7.15 (dd, 1H, J = 1.8, 9.1 Hz, $C\underline{H}$-Ph), 6.86 (dd, 2H, J = 1.7 Hz, 8.9 Hz, $C\underline{H}$-Ph), 4.49-4.45 (m, 0.5H, $NHC\underline{H}_aCO$), 4.41-4.37 (m, 0.5H, $NHC\underline{H}_bCO$), 3.78 (s, 1.5H, $OC\underline{H}_3$), 3.77 (s, 1.5H, $OC\underline{H}_3$), 3.73 (s, 1.5H, $OC\underline{H}_3$), 3.72 (s, 1.5H, $OC\underline{H}_3$), 3.57-3.49 (m, 2H, $\underline{H}$-Pro), 2.2-2.18 (m, 1H, $\underline{H}$-Pro), 2.15-2.07 (m, 1H, $\underline{H}$-Pro), 2.06-1.94 (m, 2H, $\underline{H}$-Pro) ppm.

Isopropyl (chloro(4-methoxyphenoxy)phosphoryl)-L-alaninate (4c): Prepared according to *Standard procedure 1* in > 99 %, yield as a mixture of two diastereoisomers (SS_P and SR_P). Colorless oil: 14.00 g. ^{31}P-NMR (202 MHz, $CDCl_3$): δ_P 8.76 (1P), 8.41 (1P) ppm. ^{1}H-NMR

(500 MHz, $CDCl_3$): δ_H 7.19-7.16 (m, 2H, $C\underline{H}$-Ph), 6.86 (dd, 2H, *J* = 2.4, 9.0 Hz, $C\underline{H}$-Ph), 5.11-5.04 (m, 1H, $CH_3C\underline{H}CH_3$), 4.17-4.09 (m, 1H, $NHC\underline{H}CO$), 3.78 (s, 3H, OCH_3), 1.48 (dd, 3H, *J* = 3.7, 7.1 Hz, $C\underline{H}(CH_3)_2$), 1.28-1.25 (m, 6H, $CH(C\underline{H_3})_2$) ppm.

Standard Procedure 2. **General Synthesis of 1-OBn-phosphoroamidates (5a/6a-c):** To a solution of compound **2** (1 equiv) in THF/Py (8/2, v/v), N-methylimidazole (2.5 equiv) was added dropwise under argon atmosphere at room temperature and the resulting solution was stirred for 30 minutes. A solution of an appropriate phosphorochloridate (2.5 equiv) in THF was added dropwise to the mixture and stirred overnight at room temperature. After solvent evaporation, the product was purified by column chromatography on silica using DCM/ CH_3OH (93:7) yielding the expected products **5-6**.

(α)-Isopropyl ((((2R,3S,4R,5S)-5-acetamido-6-(benzyloxy)-3,4-dihydroxytetrahydro-2H-pyran-2-yl)methoxy)(phenoxy)phosphoryl)-L-alaninate (**5a**): Colorless oil; 45 %, 1.23 g. ^{31}P-NMR (202 MHz, $CDCl_3$, mixture of R_P and S_P diastereoisomers as α anomer): δ_P 3.86 (1P), 3.45 (0.6P) ppm. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.37-7.15 (m, 10H, $C\underline{H}$-Ar), 4.97-4.90 (m, 1H, $C\underline{H}(CH_3)_2$, overlap with the solvent), 4.78-4.77 (m, 1H, $\underline{H\text{-}1}$, overlap with the solvent), 4.67-4.63 (m, 1H, $C\underline{H_{2a}}Ph$), 4.47-4.44 (m, 1H, $C\underline{H_{2b}}Ph$), 4.35-4.30 (m, 1H, H-2), 4.01-3.90 (m, 3H, $NHC\underline{H}CO$, $\underline{H\text{-}5}$ and $\underline{H\text{-}4}$), 3.83-3.77 (m, 1H, $\underline{H\text{-}3}$), 3.63-3.56 (m, 2H, $\underline{H\text{-}6}$), 1.99 (s, 3H, $NHCOC\underline{H_3}$), 1.37-1.27 (m, 3H, $NHCHC\underline{H_3}$), 1.23-1.18 (m, 6H, $CH(C\underline{H_3})_2$) ppm. ^{13}C-NMR (125 MHz, MeOD): δ_C 174.63 (d, $^3J_{C\text{-}P}$= 5.2 Hz, $\underline{C}$=O ester), 174.07 (d, $^3J_{C\text{-}P}$= 7.2 Hz, $\underline{C}$=O ester), 170.03 ($NH\underline{C}OCH_3$), 154.66 (d, $^2J_{C\text{-}P}$= 6.8 Hz, "*ipso*" $O\underline{Ph}$), 152.28 (d, $^2J_{C\text{-}P}$= 6.5 Hz, "*ipso*" $O\underline{Ph}$), 138.57 ("*ipso*" $OCH_2\underline{Ph}$), 130.72, 130.69, 130.07, 129.41, 129.19, 129.16, 128.85, 128.65, 126.03, 125.99, 123.82 ($\underline{C}HAr$), 121.54 (d, $^3J_{C\text{-}P}$= 4.9 Hz, $\underline{C}$H-OPh), 121.45 (d, $^3J_{C\text{-}P}$= 4.9 Hz, $\underline{C}$H-OPh), 99.57 ($\underline{C\text{-}1}$), 73.12 (d, $^4J_{C\text{-}P}$= 6.8 Hz, $\underline{C\text{-}4}$), 70.55, 70.39 ($\underline{C\text{-}3}$), 70.17 ($O\underline{C}H_2Ph$), 70.11, 70.03 ($\underline{C}H(CH_3)_2$), 69.19, 69.07 ($NH\underline{C}HCO$), 68.33 ($\underline{C\text{-}5}$), 68.07 (d,

$^{2}J_{C\text{-}P}$= 5.6 Hz, C-6), 67.94 (d, $^{2}J_{C\text{-}P}$= 5.5 Hz, C-6), 54.35 (C-2), 21.98, 21.95 ($NHCH\underline{C}H_3$), 9.19, 7.59 ($CH(\underline{C}H_3)_2$) ppm. MS (ES+): *m/z* 603.22 $[M+Na]^+$

(α)-Ethyl ((((2R,3S,4R,5S)-5-acetamido-6-(benzyloxy)-3,4-dihydroxytetrahydro-2H-pyran-2-yl)methoxy)(4-methoxyphenoxy)phosphoryl)-L-leucinate (**6a**): Prepared according to *Standard procedure 2* in 20 %, yield as a mixture of R_P and S_P diastereoisomers as α anomer. Light yellow oil; 0.16 g. ^{31}P-NMR (202 MHz, $CDCl_3$): δ_P 4.37 (1P), 4.30 (1P) ppm. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.35-7.31 (m, 5H, C$\underline{H}$-Ar), 7.18-7.13 (m 2H, C$\underline{H}$-Ar), 6.87-6.83 (m, 2H, C$\underline{H}$-Ar), 4.79 (d, 1H, *J* = 10.9 Hz, H-1), 4.68-4.64 (m, 1H, $C\underline{H_{2a}}Ph$), 4.49-4.41 (m, 1H, $C\underline{H_{2b}}Ph$), 4.35-4.34 (m, 1H, H-2), 4.17-4.12 (m, 2H, $OC\underline{H_2}CH_3$), 4.11-4.07 (m, 1H, H-4), 4.00-4.07 (m, $NHC\underline{H}CO$), 3.92-3.87 (m, 1H, H-3), 3.84-3.73 (m, 4H, $OC\underline{H_3}$ and H-5), 3.63-3.58 (m, 2H, H-6), 2.01 (s, 3H, $NHCOC\underline{H_3}$), 1.71-1.62 (m, 1H, $C\underline{H}(CH_3)_2$), 1.58-1.50 (m, 2H, $CHC\underline{H_2}CH$), 1.27-1.19 (m, 3H, $OCH_2C\underline{H_3}$), 0.94-0.84 (m, 6H, $CH(C\underline{H_3})_2$) ppm. ^{13}C-NMR (125 MHz, MeOD): δ_C 175.45 (d, $^{3}J_{C\text{-}P}$= 2.5 Hz, $\underline{C}$=O ester), 175.33 (d, $^{3}J_{C\text{-}P}$= 2.5 Hz, $\underline{C}$=O ester), 174.11, 174.05 ($NH\underline{C}OCH_3$), 158.25, 157.59 ("*ipso*" $\underline{Ph}OCH_3$), 145.83 (d, $^{2}J_{C\text{-}P}$= 3.8 Hz, "*ipso*" PO$\underline{Ph}$), 138.56 ("*ipso*" $OCH_2\underline{Ph}$), 129.42, 129.39, 129.27, 129.22, 128.86, 122.46, 122.42 ($\underline{C}$HAr), 122.32 (d, $^{3}J_{C\text{-}P}$= 4.4 Hz, $\underline{C}$H-POPh), 122.18 (d, $^{3}J_{C\text{-}P}$= 4.5 Hz, $\underline{C}$H-POPh), 115.58, 115.55, 115.31 ($\underline{C}$HAr), 99.56, 99.51 (C-1), 73.11 (d, $^{4}J_{C\text{-}P}$= 7.5 Hz, C-4), 73.08 (d, $^{4}J_{C\text{-}P}$= 6.3 Hz, C-4), 70.58, 70.37 ($NH\underline{C}HCO$), 70.14 ($O\underline{C}H_2Ph$), 68.40, 68.37 (C-5), 68.04 (d, $^{2}J_{C\text{-}P}$= 5.3 Hz, C-6), 67.88 (d, $^{2}J_{C\text{-}P}$= 5.4 Hz, C-6), 62.27, 62.22 ($O\underline{C}H_2CH_3$), 56.02, 55.99 (C-3), 54.56, 54.37 (C-2), 44.15, 43.96 ($CH\underline{C}H_2CH$), 25.55, 25.46 ($\underline{C}H(CH_3)_2$), 23.19, 23.16, 22.56, 22.41, 22.13, 21.90 ($NHCO\underline{C}H_3$ and $CH(\underline{C}H_3)_2$) 14.46, 14.41 ($OCH_2\underline{C}H_3$) ppm. MS (ES+): *m/z* 661.26 $[M+Na]^+$

(α)-Methyl ((((2R,3S,4R,5S)-5-acetamido-6-(benzyloxy)-3,4-dihydroxytetrahydro-2H-pyran-2-yl)methoxy)(4-methoxyphenoxy)phosphoryl)-D-prolinate (**6b**): Prepared according to

Standard procedure 2 in 32 %, yield as a mixture of *R*$_P$ and *S*$_P$ diastereoisomers as α anomer. Light yellow oil; 0.23 g. ^{31}P-NMR (202 MHz, CDCl$_3$): δ$_P$ 2.27 (0.1P), 1.87 (1P) ppm. ^{1}H-NMR (500 MHz, CDCl$_3$): δ$_H$ 7.33-7.28 (m, 5H, CH-Ar), 7.14-7.12 (m 2H, CH-Ar), 6.87-6.84 (m, 2H, CH-Ar), 4.77-4.76 (m, 1H, H-1), 4.61 (d, 1H, *J* = 11.7 Hz, CH$_{2a}$Ph), 4.55-51 (m, 1H, NHCHCO), 4.4 (m, 1H, *J* = 11.7 Hz CH$_{2b}$Ph), 4.33-4.31 (m), 4.26-4.23 (m), 3.98-3.95 (m), 3.81-3.78 (m), 3.71 (s, 3H, COOCH$_3$), 3.69 (s, 3H, PhOCH$_3$), 3.41-3.33 (m), 2.19-2.13 (m, 2H, CH$_2$Pro), 1.99 (s, 3H, NHCOCH$_3$), 1.96-1.81 (m, 2H, CH$_2$Pro) ppm. MS (ES+): *m/z* 631.21 [M+Na]$^+$

(α)-Isopropyl ((((2R,3S,4R,5S)-5-acetamido-6-(benzyloxy)-3,4-dihydroxytetrahydro-2H-pyran-2-yl)methoxy)(4-methoxyphenoxy)phosphoryl)-L-alaninate (**6c**): Prepared according to *Standard procedure 2* in 21 %, yield as a mixture of *R*$_P$ and *S*$_P$ diastereoisomers as α anomer. Colorless oil; 0.20 g. ^{31}P-NMR (202 MHz, CDCl$_3$): δ$_P$ 4.30 (1P), 4.09 (0.8P) ppm. ^{1}H-NMR (500 MHz, CDCl$_3$): δ$_H$ 7.30 (m, 5H, CH-Ar), 7.14 (d, 2H, *J* = 86 Hz, CH-Ar), 6.84-6.80 (m, 2H, CH-Ar), 4.98-4.93 (m, 1H, CH(CH$_3$)$_2$), 4.77 (s, 1H, H-1), 4.65-4.64 (m, 1H, CH$_{2a}$Ph), 4.44-4.38 (m, 1H, CH$_{2b}$Ph), 4.32-4.31 (m, 1H, H-2), 4.29-4.21 (m), 3.98-3.95 (m), 3.93-3.88 (m, 1H, NHCHCO), 3.80-3.77 (m), 3.70 (s, 1.5H, PhOCH$_3$), 3.69 (s, 1.5H, PhOCH$_3$), 3.62-3.55 (m, 2H, H-6), 1.99 (s, 3H, NHCOCH$_3$), 1.37-1.32 (m, 3H, NHCHCH$_3$), 1.23-1.20 (m, 6H, CH(CH$_3$)$_2$) ppm. ^{13}C-NMR (125 MHz, MeOD): δ$_C$ 173.34, 173.33 (C=O ester), 172.73, 172.67 (NHCOCH$_3$), 156.87 ("*ipso*" OPh), 144.38 ("*ipso*" POPh), 137.21 ("*ipso*" OCH$_2$Ph), 128.05, 127.87, 127.84, 127.49, 121.00, 120.93, 114.23, 114.20 (CHAr), 98.18, 98.14 (C-1), 73.11 (d, $^4J_{C\text{-}P}$= 7.5 Hz, C-4), 73.08 (d, $^4J_{C\text{-}P}$= 6.3 Hz, C-4), 70.58, 70.37 (NHCHCO), 70.14 (OCH$_2$Ph), 68.40, 68.37 (C-5), 68.04 (d, $^2J_{C\text{-}P}$= 5.3 Hz, C-6), 67.88 (d, $^2J_{C\text{-}P}$= 5.4 Hz, C-6), 62.27, 62.22 (OCH$_2$CH$_3$), 56.02, 55.99 (C-3), 54.56, 54.37 (C-2), 44.15, 43.96 (CHCH$_2$CH), 25.55, 25.46 (CH(CH$_3$)$_2$), 23.19, 23.16, 22.56, 22.41, 22.13, 21.90 (NHCOCH$_3$ and CH(CH$_3$)$_2$) 14.46, 14.41

$(OCH_2\underline{C}H_3)$ ppm. MS (ES+): *m/z* 661.26 $[M+Na]^+$

Standard Procedure 3. **General Synthesis of Deprotected Phosphoroamidates (8a-c):** To a solution of **5** or **6** (1 equiv) in EtOH/EtOAc (2:1) was added Pd/C (10% Pd on activated carbon) under inert atmosphere. The resulting mixture was stirred at room temperature for 5 hours under H_2 atmosphere (1 atm, ballon). The crude was filtered through celite, concentrated in vacuo and triturated with Et_2O giving the expected products **8a-c**.

Ethyl ((((2R,3S,4R,5S)-5-acetamido-3,4,6-trihydroxytetrahydro-2H-pyran-2-yl)methoxy)(4-methoxyphenoxy)phosphoryl)-L-leucinate (**8a**): Light yellow oil; 29%; 0.17 g. ^{31}P-NMR (202 MHz, $CDCl_3$, mixture of R_P and S_P diastereoisomers as α and β anomers): δ_P 4.41 (1P), 4.31 (0.7P), 4.19 (0.7P) ppm. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.14 (d, 2H, J = 9.0 Hz, C$\underline{H}$-Ar), 6.87 (d, 2H, J = 9.0 Hz, C$\underline{H}$-Ar), 5.03 (s, 1H, $\underline{H\text{-}1}$), 4.56 (s, 1H, $\underline{H\text{-}2}$), 4.34-4.26 (m, 1H, $C\underline{H_2}Ph$), 4.17-4.12 (m, 2H, $OC\underline{H_2}CH_3$), 4.11-4.07 (m, 1H, $\underline{H\text{-}4}$), 4.00-4.07 (m, NHC$\underline{H}$CO), 3.92-3.87 (m, 1H, $\underline{H\text{-}3}$), 3.84-3.73 (m, 4H, $OC\underline{H_3}$ and $\underline{H\text{-}5}$), 3.63-3.58 (m, 2H, $\underline{H\text{-}6}$), 2.01 (s, 3H, $NHCOC\underline{H_3}$), 1.71-1.62 (m, 1H, $C\underline{H}(CH_3)_2$), 1.58-1.50 (m, 2H, $CHC\underline{H_2}CH$), 1.27-1.19 (m, 3H, $OCH_2C\underline{H_3}$), 0.94-0.84 (m, 6H, $CH(C\underline{H_3})_2$) ppm. ^{13}C-NMR (125 MHz, MeOD): δ_C 174.20, 174.09 ($\underline{C}$=O ester), 172.77, 172.69 ($NH\underline{C}OCH_3$), 156.92, 156.84 ("*ipso*" $\underline{Ph}OCH_3$), 144.41 (d, $^2J_{C\text{-}P}$= 6.8 Hz, "*ipso*" PO$\underline{Ph}$), 121.06, 121.02, 120.81, 120.77, 114.21, 114.16 ($\underline{C}$HAr), 93.67, 93.37 ($\underline{C\text{-}1}$), 73.11 (d, $^4J_{C\text{-}P}$= 7.5 Hz, $\underline{C\text{-}4}$), 73.08 (d, $^4J_{C\text{-}P}$= 6.3 Hz, $\underline{C\text{-}4}$), 70.58, 70.37 (NH$\underline{C}$HCO), 70.14 ($O\underline{C}H_2Ph$), 68.40, 68.37 ($\underline{C\text{-}5}$), 68.04 (d, $^2J_{C\text{-}P}$= 5.3 Hz, $\underline{C\text{-}6}$), 67.88 (d, $^2J_{C\text{-}P}$= 5.4 Hz, $\underline{C\text{-}6}$), 62.27, 62.22 ($O\underline{C}H_2CH_3$), 56.02, 55.99 ($\underline{C\text{-}3}$), 54.56, 54.37 ($\underline{C\text{-}2}$), 44.15, 43.96 ($CH\underline{C}H_2CH$), 25.55, 25.46 ($\underline{C}H(CH_3)_2$), 23.19, 23.16, 22.56, 22.41, 22.13, 21.90 ($NHCO\underline{C}H_3$ and $CH(\underline{C}H_3)_2$) 14.46, 14.41 ($OCH_2\underline{C}H_3$) ppm. MS (ES+): *m/z* 571.21 $[M+Na]^+$

Methyl ((((2R,3S,4R,5S)-5-acetamido-3,4,6-trihydroxytetrahydro-2H-pyran-2-yl)methoxy)(4-methoxyphenoxy)phosphoryl)-D-prolinate (**8b**): Prepared according to

Standard procedure 3 in 32 %, yield as a mixture of R_P and S_P diastereoisomers as α and β anomers. Light yellow oil; 0.23 g. ^{31}P-NMR (202 MHz, $CDCl_3$): δ_P 2.36 (0.3P), 2.03 (1P), 1.90 (0.4P) ppm. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.13 (d, 2H, *J* = 9.0 Hz, CH-Ar), 6.89 (d, 2H, *J* = 9.0 Hz, CH-Ar), 5.04 (m, 1H, H-1), 4.55-51 (m, 1H, NHCHCO), 4.33-4.31 (m), 4.26-4.23 (m), 3.98-3.95 (m), 3.81-3.78 (m), 3.71 (s, 3H, $COOCH_3$), 3.69 (s, 3H, $PhOCH_3$), 3.41-3.33 (m), 2.19-2.13 (m, 2H, CH_2Pro), 1.99 (s, 3H, $NHCOCH_3$), 1.96-1.81 (m, 2H, CH_2Pro) ppm. ^{13}C-NMR (125 MHz, MeOD): δ_C 174.38 (C=O ester), 172.77 ($NHCOCH_3$), 156.94 ("*ipso*" $PhOCH_3$), 144.15 (d, $^2J_{C\text{-}P}$= 2.5 Hz, "*ipso*" POPh), 120.93 (d, $^3J_{C\text{-}P}$= 4.6 Hz, CH-POPh), 120.67 (d, $^3J_{C\text{-}P}$= 4.5 Hz, CH-POPh), 120.66, 114.33, (CHAr), 93.66, 93.33 (C-1), 68.95, 68.82, 67.05, 66.80, 60.87, 60.62, 54.68, 53.89, 51.34, 30.82 (d, $^2J_{C\text{-}P}$= 9.1 Hz, CH_2Pro), 24.81 (d, $^3J_{C\text{-}P}$= 9.0 Hz, CH_2Pro), 21.24 ($NHCOCH_3$) ppm. MS (ES+): *m/z* 541.17 $[M+Na]^+$

Isopropyl ((((2R,3S,4R,5S)-5-acetamido-3,4,6-trihydroxytetrahydro-2H-pyran-2-yl)methoxy)(4-methoxyphenoxy)phosphoryl)-L-alaninate (**8c**): Colorless oil; 42 %, 0.11 g. ^{31}P NMR (202 MHz, $CDCl_3$, mixture of R_P and S_P diastereoisomers as α and β anomers): δ_P 8.42 (0.2P), 8.33 (0.8P), 8.10 (1P) ppm. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 7.17-7.13 (m, 2H, CH-Ar), 6.89-6.87 (m, 2H, CH-Ar), 5.03 (d, 1H, *J* = 9.0 Hz, H-1), 4.98-4.93 (m, 1H, $CH(CH_3)_2$), 4.32-4.31 (m, 1H, H-2), 4.29-4.21 (m), 3.98-3.95 (m), 3.93-3.88 (m, 1H, NHCHCO), 3.80-3.77 (m), 3.70 (s, 1.5H, $PhOCH_3$), 3.69 (s, 1.5H, $PhOCH_3$), 3.62-3.55 (m, 2H, H-6), 1.99 (s, 3H, $NHCOCH_3$), 1.37-1.32 (m, 3H, $NHCHCH_3$), 1.23-1.20 (m, 6H, $CH(CH_3)_2$) ppm. ^{13}C-NMR (125 MHz, MeOD): δ_C 173.49, 173.44 (C=O ester), 172.76, ($NHCOCH_3$), 156.89 ("*ipso*" OPh), 144.38, 114.32 ("*ipso*" POPh), 121.01, 120.98, 114.21, 114.18 (CHAr), 93.79, 93.30 (C-1), 73.11 (d, $^4J_{C\text{-}P}$= 7.5 Hz, C-4), 73.08 (d, $^4J_{C\text{-}P}$= 6.3 Hz, C-4), 70.58, 70.37 (NHCHCO), 68.40, 68.37 (C-5), 68.04 (d, $^2J_{C\text{-}P}$= 5.3 Hz, C-6), 67.88 (d, $^2J_{C\text{-}P}$= 5.4 Hz, C-6), 62.27, 62.22 (OCH_2CH_3), 56.02, 55.99 (C-3), 54.56, 54.37 (C-2), 44.15, 43.96 ($CHCH_2CH$), 25.55, 25.46

1 ($\underline{C}H(CH_3)_2$), 23.19, 23.16, 22.56, 22.41, 22.13, 21.90 ($NHCO\underline{C}H_3$ and $CH(\underline{C}H_3)_2$) 14.46, 14.41

2 ($OCH_2\underline{C}H_3$) ppm. MS (ES+): *m/z* 543.18 $[M+Na]^+$

3 **Synthesis of (3*S*,4*R*,5*S*,6*R*)-3-Acetamido-6-(hydroxymethyl)tetrahydro-2H-pyran-2,4,5-**

4 **triyl triacetate (11)*:*** *N*-acetyl-D-mannosamine (22.6 mmol, 5 g) was dissolved in pyridine (73

5 mL) and trityl chloride (24.86 mmol, 6.93 g) was added to the mixture under argon atmosphere.

6 The reaction mixture was stirred at 60 °C and monitored by TLC. After two hours, the mixture

7 was concentrated in vacuo and extracted with ethyl acetate (3 x 50 mL), washed with brine and

8 dried with Na_2SO_4. The product **9** was used in the next step without any further purification.

9 To a stirred suspension of compound **9** (22.6 mmol) in pyridine (15 mL), acetic anhydride (12

10 mL) was added at 0 °C under protected atmosphere. The reaction mixture was allowed to warm

11 at rt and stirred for 12 h. The solvent was evaporated in vacuo, then the reaction crude was

12 extracted with DCM (3 x 20 mL) and purified by column chromatography (EtOAc/hexane

13 75:25). Compound **10** was isolated as light yellow powder; 65 %; 8.65 g. ^{1}H-NMR (500 MHz,

14 $CDCl_3$, mixture of diastereoisomers of α and β anomers, 1:0.3): δ_H 7.44-7.41 (m, 6H, Ph),

15 7.32-7.29 (m, 6H, Ph), 7.25-7.22 (m, 3H, Ph), 6.11 (d, 0.7H, *J* = 1.9 Hz, $N\underline{H}_\alpha$), 5.85 (d, 0.3H,

16 *J* = 1.7 Hz, $N\underline{H}_\beta$), 5.81 (d, 0.3H, *J* = 9.1 Hz, $\underline{H\text{-}1_\beta}$), 5.77 (d, 0.7H, *J* = 9.3 Hz, $\underline{H\text{-}1_\alpha}$), 5.36-5.25

17 (m, 1H, H-4, 0.7H, $\underline{H\text{-}3_\alpha}$), 4.98 (dd, 0.3H, *J* = 3.9, 9.9 Hz, $\underline{H\text{-}3_\beta}$), 4.76-4.73 (m, 0.3 H, $\underline{H\text{-}2_\beta}$),

18 4.64-4.61 (m, 0.7H, $\underline{H\text{-}2_\alpha}$), 3.92-3.89 (m, 0.7H, $\underline{H\text{-}5_\alpha}$), 3.70-3.64 (m, 0.3H, $\underline{H\text{-}5_\beta}$), 3.38-3.33

19 (m, 1H, H-6), 3.09 (dd, 0.3H, *J* = 4.1, 10.6 Hz, $\underline{H\text{-}6_\beta}$), 3.05 (dd, 0.7H, *J* = 3.8, 10.6 Hz, $\underline{H\text{-}6_\alpha}$),

20 2.16 (s, 2.1H, $OCOC\underline{H}_{3\alpha}$), 2.13 (s, 0.9H, $OCOC\underline{H}_{3\beta}$), 2.11 (s, 0.9H, $OCOC\underline{H}_{3\beta}$), 2.09 (s, 2.1H,

21 $OCOC\underline{H}_{3\alpha}$), 1.99 (s, 2.1H, $OCOC\underline{H}_{3\alpha}$), 1.98 (s, 0.9H, $OCOC\underline{H}_{3\beta}$), 1.78 (s, 0.9H, $NHCOC\underline{H}_{3\beta}$),

22 1.77 (s, 2.1H, $NHCOC\underline{H}_{3\alpha}$) ppm. Afterwards, a mixture of compound **10** (3.4 mmol, 2.0 g) in

23 80% aqueous acetic acid (46 mL) was warmed up to 60 °C and stirred for 4 h. Next, the reaction

24 mixture was concentrated under vacuum and the resulting residue was purified by column

chromatography to give the expected compound **11**. White powder; 75 %; 1.09 g. ^{1}H-NMR (500 MHz, CD_3OD, mixture of diastereoisomers of α and β anomers, 1:0.3): δ_H 5.95 (d, 0.7H, J = 1.7 Hz, H-1$_{\alpha}$), 5.92 (d, 0.3H, J = 1.7 Hz, H-1$_{\beta}$), 5.32 (t, 0.7H, J = 10.1 Hz, H-3$_{\alpha}$), 5.27-5.24 (m, 1H, H-4), 5.15-5.12 (m, 0.3H, H-3$_{\beta}$), 4.72-4.70 (m, 0.3H, H-2$_{\beta}$), 4.57-4.56 (m, 0.7H, H-2$_{\alpha}$), 3.92-3.88 (m, 0.7H, H-5$_{\alpha}$), 3.72-3.60 (m, 0.3H, H-5$_{\beta}$, 2H, H-6), 2.16 (s, 2.1 H, OCOCH$_{3\alpha}$), 2.06-2.05 (m, 4.8H, 3 × OCOCH$_3$), 2.03 (s, 2.1H, OCOCH$_{3\alpha}$), 1.95 (s, 3H, NHCOCH$_3$) ppm.

Standard Procedure 4. **General Synthesis of Phosphoramidates (12a-j):** To a solution of compound **11** (1 equiv) in anhydrous THF was added *t*BuMgCl (1.5 equiv) at 0 °C under argon atmosphere. After 30 min, a solution of an appropriate phosphorochloridates **3a-j** (1.5 equiv) in dry THF was added dropwise to the mixture at the same temperature. Then, the reaction was allowed to reach room temperature and stirred for 12 h. The solvent was evaporated in vacuo and the crude residues were purified by column chromatography (DCM/CH_3OH 98:2) to give compounds **12a-j** as mixture of S_p and R_p diastereoisomers of α and β anomers.

(3S,4R,5S,6R)-3-Acetamido-6-((((((S)-1-isopropoxy-1-oxopropan-2-yl)amino)(phenoxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12a): Light yellow powder; 68%; 0.63 g. ^{31}P-NMR (202 MHz, CD_3OD, mixture of R_P and S_P diastereoisomers as α and β anomers): δ_P 3.83 (0.5P), 3.76 (1P), 3.55 (0.5P), 3.35 (0.2P) ppm. ^{1}H-NMR (500 MHz, CD_3OD): δ_H 7.40-7.37 (m, 2H, CH-Ph), 7.27-7.21 (m, 3H, CH-Ph), 6.00-5.97 (m, 1H, H-1), 5.40-5.28 (m, 1.7H, H-3 and H-4), 5.20-5.18 (m, 0.3H, H-3), 5.04-4.97 (m, 1H, CH(CH_3)$_2$), 4.77-4.76 (m, 0.3H, H-2), 4.61-4.60 (m, 0.7H, H-2), 4.35-4.13 (m, 3H, 2H-6 and NHCHCO), 3.97-3.89 (m, 1H, H-5), 2.19-1.99 (m, 12H) (3 × OCOCH$_3$ and NHCOCH$_3$), 1.39-1.34 (m, 3H, NHCHCH$_3$), 1.28-1.24 (m, 6H, CH(CH$_3$)$_2$) ppm. ^{13}C-NMR (125 MHz, CD_3OD): δ_C 173.22 (d, $^{3}J_{C\text{-}P}$= 5.2 Hz, C=O ester), 172.94 (d, $^{3}J_{C\text{-}P}$= 4.7 Hz, C=O ester), 172.44 (d, $^{3}J_{C\text{-}P}$= 4.7 Hz, C=O ester), 170.24 (d, $^{3}J_{C\text{-}P}$= 4.4 Hz, C=O ester), 170.15, 170.12, 170.05,

170.00, 168.71, 168.62, 168.60 ($NH\underline{C}OCH_3$ and $O\underline{C}OCH_3$), 150.81 (d, $^2J_{C\text{-}P}$= 7.0 Hz, "*ipso*" O$\underline{Ph}$), 129.39, 129.32, 124.75, 124.72, 120.19, 120.03 ($\underline{C}$H-Ph), 91.75, 91.73, 90.72, 90.68 ($\underline{C\text{-}1}$), 71.37, 71.12 ($NH\underline{C}HCO$), 69.31, 69.23 ($\underline{C\text{-}3}$), 68.80, 68.75 ($\underline{C}H(CH_3)_2$), 65.56, 65.49 ($\underline{C\text{-}6}$), 65.40, 65.31 ($\underline{C\text{-}4}$), 50.38 ($\underline{C\text{-}5}$), 49.13, 48.46 ($\underline{C\text{-}2}$), 21.03, 20.99 ($NHCO\underline{C}H_3$), 20.62, 20.58, 20.51 ($CH(\underline{C}H_3)_2$), 19.32, 19.25 ($OCO\underline{C}H_3$), 19.02, 18.97 ($NHCH\underline{C}H_3$) ppm. HPLC: (gradient H_2O/ACN from 90/10 to 0/100 in 30 min, flow: 1 mL/min, λ = 210 nm): t_R 21.29 min, 21.70 min. MS (ES+): *m/z* 639.20 $[M+Na]^+$

(3S,4R,5S,6R)-3-acetamido-6-((((((S)-1-ethoxy-4-methyl-1-oxopentan-2-yl)amino)(phenoxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12b): Prepared according to *Standard procedure 4* in 43 %, yield as a mixture of R_P and S_P diastereoisomers as α and β anomers. White powder; 360 mg. ^{31}P-NMR (202 MHz, CD_3OD): δ_P 3.81 (0.6P), 3.71 (1P), 3.65 (0.4P) ppm. ^{1}H-NMR (500 MHz, CD_3OD): δ_H 7.38-7.34 (m, 2H, C$\underline{H}$-Ph), 7.22-7.18 (m, 3H, C$\underline{H}$-Ph), 5.97-5.96 (m, 1H, $\underline{H\text{-}1}$), 5.40-5.16 (m, 1.8H, $\underline{H\text{-}3}$ and $\underline{H\text{-}4}$), 5.20-5.16 (m, 0.2H, $\underline{H\text{-}3}$), 4.75-4.74 (m, 0.2H, $\underline{H\text{-}2}$), 4.58-4.57 (m, 0.8H, $\underline{H\text{-}2}$), 4.33-4.01 (m, 5H, $\underline{H\text{-}6}$, $OC\underline{H_2}CH_3$ and $NHC\underline{H}CO$), 3.88-3.83 (m, 1H, $\underline{H\text{-}5}$), 2.17-1.97 (m, 3 × $OCOC\underline{H_3}$ and $NHCO\underline{CH_3}$), 1.75-1.58 (m, 1H, $C\underline{H}(CH_3)_2$), 1.55-1.48 (m, $CHC\underline{H_2}CH$), 1.27-1.23 (q, 3H, *J* = 6.55 Hz, $OCH_2C\underline{H_3}$), 0.93-0.82 (m, 6H, $CH(C\underline{H_3})_2$) ppm. ^{13}C-NMR (125 MHz, CD_3OD): δ_C 175.52 (d, $^3J_{C\text{-}P}$= 2.9 Hz, $\underline{C}$=O ester), 175.46 (d, $^3J_{C\text{-}P}$= 1.4 Hz, $\underline{C}$=O ester), 175.19 (d, $^3J_{C\text{-}P}$= 2.7 Hz, $\underline{C}$=O ester), 173.81 (d, $^3J_{C\text{-}P}$= 4.4 Hz, $\underline{C}$=O ester), 171.65, 171.59, 171.52, 171.39, 171.36 ($O\underline{C}OCH_3$), 170.10, 169.97 ($NH\underline{C}OCH_3$), 152.25, 152.19, 152.16, 152.15 ("*ipso*" O$\underline{Ph}$), 130.78, 130.71, 130.67, 126.14, 126.10, 126.05, 121.59 (d, $^3J_{C\text{-}P}$= 4.5 Hz, $\underline{C}$H-Ph), 121.28 (d, $^3J_{C\text{-}P}$= 4.9 Hz, $\underline{C}$H-Ph), 93.14, 93.11, 92.20, 92.17 ($\underline{C\text{-}1}$), 72.88, 72.60, 70.69, 70.58 ($\underline{C\text{-}5}$, $\underline{C\text{-}4}$, $\underline{C\text{-}3}$), 66.93, 66.90 ($O\underline{C}H_2CH_3$), 66.82 ($\underline{C\text{-}2}$), 62.30 (d, $^2J_{C\text{-}P}$= 10.0 Hz, $\underline{C\text{-}6}$), 54.60 (d, $^2J_{C\text{-}P}$= 4.9 Hz, $NH\underline{C}HCO$), 50.52 ($\underline{C}H(CH_3)_2$), 44.03 (d, $^3J_{C\text{-}P}$= 7.7 Hz, $CH\underline{C}H_2CH$), 25.57,

25.41, 23.26, 23.11, 22.39, 22.07, 21.71, 21.67, 20.72, 20.64, 14.47 (NHCO$\underline{C}H_3$, 3 ×OCO$\underline{C}H_3$, $OCH_2\underline{C}H_3$, and CH($\underline{C}H_3$)$_2$) ppm. HPLC: (gradient H_2O/ACN from 90/10 to 0/100 in 30 min, flow: 1 mL/min, λ = 210 nm): t_R 18.55 min, 18.96 min. MS (ES+): *m/z* 667.23 $[M+Na]^+$

(3S,4R,5S,6R)-3-acetamido-6-(((((R)-2-(methoxycarbonyl)pyrrolidin-1-yl)(phenoxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12c): Prepared according to *Standard procedure 4* in 16 %, yield as a mixture of R_P and S_P diastereoisomers as α and β anomers. White powder; 0.12 g. ^{31}P-NMR (202 MHz, CD_3OD) δ_P 1.75 (1P), 1.65 (0.4P), 1.32 (0.6P), 1.23 (0.1P) ppm. ^{1}H-NMR (500 MHz, CD_3OD): δ_H 7.39-7.35 (m, 2H, C$\underline{H}$-Ph), 7.28-7.19 (m, 3H, C$\underline{H}$-Ph), 5.98 (d, 0.3H, *J* = 1.9 Hz, $\underline{H\text{-}1}$), 5.97 (d, 0.7H, *J* = 1.8 Hz, $\underline{H\text{-}1}$), 5.37-5.26 (m, 1.7H, $\underline{H\text{-}3}$ and $\underline{H\text{-}4}$), 5.24-5.16 (m, 0.3H, $\underline{H\text{-}3}$), 4.75-4.74 (m, 0.3H, $\underline{H\text{-}2}$), 4.60-4.58 (m, 0.7H, $\underline{H\text{-}2}$), 4.41-4.10 (m, 4H, NHC$\underline{H}$CO, $\underline{H\text{-}5}$ and $\underline{2H\text{-}6}$), 3.72 (s, 0.9H, OC$\underline{H_3}$), 3.68 (s, 2.1H, OC$\underline{H_3}$), 3.40-3.37 (m, 2H, $\underline{H}$-Pro), 2.21-1.87 (m, 16H, $\underline{4H}$-Pro, 3 × OCOC$\underline{H_3}$ and NHCO$\underline{CH_3}$) ppm. ^{13}C-NMR (125 MHz, CD_3OD): δ_C 174.30, 174.08, 172.93, 172.30 ($\underline{C}$=O ester), 170.27, 170.22, 170.11, 170.01, 169.97, 169.93, 168.69, 168.60, 168.58 (NH$\underline{C}OCH_3$ and O$\underline{C}OCH_3$), 150.73 (d, $^2J_{C\text{-}P}$= 7.1 Hz, "*ipso*" O$\underline{Ph}$), 150.59 (d, $^2J_{C\text{-}P}$= 7.4 Hz, "*ipso*" O$\underline{Ph}$), 129.51, 129.44, 124.85, 124.82, 124.76, 119.99, 119.96, 119.85, 119.81 ($\underline{C}$H-Ph), 91.84, 91.77, 90.77, 90.62 ($\underline{C\text{-}1}$), 71.34, 71.24, 71.19 ($\underline{C\text{-}5}$), 69.31, 69.26 ($\underline{C\text{-}3}$), 65.55 (d, $^2J_{C\text{-}P}$= 5.5 Hz, $\underline{C\text{-}6}$), 65.35, 65.27, 65.17 ($\underline{C\text{-}4}$), 60.63 ($^2J_{C\text{-}P}$= 6.9 Hz, NH$\underline{C}$HCO), 60.35 ($^2J_{C\text{-}P}$= 6.3 Hz) (NH$\underline{C}$HCO), 51.41, 51.37 (COO$\underline{C}H_3$), 49.18, 49.12 ($\underline{C\text{-}2}$), 47.35, 47.31 (N$\underline{C}H_2CH_2$, overlap with the solvent), 30.95, 30.87, 30.80, 30.72 ($NCH_2\underline{C}H_2$), 24.87, 24.79, 24.72 (NCH$\underline{C}H_2$), 21.02, 20.99 (NHCO$\underline{C}H_3$), 19.32, 19.30, 19.29, 19.27, 19.24, 19.23 (OCO$\underline{C}H_3$) ppm. HPLC: (gradient H_2O/ACN from 90/10 to 0/100 in 30 min, flow: 1 mL/min, λ = 210 nm): t_R 15.80 min. MS (ES+): *m/z* 637.19 $[M+Na]^+$

(3S,4R,5S,6R)-3-acetamido-6-(((((2-methoxy-2-oxoethyl)amino)(naphthalen-1-

yloxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12d): Prepared according to *Standard procedure 4* in 49 %, yield as a mixture of R_P and S_P diastereoisomers as α and β anomers. Yellow powder; 0.53 g. ^{31}P-NMR (202 MHz, CD_3OD): δ_P 5.57 (0.9P), 5.52 (0.4P), 4.75 (1P), 4.60 (0.3P) ppm. ^{1}H-NMR (500 MHz, CD_3OD): δ_H 8.20-8.17 (m, 1H, Naph), 7.89 (d, 1H, *J* = 8.0 Hz, Naph), 7.71 (d, 1H, *J* = 8.0 Hz, Naph), 7.59-7.43 (m, 4H, Naph), 6.00 (d, 0.8H, *J* = 1.7 Hz, H-1), 5.97 (d, 0.2H, *J* = 1.7 Hz, H-1), 5.40-5.28 (m, 1.8H, H-3 and H-4), 5.21-5.16 (m, 0.2H, H-3), 4.75 (ddd, 0.2H, *J* = 1.9, 4.4, 9.6 Hz, H-2), 4.59 (ddd, 0.8H, *J* = 1.9, 4.4, 9.6 Hz, H-2), 4.45-4.25 (m, 2H, H-6), 4.19-4.14 (m, 0.8H, H-5), 4.05-4.02 (m, 0.1H, H-5), 3.97-3.93 (m, 0.1H, H-5), 3.97-3.93 (m, 2H, $NHCH_2CO$), 3.69-3.67 (m, 3H, $COOCH_3$), 2.15-1.96 (m, 12H, 3 × $OCOCH_3$ and $NHCOCH_3$) ppm. ^{13}C-NMR (125 MHz, CD_3OD): δ_C 174.43 (C=O ester) 170.22, 170.08, 168.78, 168.65, 168.61 ($NHCOCH_3$ and $OCOCH_3$), 146.51, 134.91, 127.86 ("*ipso*" ONaph, Naph-C10 and Naph-C9), 127.43, 126.36, 126.11, 125.23, 124.60, 121.46 (Naph), 121.35 (d, $^3J_{C\text{-}P}$= 6.9 Hz, Naph-C1), 115.07 (d, $^3J_{C\text{-}P}$= 3.9 Hz, Naph-C1), 114.95 (d, $^3J_{C\text{-}P}$= 3.3 Hz, Naph-C1), 91.79, 91.73, 90.71 (C-1), 71.30 ($^3J_{C\text{-}P}$= 6.4 Hz, C-5), 71.22 ($^3J_{C\text{-}P}$= 6.4 Hz, C-5), 71.12 (C-5), 69.21 (C-3), 65.63 (d, $^2J_{C\text{-}P}$= 5.3 Hz, C-6), 65.50 (d, $^3J_{C\text{-}P}$= 5.7 Hz, C-6), 65.29 – 65.27 (C-4), 51.26 (OCH_3), 49.18 (C-2), 42.39, 42.33, 42.26 ($NHCH_2CO$), 21.00, 20.97 ($NHCOCH_3$), 19.28, 19.25 ($OCOCH_3$) ppm. HPLC: (gradient H_2O/ACN from 90/10 to 0/100 in 30 min, flow: 1 mL/min, λ = 210 nm): t_R 20.19 min, 20.77 min. MS (ES+): *m/z* 647.17 $[M+Na]^+$

(3S,4R,5S,6R)-3-acetamido-6-((((((S)-1-ethoxy-1-oxo-3-phenylpropan-2-yl)amino)(phenoxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12e): Prepared according to *Standard procedure 4* in 45 %, yield as a mixture of R_P and S_P diastereoisomers as α and β anomers. Yellow powder; 0.53 g. ^{31}P-NMR (202 MHz, CD_3OD): δ_P 3.40 (1P), 3.21 (0.3P), 3.02 (0.1P) ppm. ^{1}H-NMR (500 MHz, CD_3OD): δ_H 7.33-7.15 (m,

1 8H, CH-Ar), 7.11, 7.05 (m, 2H, CH-Ar), 5.96 (d, 1H, J = 1.7 Hz, H-1), 5.93 (d, 1H, J = 1.7 Hz,

2 H-1), 5.33-5.14 (2H, m, H-3 and H-4), 4.74-4.02 (m, 6H, H-5 and 2H-6 and OCH_2CH_3 and

3 NHCHCO), 3.05-3.01 (m, 1H, $CHCH_2Ph$), 2.95-2.91 (m, 1H, $CHCH_2Ph$), 2.15-1.96 (m, 12H,

4 3 × $OCOCH_3$ and $NHCOCH_3$), 1.19-1.15 (m, OCH_2CH_3) ppm. ^{13}C-NMR (125 MHz, CD_3OD):

5 δ_C 174.26, 174.05, 173.80, 171.64, 171.59, 171.46, 171.320, 170.08, 170.01 (C=O ester,

6 $NHCOCH_3$ and 3 × $OCOCH_3$), 152.04 (d, $^2J_{C-P}$= 6.2 Hz, "ipso" OPh), 137.95 ("*ipso*" CH_2Ph),

7 130.73, 130.69, 130.55, 129.50, 127.96, 127.90, 127.90, 126.06, 121.40, 121.38, 121.35 (CH-

8 Ar), 93.13, 92.16, 92.05 (C-1), 72.72, 72.61, 72.44 (d, $^2J_{C-P}$= 6.3 Hz, NHCHCO), 70.71, 70.63

9 (C-3), 66.70 (C-4), 66.65 (d, $^2J_{C-P}$= 5.5 Hz, C-6), 66.45 (d, $^2J_{C-P}$= 5.4 Hz, C-6), 62.41, 62.33

10 (OCH_2CH_3), 57.80, 57.79, 57.70 (C-5), 50.74, 50.67, 50.52 (C-2), 22.39, 22.36 ($NHCOCH_3$),

11 20.70, 20.64, 20.62 ($OCOCH_3$), 14.36 (d, J = 3.8 Hz, OCH_2CH_3) ppm. HPLC: (gradient

12 H_2O/ACN from 90/10 to 0/100 in 30 min, flow: 1 mL/min, λ = 210 nm): t_R 23.11 min, 23.44

13 min, 23.79 min, 24.23 min. MS (ES+): *m/z* 701.21 $[M+Na]^+$

14 (3S,4R,5S,6R)-3-acetamido-6-((((((S)-1-(benzyloxy)-1-oxopropan-2-yl)amino)(naphthalen-

15 1-yloxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12f): Prepared

16 according to *Standard procedure 4* in 47 %, yield as a mixture of R_P and S_P diastereoisomers

17 as α and β anomers. Yellow powder; 0.58 g. ^{31}P-NMR (202 MHz, CD_3OD): δ_P 4.11 (1P), 3.81

18 (0.8P), 3.52 (0.2P) ppm. ^{1}H-NMR (500 MHz, CD_3OD): δ_H 8.24-7.24 (12H, m, CH-Ar), 6.03

19 (d, 1H J = 1.6 Hz, H-1), 6.00 (d, 1H, J = 1.6 Hz, H-1), 5.98 (d, 1H, J = 1.6 Hz, H-1), 5.96 (d,

20 1H, J = 1.6 Hz, H-1), 5.50-5.17 (m, 2H, H-3 and H-4), 5.15-5.04 (m, 2H, CH_2Ph), 4.78-4.61

21 (m, 1H, H-2), 4.44-3.90 (m, 4H, H-5 and 2H-6 and NHCH), 1.17-1.95 (12H, m, 3 × $OCOCH_3$

22 and $NHCOCH_3$), 1.37 (3H, dd, J = 18.7, 7.4 Hz, $CHCH_3$) ppm. ^{13}C-NMR (125 MHz, CD_3OD):

23 δ_C 174.99, 174.85, 173.87, 173.81 (C=O ester), 171.63, 171.51, 171.45, 171.38, 170.04, 170.00

24 ($NHCOCH_3$ and $OCOCH_3$), 147.98, 147.93 ("*ipso*" Naph), 137.19, 136.26, 129.54, 129.27,

1 129.17, 128.88 , 127.90 127.73, 127.51, 127.46, 126.59, 125.95, 125.91, 122.85, 122.78,
2 116.42 (CH-Ar), 93.13, 92.17, 92.09 (C-1), 66.80, 66.65 (C-4), 51.90, 51.76 (C-5), 50.63,
3 50.51 (C-2), 22.63 (NHCOCH_3), 20.68, 20.64 (OCOCH_3), 20.31, 20.25 ppm. HPLC: (gradient
4 H_2O/ACN from 90/10 to 0/100 in 30 min, flow: 1 mL/min, λ = 210 nm): t_R 25.20 min, 25.45
5 min, 25.39 min, 26.25 min. MS (ES+): *m/z* 737.23 $[M+Na]^+$
6 (3S,4R,5S,6R)-3-acetamido-6-((((((S)-1-(benzyloxy)-3-methyl-1-oxobutan-2-
7 yl)amino)(phenoxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12g):
8 Prepared according to *Standard procedure 4* in 40 %, yield as a mixture of R_P and S_P
9 diastereoisomers as α and β anomers. Yellow powder; 0.39 g. ^{31}P-NMR (202 MHz, CD_3OD):
10 δ_P 4.30 (1P), 4.14 (0.2P), 3.96 (0.1P) ppm. ^{1}H-NMR (500 MHz, CD_3OD): δ_H 7.41-7.33 (m, 7H,
11 CH-Ar), 7.25-7.17 (m, 3H, CH-Ar), 6.00 (d, 0.5H, *J* = 1.5 Hz, H-1), 5.97 (d, 0.5H, *J* = 1.7 Hz,
12 H-1), 5.38-5.26 (m, 1.7H, H-4 and H-3), 5.23-5.14 (m, 2.3H, OCH_2Ph and H-3), 4.80-4.77 (m,
13 0.3H, H-2), 4.63-4.61 (m, 0.7H, H-2), 4.24-4.19 (m, 2H, H-6), 4.16-4.14 (m, 0.7H, H-5), 3.94-
14 3.91 (m, 0.3H, H-5), 3.78-3.74 (m, 0.3H, NHCHCO), 3.73-3.69 (m, 0.7H, NHCHCO), 2.17-
15 1.99 (m, 13H, 3 × OCOCH_3, CH$(CH_3)_2$ and NHCOCH_3), 0.93-0.87 (m, 6H, CH$(CH_3)_2$) ppm.
16 ^{13}C-NMR (125 MHz, CD_3OD): δ_C 172.85, 172.69, 172.41, 172.37 (C=O ester), 170.29, 170.22,
17 170.12, 170.09, 170.05, 170.01, 168.71, 168.65, 168.61 (NHCOCH_3 and OCOCH_3), 150.80
18 (d, $^2J_{C\text{-}P}$= 7.1 Hz, ("*ipso*" OPh), 150.77 (d, $^2J_{C\text{-}P}$= 7.1 Hz, "*ipso*" OPh), 135.81, 135.77 ("*ipso*"
19 OCH_2Ph), 129.41, 129.37, 129.33, 128.26, 128.23, 128.20, 128.16, 128.11, 128.06, 124.73,
20 124.71, 120.25, 120.22, 120.19, 120.09, 120.05, 120.02, 119.99 (CH-Ar), 91.75, 90.82 (C-1),
21 73.77, 73.65, 71.39, 71.25, 71.22, 71.17, 69.32, 69.23 (C-3 and C-5), 66.65, 66.60, 66.50
22 (OCH_2Ph), 65.63 (d, $^2J_{C\text{-}P}$= 5.7 Hz, C-6), 65.44, 65.41 (C-4), 60.66 (d, $^2J_{C\text{-}P}$= 7.6 Hz,
23 NHCHCO), 60.49 (d, $^2J_{C\text{-}P}$= 5.1 Hz, NHCHCO), 49.41, 49.34, 49.27, 49.16 (C-2), 31.79 (d,
24 $^3J_{C\text{-}P}$= 7.4 Hz, CH$(CH_3)_2$), 31.70 (d, $^3J_{C\text{-}P}$= 7.4 Hz, CH$(CH_3)_2$), 21.16, 21.11, 19.43, 19.35

(NHCO$\underline{C}H_3$ and OCO$\underline{C}H_3$), 18.17, 17.14 (CH($\underline{C}H_3$)$_2$) ppm. HPLC: (gradient H_2O/ACN from 90/10 to 0/100 in 30 min, flow: 1 mL/min, λ = 210 nm): t_R 25.23 min, 25.49 min, 25.90 min. MS (ES+): *m/z* 715.24 $[M+Na]^+$

(3S,4R,5S,6R)-3-acetamido-6-((((((2S,3S)-1-ethoxy-3-methyl-1-oxopentan-2-yl)amino)(phenoxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12h): Prepared according to *Standard procedure 4* in 64 %, yield as a mixture of R_P and S_P diastereoisomers as α and β anomers. Yellow powder; 0.12 g. ^{31}P-NMR (202 MHz, CD_3OD): δ_P 4.22 (1P), 4.16 (0.2P) ppm. ^{1}H-NMR (500 MHz, CD_3OD): δ_H 7.38-7.35 (m, 2H, C$\underline{H}$-Ar), 7.24-7.18 (m, 3H, C$\underline{H}$-Ar), 5.97 (d, 0.8H, J = 2.1 Hz, $\underline{H\text{-}1}$), 5.95 (d, 0.2H, J = 1.9 Hz, $\underline{H\text{-}1}$), 5.36-5.16 (m, 2H, $\underline{H\text{-}4}$ and $\underline{H\text{-}3}$), 4.76-4.74 (m, 0.2H, $\underline{H\text{-}2}$), 4.59-4.58 (m, 0.8H, $\underline{H\text{-}2}$), 4.21-4.06 (m, 5H, $\underline{H\text{-}6}$, $\underline{H\text{-}5}$ and OC$\underline{H_2}$CH$_3$), 3.75-3.68 (m, 1H, NHC$\underline{H}$CO), 2.18-1.97 (m, 12H, 3 × OCOC$\underline{H_3}$ and NHCOC$\underline{H_3}$), 1.80-1.74 (m, 1H, CH$_3$C$\underline{H}$CH$_2$), 1.57-1.43 (m, 1H, CHC$\underline{H_2}$CH$_3$), 1.27-1.21 (m, 4H, OCH$_2$C$\underline{H_3}$ and CHC$\underline{H_2}$CH$_3$), 0.92-0.85 (m, 6H, C$\underline{H_3}$CHCH$_2$ and CHCH$_2$C$\underline{H_3}$) ppm. ^{13}C-NMR (125 MHz, CD_3OD): δ_C 173.00 (d, $^3J_{C\text{-}P}$= 3.3 Hz, $\underline{C}$=O ester), 172.88 (d, $^3J_{C\text{-}P}$= 3.3 Hz, $\underline{C}$=O ester), 172.82 (d, $^3J_{C\text{-}P}$= 3.3 Hz, $\underline{C}$=O ester), 172.43 (d, $^3J_{C\text{-}P}$= 4.0 Hz, $\underline{C}$=O ester), 170.27, 170.21, 170.15, 170.08, 170.02, 169.99, 168.66, 168.59, 168.56 (NH$\underline{C}$OCH$_3$ and O$\underline{C}$OCH$_3$), 150.82 (d, $^2J_{C\text{-}P}$= 6.7 Hz, "*ipso*" O$\underline{Ph}$), 150.80 (d, $^2J_{C\text{-}P}$= 7.4 Hz, "*ipso*" O$\underline{Ph}$), 129.38, 129.33, 124.74, 124.68, 120.17 (d, $^3J_{C\text{-}P}$= 4.5 Hz, $\underline{C}$HPh), 120.01 (d, $^3J_{C\text{-}P}$= 4.6 Hz, $\underline{C}$HPh), 119.94 (d, $^3J_{C\text{-}P}$= 4.7 Hz, $\underline{C}$HPh), 91.72, 90.77 ($\underline{C\text{-}1}$), 71.18 (d, $^3J_{C\text{-}P}$= 6.9 Hz, $\underline{C\text{-}5}$), 69.29, 69.19 ($\underline{C\text{-}3}$), 65.56, 65.52 (O$\underline{C}$H$_2$CH$_3$), 65.39 ($\underline{C\text{-}4}$), 60.86, 60.77 ($\underline{C\text{-}6}$), 59.35 (NH$\underline{C}$HCO), 49.14 ($\underline{C\text{-}2}$), 38.50, 38.43 (CH$_3$$\underline{C}HCH_2$), 24.67, 24.62, 24.48 (CH$\underline{C}$H$_2$CH$_3$), 21.01, 19.34, 19.29, 19.26 (NHCO$\underline{C}$H$_3$ and OCO$\underline{C}$H$_3$), 14.51, 14.41 ($\underline{C}$H$_3$CHCH$_2$), 13.14 (OCH$_2$$\underline{C}H_3$), 10.25, 10.17 (CHCH$_2$$\underline{C}H_3$) ppm. HPLC: (gradient H_2O/ACN from 90/10 to 0/100 in 30 min, flow: 1 mL/min, λ = 210 nm): t_R 17.36 min, 17.90 min, 18.32 min. MS (ES+): *m/z*

1 667.23 [M+Na]$^+$

2 (3S,4R,5S,6R)-3-acetamido-6-(((((1-(benzyloxy)-2-methyl-1-oxopropan-2-

3 yl)amino)(phenoxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12i):

4 Prepared according to *Standard procedure 4* in 34 %, yield as a mixture of R_P and S_P

5 diastereoisomers as α and β anomers. Yellow powder; 0.07 g. ^{31}P-NMR (202 MHz, CD_3OD):

6 δ_P 2.20 (1P), 1.89 (0.4P), 1.77 (0.1P) ppm. ^{1}H-NMR (500 MHz, CD_3OD): δ_H 7.39-7.29 (m,

7 7H, CH-Ar), 7.22-7.15 (m, 3H, CH-Ar), 5.97 (d, *J* = 1.6 Hz, 0.8H, H-1), 5.94-5.93 (0.2H, H-

8 1), 5.35-5.23 (m, 1.8H, H-4 and H-3), 5.22-5.11 (m, 2.2H, OCH_2Ph and H-3), 4.78-4.75 (m,

9 0.2H, H-2), 4.60-4.58 (m, 0.8H, H-2), 4.27-4.15 (m, 2H, H-6), 4.13-4.09 (m, 0.8H, H-5), 3.95-

10 3.91 (m, 0.1H, H-5), 3.90-3.86 (m, 0.1H, H-5), 2.13-1.96 (m, 12H, 3 × $OCOCH_3$ and

11 $NHCOCH_3$), 1.52-1.44 (m, 6H, CH_3CCH_3) ppm. ^{13}C-NMR (125 MHz, CD_3OD): δ_C 176.44 (d,

12 $^3J_{C-P}$= 3.4 Hz, C=O ester), 176.62 (d, $^3J_{C-P}$= 3.5 Hz, C=O ester), 174.35, 174.27, 174.80, 171.64,

13 171.57, 171.49, 171.46, 171.31, 170.06, 169.99 ($NHCOCH_3$ and $OCOCH_3$), 152.23 (d, $^2J_{C-P}$=

14 7.0 Hz, "*ipso*" OPh), 137.38 (d, $^2J_{C-P}$= 7.0 Hz, "*ipso*" OCH_2Ph), 130.73, 130.69, 129.57,

15 129.26, 129.18, 126.02, 121.57, 121.52, 121.49, 121.46 (CHAr), 93.13, 93.08, 92.15, 92.09

16 (C-1), 75.14 (d, $^3J_{C-P}$= 7.5 Hz, C-5), 74.91 (d, $^3J_{C-P}$= 7.6 Hz, C-5), 72.57, 72.50, 70.78, 70.56

17 (C-3), 68.24, 68.22 (OCH_2Ph), 66.83 (C-4), 66.70 (d, $^2J_{C-P}$= 5.6 Hz, C-6), 50.79, 50.68, 50.54,

18 50.51 (C-2), 27.63, 27.59, 27.53, 27.48, 27.34, 27.30, 27.20, 27.16 (CH_3CCH_3), 22.42, 22.39,

19 20.73, 20.69, 20.66 ($NHCOCH_3$ and $OCOCH_3$) ppm. HPLC: (gradient H_2O/ACN from 90/10

20 to 0/100 in 30 min, flow: 1 mL/min, λ = 210 nm): t_R 18.23 min, 18.57 min. MS (ES+): *m/z*

21 701.22 [M+Na]$^+$

22 (3S,4R,5S,6R)-3-acetamido-6-((((((S)-1-(benzyloxy)-4-methyl-1-oxopentan-2-

23 yl)amino)(phenoxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12j):

24 Prepared according to *Standard procedure 4* in 61 %, yield as a mixture of R_P and S_P

diastereoisomers as α and β anomers. Light yellow powder; 0.37 g. ^{31}P-NMR (202 MHz, CD_3OD): δ_P 3.73 (0.5P), 3.68 (1P), 3.57 (0.4P) ppm. ^{1}H-NMR (500 MHz, CD_3OD): δ_H 7.37-7.31 (m, 7H, CH-Ar), 7.20-7.17 (m, 3H, CH-Ar), 5.97-5.96 (m, 0.7H, H-1), 5.94-5.93 (m, 0.3H, H-1), 5.35-5.12 (m, 4H, H-3, H-4 and OCH_2Ph), 4.58-4.57 (m, 1H, H-2), 4.20-4.10 (m, 3H, H-6 and H-5), 3.94-3.89 (m, 1H, NHCHCO), 2.16-1.97 (m, 12H, 3 × $OCOCH_3$ and $NHCOCH_3$), 1.73-1.65 (m, 1H, $CH(CH_3)_2$), 1.49-1.46 (m, 2H, $CHCH_2CH$), 1.56-1.49 (m, 6H, $CH(CH_3)_2$) ppm. ^{13}C-NMR (125 MHz, CD_3OD): δ_C 173.82, 173.79, 171.65, 171.59 (C=O ester), 171.36, 169.98, ($OCOCH_3$ and $NHCOCH_3$), 152.12 (d, $^2J_{C-P}$= 6.5 Hz, "*ipso*" OPh), 137.13 ("*ipso*" OCH_2Ph), 130.76, 130.67, 129.54, 129.40, 129.32, 129.27, 126.06, 126.01 (CH-Ar), 121.56 (d, $^3J_{C-P}$= 4.6 Hz, CH-Ar), 121.25 (d, $^3J_{C-P}$= 4.8 Hz, CH-Ar), 93.10, 93.07, 92.15 (C-1), 72.53, 72.48 (C-5), 70.66, 70.55 (C-3), 67.94, 67.85 (OCH_2Ph), 67.91 (d, $^2J_{C-P}$= 10.0 Hz, C-6), 66.72, 66.70 (C-4,), 54.66, 54.60 (NHCHCO), 50.64, 50.48 (C-2), 43.87 (d, $^3J_{C-P}$= 7.9 Hz, $CHCH_2CH$), 43.72 (d, $^3J_{C-P}$= 7.6 Hz, $CHCH_2CH$), 25.50, 25.36 ($CH(CH_3)_2$), 23.14, 23.03, 22.39, 22.34, 22.04, 22.01 21.69, 20.69, 20.63, 20.60 ($NHCOCH_3$, 3 ×$OCOCH_3$ and $CH(CH_3)_2$) ppm. MS (ES+): *m/z* 729.25 $[M+Na]^+$

Triethyl ammonium ((2R,3S,4R,5S)-5-acetamido-3,4,6-triacetoxytetrahydro-2H-pyran-2-yl)methyl ((S)-1-carboxy-3-methylbutyl)phosphoramidate (13): Prepared according to the *Standard procedure* 3. Light yellow powder; 47%; 0.37 g. ^{31}P-NMR (202 MHz, CD_3OD, mixture α and β anomers): δ_P 6.53 (1P), 6.41 (0.3P) ppm. ^{1}H-NMR (500 MHz, CD_3OD): δ_H 5.93-5.90 (m, 1H, H-1), 529.-5.16 (m, 2H, H-3 and H-4), 4.53-4.52 (m, 1H, H-2, overlap with the solvent), 4.11-4.10 (m, 1H, NHCHCO), 3.91-3.61 (m, 3H, H-6 and H-5), 3.12 (q, 6H, J = 7.4 Hz, $N(CH_2CH_3)_3$), 2.13-1.95 (m, 12H, 3 × $OCOCH_3$ and $NHCOCH_3$), 1.70-1.61 (m, 1H, $CH(CH_3)_2$), 1.47-1.44 (m, 2H, $CHCH_2CH$), 1.20 (t, 9H, J = 7.3 Hz, $N(CH_2CH_3)_3$), 0.88-0.83 (m, 6H, $CH(CH_3)_2$) ppm. ^{13}C-NMR (125 MHz, CD_3OD): δ_C 174.63, (C=O ester), 172.98,

1 172.78, 171.72, 171.54 (OCOCH$_3$ and NHCOCH$_3$), 91.97, 90.91 (C-1), 70.98, 70.92
2 (NHCHCO), 70.07, 70.02 (C-3), 65.48, (C-4), 62.37 (d, $^2J_{C-P}$= 4.6 Hz, C-6), 53.41 (C-5), 49.15,
3 49.11 (C-2), 46.65 (N(CH$_2$CH$_3$)$_3$), 42.93 (d, $^3J_{C-P}$= 3.4 Hz, CHCH$_2$CH), 24.11 (CH(CH$_3$)$_2$),
4 22.04, 21.94, 21.68, 21.37, 21.31, 20.85, 20.24, 20.22, 20.13 (NHCOCH$_3$, 3 ×OCOCH$_3$ and
5 CH(CH$_3$)$_2$), 8.20 (N(CH$_2$CH$_3$)$_3$) ppm. LC-MS: (gradient H$_2$O/ACN from 90/10 to 0/100 in 30
6 min, flow: 1 mL/min): t_R 2.5 min, 3.7 min. MS (ES-): *m/z* 574.16 [M-H]$^-$
7 **Enzymatic Activation Studies:** All phosphoramidates **12a-i** were analyzed for the enzymatic
8 activation in the presence of carboxypeptidase Y. To a solution of compound **12a-i** (5 mg) in
9 [D$_6$]acetone (150 µL),was added 0.05M Trizma buffer (pH=7.6, 300 µL) and ^{31}P-NMR was
10 recorded at 25 °C. A thawed solution of carboxypeptidase Y (0.1 mg in 150 µL of 0.05M
11 Trizma buffer) was added to the NMR sample and further analyzed by ^{31}P-NMR at 25 °C.
12 Afterwards, spectra were recorded every 3 hours and the resulting data were processed and
13 analyzed with Bruker Topspin 3.5 program and the estimated half-life was calculated. All
14 processed compounds were analyzed by mass and all active species were detected.
15 **Caco-2 Assay:** Caco-2 cells purchased from ATCC were seeded onto polyethylene membranes
16 (PET) in 96-well BD Insert plates at 1 x 105 cells/cm^2 and refreshed medium every 4~5 days
17 until to the 21st to 28th day for confluent cell monolayer formation. The transport buffer in the
18 study was HBSS with 10 mM HEPES at pH 7.40±0.05. Test compound was tested at 2 µM in
19 presence and absence of 10 µM GF120918 bi-directionally in duplicate. Digoxin was tested at
20 10 µM in presence and absence of 10 µM GF120918 bi-directionally in duplicate, while nadolol
21 and metoprolol were tested at 2 µM in absence of 10 µM GF120918 in A to B direction in
22 duplicate. Final DMSO concentration was adjusted to less than 1%. The plate was incubated
23 for 2 hours in CO$_2$ incubator at 37±1°C, with 5% CO2 at saturated humidity without shaking.
24 And all samples after mixed with acetonitrile containing internal standard were centrifuged at

1 4000 rpm for 10 min. Subsequently,100 µL supernatant solution was diluted with 100 µL
2 distilled water for LC/MS/MS analysis. Concentrations of test and control compounds in
3 starting solution, donor solution, and receiver solution were quantified by LC/MS/MS
4 methodologies, using peak area ratio of analyte/internal standard. After transport assay, lucifer
5 yellow rejection assay was applied to determine the Caco-2 cell monolayer integrity.
6 The apparent permeability coefficient P_{app} (cm/s) was calculated using the equation:
7 $$P_{app} = (dCr/dt) \times Vr / (A \times C0)$$
8 Where dCr/dt is the cumulative concentration of compound in the receiver chamber as a
9 function of time (µM/s); Vr is the solution volume in the receiver chamber (0.075 mL on the
10 apical side, 0.25 mL on the basolateral side); A is the surface area for the transport, i.e. 0.0804
11 cm^2 for the area of the monolayer; C_0 is the initial concentration in the donor chamber (µM).
12 The efflux ratio was calculated using the equation:
13 $$\text{Efflux Ratio} = P_{app}\ (BA) / P_{app}\ (AB)$$
14 Percent recovery was calculated using the equation:
15 $$\%\ \text{Recovery} = 100 \times [(Vr \times Cr) + (Vd \times Cd)] / (Vd \times C0)$$
16 Where V_d is the volume in the donor chambers (0.075 mL on the apical side, 0.25 mL on the
17 basolateral side); Cd and Cr are the final concentrations of transport compound in donor and
18 receiver chambers, respectively.
19 **Kinetic Solubility:** Test compounds (10 mM in DMSO 10 µL/well) were added into pH 7.4
20 NaH_2PO_4 buffer (490 µL/well) in a 96-well plate. The final concentration of test compound
21 and DMSO was 200 µM and 2%, respectively. Test solutions were then incubated on a shaker
22 at the speed of 600 rpm at room temperature. After 24 hours, 200 µL of each of solubility
23 solution was transferred into a new MultiScreen filter plate (polycarbonate membrane), filtered
24 by Millipore vacuum manifold and the filtrate was collected as test sample. Test compound
25 concentration was determined in the filtrate using a standard curve detected by HPLC-UV.

1 **Plasma Stability:** Pooled frozen plasma was thawed in a water bath at 37°C prior to
2 experiments. Plasma was centrifuged at 4000 rpm for 5 min and clots were removed, if any.
3 The pH was adjusted to 7.4 ± 0.1 if required. Compounds were prepared as 1 mM intermediate
4 solution by diluting 10 µL of the stock solution with 90 µL DMSO; 1 mM intermediate of
5 positive control Propantheline was prepared by diluting 10 µL of the stock solution with 90 µL
6 ultra-pure water; 100 µM dosing solution was prepared by diluting 10 µL of the intermediate
7 solution (1 mM) with 90 µL 45%MeOH/H_2O. 196 µL of blank plasma was spiked with 4 µL
8 of dosing solution (100 µM) to achieve 2 µM of the final concentration in duplicate and samples
9 were incubated at 37°C in a water bath. At each time point (0,10, 30, 60 and 120 min), 800 µL
10 of stop solution (200 ng/mL tolbutamide and 200 ng/mL Labetalol in 50% ACN/MeOH) was
11 added to precipitate protein and mixed thoroughly. Sample plates were then centrifuged at
12 4,000 rpm for 10 min. An aliquot of supernatant (100 µL) was transferred from each well and
13 mixed with 200 µL ultra-pure water. The samples were shaken at 800 rpm for about 10 min
14 before submitting to LC-MS/MS analysis.
15 The % remaining of test compound after incubation in plasma was calculated using following
16 equation: % Remaining= 100 x (PAR at appointed incubation time / PAR at T0 time), where
17 PAR is the peak area ratio of analyte versus internal standard (IS). The appointed incubation
18 time points are T_0 (0 min), T_n (n=0, 10, 30, 60, 120 min).
19 **Microsomal Stability:** Human liver microsomes were purchased from BD and were prepared
20 in solution at a final concentration of 0.5 mg protein/L in potassium phosphate buffer. The
21 NADPH regenerating system was isocitric dehydrogenase at a final concentration of 1 unit/mL
22 at incubation and the stop solution was cold ACN including 100 ng/mL Tolbutamide and 100
23 ng/mL Labetalol as internal standard (IS). Added 10 µL of compound (from 10 µM working
24 solution in buffer) or control to all plates (T0, T5, T10, T20, T30, T60, NCF60) except matrix
25 blank. Dispensed 680 µL/well microsome solution to 96-well plate as reservoir according to

1 the plate map, then added 80 μL/well to every plate by Apricot and incubated the mixture of

2 microsome solution and compound at 37°C for about 10 min. NADPH regenerating system was

3 then added. At each time point, 300 (μL/well) stop solution was added to terminate the reaction.

4 Sampling plates are then shaken for approximately 10 min. and samples were centrifuged at

5 4000 rpm for 20 min at 4°C. Transferred 100 μL supernatant to 96-well plate containing HPLC

6 water and mixed for LC/MS/MS.

7 Use equation of first order kinetics to calculate $T_{1/2}$ and $CL_{int(mic)}$:

8 $$C_t = C_0 \bullet e^{-k_e \bullet t}$$

when $C_t = \frac{1}{2} C_0$,

9 $$T_{1/2} = \frac{Ln2}{k_e} = \frac{0.693}{k_e}$$

10 $$CL_{int(mic)} = \frac{0.693}{\text{In vitro } T_{1/2}} \bullet \frac{1}{\text{mg / mL microsomal protein in reaction system}}$$

11 $$CL_{int(liver)} = CL_{int(mic)} \bullet \frac{\text{mg microsomes}}{\text{g liver}} \bullet \frac{\text{g liver}}{\text{kg body weight}}$$

12 **Cell Culture:** *Lec3* CHO cells and GNEM patient-derived myoblast cells were cultured in

13 FirmaLab Inc. *Lec3* CHO cells were grown in Dulbecco's Modified Eagle's Medium (DMEM)

14 supplemented with 5% Fetal Bovine Serum (FBS) and 1% antibiotic agent. GNEM patient-

15 derived myoblasts were grown in Nutrient Mixture Ham's F-10 supplemented with 20% FBS,

16 1% antibiotic agent, and 0.5% chick embryo extract (Accurate Chemical). Both cell lines were

17 detached from FBS prior to challenging them to compounds. Cells were incubated in a serum

18 free medium with 13b, 13c, ManNAc or media only control at 37°C in an atmosphere of 5%

19 CO_2 for 48 hours. Cell viability was determined before and after 48-hour incubation time.

20 Membrane protein fractions were extracted after the incubation period and sialic acid was

21 measured in all fractions. All cell culture reagents were obtained from Thermo Fisher

22 Scientific, except where otherwise indicated.

23 **Cell Viability Assay:** All cells and compound mixtures were treated the same. Each sample

24 was thoroughly mixed and a 1:2 dilution was made with Trypan Blue stain (Sigma Aldrich)

25 (20 μL of cell sample and 20 μL of 0.4% Trypan Blue) in a tube. The mixture was gently mixed,

10 µL of the mixture was then applied to the edge of a haemocytometer counting chamber between the cover slip and chamber. The mixture was drawn into the chamber by capillary action.

Cells were counted using a 10× objective. With the assumption that dead cells take up the stain while viable cells do not take up the stain, both viable (unstained) and dead (stained) cells were counted in each of the four corner quadrants. An average of these four readings was obtained and multiplied by 10^4 to obtain the number of viable cells per mL in the sample and doubled to account for the 1:2 dilution. Viability was calculated by taking percentage of viable cells over total number of cells (viable and dead).

Sialic Acid Quantification Assay: Cell-compound samples were pelleted after viability for extracting membrane protein. The Mem-PER plus membrane Protein Extraction Kit (Thermo Fisher Scientific) was used for protein extraction. The Mem-PER system consists of three reagents: Cell Wash Solution, Solubilization Buffer, and Permeabilization Buffer. Cell pellets were washed twice in 600 uL Cell Wash Solution. Supernatant was discarded. 150 uL Permeabilization Buffer was added to each cell pellet and vortexed to obtain a homogeneous cell suspension. Cells were incubated for 10 minutes at 4°C, then centrifuged for 15 minutes at 16,000 × g. Supernatant contained cytosolic proteins, this was a transferred to a new tube. 100 uL Solubilization Buffer was added to cell pellets and were resuspended. Samples were incubated at 4°C for 30 minutes, then centrifuged at 16,000 × g for 15 minutes. Supernatant containing solubilized membrane and membrane-associated proteins were transferred to a new tube. This fraction was used for measuring sialic acid.

Sialic acid concentration was measured with an enzymatic method using the EnzyChrom Sialic Acid Assay Kit (BioAssay Systems) on a Victor X3 plate reader (Perkin Elmer). Kit consists of 6 reagents: Assay Buffer, Enzyme, Dye Reagent, Hydrolysis Reagent, Neutralization Reagent and 10 mM Sialic Acid Standard. Sialic Acid Standard was diluted to make a standard

curve, curve points were treated as any other sample. Free and total sialic acid was measured for all samples. To measure total sialic acid, samples needed to be hydrolyzed. Samples were hydrolyzed by mixing 20 uL sample with 80 uL Hydrolysis reagent, this was incubated at 80°C for 60 minutes. 20 uL Neutralization reagent was added to mixture and briefly spun down to bring down volume. Sialic a[3]cid assay was done by preparing a working solution of 93 uL Assay buffer, 1 uL Dye reagent and 1 uL Enzyme for each sample. In a black 96-well plate, 10 uL of each sample were added to their corresponding position. 90 uL of working solution were added to 10 uL of each sample and incubated at room temperature for 60 minutes. After incubation period, fluorescence was read at λ_{ex} = 530 nm and λ_{em} = 585 nm. Sample readings were plotted against standard curve to determine sialic acid concentration. Hydrolyzed samples were multiplied by a factor of six to account for the dilution during the hydrolysis step.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website and include intermediate and final compounds characterization description, copies of ^{31}P ^{1}H, ^{13}C-NMR spectra,^{31}P deconvoluted spectra stacked plot of enzymatic reactions of compounds **12a-i**, LC-MS traces of compound **12b**, **13** and **12b** treated with CPY after 2h, cell viability bar Graphs and HPLC of compounds **12b** and **12c**. Molecular Formula String for compounds **12b** and **12c**.

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Notes

The authors declare the following competing financial interest(s): Patrick J. Crutcher and Dr. Stephen Thomas are respectively the former Chairman of the Board, President and the Chief Scientific Officer, co-founder of Ichorion therapeutics, which has provided financial support to the project. They serve now as Vice President, Business Development and Vice President, Head of Discovery at Cerecor respectively.

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ABBREVIATIONS

CDGs, Congenital Disorders of Glycosylation; GNEM, GNE myopathy; GNE, mutated gene (UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase); CPY, carboxylesterase Y; Clint, intrinsic clearance; GlcNAc, N-acetyl Glucosamine; ManNAc, N-

acetyl mannosamine; PMM2, phosphomannomutase 2;

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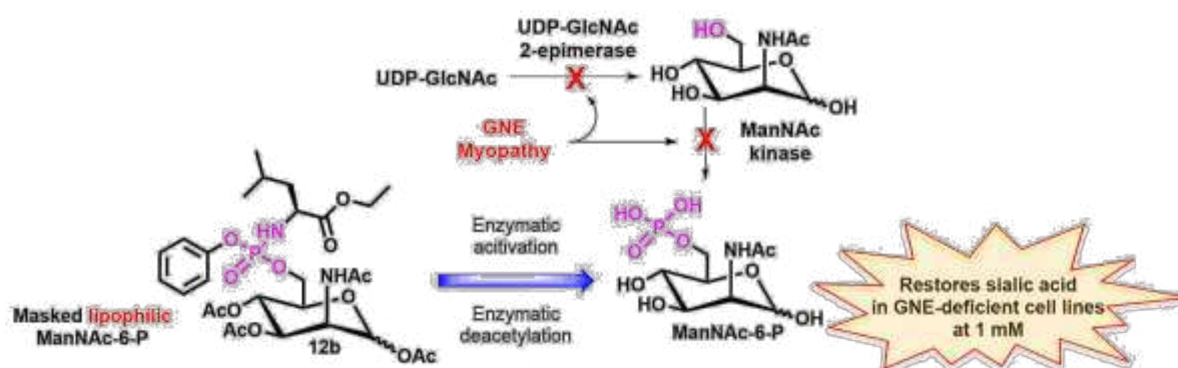
18 Table of Contents graphic



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