

Online Research @ Cardiff

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: <https://orca.cardiff.ac.uk/id/eprint/125281/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Morozzi, Chiara, Sedláková, Jana, Serpi, Michaela ORCID: <https://orcid.org/0000-0002-6162-7910>, Avigliano, Marialuce, Carbajo, Rosangela, Sandoval, Lucia, Valles-Ayoub, Yadira, Crutcher, Patrick, Thomas, Stephen and Pertusati, Fabrizio ORCID: <https://orcid.org/0000-0003-4532-9101> 2019. Targeting GNE myopathy: A dual prodrug approach for the delivery of N-acetylmannosamine 6-phosphate. *Journal of Medicinal Chemistry* 62 (17) , pp. 8178-8193. 10.1021/acs.jmedchem.9b00833 file

Publishers page: <http://dx.doi.org/10.1021/acs.jmedchem.9b00833>
<<http://dx.doi.org/10.1021/acs.jmedchem.9b00833>>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies.

See

<http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



1 Targeting GNE myopathy: A dual prodrug approach for the delivery of *N*-acetylmannosamine
2 6-phosphate.

3 Chiara Morozzi¹, Jana Sedláková¹, Michaela Serpi¹, Marialuce Avigliano¹, Rosangela
4 Carbajo², Lucia Sandoval², Yadira Valles-Ayoub², Patrick Crutcher³, Stephen Thomas³,
5 Fabrizio Pertusati^{1*}

6
7 ¹School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, King Edward
8 VII Avenue, Cardiff CF10 3NB, U.K.

9 ²FirmaLab Bio-Diagnostics, 21053 Devonshire St, 106, Chatsworth, CA 91311, USA

10 ³Cerecor Inc., 540 Gaither Rd, Suite 400, Rockville, MD 20850, USA

11

12 Abstract

13 ProTides comprise an important class of prodrugs currently marketed and developed as
14 antiviral and anticancer therapies. The ProTide technology employs a phosphate masking
15 groups capable of providing more favorable drug-like properties and an intracellular activation
16 mechanism for enzyme-mediated release of a nucleoside monophosphate. Herein we describe
17 the application of phosphoramidate chemistry to 1,3,4-*O*-acetylated *N*-acetylmannosamine
18 (Ac₃ManNAc) to deliver ManNAc-6-phosphate (ManNAc-6-P), a critical intermediate in sialic
19 acid biosynthesis. Sialic acid deficiency is a hallmark of GNE myopathy, a rare congenital
20 disorder of glycosylation (CDG) caused by mutations in *GNE* that limit the production of
21 ManNAc-6-P. Synthetic methods were developed to provide a library of Ac₃ManNAc-6-
22 phosphoramidates that were evaluated in a series of studies for their potential as a treatment
23 for GNE myopathy. Prodrug **12b** showed rapid activation in a carboxylesterase (CPY)
24 enzymatic assay and favorable ADME properties, while also being more effective than
25 ManNAc at increasing sialic acid levels in GNE-deficient cell lines than ManNAc. These

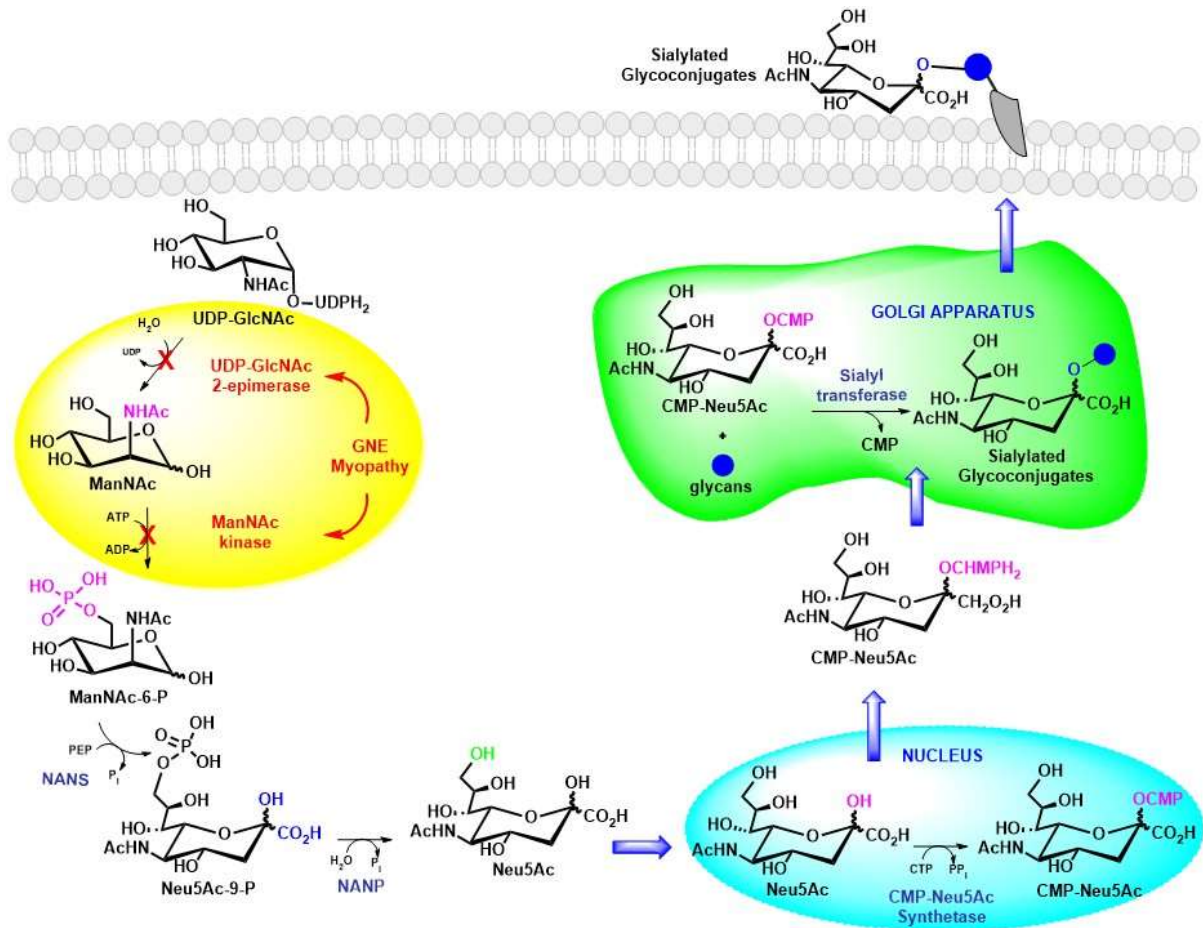
- 1 results provide a potential platform to address substrate deficiencies in GNE myopathy and
- 2 other CDGs.
- 3

1 Introduction

2 Congenital Disorders of Glycosylation (CDGs), first described as Carbohydrate Deficient
3 Glycoprotein Syndrome by Jaeken et al. in 1980¹, are a group of rare, inherited, metabolic
4 diseases characterized by defects in protein or lipid glycosylation. CDGs primarily affect
5 children and can vary in clinical presentation from disabling to life-threatening conditions.
6 Disease manifestations typically impact multiple organ systems, including liver and
7 neurological involvement. Over 100 unique CDGs have been identified to date and the number
8 continues to increase due to advances in the availability and efficiency of diagnostic
9 techniques, namely genome sequencing.^{2,3}

10 Among the CDGs, Hereditary Inclusion Body Myopathy (HIBM), also known as GNE
11 myopathy (GNEM), is an autosomal recessive neuromuscular disorder characterized by
12 symptoms that typically appear during the third decade of life, starting with skeletal muscle
13 atrophy and weakness that slowly progresses to a significant physical disability. GNEM is
14 caused by biallelic mutations in the *GNE* gene resulting in loss-of-function of uridine 5'-
15 diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) 2-epimerase and *N*-acetylmannosamine
16 (ManNAc) kinase (GNE),⁴ a key enzyme in sialic acid biosynthesis (Figure 1).⁵ Sialic acids
17 decorate cell surfaces as terminal component of glycoprotein and glycolipids,⁶ where they
18 mediate or modulate a variety of physiological and pathological processes like signal
19 transduction, cell adhesion, system embryogenesis, cancer metastasis or viral and bacterial
20 infections.⁷ GNE is a bifunctional enzyme capable of catalyzing epimerization of UDP-
21 GlcNAc at the 2-position of the hexose ring to afford ManNAc and subsequent phosphorylation
22 at the 6-position to afford ManNAc-6P (Figure 1). As the rate-limiting step in sialic acid
23 biosynthesis, GNE deficiency results in decreased sialic acid production and consequent
24 hyposialylation of glycoproteins, particularly in muscle tissue. The prevalence of GNEM is
25 estimated to be roughly 1 to 9 in 1,000,000 people. More than 140 mutations in *GNE* have been
3

1 associated with GNE myopathy and can compromise the enzymatic activity of either catalytic
 2 domain.⁸ Each domain is functional when expressed separately, but evolution of some
 3 intramolecular dependency is exemplified by mutations causing reductions in both enzyme
 4 activities to different extents in a manner independent of domain localization.⁹
 5



6
 7 **Figure 1.** Sialic acid biosynthetic cascade: the point of mutation of UDP-GlcNAc 2-
 8 epimerase/ManNAc kinase, responsible for the GNE myopathy, is highlighted in yellow.
 9 To date, there is no approved therapy for GNEM.¹⁰ Palliative treatments, such as
 10 physiotherapy, analgesic drugs, mobility devices and psychological support are available to
 11 alleviate patient symptoms and improve quality of life. Therapeutic approaches have focused
 12 on substrate replacement paradigms seeking to re-establish available pools of intracellular

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

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

19 A study conducted in a GNE myopathy mouse model demonstrated that the lipophilic ManNAc
20 analog *O*-tetra-acetylated *N*-acetylmannosamine (Ac₄ManNAc) was more effective at
21 increasing sialylation and preventing the myopathic phenotype when compared to natural sialic
22 acid metabolites after oral administration.¹⁸ Ac₄ManNAc is a prodrug of ManNAc and can
23 more readily cross cellular membranes via passive diffusion, providing evidence that synthetic
24 sugar analogs may be a practical consideration for the design of therapeutic agents for GNE
25 myopathy. However, Ac₄ManNAc still requires phosphorylation upon release of ManNAc. To

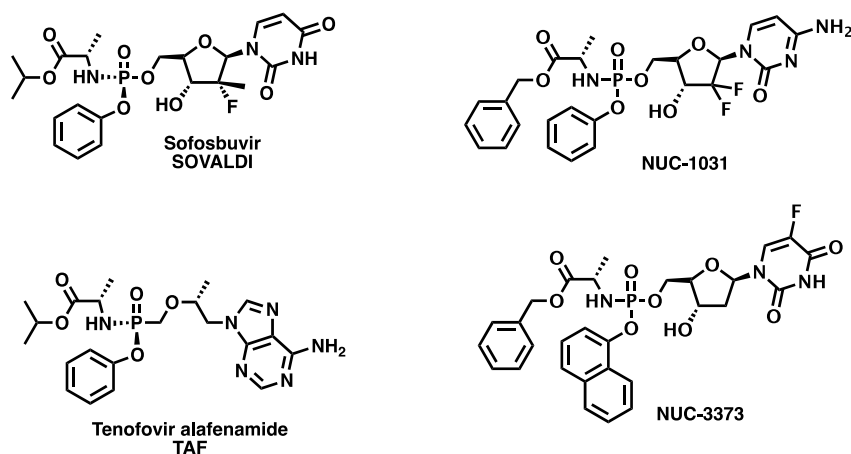
1 overcome the deficiency in both UDP-GlcNAc-2-epimerase and ManNAc kinase enzyme
2 activity observed in GNE myopathy, administration of ManAc-6-P could be envisaged as an
3 ideal substrate replacement strategy. Due to the high polarity of the phosphate moiety, a
4 prodrug approach would be required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

6 Prodrug approaches for delivering phosphate carbohydrate analogs have been reported
7 previously. Meyer *et al* designed cycloaligenyl prodrugs of *O*-tetra-acetylated mannose-1-
8 phosphate¹⁹ whereas Eklund with co-workers synthesized two mannose-1-acetoxymethylated
9 phosphates.²⁰ Both classes of phosphate prodrugs were designed for the treatment of
10 phosphomannomutase 2 (PMM2) deficiency, characteristic of the PMM2-CDG condition also
11 known as CDG-Ia. Despite encouraging results demonstrating intracellular delivery of the
12 mannose-1-monophosphate capable of correcting the CDG-Ia phenotype *in vitro*, these
13 approaches were not explored further, most likely due to toxicity and stability concerns. To
14 date, no additional studies have been reported.

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

23 Among many existing phosphate prodrug strategies,²⁴ the ProTide approach has been
24 successfully applied to nucleosides^{25, 26} as evidenced by the development and approval of the
25 currently marketed drugs Sofosbuvir²⁷ and Tenofovir Alafenamide (TAF)²⁸ (Figure 2). In

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



6
7 **Figure 2.** FDA-approved antiviral ProTides, Sofosbuvir and TAF and anticancer clinical
8 candidates ProTides, NUC-1031 and NUC-3373.

9 Given the importance of phosphoramidate prodrug technology in recent drug discovery, we
10 revived our interest in its application toward the delivery of ManNAc-6-P as a novel approach
11 to the treatment of GNE myopathy. Rationally designed ManNAc phosphoramidate prodrugs
12 could enable the bypass of deficient GNE enzyme observed in GNEM patients by directly
13 providing a source of intracellular ManNAc-6-P. Our approach sought to optimize drug-like
14 properties of ManNAc phosphoramidates to improve upon the low permeability and plasma
15 stability of ManNAc-6-P. In this work, we report the design, synthesis and evaluation of a
16 family of ManNAc-6-P and Ac₄ManNAc-6-P ProTide analogues, including ADME assays,
17 enzymatic activation studies and *in vitro* assessment of sialic acid production in cell-based
18 models of GNE myopathy. These data serve as proof-of-concept that the ProTide platform is a
19 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

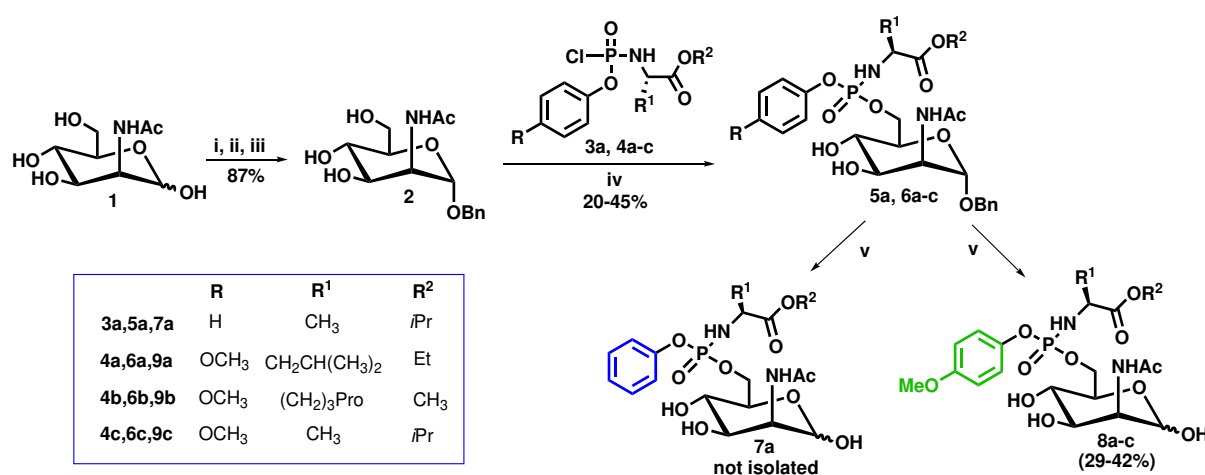
4 Our synthetic efforts began with the synthesis of ManNAc-6-phosphoramidates. We initially
5 envisaged the application of a synthetic procedure previously reported by our group for the
6 preparation of glucosamine phosphoroamidates as feasible approach.²¹ Surprisingly, when
7 ManNAc (**1**) was coupled 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 BF₃OEt₂. 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 H₂) 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
8

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

15



16

17 **Scheme 1.** Phosphoramidate synthesis through 1'-O-Bn-N-acetylmannosamine. ^aReagents and
 18 conditions: (i) Ac₂O, DMAP, Py, rt, 12h; (ii) BnOH, BF₃OEt₂ cat., ACN, 80 °C, 3h; (iii)
 19 NaOMe cat. MeOH, rt, 5h; (iv) NMI, THF/pyridine 4/2 v/v, rt, 12h; (v) H₂, 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.

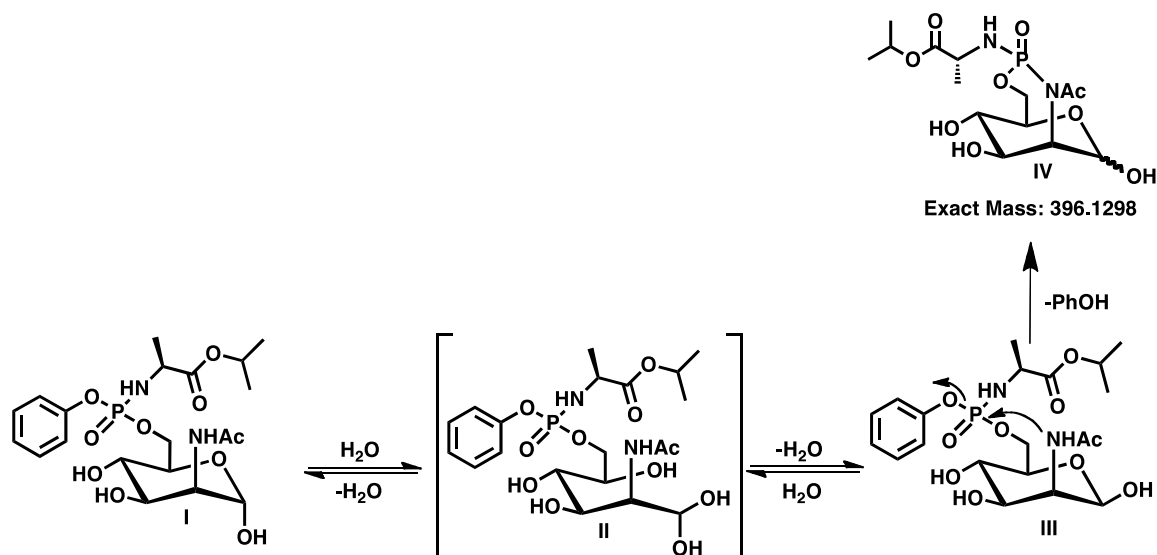
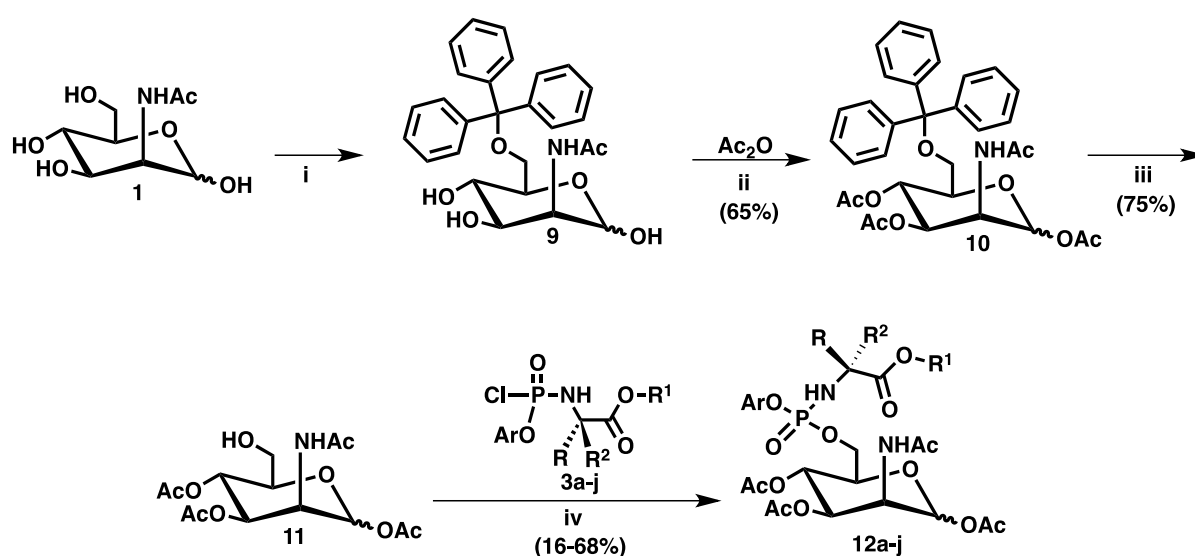


Figure 3. Proposed mechanism for the formation of the side product **IV** during the purification step.

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 phosphoramidate 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

1 ManNAc (**11**) was prepared according to a literature procedure³⁴ outlined in Scheme 2.
 2 ManNAc (**1**) was tritylated, peracetylated and selectively deprotected at the *O*-6 position to
 3 yield compound **11** as a mixture of α and β anomers (1:0.3), which was then coupled with
 4 phosphorochloridates **3a-j** in the presence of Grignard reagent³² to afford compounds **12a-j** as
 5 a mixture of S_p and R_p diastereoisomers of α and β anomers. To avoid the formation of
 6 regioisomeric phosphoramidates arising from acetyl migration,^{35, 36} low reaction temperature
 7 was required during phosphoramidate formation.

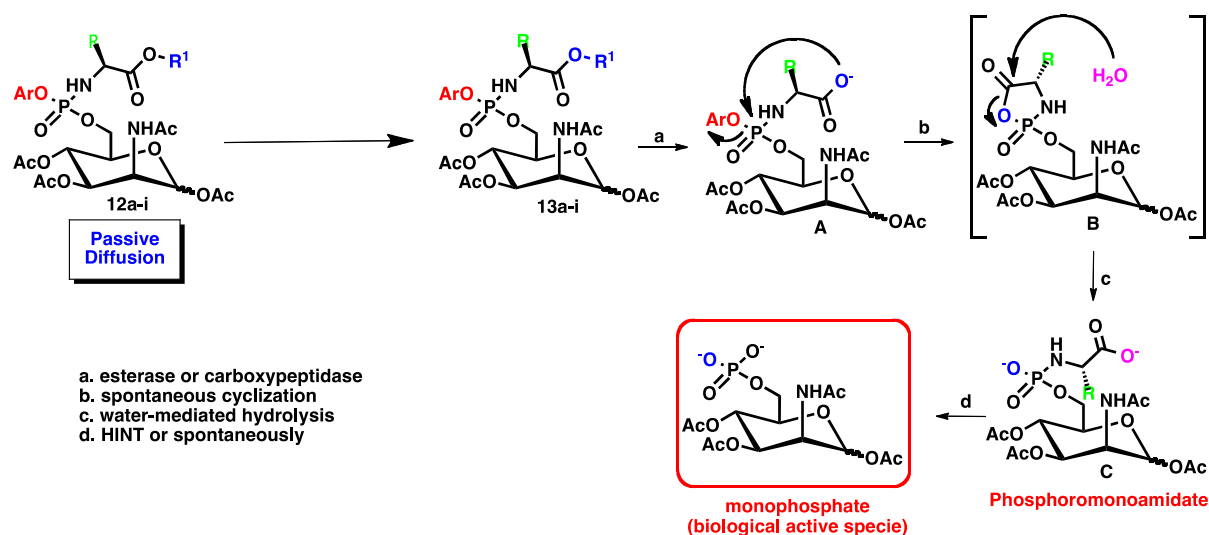


9 **Scheme 2.** Synthetic strategy for the preparation of 1,3,4-*O*-triacetylated ManNAc
 10 phosphoramidates. Reagents and conditions: (i) Pyridine, rt, 12h and 60 °C, 1h; (ii) Pyridine,
 11 0 °C to rt, 12h; (iii) glacial acetic acid, 60 °C, 2h; (iv) 1M *t*-BuMgCl, THF, 0 °C to rt, 12h.

12 A small, rationally designed library of Ac₃ManNAc phosphoramidates was built based on
 13 predicted log P values and existing knowledge of prodrug activation kinetics, using different
 14 amino acids and ester moieties to sample a diverse set of analogs (Table 1). Furthermore, since
 15 our previous report²² demonstrated that proline-based ProTides of N-acetyl glucosamine were
 16 particularly effective in cell-based models of osteoarthritis, we prepared the proline derivative
 17 **12c**.

Compound ID	Ar	R	R ¹	R ²	log P ^a	Yield ^b (%)
12a	Ph	CH ₃	<i>i</i> Pr	H	1.32	60
12b	Ph	CH ₂ CH(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 ₂ Ph	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 ₃ CH ₂ CHCH ₃	Et	H	1.95	34
12i	Ph	CH ₃	Bn	CH ₃	2.38	64
12j	Ph	CH ₂ CH(CH ₃) ₂	Bn	H	3.04	61

1 **Table 2.** Library of Ac₃ManNAc phosphoramidate prodrugs. ^aPredicted log P calculated using
2 SwissADME webservice.³⁷ ^bYield 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.



1

2 **Figure 4.** Proposed enzymatic activation mechanism for Ac₃ManNAc phosphoramidates: a)

3 carboxylesterase or carboxypeptidase catalyzed ester hydrolysis to metabolite **A**.

4 Intramolecular nucleophilic displacement of the aryloxy group provide the cyclic anhydride

5 intermediate **B**. c) water-mediated hydrolysis of **B** to phosphoromonoamidate **C**. d) P-N bond

6 cleavage mediated by HINT enzyme or by spontaneous hydrolysis.

7 To assess whether a carboxypeptidase-mediated activation mechanism to

8 phosphoromonoamidate **C** is also operative with ManNAc phosphoramidates, compounds **12a-**

9 **i** (Scheme 2 and Table 2) were subjected to enzymatic studies by incubating each compound

10 with carboxypeptidase Y (CPY, from Baker's yeast) in 0.05 M Trizma buffer (pH = 7.6) in

11 deuterated acetone, which allowed monitoring of reaction progress by ³¹P-NMR and LC-MS

12 analyses. Almost all compounds showed activation of the promoiety with less than 50% of the

13 parent phosphoramidates remaining 15 minutes following the addition of CPY. The only

14 exception was **12c**, which showed no activation (up to 24 hours). Figure 5 displays the ³¹P-

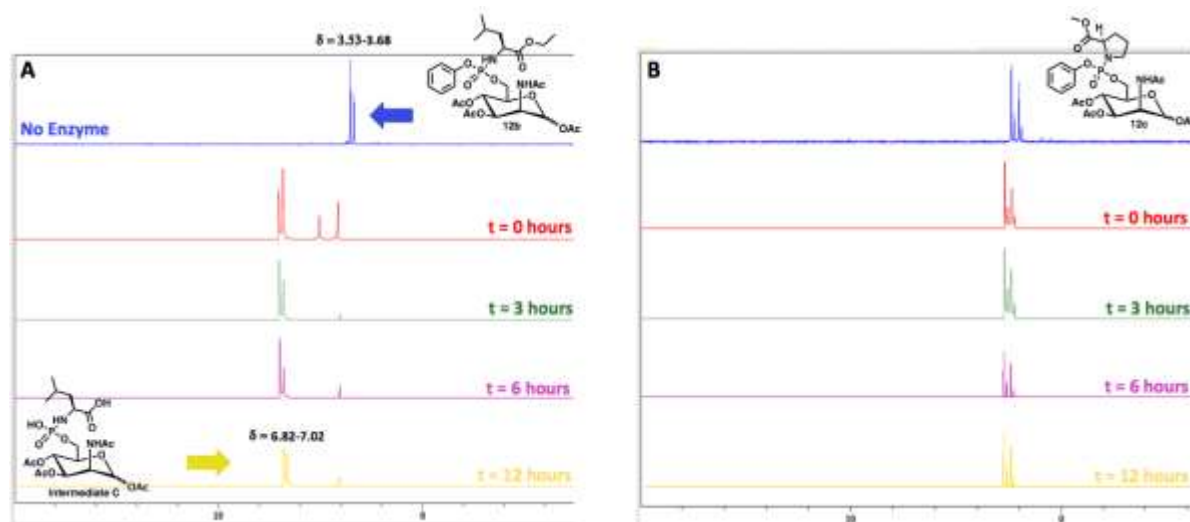
15 NMR spectra for the enzymatic activation assays of compounds **12b** and **12c**. Although the

16 exact reaction sequence cannot be established from these data alone (and, consequently, the

17 prodrug half-life), we can assert that compound **12b** demonstrates rapid conversion into a

18 species showing two signals at 6.82 and 7.02 ppm. On the other hand, replacement of leucine

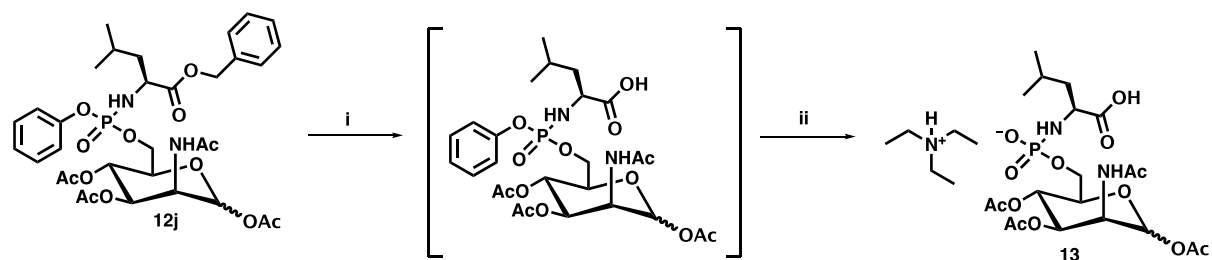
1 with proline (**12b** to **12c**) completely abolishes prodrug activation under identical incubation
2 conditions, as the minor change from ethyl to methyl ester moiety at R¹ is unlikely to cause
3 such a stark contrast in susceptibility to carboxypeptidase-mediated activation.



4
5 **Figure 5.** Deconvoluted ³¹P-NMR spectra of phosphoroamidates **12b** (A) and **12c** (B) over
6 time after enzymatic incubation with Carboxypeptidase Y. Conditions: 202MHz, [D₆]acetone,
7 0.05M Trizma buffer, pH=7.6.

8 Crude enzymatic reaction mixture of **12b** was further analyzed by LC-MS confirming the
9 presence of intermediate **C** (see Figure 31 in Supporting Information). To independently
10 confirm the real structure of intermediate **C** (two ³¹P-NMR peaks at 6.82 and 7.02 ppm), a
11 sample of this compound was prepared synthetically. Compound **12j** was debenzylated via
12 catalytic hydrogenation to the corresponding acid, which formed the expected intermediate **C**
13 as a triethyl ammonium salt (**13**) (Scheme 3) after treatment with 0.1 M triethylammonium
14 bicarbonate buffer. Comparison of LC-MS (t_R = 2.5, 3.7 min) and ³¹P-NMR (7.02, 6.97 ppm)
15 data for compound **13** and those obtained from incubation of compound **12b** confirmed the
16 effective activation of **12b** into the corresponding phosphoromonoamidate **C**. This result
17 demonstrates for the first time that carbohydrate phosphoramidates undergo a similar activation
18 pathway observed for nucleoside phosphoramidates. In addition, these studies suggest that
15

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.



6 **Scheme 3.** Synthesis of compound **13**. Reagents and conditions: (i) H₂, 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 Ac₃ManNAc 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 promoity 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 (μ M, 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 μ M). 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.

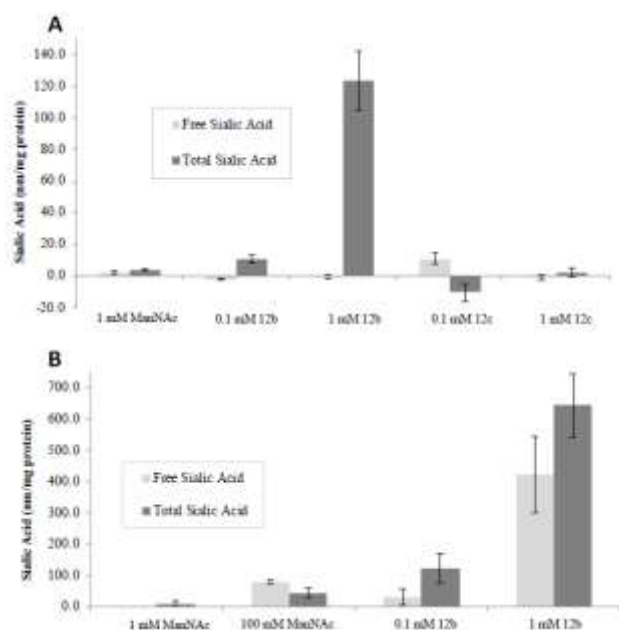
16 Sialic Acid Quantification in Cell-Based Models of GNE Myopathy

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

1 conducted using two different cell lines: *Lec3* CHO cells and GNEM patient-derived
2 myoblasts. Both cell types present with reduced sialic acid levels due to mutations in *GNE* that
3 mimic the hyposialylation defects observed in patients with GNE myopathy. In particular, *Lec3*
4 mutant CHO cells lack any detectable GNE activity³⁸, whereas the patient-derived myoblasts
5 contained a D378Y mutation that cause an 80 % reduction in GNE activity.⁹ Compounds **12b**
6 and **12c** were initially assessed for cytotoxicity in *GNE* mutant cell lines relative to ManNAc
7 using Trypan Blue staining as an assay for cell viability after 48 hours incubation. At 0.1 mM,
8 **12b** and **12c** induced no observable difference in cell viability relative to controls and at 1 mM,
9 cell viability was comparable to 1 mM ManNAc (see Supporting Info, Figure 32).

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

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



1
2 **Figure 6.** Sialic acid levels in membrane protein fraction. Sialic acid levels were determined
3 using Sialic Acid Assay kit on isolated membrane protein fractions from *Lec3* CHO cells (**A**)
4 and GNEM patient-derived fibroblasts (**B**) incubated with **12b**, **12c**, ManNAc for 48 hours and
5 normalized to control (media only) (n = 2, +/- SEM).

6 CONCLUSION

7 A substrate replacement paradigm was applied to the design of novel ProTide prodrugs of
8 ManNAc-6-P as a therapeutic approach for GNE myopathy. A focused library of ManNAc-6-
9 P phosphoramidates was synthesized and evaluated for ADME properties to select lead
10 compounds for profiling in cell-based models of GNE myopathy. Two leads, **12b** and **12c**,
11 displayed the most promising ADME properties, although **12c** did not show activation in a
12 carboxypeptidase assay. The demonstration and independent confirmation of
13 carboxypeptidase-mediated activation of **12b** serves as the first example of a non-nucleoside
14 phosphoramidate activation mechanism. The contrast in prodrug activation between **12b** and
15 **12c** further emphasizes the importance of selecting the appropriate amino acid side chain for
16 the ProTide moiety. Compounds **12b** and **12c** were evaluated in two separate GNE-deficient
17 cell lines for their ability to increase sialic acid production in membrane protein fractions
18
19

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

6 Experimental Section

7 General Experimental Information

8 All chemicals were purchased from Sigma-Aldrich and Carbosynth and used without any
9 further purification. All ¹H-NMR (500MHz), ³¹P-NMR (202MHz) and ¹³C-NMR (125MHz)
10 spectra were recorded on a Bruker AVANCE III HD 500 MHz spectrometer at 25 °C. The
11 deuterated solvent used were CDCl₃ and CD₃OD and the spectra were calibrated using their
12 residual peaks: CDCl₃, ¹H: δ=7.26 ppm; ¹³C: δ=77.36 ppm and CD₃OD, ¹H: δ=3.34 ppm; ¹³C:
13 δ=49.86 ppm. Chemical shifts (δ) are reported in parts per million (ppm) and coupling
14 constants (J) in Hertz. The following abbreviations are used in the assignment of NMR signals:
15 s (singlet), d (doublet), t (triplet), q (quartet), spt (septet), m (multiplet), br (broad), dd (doublet
16 of doublets) and ddd (doublet of doublet of doublets). The signals in the ¹H and ¹³C NMR
17 spectra were assigned based on analysis of coupling constants and additional two-dimensional
18 experiments (COSY and HSQC). Some peaks in all NMR analyses may be overlapped.
19 Reactions followed by thin-layer chromatography (precoated aluminium-backed plates, 60
20 F254, 0.2 mm thickness, Merck, were visualized under short-wave ultraviolet light (254 nm)
21 and/or via Pancaldi's solution (molybdate ammonium cerium sulfate solution).

22 Chromatography purifications were performed through ISOLERA™ 3.0 BIOTAGE OS 852M
23 both with the sample being loaded as a concentrated solution in the same eluent and pre-
24 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 °C, 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, NaHCO₃ and brine. The residue was concentrated in vacuo and purified
18 by column chromatography eluting with DCM/CH₃OH (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 BF₃·Et₂O (1.83 mmol, 230 μ L). 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 NaHCO₃. 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

1 g) dissolved in anhydrous MeOH (50 mL) with a solution of NaOMe (0.23 mmol, 0.012 g) in
2 300 μ L of dry CH₃OH under argon atmosphere. The resulting solution was stirred at rt for 5 h
3 and then Amberlite IR-120 hydrogen form was added leaving the mixture under stirring for
4 another 1 h. The mixture was then filtered through celite and the solvent was removed under
5 reduced pressure. The resulting solid was triturated with Et₂O and further purified by column
6 chromatography on silica using EtOAc/MeOH (4:1) to obtain the final product (**2**) in 87 % as
7 two anomers α and β anomers (0.62 g). White solid. ¹H-NMR (500 MHz, CDCl₃): δ _H 7.35-
8 7.28 (m, 5H, Ph), 4.81 (s, 1H, H-1), 4.64 (d, 1H, $J = 11.7$ Hz, CH_{2a}Ph), 4.47-4.44 (m, 2H,
9 CH_{2b}Ph and H-2), 4.11-4.09 (m, 1H, H-3), 3.95 (d, 1H, $J = 10.8$ Hz, H-4), 3.79-3.730 (m, 2H,
10 H-5, H-6), 3.61 (d, 1H, $J = 9.4$ Hz, H-6), 2.00 (s, 3H, OCOCH₃) ppm. MS (ES⁺): m/z 334.15
11 [M+Na]⁺

12 **Standard Procedure 1. General Synthesis of Phosphorochloridate (3a-i/4a-c):** A solution
13 of aryl dichlorophosphate (1 equiv) and an appropriate amino acid ester (1 equiv) in anhydrous
14 DCM under argon atmosphere was cooled down to -78 °C. Afterwards, Et₃N (2 equiv) was
15 added dropwise and the resulting mixture was stirred for 15 minutes at -78 °C. Then, the
16 reaction mixture was allowed to reach room temperature and left under stirring for 1 hour. The
17 solvent was removed under reduced pressure, the solid residue was redissolved in diethyl ether
18 and the resulting mixture was stirred under argon atmosphere for 20 min. The solid was filtered
19 off and the solvent was evaporated giving the pure compound as a colorless oil which was used
20 in the next step without further purification.

21 **Isopropyl (chloro(phenoxy)phosphoryl)-L-alaninate (3a):** Colorless oil; 99 %, 8.36 g. ³¹P-
22 NMR (202 MHz, CDCl₃, mixture of *R*_P and *S*_P diastereoisomers, a:b): δ _P 8.09 (1P), 7.71 (0.9)
23 ppm. ¹H-NMR (500 MHz, CDCl₃): δ _H 7.39-7.36 (m, 2H, CH-Ph), 7.28-7.22 (m, 3H, CH-Ph),
24 5.10-5.05 (m, 1H, CH(CH₃)₂), 4.16-4.08 (m, 1H, NHCHCO), 1.50 (dd, 3H, $J = 2.4, 7.1$ Hz,

1 NHCHCH₃), 1.30-1.26 (m, 6H, CH(CH₃)₂) ppm.

2 **Ethyl (chloro(phenoxy)phosphoryl)-L-leucinate (3b):** Prepared according to *Standard*
3 *procedure 1* in 98 %, yield as a mixture of two diastereoisomers (*SS_P* and *SR_P*). Colorless oil;
4 4.20 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 8.51 (1P), 8.27 (1P) ppm. ¹H-NMR (500 MHz,
5 CDCl₃): δ_H 7.38-7.34 (m, 2H, CH-Ph), 7.27-7.21 (m, 3H, CH-Ph), 4.34-4.19 (m, 3H, NH,
6 OCH₂CH₃), 4.14-4.02 (m, 1H, NHCHCO), 1.86 (spt, 0.5H, *J* = 6.6 Hz, CH₃(CH₃)₂), 1.79 (spt,
7 0.5H, *J* = 6.6 Hz, CH_b(CH₃)₂), 1.64-1.59 (m, 2H, CHCH₂CH), 1.30-1.26 (m, 3 H, OCH₂CH₃),
8 0.96-0.93 (m, 6H, CH₃CHCH₃) ppm.

9 **Methyl (chloro(phenoxy)phosphoryl)-D-prolinate (3c):** Prepared according to *Standard*
10 *procedure 1* in 80 %, yield as a mixture of two diastereoisomers (*SS_P* and *SR_P*). Light yellow
11 oil; 1.20 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 7.95 (1P), 7.76 (0.9P) ppm. ¹H-NMR (500 MHz,
12 CDCl₃): δ_H 7.38-7.34 (m, 2H, CH-Ph), 7.30-7.28 (m, 1H, CH-Ph), 7.24-7.20 (m, 2H, CH-Ph),
13 4.50-4.46 (m, 0.5H, NHCH₃CO), 4.32-4.39 (m, 0.5H, NHCH_bCO), 3.73 (s, 1.5H, COOCH₃),
14 3.72 (s, 1.5H, COOCH₃), 3.58-3.49 (m, 2H, H-Pro), 2.28-2.19 (m, 1H, H-Pro), 2.15-2.08 (m,
15 1H, H-Pro), 2.07-1.95 (m, 2H, H-Pro) ppm.

16 **Methyl (chloro(naphthalen-1-yloxy)phosphoryl)glycinate (3d):** Prepared according to
17 *Standard procedure 1* in 78 %, yield as a mixture of two stereoisomers (*SS_P* and *SR_P*). Light
18 yellow oil; 2.30 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 9.08 ppm. ¹H-NMR (500 MHz, CDCl₃):
19 δ_H 8.06 (d, 1H, *J* = 8.3 Hz, CH-Naph), 7.87 (d, 1H, *J* = 7.7 Hz, CH-Naph), 7.72 (d, 1H, *J* = 8.3
20 Hz, CH-Naph), 7.60-7.52 (m, 3H, CH-Naph), 7.43 (t, 1H, *J* = 8.3 Hz, CH-Naph), 4.43-4.40
21 (br, 1H, NH), 4.00 (dd, 2H, *J* = 4.2, 9.7 Hz, NHCH₂CO), 3.80 (s, 3H, COOCH₃) ppm.

22 **Ethyl (chloro(phenoxy)phosphoryl)-L-phenylalaninate (3e):** Prepared according to
23 *Standard procedure 1* in > 99 %, yield as a mixture of two diastereoisomers (*SS_P* and *SR_P*).
24 Colorless oil; 6.30 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 8.03 (1P), 7.96 (1P) ppm. ¹H-NMR (500
25 MHz, CDCl₃): δ_H 7.39-7.15 (m, 10H, CH-Ar), 4.48-4.35 (m, 1H, NHCHCO), 4.23-4.16 (m,
23

1 2H, OCH₂CH₃), 3.21-3.10 (m, 2H, CH₂Ph), 1.26-1.21 (m, 3H, OCH₂CH₃) ppm.

2 **Benzyl (chloro(naphthalen-1-yloxy)phosphoryl)-L-alaninate (3f):** Prepared according to
3 *Standard procedure 1* in 96 %, yield as a mixture of two diastereoisomers (SS_P and SR_P).
4 Colorless oil; 7.2 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 8.17 (0.8P), 7.90 (1P) ppm. ¹H-NMR
5 (500 MHz, CDCl₃): δ_H 8.06-8.04 (m, 1H, CH-Ar), 7.88-7.86 (m, 1H, CH-Ar), 7.74-7.72 (d,
6 1H, *J* = 8.3 Hz, CH-Ar), 7.60-7.52 (m, 3H, CH-Ar), 7.44-7.40 (m, 1H, CH-Ar), 7.39-7.35 (m,
7 2H, CH-Ar), 7.34-7.32 (m, 3H, CH-Ar), 5.23-5.15 (m, 2H, OCH₂Ph), 4.52-4.39 (m, 1H, NH),
8 4.37-4.31 (br, 1H, NHCHCO), 1.55 (dd, 3H, *J* = 7.3, 10.2 Hz, CHCH₃) ppm.

9 **Benzyl (chloro(phenoxy)phosphoryl)-L-valinate (3g):** Prepared according to *Standard*
10 *procedure 1* in 89 %, yield as a mixture of two diastereoisomers (SS_P and SR_P). Light yellow
11 oil; 4.40 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 9.53 (1P), 9.02 (1P) ppm. ¹H-NMR (500 MHz,
12 CDCl₃): δ_H 7.38-7.22 (m, 10H, CH-Ar), 5.32-5.07 (AB system, 2H, *J* = 7.6 Hz, OCH₂Ph),
13 4.32-4.16 (m, 1H, NH), 4.04-3.93 (m, 1H, NHCHCO), 2.21-2.13 (m, 1H, 3H, CH(CH₃)₂), 1.00
14 (m, 3H, CH(CH₃)₂), 0.90 (m, 3H, CH(CH₃)₂) ppm.

15 **Ethyl (chloro(phenoxy)phosphoryl)-L-isoleucinate (3h):** Prepared according to *Standard*
16 *procedure 1* in 98 %, yield as a mixture of two diastereoisomers (SS_P and SR_P). Light yellow
17 oil; 8.40 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 9.47 (1P), 8.91 (0.9P) ppm. ¹H-NMR (500 MHz,
18 CDCl₃): δ_H 7.39-7.35 (m, 2H, CH-Ph), 7.29-7.22 (m, 3H, CH-Ph), 4.56-4.48 (m, 1H, NH),
19 4.28-4.20 (m, 2H, OCH₂CH₃), 4.03-3.93 (m, 1H, NHCHCO), 1.91-1.88 (m, 1H, CH₃CHCH₂),
20 1.56-1.48 (m, 1H, CHCH_{2a}CH₃), 1.30 (m, 3H, OCH₂CH₃), 1.27-1.23 (m, 1H, CHCH_{2b}CH₃),
21 1.00-0.92 (m, 6H, CH(CH₃)₂) ppm.

22 **Benzyl 2-((chloro(phenoxy)phosphoryl)amino)-2-methylpropanoate (3i):** Prepared
23 according to *Standard procedure 1* in 96 %, yield as a mixture of two stereoisomers (SS_P and
24 SR_P). Light yellow oil; 96 %; 3.84 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 5.62 ppm. ¹H-NMR (500
25 MHz, CDCl₃): δ_H 7.28-7.11 (m, 10H, CH-Ph), 5.12 (s, 2H, OCH₂Ph), 1.62 (s, 3H, C(CH₃)₂),
24

1 1.60 (s, 3H, C(CH₃)₂) ppm.

2 **Benzyl (chloro(phenoxy)phosphoryl)-L-leucinate (3j)**: Prepared according to *Standard*
3 *procedure 1* in > 99 %, yield as a mixture of two diastereoisomers (SS_P and SR_P). Colorless oil;
4 2.43 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 8.31 (0.9P), 8.00 (1P) ppm. ¹H-NMR (500 MHz,
5 CDCl₃): δ_H 7.30-7.26 (m, 7H, CH-Ar), 7.18-7.13 (m, 3H, CH-Ar), 5.16-5.08 (m, 2H, OCH₂Ph),
6 4.14-4.01 (m, 2H, NH and NHCHCO), 1.81-1.67 (m, 1H, CH(CH₃)₂), 1.61-1.49 (m, 2H,
7 CHCH₂CH), 0.88-0.85 (m, 6H, CH(CH₃)₂) ppm.

8 **Ethyl (chloro(4-methoxyphenoxy)phosphoryl)-L-leucinate (4a)**: Prepared according to
9 *Standard procedure 1* in > 99 %, yield as a mixture of two diastereoisomers (SS_P and SR_P).
10 Colorless oil; 4.58 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 9.03 (1P), 8.84 (1P) ppm. ¹H-NMR (500
11 MHz, CDCl₃): δ_H 7.18-7.16 (m, 2H, CH-Ph), 6.87-6.85 (m, 2H, CH-Ph), 4.22 (m, 2H,
12 OCH₂CH₃), 4.15-4.00 (m, 2H, NH and NHCHCO), 3.78 (s, OCH₃), 1.86 (spt, 0.5H, J = 6.7
13 Hz, CH_a(CH₃)₂), 1.79 (p, 0.5H, J = 6.7 Hz, CH_b(CH₃)₂), 1.66-1.51 (m, 2H, CHCH₂CH), 1.29
14 (m, 3 H, OCH₂CH₃), 0.97-0.94 (m, 6H, CH(CH₃)₂) ppm.

15 **Methyl (chloro(4-methoxyphenoxy)phosphoryl)-D-prolinate (4b)**: Prepared according to
16 *Standard procedure 1* in > 99 %, yield as a mixture of two diastereoisomers (SS_P and SR_P).
17 Light yellow oil; 10.00 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 8.60 (1P), 8.42 (0.9P) ppm. ¹H-
18 NMR (500 MHz, CDCl₃): δ_H 7.20 (dd, 1H, J = 1.8, 9.3 Hz, CH-Ph), 7.15 (dd, 1H, J = 1.8, 9.1
19 Hz, CH-Ph), 6.86 (dd, 2H, J = 1.7 Hz, 8.9 Hz, CH-Ph), 4.49-4.45 (m, 0.5H, NHCH₃CO), 4.41-
20 4.37 (m, 0.5H, NHCH₃CO), 3.78 (s, 1.5H, OCH₃), 3.77 (s, 1.5H, OCH₃), 3.73 (s, 1.5H, OCH₃),
21 3.72 (s, 1.5H, OCH₃), 3.57-3.49 (m, 2H, H-Pro), 2.2-2.18 (m, 1H, H-Pro), 2.15-2.07 (m, 1H,
22 H-Pro), 2.06-1.94 (m, 2H, H-Pro) ppm.

23 **Isopropyl (chloro(4-methoxyphenoxy)phosphoryl)-L-alaninate (4c)**: Prepared according to
24 *Standard procedure 1* in > 99 %, yield as a mixture of two diastereoisomers (SS_P and SR_P).
25 Colorless oil: 14.00 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 8.76 (1P), 8.41 (1P) ppm. ¹H-NMR
25

1 (500 MHz, CDCl₃): δ_H 7.19-7.16 (m, 2H, CH-Ph), 6.86 (dd, 2H, *J* = 2.4, 9.0 Hz, CH-Ph), 5.11-
2 5.04 (m, 1H, CH₃CHCH₃), 4.17-4.09 (m, 1H, NHCHCO), 3.78 (s, 3H, OCH₃), 1.48 (dd, 3H, *J*
3 = 3.7, 7.1 Hz, CH(CH₃)₂), 1.28-1.25 (m, 6H, CH(CH₃)₂) ppm.

4 **Standard Procedure 2. General Synthesis of 1-OBn-phosphoramidates (5a/6a-c):** To a
5 solution of compound **2** (1 equiv) in THF/Py (8/2, v/v), N-methylimidazole (2.5 equiv) was
6 added dropwise under argon atmosphere at room temperature and the resulting solution was
7 stirred for 30 minutes. A solution of an appropriate phosphorochloridate (2.5 equiv) in THF
8 was added dropwise to the mixture and stirred overnight at room temperature. After solvent
9 evaporation, the product was purified by column chromatography on silica using DCM/
10 CH₃OH (93:7) yielding the expected products **5-6**.

11 (α)-Isopropyl (((((2R,3S,4R,5S)-5-acetamido-6-(benzyloxy)-3,4-dihydroxytetrahydro-2H-
12 pyran-2-yl)methoxy)(phenoxy)phosphoryl)-L-alaninate (**5a**): Colorless oil; 45 %, 1.23 g. ³¹P-
13 NMR (202 MHz, CDCl₃, mixture of *R_P* and *S_P* diastereoisomers as α anomer): δ_P 3.86 (1P),
14 3.45 (0.6P) ppm. ¹H-NMR (500 MHz, CDCl₃): δ_H 7.37-7.15 (m, 10H, CH-Ar), 4.97-4.90 (m,
15 1H, CH(CH₃)₂, overlap with the solvent), 4.78-4.77 (m, 1H, H-1, overlap with the solvent),
16 4.67-4.63 (m, 1H, CH_{2a}Ph), 4.47-4.44 (m, 1H, CH_{2b}Ph), 4.35-4.30 (m, 1H, H-2), 4.01-3.90 (m,
17 3H, NHCHCO, H-5 and H-4), 3.83-3.77 (m, 1H, H-3), 3.63-3.56 (m, 2H, H-6), 1.99 (s, 3H,
18 NHCOCH₃), 1.37-1.27 (m, 3H, NHCHCH₃), 1.23-1.18 (m, 6H, CH(CH₃)₂) ppm. ¹³C-NMR
19 (125 MHz, MeOD): δ_C 174.63 (d, ³*J*_{C-P} = 5.2 Hz, C=O ester), 174.07 (d, ³*J*_{C-P} = 7.2 Hz, C=O
20 ester), 170.03 (NHCOCH₃), 154.66 (d, ²*J*_{C-P} = 6.8 Hz, “*ipso*” OPh), 152.28 (d, ²*J*_{C-P} = 6.5 Hz,
21 “*ipso*” OPh), 138.57 (“*ipso*” OCH₂Ph), 130.72, 130.69, 130.07, 129.41, 129.19, 129.16,
22 128.85, 128.65, 126.03, 125.99, 123.82 (CHAr), 121.54 (d, ³*J*_{C-P} = 4.9 Hz, CH-OPh), 121.45
23 (d, ³*J*_{C-P} = 4.9 Hz, CH-OPh), 99.57 (C-1), 73.12 (d, ⁴*J*_{C-P} = 6.8 Hz, C-4), 70.55, 70.39 (C-3),
24 70.17 (OCH₂Ph), 70.11, 70.03 (CH(CH₃)₂), 69.19, 69.07 (NHCHCO), 68.33 (C-5), 68.07 (d,

1 $^2J_{C-P} = 5.6$ Hz, C-6), 67.94 (d, $^2J_{C-P} = 5.5$ Hz, C-6), 54.35 (C-2), 21.98, 21.95 (NHCHCH₃), 9.19,
2 7.59 (CH(CH₃)₂) ppm. MS (ES+): m/z 603.22 [M+Na]⁺
3 (α)-Ethyl (((2R,3S,4R,5S)-5-acetamido-6-(benzyloxy)-3,4-dihydroxytetrahydro-2H-pyran-
4 2-yl)methoxy)(4-methoxyphenoxy)phosphoryl)-L-leucinate (**6a**): Prepared according to
5 *Standard procedure 2* in 20 %, yield as a mixture of *R_P* and *S_P* diastereoisomers as α anomer.
6 Light yellow oil; 0.16 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 4.37 (1P), 4.30 (1P) ppm. ¹H-NMR
7 (500 MHz, CDCl₃): δ_H 7.35-7.31 (m, 5H, CH-Ar), 7.18-7.13 (m 2H, CH-Ar), 6.87-6.83 (m,
8 2H, CH-Ar), 4.79 (d, 1H, $J = 10.9$ Hz, H-1), 4.68-4.64 (m, 1H, CH_{2a}Ph), 4.49-4.41 (m, 1H,
9 CH_{2b}Ph), 4.35-4.34 (m, 1H, H-2), 4.17-4.12 (m, 2H, OCH₂CH₃), 4.11-4.07 (m, 1H, H-4), 4.00-
10 4.07 (m, NHCHCO), 3.92-3.87 (m, 1H, H-3), 3.84-3.73 (m, 4H, OCH₃ and H-5), 3.63-3.58 (m,
11 2H, H-6), 2.01 (s, 3H, NHCOCH₃), 1.71-1.62 (m, 1H, CH(CH₃)₂), 1.58-1.50 (m, 2H,
12 CHCH₂CH), 1.27-1.19 (m, 3H, OCH₂CH₃), 0.94-0.84 (m, 6H, CH(CH₃)₂) ppm. ¹³C-NMR (125
13 MHz, MeOD): δ_C 175.45 (d, $^3J_{C-P} = 2.5$ Hz, C=O ester), 175.33 (d, $^3J_{C-P} = 2.5$ Hz, C=O ester),
14 174.11, 174.05 (NHCOCH₃), 158.25, 157.59 (“*ipso*” PhOCH₃), 145.83 (d, $^2J_{C-P} = 3.8$ Hz,
15 “*ipso*” POPh), 138.56 (“*ipso*” OCH₂Ph), 129.42, 129.39, 129.27, 129.22, 128.86, 122.46,
16 122.42 (CHAr), 122.32 (d, $^3J_{C-P} = 4.4$ Hz, CH-POPh), 122.18 (d, $^3J_{C-P} = 4.5$ Hz, CH-POPh),
17 115.58, 115.55, 115.31 (CHAr), 99.56, 99.51 (C-1), 73.11 (d, $^4J_{C-P} = 7.5$ Hz, C-4), 73.08 (d,
18 $^4J_{C-P} = 6.3$ Hz, C-4), 70.58, 70.37 (NHCHCO), 70.14 (OCH₂Ph), 68.40, 68.37 (C-5), 68.04 (d,
19 $^2J_{C-P} = 5.3$ Hz, C-6), 67.88 (d, $^2J_{C-P} = 5.4$ Hz, C-6), 62.27, 62.22 (OCH₂CH₃), 56.02, 55.99 (C-
20 3), 54.56, 54.37 (C-2), 44.15, 43.96 (CHCH₂CH), 25.55, 25.46 (CH(CH₃)₂), 23.19, 23.16,
21 22.56, 22.41, 22.13, 21.90 (NHCOCH₃ and CH(CH₃)₂) 14.46, 14.41 (OCH₂CH₃) ppm. MS
22 (ES+): m/z 661.26 [M+Na]⁺
23 (α)-Methyl (((2R,3S,4R,5S)-5-acetamido-6-(benzyloxy)-3,4-dihydroxytetrahydro-2H-pyran-
24 2-yl)methoxy)(4-methoxyphenoxy)phosphoryl)-D-prolinate (**6b**): Prepared according to

1 *Standard procedure 2* in 32 %, yield as a mixture of *R_P* and *S_P* diastereoisomers as α anomer.
2 Light yellow oil; 0.23 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 2.27 (0.1P), 1.87 (1P) ppm. ¹H-NMR
3 (500 MHz, CDCl₃): δ_H 7.33-7.28 (m, 5H, CH-Ar), 7.14-7.12 (m 2H, CH-Ar), 6.87-6.84 (m,
4 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,
5 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),
6 3.81-3.78 (m), 3.71 (s, 3H, COOCH₃), 3.69 (s, 3H, PhOCH₃), 3.41-3.33 (m), 2.19-2.13 (m, 2H,
7 CH₂Pro), 1.99 (s, 3H, NHCOCH₃), 1.96-1.81 (m, 2H, CH₂Pro) ppm. MS (ES⁺): *m/z* 631.21
8 [M+Na]⁺
9 (α)-Isopropyl (((2*R*,3*S*,4*R*,5*S*)-5-acetamido-6-(benzyloxy)-3,4-dihydroxytetrahydro-2H-
10 pyran-2-yl)methoxy)(4-methoxyphenoxy)phosphoryl)-L-alaninate (**6c**): Prepared according to
11 *Standard procedure 2* in 21 %, yield as a mixture of *R_P* and *S_P* diastereoisomers as α anomer.
12 Colorless oil; 0.20 g. ³¹P-NMR (202 MHz, CDCl₃): δ_P 4.30 (1P), 4.09 (0.8P) ppm. ¹H-NMR
13 (500 MHz, CDCl₃): δ_H 7.30 (m, 5H, CH-Ar), 7.14 (d, 2H, *J* = 86 Hz, CH-Ar), 6.84-6.80 (m,
14 2H, CH-Ar), 4.98-4.93 (m, 1H, CH(CH₃)₂), 4.77 (s, 1H, H-1), 4.65-4.64 (m, 1H, CH_{2a}Ph),
15 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
16 (m, 1H, NHCHCO), 3.80-3.77 (m), 3.70 (s, 1.5H, PhOCH₃), 3.69 (s, 1.5H, PhOCH₃), 3.62-
17 3.55 (m, 2H, H-6), 1.99 (s, 3H, NHCOCH₃), 1.37-1.32 (m, 3H, NHCHCH₃), 1.23-1.20 (m, 6H,
18 CH(CH₃)₂) ppm. ¹³C-NMR (125 MHz, MeOD): δ_C 173.34, 173.33 (C=O ester), 172.73, 172.67
19 (NHCOCH₃), 156.87 (“*ipso*” OPh), 144.38 (“*ipso*” POPh), 137.21 (“*ipso*” OCH₂Ph), 128.05,
20 127.87, 127.84, 127.49, 121.00, 120.93, 114.23, 114.20 (CHAr), 98.18, 98.14 (C-1), 73.11 (d,
21 ⁴*J*_{C-P} = 7.5 Hz, C-4), 73.08 (d, ⁴*J*_{C-P} = 6.3 Hz, C-4), 70.58, 70.37 (NHCHCO), 70.14 (OCH₂Ph),
22 68.40, 68.37 (C-5), 68.04 (d, ²*J*_{C-P} = 5.3 Hz, C-6), 67.88 (d, ²*J*_{C-P} = 5.4 Hz, C-6), 62.27, 62.22
23 (OCH₂CH₃), 56.02, 55.99 (C-3), 54.56, 54.37 (C-2), 44.15, 43.96 (CHCH₂CH), 25.55, 25.46
24 (CH(CH₃)₂), 23.19, 23.16, 22.56, 22.41, 22.13, 21.90 (NHCOCH₃ and CH(CH₃)₂) 14.46, 14.41

1 (OCH₂CH₃) ppm. MS (ES⁺): *m/z* 661.26 [M+Na]⁺

2 **Standard Procedure 3. General Synthesis of Deprotected Phosphoramidates (8a-c):** To a
3 solution of **5** or **6** (1 equiv) in EtOH/EtOAc (2:1) was added Pd/C (10% Pd on activated carbon)
4 under inert atmosphere. The resulting mixture was stirred at room temperature for 5 hours
5 under H₂ atmosphere (1 atm, ballon). The crude was filtered through celite, concentrated in
6 vacuo and triturated with Et₂O giving the expected products **8a-c**.

7 Ethyl (((((2R,3S,4R,5S)-5-acetamido-3,4,6-trihydroxytetrahydro-2H-pyran-2-yl)methoxy)(4-
8 methoxyphenoxy)phosphoryl)-L-leucinate (**8a**): Light yellow oil; 29%; 0.17 g. ³¹P-NMR (202
9 MHz, CDCl₃, mixture of *R*_P and *S*_P diastereoisomers as α and β anomers): δ_P 4.41 (1P), 4.31
10 (0.7P), 4.19 (0.7P) ppm. ¹H-NMR (500 MHz, CDCl₃): δ_H 7.14 (d, 2H, *J* = 9.0 Hz, CH-Ar), 6.87
11 (d, 2H, *J* = 9.0 Hz, CH-Ar), 5.03 (s, 1H, H-1), 4.56 (s, 1H, H-2), 4.34-4.26 (m, 1H, CH₂Ph),
12 4.17-4.12 (m, 2H, OCH₂CH₃), 4.11-4.07 (m, 1H, H-4), 4.00-4.07 (m, NHCHCO), 3.92-3.87
13 (m, 1H, H-3), 3.84-3.73 (m, 4H, OCH₃ and H-5), 3.63-3.58 (m, 2H, H-6), 2.01 (s, 3H,
14 NHCOCH₃), 1.71-1.62 (m, 1H, CH(CH₃)₂), 1.58-1.50 (m, 2H, CHCH₂CH), 1.27-1.19 (m, 3H,
15 OCH₂CH₃), 0.94-0.84 (m, 6H, CH(CH₃)₂) ppm. ¹³C-NMR (125 MHz, MeOD): δ_C 174.20,
16 174.09 (C=O ester), 172.77, 172.69 (NHCOCH₃), 156.92, 156.84 (“*ipso*” PhOCH₃), 144.41 (d,
17 ²*J*_{C-P} = 6.8 Hz, “*ipso*” POPh), 121.06, 121.02, 120.81, 120.77, 114.21, 114.16 (CHAr), 93.67,
18 93.37 (C-1), 73.11 (d, ⁴*J*_{C-P} = 7.5 Hz, C-4), 73.08 (d, ⁴*J*_{C-P} = 6.3 Hz, C-4), 70.58, 70.37
19 (NHCHCO), 70.14 (OCH₂Ph), 68.40, 68.37 (C-5), 68.04 (d, ²*J*_{C-P} = 5.3 Hz, C-6), 67.88 (d, ²*J*_{C-}
20 _P = 5.4 Hz, C-6), 62.27, 62.22 (OCH₂CH₃), 56.02, 55.99 (C-3), 54.56, 54.37 (C-2), 44.15, 43.96
21 (CHCH₂CH), 25.55, 25.46 (CH(CH₃)₂), 23.19, 23.16, 22.56, 22.41, 22.13, 21.90 (NHCOCH₃
22 and CH(CH₃)₂) 14.46, 14.41 (OCH₂CH₃) ppm. MS (ES⁺): *m/z* 571.21 [M+Na]⁺

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

1 Standard procedure 3 in 32 %, yield as a mixture of R_P and S_P diastereoisomers as α and β
2 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
3 (0.4P) ppm. ^1H -NMR (500 MHz, CDCl_3): δ_H 7.13 (d, 2H, $J = 9.0$ Hz, $\underline{\text{CH}}$ -Ar), 6.89 (d, 2H, $J =$
4 9.0 Hz, $\underline{\text{CH}}$ -Ar), 5.04 (m, 1H, $\underline{\text{H-1}}$), 4.55-5.1 (m, 1H, NHCHCO), 4.33-4.31 (m), 4.26-4.23 (m),
5 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),
6 2.19-2.13 (m, 2H, $\underline{\text{CH}_2\text{Pro}}$), 1.99 (s, 3H, NHCOCH_3), 1.96-1.81 (m, 2H, $\underline{\text{CH}_2\text{Pro}}$) ppm. ^{13}C -
7 NMR (125 MHz, MeOD): δ_C 174.38 ($\underline{\text{C}}=\text{O}$ ester), 172.77 (NHCOCH_3), 156.94 (“*ipso*”
8 $\underline{\text{PhOCH}_3}$), 144.15 (d, $^2J_{\text{C-P}} = 2.5$ Hz, “*ipso*” POPh), 120.93 (d, $^3J_{\text{C-P}} = 4.6$ Hz, $\underline{\text{CH-POPh}}$), 120.67
9 (d, $^3J_{\text{C-P}} = 4.5$ Hz, $\underline{\text{CH-POPh}}$), 120.66, 114.33, ($\underline{\text{CHAr}}$), 93.66, 93.33 ($\underline{\text{C-1}}$), 68.95, 68.82, 67.05,
10 66.80, 60.87, 60.62, 54.68, 53.89, 51.34, 30.82 (d, $^2J_{\text{C-P}} = 9.1$ Hz, $\underline{\text{CH}_2\text{Pro}}$), 24.81 (d, $^3J_{\text{C-P}} = 9.0$
11 Hz, $\underline{\text{CH}_2\text{Pro}}$), 21.24 (NHCOCH_3) ppm. MS (ES+): m/z 541.17 [$\text{M}+\text{Na}$] $^+$

12 Isopropyl (((((2R,3S,4R,5S)-5-acetamido-3,4,6-trihydroxytetrahydro-2H-pyran-2-
13 yl)methoxy)(4-methoxyphenoxy)phosphoryl)-L-alaninate (**8c**): Colorless oil; 42 %, 0.11 g. ^{31}P
14 NMR (202 MHz, CDCl_3 , mixture of R_P and S_P diastereoisomers as α and β anomers): δ_P 8.42
15 (0.2P), 8.33 (0.8P), 8.10 (1P) ppm. ^1H -NMR (500 MHz, CDCl_3): δ_H 7.17-7.13 (m, 2H, $\underline{\text{CH}}$ -
16 Ar), 6.89-6.87 (m, 2H, $\underline{\text{CH}}$ -Ar), 5.03 (d, 1H, $J = 9.0$ Hz, $\underline{\text{H-1}}$), 4.98-4.93 (m, 1H, $\underline{\text{CH}}(\text{CH}_3)_2$),
17 4.32-4.31 (m, 1H, $\underline{\text{H-2}}$), 4.29-4.21 (m), 3.98-3.95 (m), 3.93-3.88 (m, 1H, NHCHCO), 3.80-3.77
18 (m), 3.70 (s, 1.5H, PhOCH_3), 3.69 (s, 1.5H, PhOCH_3), 3.62-3.55 (m, 2H, $\underline{\text{H-6}}$), 1.99 (s, 3H,
19 NHCOCH_3), 1.37-1.32 (m, 3H, NHCHCH_3), 1.23-1.20 (m, 6H, $\text{CH}(\text{CH}_3)_2$) ppm. ^{13}C -NMR
20 (125 MHz, MeOD): δ_C 173.49, 173.44 ($\underline{\text{C}}=\text{O}$ ester), 172.76, (NHCOCH_3), 156.89 (“*ipso*”
21 OPh), 144.38, 114.32 (“*ipso*” POPh), 121.01, 120.98, 114.21, 114.18 ($\underline{\text{CHAr}}$), 93.79, 93.30 ($\underline{\text{C-}}$
22 $\underline{1}$), 73.11 (d, $^4J_{\text{C-P}} = 7.5$ Hz, $\underline{\text{C-4}}$), 73.08 (d, $^4J_{\text{C-P}} = 6.3$ Hz, $\underline{\text{C-4}}$), 70.58, 70.37 (NHCHCO), 68.40,
23 68.37 ($\underline{\text{C-5}}$), 68.04 (d, $^2J_{\text{C-P}} = 5.3$ Hz, $\underline{\text{C-6}}$), 67.88 (d, $^2J_{\text{C-P}} = 5.4$ Hz, $\underline{\text{C-6}}$), 62.27, 62.22
24 (OCH_2CH_3), 56.02, 55.99 ($\underline{\text{C-3}}$), 54.56, 54.37 ($\underline{\text{C-2}}$), 44.15, 43.96 (CHCH_2CH), 25.55, 25.46

1 ($\underline{\text{C}}\text{H}(\underline{\text{C}}\text{H}_3)_2$), 23.19, 23.16, 22.56, 22.41, 22.13, 21.90 ($\text{NHCO}\underline{\text{C}}\text{H}_3$ and $\text{CH}(\underline{\text{C}}\text{H}_3)_2$) 14.46, 14.41
2 ($\text{OCH}_2\underline{\text{C}}\text{H}_3$) ppm. MS (ES+): m/z 543.18 $[\text{M}+\text{Na}]^+$

3 **Synthesis of (3*S*,4*R*,5*S*,6*R*)-3-Acetamido-6-(hydroxymethyl)tetrahydro-2H-pyran-2,4,5-**
4 **triyyl triacetate (11):** *N*-acetyl-D-mannosamine (22.6 mmol, 5 g) was dissolved in pyridine (73
5 mL) and triyl 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. $^1\text{H-NMR}$ (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, NH α), 5.85 (d, 0.3H,
16 $J = 1.7$ Hz, NH β), 5.81 (d, 0.3H, $J = 9.1$ Hz, H-1 β), 5.77 (d, 0.7H, $J = 9.3$ Hz, H-1 α), 5.36-5.25
17 (m, 1H, H-4, 0.7H, H-3 α), 4.98 (dd, 0.3H, $J = 3.9, 9.9$ Hz, H-3 β), 4.76-4.73 (m, 0.3 H, H-2 β),
18 4.64-4.61 (m, 0.7H, H-2 α), 3.92-3.89 (m, 0.7H, H-5 α), 3.70-3.64 (m, 0.3H, H-5 β), 3.38-3.33
19 (m, 1H, H-6), 3.09 (dd, 0.3H, $J = 4.1, 10.6$ Hz, H-6 β), 3.05 (dd, 0.7H, $J = 3.8, 10.6$ Hz, H-6 α),
20 2.16 (s, 2.1H, OCOCH 3α), 2.13 (s, 0.9H, OCOCH 3β), 2.11 (s, 0.9H, OCOCH 3β), 2.09 (s, 2.1H,
21 OCOCH 3α), 1.99 (s, 2.1H, OCOCH 3α), 1.98 (s, 0.9H, OCOCH 3β), 1.78 (s, 0.9H, NHCOCH 3β),
22 1.77 (s, 2.1H, NHCOCH 3α) 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

1 chromatography to give the expected compound **11**. White powder; 75 %; 1.09 g. ¹H-NMR
2 (500 MHz, CD₃OD, mixture of diastereoisomers of α and β anomers, 1:0.3): δ_H 5.95 (d, 0.7H,
3 *J* = 1.7 Hz, H-1_α), 5.92 (d, 0.3H, *J* = 1.7 Hz, H-1_β), 5.32 (t, 0.7H, *J* = 10.1 Hz, H-3_α), 5.27-5.24
4 (m, 1H, H-4), 5.15-5.12 (m, 0.3H, H-3_β), 4.72-4.70 (m, 0.3H, H-2_β), 4.57-4.56 (m, 0.7H, H-
5 2_α), 3.92-3.88 (m, 0.7H, H-5_α), 3.72-3.60 (m, 0.3H, H-5_β, 2H, H-6), 2.16 (s, 2.1 H, OCOCH₃α),
6 2.06-2.05 (m, 4.8H, 3 × OCOCH₃), 2.03 (s, 2.1H, OCOCH₃α), 1.95 (s, 3H, NHCOCH₃) ppm.

7 **Standard Procedure 4. General Synthesis of Phosphoramidates (12a-j):** To a solution of
8 compound **11** (1 equiv) in anhydrous THF was added *t*BuMgCl (1.5 equiv) at 0 °C under argon
9 atmosphere. After 30 min, a solution of an appropriate phosphorochloridates **3a-j** (1.5 equiv)
10 in dry THF was added dropwise to the mixture at the same temperature. Then, the reaction was
11 allowed to reach room temperature and stirred for 12 h. The solvent was evaporated in vacuo
12 and the crude residues were purified by column chromatography (DCM/CH₃OH 98:2) to give
13 compounds **12a-j** as mixture of *S_p* and *R_p* diastereoisomers of α and β anomers.

14 (3*S*,4*R*,5*S*,6*R*)-3-Acetamido-6-((((((*S*)-1-isopropoxy-1-oxopropan-2-
15 yl)amino)(phenoxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12a):
16 Light yellow powder; 68%; 0.63 g. ³¹P-NMR (202 MHz, CD₃OD, mixture of *R_p* and *S_p*
17 diastereoisomers as α and β anomers): δ_P 3.83 (0.5P), 3.76 (1P), 3.55 (0.5P), 3.35 (0.2P) ppm.
18 ¹H-NMR (500 MHz, CD₃OD): δ_H 7.40-7.37 (m, 2H, CH-Ph), 7.27-7.21 (m, 3H, CH-Ph), 6.00-
19 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,
20 1H, CH(CH₃)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
21 and NHCHCO), 3.97-3.89 (m, 1H, H-5), 2.19-1.99 (m, 12H) (3 × OCOCH₃ and NHCOCH₃),
22 1.39-1.34 (m, 3H, NHCHCH₃), 1.28-1.24 (m, 6H, CH(CH₃)₂) ppm. ¹³C-NMR (125 MHz,
23 CD₃OD): δ_C 173.22 (d, ³*J*_{C-P} = 5.2 Hz, C=O ester), 172.94 (d, ³*J*_{C-P} = 4.7 Hz, C=O ester), 172.44
24 (d, ³*J*_{C-P} = 4.7 Hz, C=O ester), 170.24 (d, ³*J*_{C-P} = 4.4 Hz, C=O ester), 170.15, 170.12, 170.05,

1 170.00, 168.71, 168.62, 168.60 (NHCOCH₃ and OCOCH₃), 150.81 (d, ²J_{C-P}= 7.0 Hz, “*ipso*”
2 OPh), 129.39, 129.32, 124.75, 124.72, 120.19, 120.03 (CH-Ph), 91.75, 91.73, 90.72, 90.68 (C-
3 1), 71.37, 71.12 (NHCHCO), 69.31, 69.23 (C-3), 68.80, 68.75 (CH(CH₃)₂), 65.56, 65.49 (C-
4 6), 65.40, 65.31 (C-4), 50.38 (C-5), 49.13, 48.46 (C-2), 21.03, 20.99 (NHCOCH₃), 20.62,
5 20.58, 20.51 (CH(CH₃)₂), 19.32, 19.25 (OCOCH₃), 19.02, 18.97 (NHCHCH₃) ppm. HPLC:
6 (gradient H₂O/ACN from 90/10 to 0/100 in 30 min, flow: 1 mL/min, λ = 210 nm): t_R 21.29
7 min, 21.70 min. MS (ES⁺): m/z 639.20 [M+Na]⁺
8 (3S,4R,5S,6R)-3-acetamido-6-((((((S)-1-ethoxy-4-methyl-1-oxopentan-2-
9 yl)amino)(phenoxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12b):
10 Prepared according to *Standard procedure 4* in 43 %, yield as a mixture of R_P and S_P
11 diastereoisomers as α and β anomers. White powder; 360 mg. ³¹P-NMR (202 MHz, CD₃OD):
12 δ_P 3.81 (0.6P), 3.71 (1P), 3.65 (0.4P) ppm. ¹H-NMR (500 MHz, CD₃OD): δ_H 7.38-7.34 (m, 2H,
13 CH-Ph), 7.22-7.18 (m, 3H, CH-Ph), 5.97-5.96 (m, 1H, H-1), 5.40-5.16 (m, 1.8H, H-3 and H-
14 4), 5.20-5.16 (m, 0.2H, H-3), 4.75-4.74 (m, 0.2H, H-2), 4.58-4.57 (m, 0.8H, H-2), 4.33-4.01
15 (m, 5H, H-6, OCH₂CH₃ and NHCHCO), 3.88-3.83 (m, 1H, H-5), 2.17-1.97 (m, 3 × OCOCH₃
16 and NHCOCH₃), 1.75-1.58 (m, 1H, CH(CH₃)₂), 1.55-1.48 (m, CHCH₂CH), 1.27-1.23 (q, 3H,
17 J = 6.55 Hz, OCH₂CH₃), 0.93-0.82 (m, 6H, CH(CH₃)₂) ppm. ¹³C-NMR (125 MHz, CD₃OD):
18 δ_C 175.52 (d, ³J_{C-P}= 2.9 Hz, C=O ester), 175.46 (d, ³J_{C-P}= 1.4 Hz, C=O ester), 175.19 (d, ³J<sub>C-
19 P</sub>= 2.7 Hz, C=O ester), 173.81 (d, ³J_{C-P}= 4.4 Hz, C=O ester), 171.65, 171.59, 171.52, 171.39,
20 171.36 (OCOCH₃), 170.10, 169.97 (NHCOCH₃), 152.25, 152.19, 152.16, 152.15 (“*ipso*”
21 OPh), 130.78, 130.71, 130.67, 126.14, 126.10, 126.05, 121.59 (d, ³J_{C-P}= 4.5 Hz, CH-Ph),
22 121.28 (d, ³J_{C-P}= 4.9 Hz, CH-Ph), 93.14, 93.11, 92.20, 92.17 (C-1), 72.88, 72.60, 70.69, 70.58
23 (C-5, C-4, C-3), 66.93, 66.90 (OCH₂CH₃), 66.82 (C-2), 62.30 (d, ²J_{C-P}= 10.0 Hz, C-6), 54.60
24 (d, ²J_{C-P}= 4.9 Hz, NHCHCO), 50.52 (CH(CH₃)₂), 44.03 (d, ³J_{C-P}= 7.7 Hz, CHCH₂CH), 25.57,

1 25.41, 23.26, 23.11, 22.39, 22.07, 21.71, 21.67, 20.72, 20.64, 14.47 (NHCOCH₃, 3 ×OCOCH₃,
2 OCH₂CH₃, and CH(CH₃)₂) ppm. HPLC: (gradient H₂O/ACN from 90/10 to 0/100 in 30 min,
3 flow: 1 mL/min, λ = 210 nm): t_R 18.55 min, 18.96 min. MS (ES⁺): m/z 667.23 [M+Na]⁺
4 (3S,4R,5S,6R)-3-acetamido-6-((((R)-2-(methoxycarbonyl)pyrrolidin-1-
5 yl)(phenoxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12c):
6 Prepared according to *Standard procedure 4* in 16 %, yield as a mixture of R_P and S_P
7 diastereoisomers as α and β anomers. White powder; 0.12 g. ³¹P-NMR (202 MHz, CD₃OD) δ_P
8 1.75 (1P), 1.65 (0.4P), 1.32 (0.6P), 1.23 (0.1P) ppm. ¹H-NMR (500 MHz, CD₃OD): δ_H 7.39-
9 7.35 (m, 2H, CH-Ph), 7.28-7.19 (m, 3H, CH-Ph), 5.98 (d, 0.3H, J = 1.9 Hz, H-1), 5.97 (d, 0.7H,
10 J = 1.8 Hz, H-1), 5.37-5.26 (m, 1.7H, H-3 and H-4), 5.24-5.16 (m, 0.3H, H-3), 4.75-4.74 (m,
11 0.3H, H-2), 4.60-4.58 (m, 0.7H, H-2), 4.41-4.10 (m, 4H, NHCHCO, H-5 and 2H-6), 3.72 (s,
12 0.9H, OCH₃), 3.68 (s, 2.1H, OCH₃), 3.40-3.37 (m, 2H, H-Pro), 2.21-1.87 (m, 16H, 4H-Pro, 3
13 ×OCOCH₃ and NHCOCH₃) ppm. ¹³C-NMR (125 MHz, CD₃OD): δ_C 174.30, 174.08, 172.93,
14 172.30 (C=O ester), 170.27, 170.22, 170.11, 170.01, 169.97, 169.93, 168.69, 168.60, 168.58
15 (NHCOCH₃ and OCOCH₃), 150.73 (d, ²J_{C-P} = 7.1 Hz, “*ipso*” OPh), 150.59 (d, ²J_{C-P} = 7.4 Hz,
16 “*ipso*” OPh), 129.51, 129.44, 124.85, 124.82, 124.76, 119.99, 119.96, 119.85, 119.81 (CH-Ph),
17 91.84, 91.77, 90.77, 90.62 (C-1), 71.34, 71.24, 71.19 (C-5), 69.31, 69.26 (C-3), 65.55 (d, ²J_{C-}
18 _P = 5.5 Hz, C-6), 65.35, 65.27, 65.17 (C-4), 60.63 (²J_{C-P} = 6.9 Hz, NHCHCO), 60.35 (²J_{C-P} = 6.3
19 Hz) (NHCHCO), 51.41, 51.37 (COOCH₃), 49.18, 49.12 (C-2), 47.35, 47.31 (NCH₂CH₂,
20 overlap with the solvent), 30.95, 30.87, 30.80, 30.72 (NCH₂CH₂), 24.87, 24.79, 24.72
21 (NCHCH₂), 21.02, 20.99 (NHCOCH₃), 19.32, 19.30, 19.29, 19.27, 19.24, 19.23 (OCOCH₃)
22 ppm. HPLC: (gradient H₂O/ACN from 90/10 to 0/100 in 30 min, flow: 1 mL/min, λ = 210
23 nm): t_R 15.80 min. MS (ES⁺): m/z 637.19 [M+Na]⁺
24 (3S,4R,5S,6R)-3-acetamido-6-((((2-methoxy-2-oxoethyl)amino)(naphthalen-1-

1 yloxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12d): Prepared
2 according to *Standard procedure 4* in 49 %, yield as a mixture of R_P and S_P diastereoisomers
3 as α and β anomers. Yellow powder; 0.53 g. ^{31}P -NMR (202 MHz, CD_3OD): δ_P 5.57 (0.9P),
4 5.52 (0.4P), 4.75 (1P), 4.60 (0.3P) ppm. ^1H -NMR (500 MHz, CD_3OD): δ_H 8.20-8.17 (m, 1H,
5 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,
6 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-
7 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,
8 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
9 (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,
10 COOCH_3), 2.15-1.96 (m, 12H, $3 \times \text{OCOCH}_3$ and NHCOCH_3) ppm. ^{13}C -NMR (125 MHz,
11 CD_3OD): δ_C 174.43 ($\text{C}=\text{O}$ ester) 170.22, 170.08, 168.78, 168.65, 168.61 (NHCOCH_3 and
12 OCOCH_3), 146.51, 134.91, 127.86 (“*ipso*” ONaph, Naph-C10 and Naph-C9), 127.43, 126.36,
13 126.11, 125.23, 124.60, 121.46 (Naph), 121.35 (d, $^3J_{\text{C-P}} = 6.9$ Hz, Naph-C1), 115.07 (d, $^3J_{\text{C-P}} =$
14 3.9 Hz, Naph-C1), 114.95 (d, $^3J_{\text{C-P}} = 3.3$ Hz, Naph-C1), 91.79, 91.73, 90.71 (C-1), 71.30 ($^3J_{\text{C-P}} =$
15 6.4 Hz, C-5), 71.22 ($^3J_{\text{C-P}} = 6.4$ Hz, C-5), 71.12 (C-5), 69.21 (C-3), 65.63 (d, $^2J_{\text{C-P}} = 5.3$ Hz,
16 C-6), 65.50 (d, $^3J_{\text{C-P}} = 5.7$ Hz, C-6), 65.29 – 65.27 (C-4), 51.26 (OCH_3), 49.18 (C-2), 42.39,
17 42.33, 42.26 (NHCH_2CO), 21.00, 20.97 (NHCOCH_3), 19.28, 19.25 (OCOCH_3) ppm. HPLC:
18 (gradient $\text{H}_2\text{O}/\text{ACN}$ from 90/10 to 0/100 in 30 min, flow: 1 mL/min, $\lambda = 210$ nm): t_R 20.19
19 min, 20.77 min. MS (ES+): m/z 647.17 $[\text{M}+\text{Na}]^+$
20 (3S,4R,5S,6R)-3-acetamido-6-((((((S)-1-ethoxy-1-oxo-3-phenylpropan-2-
21 yl)amino)(phenoxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12e):
22 Prepared according to *Standard procedure 4* in 45 %, yield as a mixture of R_P and S_P
23 diastereoisomers as α and β anomers. Yellow powder; 0.53 g. ^{31}P -NMR (202 MHz, CD_3OD):
24 δ_P 3.40 (1P), 3.21 (0.3P), 3.02 (0.1P) ppm. ^1H -NMR (500 MHz, CD_3OD): δ_H 7.33-7.15 (m,

1 8H, $\underline{\text{CH}}$ -Ar), 7.11, 7.05 (m, 2H, $\underline{\text{CH}}$ -Ar), 5.96 (d, 1H, $J = 1.7$ Hz, $\underline{\text{H-1}}$), 5.93 (d, 1H, $J = 1.7$ Hz,
2 $\underline{\text{H-1}}$), 5.33-5.14 (2H, m, $\underline{\text{H-3}}$ and $\underline{\text{H-4}}$), 4.74-4.02 (m, 6H, H-5 and $\underline{2\text{H-6}}$ 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 $3 \times \text{OCOCH}_3$ 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 ($\underline{\text{C=O}}$ ester,
6 NHCOCH_3 and $3 \times \text{OCOCH}_3$), 152.04 (d, $^2J_{\text{C-P}} = 6.2$ 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 ($\underline{\text{CH}}$ -
8 Ar), 93.13, 92.16, 92.05 ($\underline{\text{C-1}}$), 72.72, 72.61, 72.44 (d, $^2J_{\text{C-P}} = 6.3$ Hz, NHCHCO), 70.71, 70.63
9 ($\underline{\text{C-3}}$), 66.70 ($\underline{\text{C-4}}$), 66.65 (d, $^2J_{\text{C-P}} = 5.5$ Hz, $\underline{\text{C-6}}$), 66.45 (d, $^2J_{\text{C-P}} = 5.4$ Hz, $\underline{\text{C-6}}$), 62.41, 62.33
10 (OCH_2CH_3), 57.80, 57.79, 57.70 ($\underline{\text{C-5}}$), 50.74, 50.67, 50.52 ($\underline{\text{C-2}}$), 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 $\text{H}_2\text{O}/\text{ACN}$ from 90/10 to 0/100 in 30 min, flow: 1 mL/min, $\lambda = 210$ nm): t_{R} 23.11 min, 23.44
13 min, 23.79 min, 24.23 min. MS (ES+): m/z 701.21 $[\text{M}+\text{Na}]^+$
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. ^1H -NMR (500 MHz, CD_3OD): δ_{H} 8.24-7.24 (12H, m, $\underline{\text{CH}}$ -Ar), 6.03
19 (d, 1H $J = 1.6$ Hz, $\underline{\text{H-1}}$), 6.00 (d, 1H, $J = 1.6$ Hz, $\underline{\text{H-1}}$), 5.98 (d, 1H, $J = 1.6$ Hz, $\underline{\text{H-1}}$), 5.96 (d,
20 1H, $J = 1.6$ Hz, $\underline{\text{H-1}}$), 5.50-5.17 (m, 2H, H-3 and H-4), 5.15-5.04 (m, 2H, $\underline{\text{CH}_2}\text{Ph}$), 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, $3 \times \text{OCOCH}_3$
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 ($\underline{\text{C=O}}$ ester), 171.63, 171.51, 171.45, 171.38, 170.04, 170.00
24 (NHCOCH_3 and OCOCH_3), 147.98, 147.93 (“ipso” $\underline{\text{Naph}}$), 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 (NHCOCH₃), 20.68, 20.64 (OCOCH₃), 20.31, 20.25 ppm. HPLC: (gradient
4 H₂O/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. ³¹P-NMR (202 MHz, CD₃OD):
10 δ_P 4.30 (1P), 4.14 (0.2P), 3.96 (0.1P) ppm. ¹H-NMR (500 MHz, CD₃OD): 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, OCH₂Ph 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 \times OCOCH₃, CH(CH₃)₂ and NHCOCH₃), 0.93-0.87 (m, 6H, CH(CH₃)₂) ppm.
16 ¹³C-NMR (125 MHz, CD₃OD): 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 (NHCOCH₃ and OCOCH₃), 150.80
18 (d, ²J_{C-P} = 7.1 Hz, (“*ipso*” OPh), 150.77 (d, ²J_{C-P} = 7.1 Hz, “*ipso*” OPh), 135.81, 135.77 (“*ipso*”
19 OCH₂Ph), 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 (OCH₂Ph), 65.63 (d, ²J_{C-P} = 5.7 Hz, C-6), 65.44, 65.41 (C-4), 60.66 (d, ²J_{C-P} = 7.6 Hz,
23 NHCHCO), 60.49 (d, ²J_{C-P} = 5.1 Hz, NHCHCO), 49.41, 49.34, 49.27, 49.16 (C-2), 31.79 (d,
24 ³J_{C-P} = 7.4 Hz, CH(CH₃)₂), 31.70 (d, ³J_{C-P} = 7.4 Hz, CH(CH₃)₂), 21.16, 21.11, 19.43, 19.35

1 (NHCOCH₃ and OCOCH₃), 18.17, 17.14 (CH(CH₃)₂) ppm. HPLC: (gradient H₂O/ACN from
2 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.
3 MS (ES+): m/z 715.24 [M+Na]⁺
4 (3S,4R,5S,6R)-3-acetamido-6-((((((2S,3S)-1-ethoxy-3-methyl-1-oxopentan-2-
5 yl)amino)(phenoxy)phosphoryl)oxy)methyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (12h):
6 Prepared according to *Standard procedure 4* in 64 %, yield as a mixture of R_P and S_P
7 diastereoisomers as α and β anomers. Yellow powder; 0.12 g. ³¹P-NMR (202 MHz, CD₃OD):
8 δ_P 4.22 (1P), 4.16 (0.2P) ppm. ¹H-NMR (500 MHz, CD₃OD): δ_H 7.38-7.35 (m, 2H, CH-Ar),
9 7.24-7.18 (m, 3H, CH-Ar), 5.97 (d, 0.8H, J = 2.1 Hz, H-1), 5.95 (d, 0.2H, J = 1.9 Hz, H-1),
10 5.36-5.16 (m, 2H, H-4 and H-3), 4.76-4.74 (m, 0.2H, H-2), 4.59-4.58 (m, 0.8H, H-2), 4.21-
11 4.06 (m, 5H, H-6, H-5 and OCH₂CH₃), 3.75-3.68 (m, 1H, NHCHCO), 2.18-1.97 (m, 12H, 3 ×
12 OCOCH₃ and NHCOCH₃), 1.80-1.74 (m, 1H, CH₃CHCH₂), 1.57-1.43 (m, 1H, CHCH₂CH₃),
13 1.27-1.21 (m, 4H, OCH₂CH₃ and CHCH₂CH₃), 0.92-0.85 (m, 6H, CH₃CHCH₂ and
14 CHCH₂CH₃) ppm. ¹³C-NMR (125 MHz, CD₃OD): δ_C 173.00 (d, ³J_{C-P} = 3.3 Hz, C=O ester),
15 172.88 (d, ³J_{C-P} = 3.3 Hz, C=O ester), 172.82 (d, ³J_{C-P} = 3.3 Hz, C=O ester), 172.43 (d, ³J_{C-P} =
16 4.0 Hz, C=O ester), 170.27, 170.21, 170.15, 170.08, 170.02, 169.99, 168.66, 168.59, 168.56
17 (NHCOCH₃ and OCOCH₃), 150.82 (d, ²J_{C-P} = 6.7 Hz, “*ipso*” OPh), 150.80 (d, ²J_{C-P} = 7.4 Hz,
18 “*ipso*” OPh), 129.38, 129.33, 124.74, 124.68, 120.17 (d, ³J_{C-P} = 4.5 Hz, CHPh), 120.01 (d, ³J<sub>C-
19 P</sub> = 4.6 Hz, CHPh), 119.94 (d, ³J_{C-P} = 4.7 Hz, CHPh), 91.72, 90.77 (C-1), 71.18 (d, ³J_{C-P} = 6.9
20 Hz, C-5), 69.29, 69.19 (C-3), 65.56, 65.52 (OCH₂CH₃), 65.39 (C-4), 60.86, 60.77 (C-6), 59.35
21 (NHCHCO), 49.14 (C-2), 38.50, 38.43 (CH₃CHCH₂), 24.67, 24.62, 24.48 (CHCH₂CH₃),
22 21.01, 19.34, 19.29, 19.26 (NHCOCH₃ and OCOCH₃), 14.51, 14.41 (CH₃CHCH₂), 13.14
23 (OCH₂CH₃), 10.25, 10.17 (CHCH₂CH₃) ppm. HPLC: (gradient H₂O/ACN from 90/10 to 0/100
24 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. ³¹P-NMR (202 MHz, CD₃OD):
6 δ_P 2.20 (1P), 1.89 (0.4P), 1.77 (0.1P) ppm. ¹H-NMR (500 MHz, CD₃OD): 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, OCH₂Ph 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 × OCOCH₃ and
11 NHCOCH₃), 1.52-1.44 (m, 6H, CH₃CCH₃) ppm. ¹³C-NMR (125 MHz, CD₃OD): δ_C 176.44 (d,
12 ³*J*_{C-P} = 3.4 Hz, C=O ester), 176.62 (d, ³*J*_{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 (NHCOCH₃ and OCOCH₃), 152.23 (d, ²*J*_{C-P} =
14 7.0 Hz, “*ipso*” OPh), 137.38 (d, ²*J*_{C-P} = 7.0 Hz, “*ipso*” OCH₂Ph), 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, ³*J*_{C-P} = 7.5 Hz, C-5), 74.91 (d, ³*J*_{C-P} = 7.6 Hz, C-5), 72.57, 72.50, 70.78, 70.56
17 (C-3), 68.24, 68.22 (OCH₂Ph), 66.83 (C-4), 66.70 (d, ²*J*_{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 (CH₃CCH₃), 22.42, 22.39,
19 20.73, 20.69, 20.66 (NHCOCH₃ and OCOCH₃) ppm. HPLC: (gradient H₂O/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*

1 diastereoisomers as α and β anomers. Light yellow powder; 0.37 g. ^{31}P -NMR (202 MHz,
2 CD_3OD): δ_{P} 3.73 (0.5P), 3.68 (1P), 3.57 (0.4P) ppm. ^1H -NMR (500 MHz, CD_3OD): δ_{H} 7.37-
3 7.31 (m, 7H, $\underline{\text{CH}}$ -Ar), 7.20-7.17 (m, 3H, $\underline{\text{CH}}$ -Ar), 5.97-5.96 (m, 0.7H, $\underline{\text{H-1}}$), 5.94-5.93 (m, 0.3H,
4 $\underline{\text{H-1}}$), 5.35-5.12 (m, 4H, $\underline{\text{H-3}}$, $\underline{\text{H-4}}$ and OCH_2Ph), 4.58-4.57 (m, 1H, $\underline{\text{H-2}}$), 4.20-4.10 (m, 3H, $\underline{\text{H-}}$
5 $\underline{6}$ and $\underline{\text{H-5}}$), 3.94-3.89 (m, 1H, NHCHCO), 2.16-1.97 (m, 12H, 3 \times OCOCH_3 and NHCOCH_3),
6 1.73-1.65 (m, 1H, $\underline{\text{CH}}(\text{CH}_3)_2$), 1.49-1.46 (m, 2H, CHCH_2CH), 1.56-1.49 (m, 6H, $\text{CH}(\underline{\text{CH}_3})_2$)
7 ppm. ^{13}C -NMR (125 MHz, CD_3OD): δ_{C} 173.82, 173.79, 171.65, 171.59 ($\underline{\text{C}}=\text{O}$ ester), 171.36,
8 169.98, (OCOCH_3 and NHCOCH_3), 152.12 (d, $^2J_{\text{C-P}} = 6.5$ Hz, “*ipso*” OPh), 137.13 (“*ipso*”
9 OCH_2Ph), 130.76, 130.67, 129.54, 129.40, 129.32, 129.27, 126.06, 126.01 ($\underline{\text{CH}}$ -Ar), 121.56
10 (d, $^3J_{\text{C-P}} = 4.6$ Hz, $\underline{\text{CH}}$ -Ar), 121.25 (d, $^3J_{\text{C-P}} = 4.8$ Hz, $\underline{\text{CH}}$ -Ar), 93.10, 93.07, 92.15 ($\underline{\text{C-1}}$), 72.53,
11 72.48 ($\underline{\text{C-5}}$), 70.66, 70.55 ($\underline{\text{C-3}}$), 67.94, 67.85 (OCH_2Ph), 67.91 (d, $^2J_{\text{C-P}} = 10.0$ Hz, $\underline{\text{C-6}}$), 66.72,
12 66.70 ($\underline{\text{C-4}}$), 54.66, 54.60 (NHCHCO), 50.64, 50.48 ($\underline{\text{C-2}}$), 43.87 (d, $^3J_{\text{C-P}} = 7.9$ Hz,
13 CHCH_2CH), 43.72 (d, $^3J_{\text{C-P}} = 7.6$ Hz, CHCH_2CH), 25.50, 25.36 ($\underline{\text{CH}}(\text{CH}_3)_2$), 23.14, 23.03,
14 22.39, 22.34, 22.04, 22.01 21.69, 20.69, 20.63, 20.60 (NHCOCH_3 , 3 \times OCOCH_3 and
15 $\text{CH}(\underline{\text{CH}_3})_2$) ppm. MS (ES+): m/z 729.25 $[\text{M}+\text{Na}]^+$

16 Triethyl ammonium ((2R,3S,4R,5S)-5-acetamido-3,4,6-triacetoxytetrahydro-2H-pyran-2-
17 yl)methyl ((S)-1-carboxy-3-methylbutyl)phosphoramidate (13): Prepared according to the
18 *Standard procedure 3*. Light yellow powder; 47%; 0.37 g. ^{31}P -NMR (202 MHz, CD_3OD ,
19 mixture α and β anomers): δ_{P} 6.53 (1P), 6.41 (0.3P) ppm. ^1H -NMR (500 MHz, CD_3OD): δ_{H}
20 5.93-5.90 (m, 1H, $\underline{\text{H-1}}$), 5.29-5.16 (m, 2H, $\underline{\text{H-3}}$ and $\underline{\text{H-4}}$), 4.53-4.52 (m, 1H, $\underline{\text{H-2}}$, overlap with
21 the solvent), 4.11-4.10 (m, 1H, NHCHCO), 3.91-3.61 (m, 3H, $\underline{\text{H-6}}$ and $\underline{\text{H-5}}$), 3.12 (q, 6H, $J =$
22 7.4 Hz, $\text{N}(\underline{\text{CH}_2\text{CH}_3})_3$), 2.13-1.95 (m, 12H, 3 \times OCOCH_3 and NHCOCH_3), 1.70-1.61 (m, 1H,
23 $\underline{\text{CH}}(\text{CH}_3)_2$), 1.47-1.44 (m, 2H, CHCH_2CH), 1.20 (t, 9H, $J = 7.3$ Hz, $\text{N}(\underline{\text{CH}_2\text{CH}_3})_3$), 0.88-0.83
24 (m, 6H, $\text{CH}(\underline{\text{CH}_3})_2$) ppm. ^{13}C -NMR (125 MHz, CD_3OD): δ_{C} 174.63, ($\underline{\text{C}}=\text{O}$ 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, $^2J_{\text{C-P}}=4.6$ Hz, C-6), 53.41 (C-5), 49.15,
3 49.11 (C-2), 46.65 ($\text{N(CH}_2\text{CH}_3)_3$), 42.93 (d, $^3J_{\text{C-P}}=3.4$ Hz, CHCH_2CH), 24.11 ($\text{CH(CH}_3)_2$),
4 22.04, 21.94, 21.68, 21.37, 21.31, 20.85, 20.24, 20.22, 20.13 (NHCOCH_3 , $3 \times \text{OCOCH}_3$ and
5 $\text{CH(CH}_3)_2$), 8.20 ($\text{N(CH}_2\text{CH}_3)_3$) ppm. LC-MS: (gradient $\text{H}_2\text{O/ACN}$ 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 1×10^5 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 $37 \pm 1^\circ\text{C}$, with 5% CO_2 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 μ L supernatant solution was diluted with 100 μ L
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:

$$7 \quad P_{app} = (dC_r/dt) \times V_r / (A \times C_0)$$

8 Where dC_r/dt is the cumulative concentration of compound in the receiver chamber as a
9 function of time (μ M/s); V_r 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 (μ M).

12 The efflux ratio was calculated using the equation:

$$13 \quad \text{Efflux Ratio} = P_{app} (BA) / P_{app} (AB)$$

14 Percent recovery was calculated using the equation:

$$15 \quad \% \text{ Recovery} = 100 \times [(V_r \times C_r) + (V_d \times C_d)] / (V_d \times C_0)$$

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); C_d and C_r are the final concentrations of transport compound in donor and
18 receiver chambers, respectively.

19 **Kinetic Solubility:** Test compounds (10 mM in DMSO 10 μ L/well) were added into pH 7.4
20 NaH_2PO_4 buffer (490 μ L/well) in a 96-well plate. The final concentration of test compound
21 and DMSO was 200 μ M 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 μ L 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/H₂O. 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= $100 \times (\text{PAR at appointed incubation time} / \text{PAR at T}_0 \text{ time})$, where
17 PAR is the peak area ratio of analyte versus internal standard (IS). The appointed incubation
18 time points are T₀ (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
43

1 the plate map, then added 80 $\mu\text{L}/\text{well}$ 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 ($\mu\text{L}/\text{well}$) 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 $CL_{\text{int(mic)}}$:

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

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

$$9 \quad T_{1/2} = \frac{\text{Ln}2}{k_e} = \frac{0.693}{k_e}$$

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

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

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,

1 10 μ L of the mixture was then applied to the edge of a haemocytometer counting chamber
2 between the cover slip and chamber. The mixture was drawn into the chamber by capillary
3 action.

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

10 **Sialic Acid Quantification Assay:** Cell-compound samples were pelleted after viability for
11 extracting membrane protein. The Mem-PER plus membrane Protein Extraction Kit (Thermo
12 Fisher Scientific) was used for protein extraction. The Mem-PER system consists of three
13 reagents: Cell Wash Solution, Solubilization Buffer, and Permeabilization Buffer. Cell pellets
14 were washed twice in 600 μ L Cell Wash Solution. Supernatant was discarded. 150 μ L
15 Permeabilization Buffer was added to each cell pellet and vortexed to obtain a homogeneous
16 cell suspension. Cells were incubated for 10 minutes at 4 $^{\circ}$ C, then centrifuged for 15 minutes at
17 16,000 \times g. Supernatant contained cytosolic proteins, this was transferred to a new tube. 100
18 μ L Solubilization Buffer was added to cell pellets and were resuspended. Samples were
19 incubated at 4 $^{\circ}$ C for 30 minutes, then centrifuged at 16,000 \times g for 15 minutes. Supernatant
20 containing solubilized membrane and membrane-associated proteins were transferred to a new
21 tube. This fraction was used for measuring sialic acid.

22 Sialic acid concentration was measured with an enzymatic method using the EnzyChrom Sialic
23 Acid Assay Kit (BioAssay Systems) on a Victor X3 plate reader (Perkin Elmer). Kit consists
24 of 6 reagents: Assay Buffer, Enzyme, Dye Reagent, Hydrolysis Reagent, Neutralization
25 Reagent and 10 mM Sialic Acid Standard. Sialic Acid Standard was diluted to make a standard
45

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

12

1 ASSOCIATED CONTENT

2 Supporting Information

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

9

10 AUTHOR INFORMATION

11 Corresponding Author

12 *Email: pertusatif1@cardiff.ac.uk

13 Notes

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

19 ORCID: **Fabrizio Pertusati**: 0000-0003-4532-9101

20 ACKNOWLEDGMENTS

21 We thank Wuxi AppTec for the ADME data.

22 ABBREVIATIONS

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

1 acetyl mannosamine; PMM2, phosphomannomutase 2;

2 REFERENCES

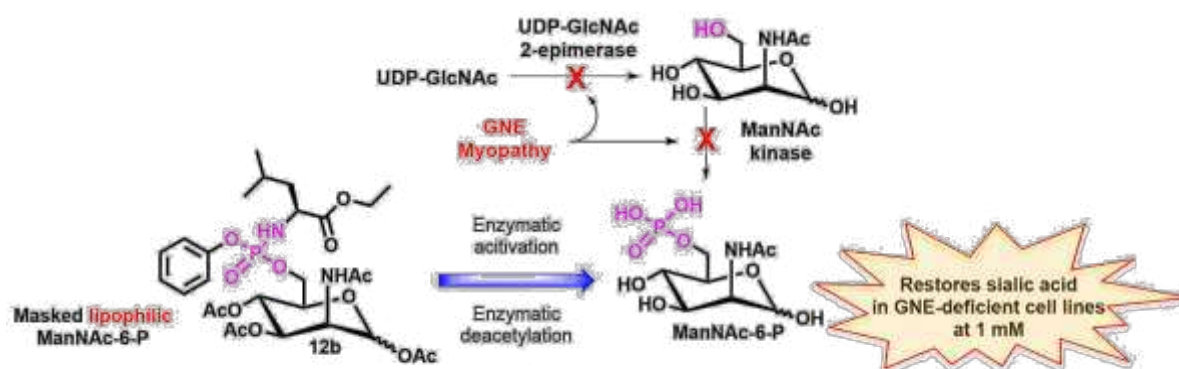
- 3 1. Jaeken, J.; Vanderschueren-Lodeweyckx, M.; Casaer, P.; Snoeck, L.; Corbeel, L.;
4 Eggermont, E.; Eeckels, R. Familial psychomotor retardation with markedly fluctuating serum
5 prolactin, FSH and GH levels, partial TBG-deficiency, increased serum arylsulphatase A and
6 increased CSF protein: a new syndrome?: 90. *Pediatric Research* **1980**, 14, 179.
- 7 2. Hennet, T.; Cabalzar, J. Congenital disorders of glycosylation: a concise chart of
8 glycoalkyl dysfunction. *Trends Biochem. Sci* **2015**, 40, 377-384.
- 9 3. Brasil, S.; Pascoal, C.; Francisco, R.; Marques-da-Silva, D.; Andreotti, G.; Videira, P.
10 A.; Morava, E.; Jaeken, J.; Dos Reis Ferreira, V. CDG Therapies: From Bench to Bedside. *Int*
11 *J Mol Sci* **2018**, 19.
- 12 4. Luchansky, S. J.; Yarema, K. J.; Takahashi, S.; Bertozzi, C. R. GlcNAc 2-epimerase
13 can serve a catabolic role in sialic acid metabolism. *J. Biol. Chem.* **2003**, 278, 8035-42.
- 14 5. Pogoryelova, O.; Gonzalez Coraspe, J. A.; Nikolenko, N.; Lochmuller, H.; Roos, A.
15 GNE myopathy: from clinics and genetics to pathology and research strategies. *Orphanet J*
16 *Rare Dis* **2018**, 13, 70.
- 17 6. Charter, N. W.; Mahal, L. K.; Koshland, D. E., Jr.; Bertozzi, C. R. Biosynthetic
18 incorporation of unnatural sialic acids into polysialic acid on neural cells. *Glycobiology* **2000**,
19 10, 1049-56.
- 20 7. Li, Y.; Chen, X. Sialic acid metabolism and sialyltransferases: natural functions and
21 applications. *Appl. Microbiol. Biotechnol.* **2012**, 94, 887-905.
- 22 8. Celeste, F. V.; Vilboux, T.; Ciccone, C.; de Dios, J. K.; Malicdan, M. C.; Leoyklang,
23 P.; McKew, J. C.; Gahl, W. A.; Carrillo-Carrasco, N.; Huizing, M. Mutation update for GNE
24 gene variants associated with GNE myopathy. *Hum Mutat* **2014**, 35, 915-26.
- 25 9. Penner, J.; Mantey, L. R.; Elgavish, S.; Ghaderi, D.; Cirak, S.; Berger, M.; Krause, S.;
26 Lucka, L.; Voit, T.; Mitrani-Rosenbaum, S.; Hinderlich, S. Influence of UDP-GlcNAc 2-
27 epimerase/ManNAc kinase mutant proteins on hereditary inclusion body myopathy. *Biochem.*
28 **2006**, 45, 2968-77.
- 29 10. Carrillo, N.; Malicdan, M. C.; Huizing, M. GNE Myopathy: Etiology, Diagnosis, and
30 Therapeutic Challenges. *Neurotherapeutics* **2018**, 15, 900-914.
- 31 11. Noguchi, S.; Keira, Y.; Murayama, K.; Ogawa, M.; Fujita, M.; Kawahara, G.; Oya, Y.;
32 Imazawa, M.; Goto, Y.; Hayashi, Y. K.; Nonaka, I.; Nishino, I. Reduction of UDP-N-
33 acetylglucosamine 2-epimerase/N-acetylmannosamine kinase activity and sialylation in distal
34 myopathy with rimmed vacuoles. *J. Biol. Chem.* **2004**, 279, 11402-7.
- 35 12. Nishino, I.; Carrillo-Carrasco, N.; Argov, Z. GNE myopathy: current update and future
36 therapy. *J Neurol Neurosurg Psychiatry* **2015**, 86, 385-92.
- 37 13. <https://ClinicalTrials.gov/show/NCT02346461>. An Open Label Phase 2 Study of
38 ManNAc in Subjects With GNE Myopathy. (accessed Jan 7, 2019).
- 39 14. Xu, X.; Wang, A. Q.; Latham, L. L.; Celeste, F.; Ciccone, C.; Malicdan, M. C.;
40 Goldspiel, B.; Terse, P.; Craddock, J.; Yang, N.; Yorke, S.; McKew, J. C.; Gahl, W. A.; Huizing,
41 M.; Carrillo, N. Safety, pharmacokinetics and sialic acid production after oral administration
42 of N-acetylmannosamine (ManNAc) to subjects with GNE myopathy. *Mol Genet Metab* **2017**,
43 122, 126-134.
- 44 15. <https://clinicaltrials.gov/ct2/show/NCT02377921>. (accessed Jan 7, 2019).
- 45 16. Nemunaitis, G.; Jay, C. M.; Maples, P. B.; Gahl, W. A.; Huizing, M.; Yardeni, T.; Tong,
46 A. W.; Phadke, A. P.; Pappen, B. O.; Bedell, C.; Allen, H.; Hernandez, C.; Templeton, N. S.;
47 Kuhn, J.; Senzer, N.; Nemunaitis, J. Hereditary inclusion body myopathy: single patient

- 1 response to intravenous dosing of GNE gene lipoplex. *Hum Gene Ther* **2011**, 22, 1331-41.
- 2 17. Huizing, M.; Krasnewich, D. M. Hereditary inclusion body myopathy: a decade of
3 progress. *Biochim. Biophys. Acta* **2009**, 1792, 881-7.
- 4 18. Malicdan, M. C.; Noguchi, S.; Tokutomi, T.; Goto, Y.; Nonaka, I.; Hayashi, Y. K.;
5 Nishino, I. Peracetylated N-acetylmannosamine, a synthetic sugar molecule, efficiently rescues
6 muscle phenotype and biochemical defects in mouse model of sialic acid-deficient myopathy.
7 *J. Biol. Chem.* **2012**, 287, 2689-705.
- 8 19. Muus, U.; Kranz, C.; Marquardt, T.; Meier, C. cycloSaligenyl-mannose-1-
9 monophosphates as a New Strategy in CDG-Ia Therapy: Hydrolysis, Mechanistic Insights and
10 Biological Activity. **2004**, 2004, 1228-1235.
- 11 20. Eklund, E. A.; Merbouh, N.; Ichikawa, M.; Nishikawa, A.; Clima, J. M.; Dorman, J.
12 A.; Norberg, T.; Freeze, H. H. Hydrophobic Man-1-P derivatives correct abnormal
13 glycosylation in Type I congenital disorder of glycosylation fibroblasts. *Glycobiology* **2005**,
14 15, 1084-93.
- 15 21. McGuigan, C.; Serpi, M.; Bibbo, R.; Roberts, H.; Hughes, C.; Caterson, B.; Gibert, A.
16 T.; Verson, C. R. A. Phosphate Prodrugs Derived from N-Acetylglucosamine Have Enhanced
17 Chondroprotective Activity in Explant Cultures and Represent a New Lead in
18 Antiosteoarthritis Drug Discovery. *J. Med. Chem.* **2008**, 51, 5807-5812.
- 19 22. Serpi, M.; Bibbo, R.; Rat, S.; Roberts, H.; Hughes, C.; Caterson, B.; Alcaraz, M. J.;
20 Gibert, A. T.; Verson, C. R.; McGuigan, C. Novel phosphoramidate prodrugs of N-acetyl-(D)-
21 glucosamine with antidegenerative activity on bovine and human cartilage explants. *J Med*
22 *Chem* **2012**, 55, 4629-39.
- 23 23. Hamon, N.; Quintiliani, M.; Balzarini, J.; McGuigan, C. Synthesis and biological
24 evaluation of prodrugs of 2-fluoro-2-deoxyribose-1-phosphate and 2,2-difluoro-2-
25 deoxyribose-1-phosphate. *Bioorg. Med. Chem. Lett.* **2013**, 23, 2555-9.
- 26 24. Wiemer, A. J.; Wiemer, D. F. Prodrugs of phosphonates and phosphates: crossing the
27 membrane barrier. *Top. Curr. Chem.* **2015**, 360, 115-160.
- 28 25. Mehellou, Y.; Rattan, H. S.; Balzarini, J. The ProTide Prodrug Technology: From the
29 Concept to the Clinic. *J Med Chem* **2018**, 61, 2211-2226.
- 30 26. Slusarczyk, M.; Serpi, M.; Pertusati, F. Phosphoramidates and phosphonamidates
31 (ProTides) with antiviral activity. *Antivir Chem Chemother* **2018**, 26, 2040206618775243.
- 32 27. Bhatia, H. K.; Singh, H.; Grewal, N.; Natt, N. K. Sofosbuvir: A novel treatment option
33 for chronic hepatitis C infection. *J Pharmacol Pharmacother* **2014**, 5, 278-84.
- 34 28. Ray, A. S.; Fordyce, M. W.; Hitchcock, M. J. Tenofovir alafenamide: A novel prodrug
35 of tenofovir for the treatment of Human Immunodeficiency Virus. *Antiviral Res.* **2016**, 125,
36 63-70.
- 37 29. Slusarczyk, M.; Lopez, M. H.; Balzarini, J.; Mason, M.; Jiang, W. G.; Blagden, S.;
38 Thompson, E.; Ghazaly, E.; McGuigan, C. Application of ProTide Technology to
39 Gemcitabine: A Successful Approach to Overcome the Key Cancer Resistance Mechanisms
40 Leads to a New Agent (NUC-1031) in Clinical Development. *J. Med. Chem.* **2014**, 57, 1531-
41 1542.
- 42 30. Vande Voorde, J.; Liekens, S.; McGuigan, C.; Murziani, P. G.; Slusarczyk, M.;
43 Balzarini, J. The cytostatic activity of NUC-3073, a phosphoramidate prodrug of 5-fluoro-2'-
44 deoxyuridine, is independent of activation by thymidine kinase and insensitive to degradation
45 by phosphorolytic enzymes. *Biochem Pharmacol* **2011**, 82, 441-52.
- 46 31. James, E.; Pertusati, F.; Brancale, A.; McGuigan, C. Kinase-independent
47 phosphoramidate S1P1 receptor agonist benzyl ether derivatives. *Bioorg. Med. Chem. Lett.*
48 **2017**, 27, 1371-1378.
- 49 32. Serpi, M.; Madela, K.; Pertusati, F.; Slusarczyk, M. Synthesis of phosphoramidate
- 49

- 1 prodrugs: ProTide approach. *Curr Protoc Nucleic Acid Chem* **2013**, Chapter 15, Unit15.5.
 2 33. Halcomb, R. L. F., W.; Wong, C. . Enzymatic synthesis of 7-deoxy-N-acetylneuraminic
 3 acid and 7-O-methyl-N-acetylneuraminic acid. *Tetrahedron-asymmetry* **1994**, *5*, 2437-2442.
 4 34. Aich, U.; Campbell, C. T.; Elmouelhi, N.; Weier, C. A.; Sampathkumar, S. G.; Choi,
 5 S. S.; Yarema, K. J. Regioisomeric SCFA attachment to hexosamines separates metabolic flux
 6 from cytotoxicity and MUC1 suppression. *ACS Chem. Biol.* **2008**, *3*, 230-40.
 7 35. Chevallier, O. P.; Migaud, M. E. Investigation of acetyl migrations in furanosides.
 8 *Beilstein journal of organic chemistry* **2006**, *2*, 14-14.
 9 36. I. Gorbach, V.; N. Krasikova, I.; Lukyanov, P.; Solov'eva, T.; Ovodov, Y. *Migration of*
 10 *an acetyl group in glucosamine and glucose derivatives by the action of butyllithium. The*
 11 *synthesis of an N-acetylglucosamine 4-phosphate derivative.* 1987; Vol. 36, p 1957-1960.
 12 37. <http://www.swissadme.ch>. (accessed May 24, 2019).
 13 38. Hong, Y.; Stanley, P. Lec3 Chinese hamster ovary mutants lack UDP-N-
 14 acetylglucosamine 2-epimerase activity because of mutations in the epimerase domain of the
 15 Gne gene. *J. Biol. Chem.* **2003**, *278*, 53045-54.

16

17
 18 Table of Contents graphic



19

20

21

22

23

24

25

26