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Novel Phosphoramidate Prodrugs of *N*-Acetyl-(D)-Glucosamine with Antidegenerative Activity on Bovine and Human Cartilage Explants

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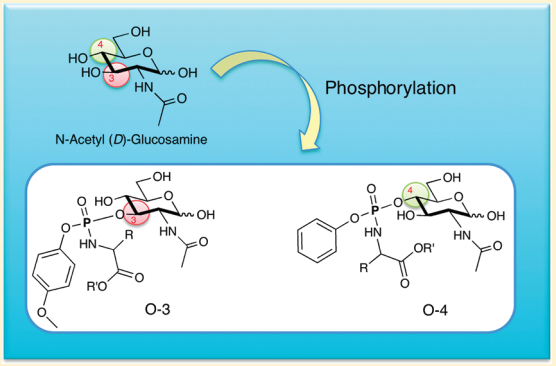
Supporting Information

ABSTRACT: (D)-Glucosamine and other nutritional supplements have emerged as safe alternative therapies for osteoarthritis (OA), a chronic and degenerative articular joint disease. In our preceding paper, a series of novel O-6 phosphate *N*-acetyl (D)-glucosamine prodrugs aimed at improving the oral bioavailability of *N*-acetyl-(D)-glucosamine as its putative bioactive phosphate form were shown to have greater chondroprotective activity in vitro when compared to the parent agent. In order to extend the SAR studies, this work focuses on the O-3 and O-4 phosphate prodrugs of *N*-acetyl-(D)-glucosamine bearing a 4-methoxy phenyl group and different amino acid esters on the phosphate moiety. Among the compounds, the (L)-proline amino acid-containing prodrugs proved to be the most active of the series, more effective than the prior O-6 compounds, and well processed in chondrocytes in vitro. Data on human cartilage support the notion that these novel O-3 and O-4 regioisomers may represent novel promising leads for drug discovery for osteoarthritis.



INTRODUCTION

2-Amino-2-deoxy-(D)-glucose ((D)-glucosamine, **1**) (Figure 1) is an amino monosaccharide derivative of (D)-glucose. It is found in numerous biologically potent molecules such as cell surface *N*-glycoproteins, hyaluronic acid, glycosphingolipids (Lewis a/x), glycosylphosphatidylinositol (GPI) anchors, blood group antigens, bacterial cell wall, lipopolysaccharides, chitin/chitosan, and glycosaminoglycan (GAGs) chains. Most importantly, it is a precursor in the biosynthesis of the GAGs[1] that, covalently linked with a protein core, form the proteoglycans (PGs). Proteoglycans[2] are major components of the extracellular matrix of the articular cartilage and, together with the chondrocytes, the cellular components play a pivotal role in the functioning of joints.[3] Failure of chondrocytes to maintain the balance between synthesis and degradation of the extracellular matrix may lead to osteoarthritis (OA), which is a degenerative and progressive joint disorder characterized by joint pain, tenderness, limitation of movement, crepitus, occasional effusion, and variable degrees of joint inflammation.[4] Cartilage is responsible for providing the shock-absorption at the end of bones, and it is indeed the loss of this cushioning effect due to the loss of GAGs that results in pain and loss of range of movement. Osteoarthritis (OA) is a major cause of disability and, being strongly associated with aging, its medical relevance is rising given the increasing proportion of older people. It can be treated with analgesics and nonsteroidal anti-inflammatory drugs, but these drugs can cause serious gastrointestinal and cardiovascular adverse events, especially with long-term use,[5] and they do not address the underlying physiology of the disease. Disease-modifying drugs that

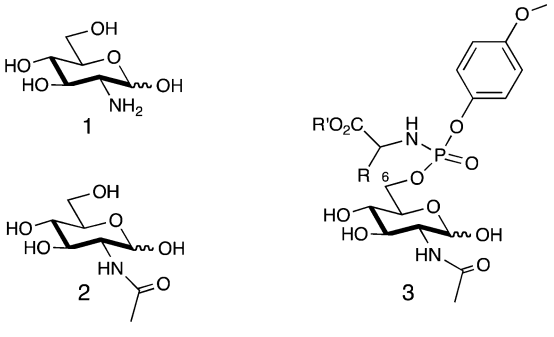


Figure 1. Structures of (D)-glucosamine (**1**), *N*-acetyl-(D)-glucosamine (**2**), and general structure of the O-6 aryloxy phosphoramidates of **2** (**3**). Stereochemistry at the phosphorus and amino acid α-carbon is omitted.

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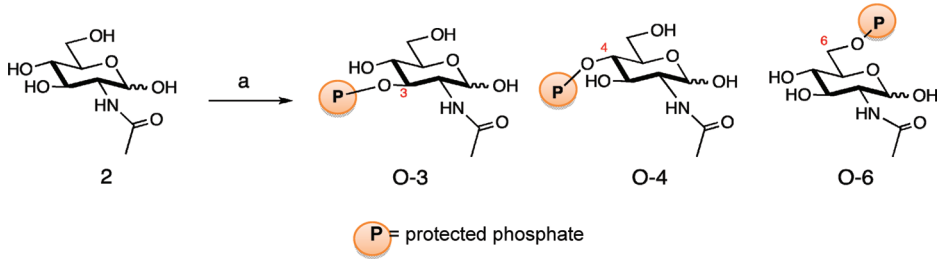
Scheme 1[a]



[a]Reagents and conditions: (a) Phosphorochloridate, NMI, pyridine, THF, −30 °C to ambient, 5 h.

interfere with the progression of the condition would be strongly desired.

Worldwide, over the past 10 years, products containing chondroitin (GAGs) and **1** have been increasingly recommended by general practitioners and rheumatologists, and nowadays **1** (either as sulfate or chloride salts) represents one of the most commonly used agents to treat OA.[6−11] Despite the increased use of **1** in the treatment of OA, the mechanisms accounting for its in vivo and in vitro activity are still poorly understood. The most common notion is that augmenting the intake of the precursor molecule **1** may directly stimulate articular PG synthesis,[12,13] although the results of these investigations have been controversial.[14] Different studies have also presented experimental evidence that **1** and, to a larger degree, *N*-acetyl-(D)-glucosamine (**2**) (Figure 1), possess a unique range of anti-inflammatory activities.[15] **2** is one of the building blocks for glycoproteins, formed from (D)-glucose and/or **1** during the in vivo biosynthesis of PGs.[1] (D)-Glucose is a general precursor for cellular GAG biosynthesis. Inside the cell, (D)-glucose is converted into (D)-glucose 6-phosphate and (D)-fructose-6-phosphate. The conversion of fructose-6-phosphate to glucosamine-6-phosphate takes place by the enzyme glutamine fructose-6-phosphate aminotransferase. (D)-Glucosamine-6-phosphate is rapidly converted into the *N*-acetyl-(D)-glucosamine-6-phosphate by acetyl-CoA glucosamine-6-phosphate *N*-acetyltransferase. However, exogenous **1** or **2** supplemented to the cultured cells can enter this metabolic pathway by conversion into (D)-glucosamine-6-phosphate. *N*-Acetyl-glucosamine-6-phosphate is further converted via *N*-acetylglucosamine-1-phosphate into uridine diphosphate (UDP) *N*-acetyl-(D)-glucosamine, and by epimerase into UDP-*N*-acetyl-(D)-galactosamine.[1] These nucleotide-activated sugars, together with UDP-glucuronic acid, are utilized in the assembly of GAG chain. By analogy to **1**, **2** has been found to decrease pain and inflammation and to aid cartilage repair, increasing the range of motion in osteoarthritic patients.[16]

One of the aims in the development of our phosphate prodrugs was to deliver the putative bioactive O6 monophosphate directly and to increase the lipophilicity of **1** which, due to the highly polar character ($ClogD_{(pH=6.50)} = -3.93$),[17] has an inadequate oral bioavailability (~5−20%).[18−20] Our group had previously investigated a family of aryloxy phosphoramidates at the O-6 position of **2** (see general structure **3** in Figure 1).[21] Several of these prodrugs at concentrations as low as 0.1 mM were noncytotoxic and ca. ≥ 100-fold more active than the parent compound in a biological assay for inhibition of interleukin 1 (IL-1) induced GAG release from bovine articular cartilage in in vitro explant cultures.

On the basis of these encouraging results and as a continuation of our study on *N*-acetyl-(D)-glucosamine monophosphate prodrugs, we decided to investigate the regioisomeric O-3 and O-4 prodrugs of **2**. These were originally identified as synthetic byproduct, but we herein report the surprising biological activity of these analogues.

RESULTS AND DISCUSSION

Chemistry. We have previously reported[21] that reaction of **2** with different phosphorochloridates in THF/pyridine in the presence of *N*-methyl imidazole (NMI) at −30 °C to ambient yields a complex mixture of regio (mono phosphorylated at O-3, O-4, or O-6) and stereoisomers (R_p and S_p, α and β anomers). In the first instance, we were able to isolate from the mixtures pure O-6 derivatives in a range of yield between ≤1−21%. Besides the desired O-6 isomers, formation of the O-3 and O-4 regioisomers were also detected, but at such time they were not isolated in pure form (Scheme 1).

More recently, we were able to isolate the O-3 and O-4 phosphoramidate regioisomers in addition to the O-6 prodrugs by reacting **2** with a series of freshly prepared phosphorochloridates (**6a−v**) in the presence of NMI in THF/pyridine at −30 °C to ambient. At first, except for **9d** and **9o**, which were isolated as pure O-3 isomers, O-4 and O-3 phosphoramidate regioisomers were recovered as mixtures (**8a−v**/**9a−v**) in a range of isolated yields between ≤1−27% (Scheme 2, Table 1). According to the observed ratios of regioisomers, the formation of O-4/O-3 isomers was in general more favored than the O-6 derivatives, which were found to be predominant only in reactions with α,α-dimethylglycine or (L)-proline amino acid-containing phosphorochloridates. ^{1}H NMR spectra of the O-4/O-3 isomer mixtures suggested that the O-3 derivatives were always the predominant regioisomers. This result is in good accordance with the literature, which provides much evidence of the poor reactivity of the 4-OH[22] of **2**, when compared to the 3-OH. Several explanations have been provided, including steric hindrance at this position,[22] formation of either intermolecular or intramolecular hydrogen bonding, involving the glucosamine amide group and lowering the reactivity of the hydroxyl groups,[23] and NH group acting as a competitive nucleophile.[24]

There was no significant increase in yield or any major change in the ratio of regioisomers when the temperature was varied from −30 °C to room temperature.

Preliminary biological evaluation indicates that several of the O-4/O-3 regioisomeric mixtures (**8a−s**/**9a−s**) are able to significantly reduce the in vitro IL-1 induced GAG release (see the later section on Biological Activity), suggesting that testing O-3 and O-4 regioisomers individually was essential. Separation of O-4/O-3 isomers by extensive and repeated column chromatography was achieved in a few cases (Table 2).

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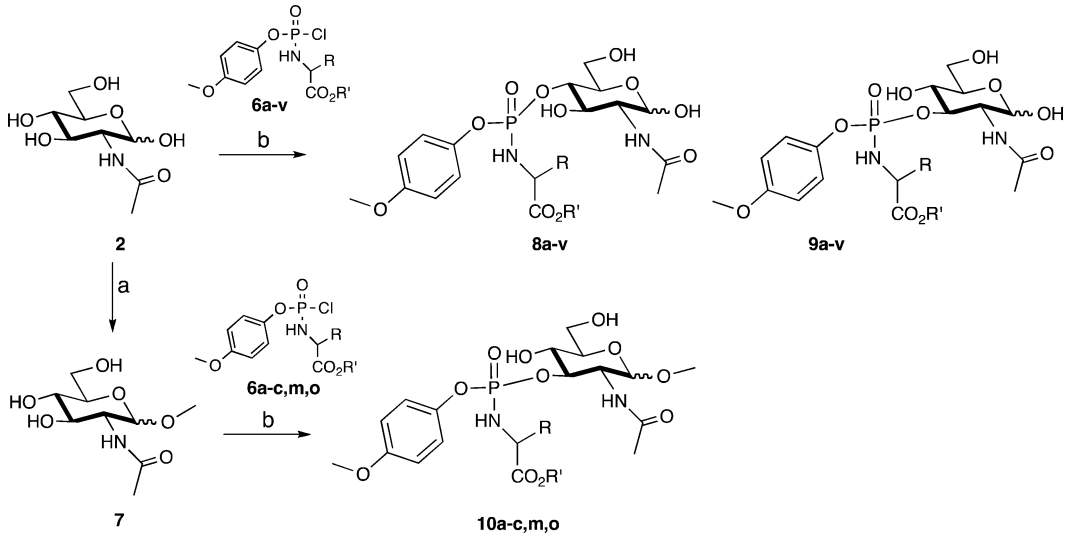
Scheme 2[a]



[a]Reagents and conditions: (a) Dowex 50 WXS 200, methanol, reflux, 24h; (b) NMI, pyridine, THF, −30 °C to rt, 5 h. O-6 isomers are also formed (not shown).

Table 1. Substitution Pattern and Isolated Yields of *N*-Acetyl-(D)-Glucosamine O-4/O-3 Phosphoramidate Derivatives 8a−v/9a−v and 1-*O*-Methyl-*N*-Acetyl-(D)-Glucosamine O-3 Phosphoramidate Derivatives 10a−c,m,o

compd	AA	R′	yield (%)	compd	AA	R′	yield (%)
8a/9a	(L)-Ala	Bn	16	**9o**	(L)-Sar	Et	2
8b/9b	(L)-Val	Bn	28	**8p/9p**	(L)-Pro	*n*Bu	8
8c/9c	(L)-Pro	Bn	2	**8q/9q**	(L)-Val	*t*Bu	9
9d	(L)-Phe	Bn	3	**8r/9r**	(L)-Val	cyclohex	5
8e/9e	(L)-Leu	Bn	16	**8s/9s**	(L)-Val	2-Bu	5
8f/9f	(L)-ILe	Bn	13	**8t/9t**	(L)-Pro	*i*Pr	1.5
8g/9g	$(Me)_2$Gly	Bn	7	**8u/9u**	(L)-Pro	2-Bu	2.4
8h/9h	Gly	Bn	6	**8v/9v**	(L)-Pro	cyclohex	1.8
8i/9i	(L)-Met	Bn	5	**10a**	(L)-Ala	Bn	13
8j/9j	(D)-Ala	Bn	1	**10b**	(L)-Val	Bn	3
8k/9k	(L)-Gly	Et	1	**10c**	(L)-Pro	Et	3
8l/9l	(L)-Pro	Et	1	**10m**	(L)-Val	Et	0.3
8m/9m	(L)-Val	Et	5	**10o**	(L)-Sar	Et	1
8n/9n	(L)-Val	*i*Pr	9				

Table 2. Substitution Pattern and Isolated Yields of *N*-Acetyl-(D)-Glucosamine O-3 Phosphoramidate Derivatives 8c,l,p,t−v and *N*-Acetyl-(D)-Glucosamine O-4 Phosphoramidate Derivatives 9c,l,p,t−v

compd	AA	R′	yield (%)	compd	AA	R′	yield (%)
8c	(L)-Pro	Bn	0.5	**8t**	(L)-Pro	*i*Pr	05
9c	(L)-Pro	Bn	1.3	**9t**	(L)-Pro	*i*Pr	1.0
8l	(L)-Pro	Et	0.8	**8u**	(L)-Pro	2-Bu	0.8
9l	(L)-Pro	Et	1.3	**9u**	(L)-Pro	2-Bu	1.6
8p	(L)-Pro	*n*Bu	1.0	**8v**	(L)-Pro	cyclohex	0.5
9p	(L)-Pro	*n*Bn	1.1	**9v**	(L)-Pro	cyclohex	1.3

However, because the separation proved to be slow and inefficient, it was decided to investigate a regioselective synthetic procedure. Considering that the 4-OH is reported to be the least reactive secondary OH group and that the O-3 phosphoramidates appeared to be the most abundant in the mixture, our initial effort was focused on obtaining the O-3 regioisomers selectively.

Interestingly, it has been demonstrated that in a sugar moiety the hydroxyl group reactivity pattern usually depends upon the nature of the surrounding protecting groups. In the case of glucosamine, many publications showed the strong influence of the protecting groups at the O-6 and N-2 positions as well as the configuration of the anomeric carbon on the observed regioselectivity of reaction.[25−27] In particular, it has been noted that bulky protecting groups at O-6 position could hinder the topside of the 3-OH,[28,29] whereas large N-2 groups may impart steric hindrance on the neighboring O-3 position,[30] in both cases preventing the possibility to approach the 3-OH. Previous studies[26] have also shown that O-6 protected methyl α-glycoside derivatives tend to give preferentially O-3 substitution,

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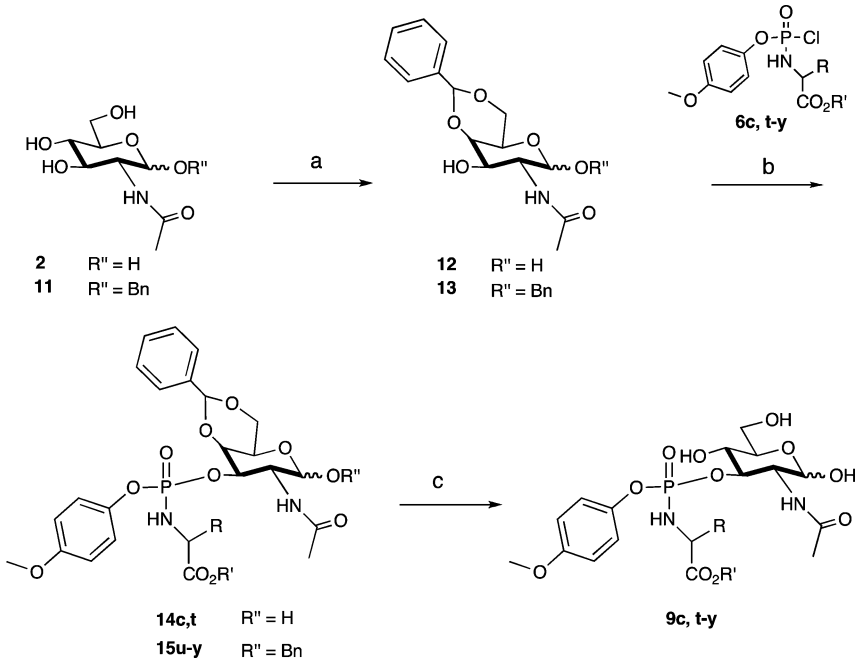
Scheme 3[a]



[a]Reagents and conditions: (a) cat. *p*TsOH, $PhCH(OMe)_2$, DMF, 55 °C 24h; (b) NMI, pyridine, THF, −30 °C to rt, 5 h or 1M *t*BuMgCl, THF, rt, 5 h; (c) I_2, MeOH at reflux or H_2, Pd/C, EtOH, 55 psi rt.

whereas the corresponding β anomers gave mainly substitution at the O-4 position.

As a first attempt to pursue selective phosphorylation at the O-3 over the O-4 position, protection at the O-1 position of **2**, favoring mainly formation of the α anomer, was investigated. Thus 1-*O*-methyl-*N*-acetyl-D-glucosamine (**7**) (ratio α/β 8:1), selected for these studies, was synthesized by treatment of **2** with Dowex 50 WXS 200 in methanol at reflux, and then it was reacted with different phosphorochloridates using the same conditions described above (Scheme 2, Table 1). In all cases, besides the O-6 derivatives (data not shown), exclusively O-3 regioisomers were obtained only as α anomers (**10a−c,m,o**) as determined on the basis of the C-3-P and H-1−H-2 coupling constant values ($J_{(C\text{-}3\text{-}P)}$ ~7.0 Hz and $J_{(H\text{-}1,\ H\text{-}2)}$ = 3.0 Hz). No traces of O-4 derivatives were detected. Because the O-6 isomers were predominant, regardless of which amino acid-containing phosphorochloridate was used, it is clear how manipulation of the protecting group on a sugar moiety can not only increase the usual selectivity (3-OH ≫ 4-OH) but also reverse it (6-OH > 3-OH). Unfortunately, no significant improvement in the yield of O-3 phosphate regioisomers was achieved (≤1−13%).

Therefore, in view of these results and in order to achieve regioselective phosphorylation, a regioselective-protecting strategy had to be applied. It was decided to introduce a 4,6-*O*-benzylidene protecting group onto the sugar, the advantages of which were to block both the O-4 and O-6 positions. To this end, **2** was transformed into the *N*-acetyl-4,6-*O*-benzylidene-D-glucosamine (**12**) by treatment with benzaldehyde dimethylacetal in the presence of *p*-toluenesulfonic acid (*p*-TsOH) as catalyst [Scheme 3].[31] Then derivatives **9c** and **9t** were synthesized, reacting **12** with the appropriate phosphorochloridate (**6c** or **6t**) in THF/pyridine at −30 °C to rt, followed by either treatment with I_2 in methanol at reflux[32] or catalytic hydrogenation under pressure at rt (Scheme 3, Table 2). By following the latter strategy, only the desired O-3 regioisomer was produced, however, there was not a satisfactory increase in yield for the coupling reaction (3.4−8.4% yield).

As a final synthetic strategy, it was decided to protect the 1-OH, 4-OH, and 6-OH groups simultaneously. Therefore, the reaction of 1-*O*-benzyl-*N*-acetyl-D-glucosamine (**11**) with benzaldheyde dimethylacetal in the presence of *p*-TsOH as a catalyst afforded regioselectively 1-*O*-benzyl-4,6-di-*O*-benzylidene-*N*-acetyl-D-glucosamine (**13**) (ratio α/β 8:2) (Scheme 3).[31] Having protected all the hydroxyl groups, which could possibly compete with the 3-OH for phosphorylation, it was decided to use the Grignard reagent, *tert*-butyl magnesium chloride (*t*BuMgCl), as base. Because of the higher solubility of **13** in organic solvent when compared to **2**, it was possible to replace pyridine with tetrahydrofuran (THF). Reaction of **13** with different phosphorochloridates (**6u−y**) furnished O-4, O-6-protected O-3 phosphoramidates **15u−y** in moderate yields (30−69%) (Scheme 3, Table 3). Both the 4,6-di-*O*-benzylidene and benzyl groups were removed in one pot by hydrogenation under pressure in the presence of a palladium catalyst. In some cases, partial deprotection along with decomposition was observed, causing lowering of the yield and requiring further purification on silica gel in order to afford the final compounds **9u−y**.

Biological Activity. To examine the effect of the glucosamine derivatives on the loss of aggrecan (cartilage proteoglycan) from articular cartilage (an early event in the development of matrix degradation in osteoarthritis), we used the previously established[21] in vitro culture system where cartilage explants were exposed to IL-1 (to induce the loss of aggrecan from the tissue) in the presence or absence of the glucosamine derivatives. Exposure of bovine articular cartilage explant cultures to IL-1 led to an approximately 3- to 4-fold increase in levels of glycosaminoglycan (GAG) released (which is representative of a loss of aggrecan from the tissue due to degradation by matrix proteases) relative to untreated controls.

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Table 3. Substitution Pattern and Isolated Yields of Protected *N*-Acetyl-(D)-Glucosamine O-3 Phosphoramidate Derivatives 14c,t and 15u−y and *N*-Acetyl-(D)-Glucosamine O-3 Phosphoramidate Derivatives 9c,t−y

compd	R″	AA	R′	yield (%)	compd	R″	AA	R′	yield (%)
14c	H	(L)-Pro	Bn	8	**9c**	H	(L)-Pro	Bn	20
14t	H	(L)-Pro	*i*Pr	3	**9t**	H	(L)-Pro	*i*Pr	70
15u	Bn	(L)-Pro	2-Bu	30	**9u**	H	(L)-Pro	2-Bu	30
15v	Bn	(L)-Pro	cyclohex	41	**9v**	H	(L)-Pro	cyclohex	12
15w	Bn	(L)-NorVal	Et	69	**9w**	H	(L)-NorVal	Et	77
15x	Bn	(L)-Pro	*t*Bu	47	**9x**	H	(L)-Pro	*t*Bu	10
15y	Bn	(L)-Val	Me	38	**9y**	H	(L)-Val	Me	30

Table 4. Efficacy and Toxicity Data of (D)-Glucosamine (1), *N*-acetyl-(D)-Glucosamine (2) Representative Phosphoramidate of 2 from O-6 Series (16)[b] and *N*-Acetyl-(D)-Glucosamine Phosphoramidate Derivatives 8a−s/9a−s, 9t−y, and 10a−c,m−o in Bovine Cartilage Explant[a]

	10 mM			1 mM			0.1 mM		
compd	*n*	% reduction GAG fold (±SE)	MTT (±SE)	*n*	% reduction GAG fold (±SE)	MTT (±SE)	*n*	% reduction GAG fold (±SE)	MTT (±SE)
1	2	19 (30.42)	56 (0.5)	5	3.4 (4.5)	88 (2.51)	4	0.8 (5.9)	106 (4.18)
2	1	9.3	61(1.6)	3	−1.0 (7.6)	70 (1.68)	2	5.4 (0.4)	107 (2.03)
16[b]				2	31.0 (6.38)	66 (1.92)	4	24.3 (16.28)	96 (2.91)
8a/9a	1	71.2	44			36	4	−2.7 (16.0)	79
8b/9b				4	61.0 (4.4)	100	1	26.9 (4.0)	
8c/9c	1	27.2	60	5	66.2(6.9)	87	1	31.6 (4.5)	94
9d									
8e/9e	1	68.3	52			22	4	−7.0 (6.4)	96
8f/9f	1	25.3	46			47	2	−8.9 (17.2)	100
8g/9g						33	2	−5.5 (9.8)	56
8h/9h						81	5	−9.6 (10.1)	100
8i/9i				1	70.4	100	5	12.9 (11.3)	99
8j/9j				2	54.3 (13.6)	40	6	15.1 (4.0)	83
8k/9k									
8l/9l						96	6	15.6 (12.5)	103
8m/9m							2	0.2 (66.5)	98
8n/9n									
9o						80	2	−24.9 (5.0)	32
8p/9p								−5.4 (8.0)	91
8q/9q						22	3	−14.4 (7.2)	92
8r/9r						8	4	5.1 (18.1)	60
8s/9s						26	6	41.1 (6.4)	84
9t							2	17.5 (6.4)	90
9u						7	3	−4.4 (15.7)	96
9v								−9.6	
9w							2	11.8 (11.5)	93
9x						5	3	−8.0 (9.4)	91
9y						7	3	−4.4 (15.7)	96
10a							5	−19.0 (17.2)	78
10b							2	9.4 (25.2)	99
10c							4	−53.7 (33.4)	62
10m							4	−52.5 (28.4)	85
10o							2	−23.2 (5.4)	100

[a]The average fold increase in GAG release into the culture media in IL-1 treated cultures is calculated using the appropriate control (minus IL-1) for explants cultured in the absence (control GAG fold) and presence (GAG fold) of glucosamine compounds at concentrations ranging from 10 to 0.1 mM. The percent reduction in GAG fold was calculated for each experiment as the percent difference observed for each experiment using the following calculation: {[(control GAG fold) − (sample GAG fold)]/(control GAG fold)} × 100. The figures described above are the mean values of the calculated results from individual experiments within the sample group. The number of experiments performed using cartilage explants from different cows are indicated by *n*, where experiments were performed in triplicate for each number. The effects of different concentrations of glucosamine compounds on chondrocyte viability were assessed using the MTT assay. The percentage cell viability was calculated compared to the control cells (absence of glucosamine compounds, taken as 100%). Standard error SE was calculated from the data generated from the number of experiments *n*; some high standard error readings are due to biological variability of the cartilage obtained. [b]**3j** from ref 21.

In each experiment, a control level of fold-increase in GAG released from the explant as a result of exposure to IL-1 only was determined. A fold-increase was also calculated for cartilage explants cultured in the presence of glucosamine derivatives

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with or without IL-1. This GAG-fold was then taken and compared to the individual control GAG-fold for each set of experiments. From these two figures, a percent reduction in GAG-fold release was calculated and the values are reported herein (Table 4). Thus, in the first set of experiments, where drug substance was dosed at 10 mM, the mean increase in GAG released in controls (i.e., IL-1 alone, no glucosamine derivatives added) was 2.6-fold. Drug efficacy was measured as the reduction in this fold-increase in GAG release into the culture media. Thus, treatment with **1** at 10 mM led to a reduction in GAG release (before/after exposure) to 2-fold, representing a 19% reduction relative to the control (IL-1 only treated explant culture), while **2** caused only 9% reduction relative to the control. It is also notable that in the parallel cytotoxicity assays, conducted using the MTT assay on chondrocyte monolayer cultures, both **1** and **2** were cytotoxic at 10 mM with only an approximately 60% cell viability relative to the control. Whether the apparent inhibition of GAG release by these agents was in part attributable to cytoxicity is at present unclear. However, we were able to ascertain that these compounds were considerably less cytotoxic at 1 and 0.1 mM, but at these lower concentrations, both of these compounds were unable to inhibit IL-1 induced aggrecan release from the tissue (% reduction GAG fold). Thus, it is not impossible that all of the chondroprotective effect of (**1**) and (**2**) at 10 mM is simply due to cytotoxicity. There was no nontoxic concentration of (**1**) or (**2**) at which we were able to demonstrate a protective effect in the assay.

By contrast, phosphate derivatives when tested at 0.1 mM concentration showed low or no cytoxicity. At the same concentration, several of the O-4/O-3 phosphoramidate mixtures synthesized were much more active than the parent compounds **1** and **2**, indeed showing a reduction of GAG release to the same or greater extent when compared to the most active O-6 phosphoramidate **16** previously reported by us (**3j** from reference 21). At first sight, the increased activity of prodrugs with the phosphate attached to the "wrong" site, with respect to the putative pharmacophore, is surprising. As previously mentioned, *N*-acetyl-(D)-glucosamine-6-phosphate is indeed the active intermediate formed during the in vivo biosynthesis of PGs. However, the identification and cloning of the human phosphoacetylglucosamine mutase,[33] which has been reported to be able to interconvert *N*-acetyl-(D)-glucosamine-6-phosphate into *N*-acetyl-(D)-glucosamine-1-phosphate during the in vivo biosynthesis of GAGs, could explain how activity is not solely found in phosphoramidates where the phosphate is attached to a specific hydroxyl group. It cannot be excluded that the phosphohexose mutase may be able to transfer the phosphate group to the O-1 position from either O-3 or O-4 positions of the phosphoramidate or a metabolite thereof. Among the active compounds, the percentage reduction of GAG release varied over a range of 9−41%. Analysis of the data shows the importance of the amino acid side chain. The (L)-valine and (L)-proline emerged as the amino acids of choice, the compounds **8s**/**9s** and **8c**/**9c** being the most active among the series. The low or no activity found using the 1-O methylated compounds indicate that a free 1-OH is an essential requisite for maintaining activity.

Additionally, the chondroprotective activity of several proline amino acid-containing phosphoramidates as either pure O-3 or O-4 isomer was investigated in human cartilage explants. Table 5 summarizes the preliminary results of the GAG release inhibition assay for compounds **9c**, **9l**, **9p**, **9t**, **9u**, and **8l**.

Table 5. Efficacy and Toxicity Data of (D)-Glucosamine (1) and *N*-Acetyl-(D)-Glucosamine O-3 Phosphoramidate Derivatives 9c, 9l, 9p, 9t, and 9u and *N*-Acetyl-(D)-Glucosamine O-4 Phosphoramidate Derivative 81 in Human Cartilage Explant[a]

				% reduction in GAG release	% metabolic activity
compd	AA	ester	isomer	10 μM	10 μM
9c	(L)-Pro	Bn	O3	28.8	115.4
9l	(L)-Pro	Et	O3	38	66.4
8l	(L)-Pro	Et	O4	17.4	106.7
9p	(L)-Pro	*n*Bu	O3	30.4	87.1
9t	(L)-Pro	*i*Pr	O3	24.4	79.4
9u	(L)-Pro	2-Bu	O3	27.1	53.9

	% reduction in GAG release		% metabolic activity	
compd	250 μM	10 μM	250 μM	10 μM
1	14.1	10.3	85.3	106.7

[a]The percentage of reduction in GAGs release for each compound was calculated using the following calculation: {[(mean control GAG's release) − (mean sample GAG's release)]/(control GAG's release)} × 100.

Compounds **9c**, **9l**, and **9p** showed a statistically significant activity in the reduction of GAG release at 10 μM, whereas **9t** and **9u** were slightly less active at the same concentration. The O-4 isomer **8l** showed substantial decrease in activity compared to its corresponding O-3 isomer **9l**, suggesting that the O-4 isomer series is most probably less active than the O-3 isomers. Most importantly, **1** was inactive at the same or even higher doses (see Table 5).

Some of the compounds showed some degree of cytotoxic effects in WST-1 assay at the studied doses. Derivative **9c**, the proline benzyl ester, showed the best chondroprotective effect/cytoxicity ratio among the series, being significantly more active and less cytotoxic than **1**.

The CLogP values were also evaluated using the program Marvin Sketch v. 5.2.0. Log *P* is an index of molecular hydrophobicity, a parameter that affects the compound bioavailability, besides interaction with biological targets, metabolism as well as the toxicity. It has become one of the key parameters used to study the fate and behavior of bioactive compounds. According to Lipinski's rule of five, Log *P* should optimally be not greater than 5. The prediction results showed that Log *P* values of the identified active compounds are between −0.99 and 0.99 (Table 6), confirming that they represent promising candidates for further lead optimization and development.

Table 6. Predicted Log *P* Values for (D)-Glucosamine (1) *N*-Acetyl-(D)-Glucosamine (2) and the Active Compounds 8b/9b, 9c, 9l, 9p, and 8s/9s

compd	AA	ester	Log *P*
1			−3.04
2			−3.22
8b/9b	(L)-Val	Bn	0.37
9c	(L)-Pro	Bn	0.99
9l	(L)-Pro	Et	−0.99
9p	(L)-Pro	*n*Bu	−0.17
8s/9s	(L)-Val	2-Bu	0.56

Stability Studies. The stability of several compounds (**8c**/**9c**, **8b**/**9b**, **9l**, and **8s**/**9s**, **9y**) was studied in biological media

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using ^{31}P NMR analysis. First, we investigated the stability in guinea pig and human serum to assess the potential of these compounds as drugs, and then we studied their stability in chondrocyte cell lysate in order to verify whether or not the phosphoramidates may be activated once delivered to the cell. The disappearance of the phosphoramidate and the appearance of metabolites were monitored by ^{31}P NMR for several hours at 15 min intervals at 37 °C. Because of excess noise and poor shimming profiles (most likely due to the biological media and concentration), individual spectra were processed further. After normal Fourier transform processing, each spectrum was deconvoluted (Lorentz−Gauss deconvolution) to reveal solely the frequency and area of spectral peaks without the baseline. To assess the enzymatic stability, the rate of disappearance of each prodrug was determined in guinea pig or human serum and in chondrocytes cell lysate at 37 °C (pH = 7.0). Half-lives of the compounds, reported in Table 7, were determined from the apparent first-order rate constant derived from linear regression of pseudo-first-order plots of prodrug concentration versus time.

Table 7. Half-Lives for *N*-Acetyl-(D)-Glucosamine O-3/O-4 Phosphoramidate Derivatives 8b/9b, 8c/9c, 8l, 8s/9s, and 8u in Guinea Pig and Human Serum and in Chondrocyte Cell Lysate at 37 °C

			half-lives, $t_{1/2}$ (h)		
compd	AA	ester	guinea pig serum	human serum	chondrocyte cell lysate
8b/9b	Bn	(L)-Val	0.55	0.80	13
8c/9c	Bn	(L)-Pro	107	164	39
8l/9l	Et	(L)-Pro	57	42	nd[a]
8s/9s	2-Bu	(L)-Val	1	1.3	nd
9x	*t*Bu	(L)-Pro	stable	stable	nd

[a]nd = not determined.

The (L)-proline amino acid-containing phosphoramidates tested proved to be in general significantly much more stable either in guinea pig or human serum when compared to those containing (L)-valine amino acid. No obvious difference was instead observed by changing the benzyl ester with the 2-butyl in the valine analogues as **8b/9b** and **8s/9s** showed similar stability profiles in both human and guinea pig serum. Compound **9x** was extremely stable in serum, with no sign of any hydrolysis after 17 h. This stability, likely due to the poor susceptibility of the *t*-butyl ester to esterase, may account for its inactivity, confirming that the initial ester cleavage is important in the activation of the phosphoramidates, as was previously found within our research group working on 5′ phenoxy, alaninyl *tert*-butyl ester phosphoramidate derivatives of d4T, and other nucleosides.[34] The proline series emerged as approximately 200 times more stable than the valine one in guinea pig or human serum, where as in the chondrocytes cell lysate model this difference is reduced and both of the compounds were processed. In Figure 2 is reported the ^{31}P NMR spectra of **8c/9c**, 12 h after the addition of chondrocytes cell lysate at 37 °C. The spectrum clearly shows the formation of a peak at 0.84 ppm, which may correspond to the glucosamine monophosphate.

CONCLUSION

In conclusion, we prepared a new series of O-4/O-3 *N*-acetyl-(D)-glucosamine phosphoramidates, the structural isomers of our previously reported O-6 *N*-acetyl-(D)-glucosamine phosphoramidates. Several of these compounds were able to significantly decrease the loss of GAG at noncytotoxic concentrations. In addition, we reported several examples of 1-O methylated O-3 phosphoramidates prodrugs. However, in general, these methylated prodrugs appear to be inactive in the biological assay, suggesting that a free 1-OH is required for activity. Finally, we elaborated a protection−deprotection strategy, which made it possible to achieve exclusively O-3 phosphoramidate regioisomers. Although this strategy is a longer process, in our case it was found to be the most efficient synthetic route to afford pure O-3 regiosomer phosphate *N*-acetylglucosamine prodrugs in moderate yield. We are now

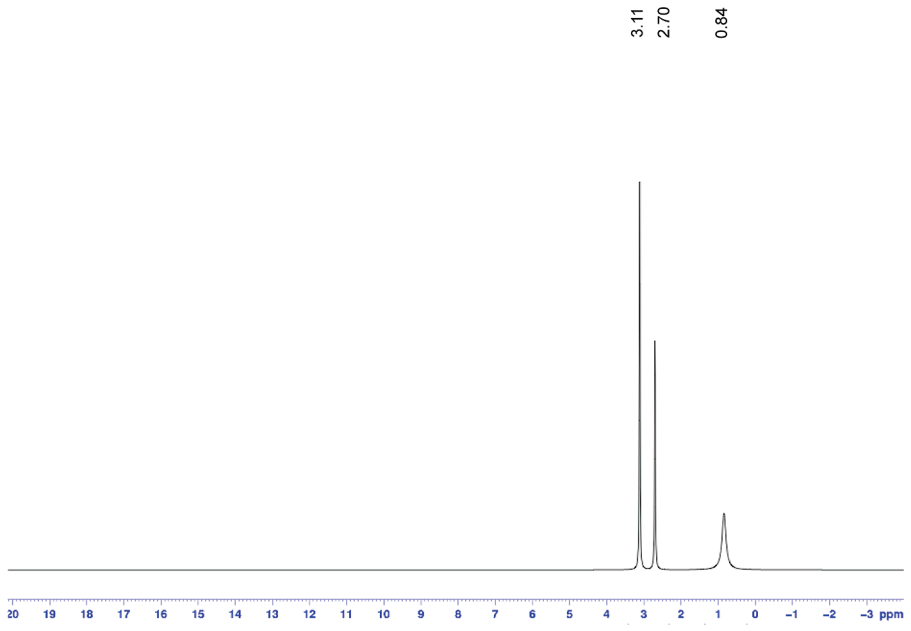

Figure 2. Stability of compound **8c/9c** after 12 h incubation in chondrocyte cell lysate at 37 °C, monitored by ^{31}P NMR.

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actively working toward minimizing the protecting group manipulations in order to influence the outcome of the coupling reaction in a desirable way. Considering the reduction in the GAG release induced by IL-1 in both bovine and human cartilages, in addition to good biological stability and the desired activation in chondrocyte cell lysate, the (L)- proline amino acid-containing phosphoramidate mixture **8c**/**9c** emerged as a promising candidate for further development for the treatment of osteoarthritis and other musculoskeletal diseases. It is a striking outcome of this research that the prodrugs of the O-3 and O-4 phosphate are more active than those of the O-6 phosphate, which is more closely related to the suggested bioactive species. Thus the O-3 and O-4 regioisomers emerge as promising novel leads, with activity in human cartilage ex vivo exceeding that of **1**.

EXPERIMENTAL SECTION

General Experimental Details. *Chemistry. General Procedures. Solvents and Reagents.* The following anhydrous solvents were bought from Sigma-Aldrich: dichloromethane (CH_2Cl_2), diethyl ether (Et_2O), *N*-methylimidazole (NMI), pyridine (pyr), tetrahydrofuran (THF), triethylamine (TEA), para-toluensulfonic acid (*p*TsOH), amino acid ester salts, *N*-acetyl-(D)-glucosamine, and any other reagents used. All reagents commercially available were used without further purification.

Thin Layer Chromatography (TLC). Precoated aluminum backed plates (60 F254, 0.2 mm thickness, Merck) were visualized under both short and long wave ultraviolet light (254 and 366 nm) or by burning using the following TLC indicators: (i) molybdate ammonium cerium sulfate, (ii) potassium permanganate solution. Preparative TLC plates (20 cm × 20 cm, 500−2000 μm) were purchased from Merck.

Flash Column Chromatography (FCC). Flash column chromatography was carried out using silica gel supplied by Fisher (60A, 35−70 μm). Glass columns were slurry packed using the appropriate eluent, with the sample being loaded as a concentrated solution in the same eluent or preadsorbed onto silica gel. Fractions containing the product were identified by TLC and pooled and the solvent was removed in vacuo.

High Performance Liquid Chromatography (HPLC). Analytical and semipreparative HPLC were conducted on Varian Prostar LC workstation, Varian Prostar 335 LC detector, Varian fraction collector (model 701), and Prostar 210 solvent delivery system, with Varian Polaris C18-A (10 μm) as an analytical column and Varian Polaris C18-A (10 μm) as a semipreparative column. The software used was Galaxie Chromatography Data System.

Nuclear Magnetic Resonance (NMR). ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), and ^{31}P NMR (202 MHz) were recorded on a Bruker Avance 500 MHz spectrometer at 25 °C. Chemical shifts (δ) are quoted in parts per million (ppm) relative to internal CD_3OD (δ 3.34 ^{1}H NMR, δ 49.86 ^{13}C NMR) and $CDCl_3$ (δ 7.26 ^{1}H NMR, δ 77.36 ^{13}C NMR) or external 85% H_3PO_4 (δ 0.00 ^{31}P NMR). Coupling constants (J) are measured in hertz. The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), dd (doublet of doublet), dt (doublet of triplet). The characterization of phosphoramidates involves the identification of α and β sugar derivatives. The ratio of diastereisomers was determined based on ^{31}P NMR. The assignment of the signals in ^{1}H NMR and ^{13}C NMR was done based on the analysis of coupling constants and additional two-dimensional experiments (COSY, HSQC, HMBC, PENDANT).

Mass Spectrometry (MS). Low resolution mass spectra was performed on Bruker Daltonics microTof-LC, (atmospheric pressure ionization, electron spray mass spectroscopy) in either positive or negative mode. High resolution mass spectroscopy was performed as a service by Birmingham University, using fast atom bombardment (FAB).

Elemental Analysis (CHN). CHN microanalysis was performed as a service by the School of Pharmacy at the University of London.

Purity of Final Compounds. The ≥95% purity of the final compounds **8a−b**/**9a−b**, **8c**, **9c**, **8e−g**/**9e−g**, **8j**/**9j**, **81**, **91**, **8m**/**9m**, **9o**, **8p**, **9p**, **8t**, **9t**, **8u**, **8v**, **9v**, **9x**, **10a−b**, and **10o**, and the <95% purity of the final compounds **8c**/**9c**, **8h−i**/**9h−i**, **9u**, and **10c** was confirmed using HPLC analysis. The ≥95% purity of the final compounds **9d**, **8r**/**9r**, **8s**/**9s**, and **9w** was confirmed by elemental analyses, which were within ±0.4 of calculated values.

Standard Procedures. For practical purposes, standard procedures are given. Any variations from these procedures are discussed individually. Procedures that differ from the standard ones are described in full.

*4-Methoxyphenyl Phosphorodichloridate (**5**).* Triethylamine (4.75 mL 33.83 mmol) was added dropwise to a −78 °C stirred solution of phosphorus oxychloride (5.19 g, 33.83 mmol) and 4-methoxyphenol (4.20 g, 33.83 mmol) in anhydrous diethyl ether (100 mL) under nitrogen. Following the addition, the reaction mixture was allowed to slowly warm to room temperature and stirred for 2 h. The mixture was filtered under nitrogen and the solvent removed under reduced pressure to give the title compound (8.1 g, 99% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.70 (3H, s, $-OCH_3$), 6.80 (2H, d J = 9.15 Hz, −OPh), 7.12 (2H, d J = 9.15 Hz, −OPh). ^{31}P NMR ($CDCl_3$, 202 MHz): δ 4.31.

*Standard Procedure 1: Synthesis of Phosphochloridates **6a−v**.* **5** (1.0 mol) and the appropriate amino ester hydrochloride salt (1.0 mol) were suspended in anhydrous dichloromethane (61.6 mol). Anhydrous triethylamine (2.0 mol) was added dropwise at −78 °C, and after 15 min the reaction was left to rise to room temperature and stirred overnight. The formation of phosphorochloridate was monitored by ^{31}P NMR. The solvent was removed under reduced pressure, anhydrous ethyl ether (Et_2O) was added to solubilize the phosphorochloridate, and the triethylamine salt was removed by filtration, the filtrate was reduced to dryness and the product purified by flash chromatography using like eluent ethyl acetate/hexane 1/1 (V/V).

*4-Methoxyphenyl(cyclohexoxy-(l)-prolinyl) phosphorochloridate (**6v**).* Prepared according to standard procedure 1 in 30% yield as a mixture of two diastereoisomers (SS_p and SR_p). ^{1}H NMR (CD_3OD, 500 MHz): δ 7.08 (2H, m, −OPh), 6.76 (2H, m, −OPh), 6.87 (2H,m, −OPh), 4.73 (1H, m, $-OC\underline{H}CH_2$), 4.35 (0.5H, m, $-CHCO_2$, one diastereoisomer), 4.26 (0.5H, m, $-CHCO_2$, one diastereoisomer), 3.69 (1.5H, s, $-OCH_3$, one diastereoisomer), 3.68 (1.5H, s, $-OCH_3$, one diastereoisomer), 3.66 (1.5H, s, $-OCH_3$, one diastereoisomer), 3.40 (2H, m, $-CH_2N$), 2.13 (1H, m, $-C\underline{H}_{2a}CHCO_2$), 1.94 (3H, m, $-C\underline{H}_{2b}CHCO_2$ and $C\underline{H}_2CH_2N$), 1.73 (2H, m, $-C\underline{H}_2$ (cyclohex)), 1.71 (2H, m, $-C\underline{H}_2$ (cyclohex)), 1.31 (6H, m, $-C\underline{H}_2$ (cyclohex)). ^{31}P NMR ($CDCl_3$, 202 MHz): δ 8.46, 8.38 (ratio 1:1).

*Standard Procedure 2: Synthesis of Phosphoramidates **8a−v**/**9a−v**, **10a−o**, and **14c,t** using NMI.* A solution of appropriate phosphorochloridate (15.63 mmol) in anhydrous THF (15 mL) was added to a −30 °C stirred solution of *N*-acetyl-(D)-glucosamine or 1-*O*-methyl-*N*-acetyl-(D)-glucosamine or *N*-acetyl-4,6-*O*-benzylidene-(D)-glucosamine (13.56 mmol) and NMI (6.2 mL, 78.15 mmol) in pyridine (100 mL). After 15 min, the reaction was allowed to slowly warm to room temperature and stirred for 3 h. Then methanol was added, and the solvent was removed under reduced pressure, the crude residue purified by flash chromatography using like eluent a gradient of CH_2Cl_2/MeOH from 98/2 to 95/5 (V/V).

*2-Acetamido-2-deoxy-4-O-[4-methoxyphenyl-(cyclohexyloxy-(L)-prolinyl)-phosphate-(D)-glucopyranose (**8v**) and 2-Acetamido-2-deoxy-3-O-[4-methoxyphenyl-(cyclohexyloxy-(L)-prolinyl)-phosphate-(D)-glucopyranose (**9v**).* The compounds were obtained as described in standard procedure 2. However the crude product was purified twice by flash chromatography on silica gel under gradient elution system of CH_2Cl_2/MeOH from 100/0 to 97/3, affording **8v** as a mixture of *R*p and *S*p diastereoisomers as α and β anomers in 0.5% yield. ^{1}H NMR (CD_3OD, 500 MHz) δ 7.18 (1.1H, m, $-O\underline{P}h$), 7.09 (0.9H, m, $-O\underline{P}h$), 6.98 (1.1H, m, $-O\underline{P}h$), 6.81 (0.9H, m, $-O\underline{P}h$), 5.17 (1H, d J = 3.7 Hz, $-C\underline{H}$-1), 4.78 (1H, m, $-OC\underline{H}CH_2$), 4.67 (1H, m, $-C\underline{H}$-4), 4.55 (1H, m, $-CH_{2a}$-6), 4.49 (1H, m, $-CH_{2b}$-6), 4.38 (1H, m, $-NC\underline{H}CO_2$), 4.04 (2H, m, $-C\underline{H}$-2, $-C\underline{H}$-5), 3.81 (3H, s, $PhOC\underline{H}_3$), 3.62 (1H, m, $-C\underline{H}$-3), 3.46 (1H, m, $-C\underline{H}_{2a}NH$), 3.38 (1H, m, $-C\underline{H}_{2b}NH$), 2.21 (1H, m, $C\underline{H}_{2a}$-CH-CO_2), 2.06−1.98 (3H,

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m, $-C\underline{H}_{2b}$-CH-CO_2, $-C\underline{H}_2CH_2NH$), 1.79 (4H, m, $-C\underline{H}_2CHO$ and $-C\underline{H}_2CH_2C\underline{H}_2$), 1.78 (3H, s, $-NHCOC\underline{H}_3$), 1.38 (6H, m, $-OCHC\underline{H}_2$, $CH_2C\underline{H}_2CH_2$ and $C\underline{H}_2C\underline{H}_2C\underline{H}_2$). ^{13}C NMR ($CD_3OD$, 125 MHz) δ 24.68 ($-NHCOC\underline{H}_3$), 26.13 (d $J_{(C-P)}$ = 8.2 Hz, $-C\underline{H}_2$-CH_2-NH), 26.45 ($-C\underline{H}_2$-CH_2), 32.44 (m, $-C\underline{H}_2$-CH-$CO_2C\underline{H}_2Ph$, $-C\underline{H}_2CHO$, and $-OCHC\underline{H}_2$), 48.50 (d $J_{(C-P)}$ = 3.7 Hz, $-CH_2-NH$, overlap with the solvent), 54.93 (d $J_{(C-P)}$ = 3.0 Hz, C-2), 56.11, 56.16 ($-OCH_3$), 62.01 (d $J_{(C-P)}$ = 6.5 Hz, $-\underline{C}HNH$), 66.84 (CH_2-6), 70.18 (C-3), 71.80 (C-5), 74.41 ($-O\underline{C}HCH_2$), 79.88 (d $J_{(C-P)}$ = 6.4 Hz, C-4), 92.70 (C-1), 97.65 (C-1), 115.78 ($-\underline{Ph}OCH_3$), 122.29 (d $J_{(C-P)}$ = 4.4 Hz, $-\underline{Ph}OCH_3$), 145.51 (d $J_{(C-P)}$ = 8.1 Hz, "*ipso*" $-PO\underline{Ph}$), 158.43 ("*ipso*" $-\underline{Ph}OCH_3$), 173.25 ($-COCH_3$), 175.25, 176.31 ($-\underline{C}O_2CH_2$). ^{31}P NMR (CD_3OD, 202 MHz): 3.16, 2.53, 2.24, ratio (6.1:5.9:1). MS (E/I) 587.11 (MNa^+). HPLC: (gradient H_2O/CH_3CN from 70/30 to H_2O/CH_3CN: 0/100 in 20 min, flow = 1 mL/min, λ = 275 nm): t_R 12.51 min, 12.84 min.

Further elution with CH_2Cl_2/MeOH 97/3 afforded **9v** as a mixture of *Rp* and *Sp* diastereoisomers as α and β anomers in 1.3% yield. ^{1}H NMR (CD_3OD, 500 MHz): δ 7.15 (1H, m, $-OPh$), 7.01 (1H, d, J = 9.2 Hz, $-OPh$), 6.78 (1H, d, J = 9.2 Hz, $-OPh$), 6.70 (1H, d, J = 9.2 Hz, $-OPh$), 4.98 (0.8H, d, J = 3.9 Hz, $-CH$-1), 4.62 (1.2H, m, CH-1 and $-OC\underline{H}CH_2$), 4.39 (0.8H, m, $-CH$-3), 4.22 (0.2H, m, $-CH$-3), 4.11 (1H, m, $-NCHCO_2$) 3.99 (1H, m, $-CH$-2), 3.75 (1H, m, $-CH$-5), 3.67 (5H, m, $-CH_2$-6 and $PhOC\underline{H}_3$), 3.51 (0.8H, t J = 9.6 Hz, $-CH$-4), 3.44 (0.2H, d J = 9.6 Hz, $-CH$-4), 3.30 (1H, m, $-C\underline{H}_{2a}NH$), 3.20 (1H, m, $-C\underline{H}_{2b}NH$, overlap with the solvent), 2.10 (1H, m, $C\underline{H}_{2a}$-CH-CO_2) 1.91, 1.90 (3H, s, $-NHCOC\underline{H}_3$), 1.81 (3H, $C\underline{H}_{2b}CHCO_2$, $-C\underline{H}_2CH_2NH$), 1.63 (4H, m, $-C\underline{H}_2CHO$ and $-C\underline{H}_2CH_2C\underline{H}_2$), 1.28 (6H, m, $-OCHC\underline{H}_2$, $CH_2C\underline{H}_2CH_2$, and $C\underline{H}_2C\underline{H}_2C\underline{H}_2$). ^{13}C NMR ($CD_3OD$, 125 MHz): δ 23.01, 23.33 ($-NHCOC\underline{H}_3$), 24.62 ($-C\underline{H}_2CH_2CH_2$), 26.10, 26.25 (d $J_{(C-P)}$ = 8.2 Hz, $-C\underline{H}_2$-CH_2-NH), 26.46 ($-CH_2C\underline{H}_2CH_2$), 32.46 (m, $-C\underline{H}_2$-CH-$CO_2C\underline{H}_2Ph$, $-C\underline{H}_2CHO$, and $-OCHC\underline{H}_2$), 48.80 (d $J_{(C-P)}$ = 3.7 Hz, $-CH_2$-NH, overlap with the solvent), 54.59 (d $J_{(C-P)}$ = 3.8 Hz, C-2), 56.05, 56.09 ($-OCH_3$), 61.96 (d $J_{(C-P)}$ = 7.3 Hz, $-CH_2\underline{C}HNH$), 66.46, 62.61 ($-\underline{C}H_2$-6), 70.80, 70.90 (d $J_{(C-P)}$ = 3.1 Hz, C-4), 74.44, 74.74 ($-OC\underline{H}CH_2$), 74.82 (C-5), 81.05 (d $J_{(C-P)}$ = 7.0 Hz, C-3), 82.87 (d $J_{(C-P)}$ = 8.7 Hz, C-3), 92.83 (C-1), 96.29 (C-1), 115.19, 115.58 ($-\underline{Ph}OCH_3$), 122.32, 122.56 (d $J_{(C-P)}$ = 4.7 Hz, $-\underline{Ph}OCH_3$), 145.94 (d $J_{(C-P)}$ = 7.1 Hz, "*ipso*" $-PO\underline{Ph}$), 158.28 ("*ipso*" $-\underline{Ph}OCH_3$), 173.46, 173.78 ($-NH\underline{C}OCH_3$), 174.50, 175.06 (d $J_{(C-P)}$ = 1.5 Hz, $-\underline{C}O_2CH_2$). ^{31}P NMR (CD_3OD, 202 MHz): 2.42, 1.80 ratio (4.4:1). MS (E/I) 587.11 (MNa^+). HPLC: (gradient H_2O/CH_3CN from 70/30 to H_2O/CH_3CN: 0/100 in 20 min, flow = 1 mL/min, λ = 275 nm): t_R 11.97 min, 12.24 min.

9v was also obtained from **15v** according to standard procedure 4 (see later section).

*Standard Procedure 3: Synthesis of Protected Phosphoramidates **15u–y** Using t-BuMgCl.* To a solution of 1-*O*-benzyl-*N*-acetyl-4,6-*O*-benzylidene-D-glucosamine (1.98 mmol) in THF (20 mL), *t*-BuMgCl (3.97 mL, 3.96 mmol) was added at rt. Then a solution of appropriate phosphorochloridate (5.98 mmol) in THF (5 mL) was added dropwise. After 1 h, the solvent was removed under vacuum and the crude purified by flash chromatography using like eluent a gradient of EtPt/EtOAc 5/5 to EtOAc (V/V).

*1-O-Benzyl-2-acetamido-2-deoxy-4,6-O-benzylidene-3-O-[4-methoxyphenyl-(cyclohexyloxy-(L)-prolinyl)-phosphate-(D)-glucopyranose (**15v**).* The compound was obtained as described in standard procedure 3 in 41% yield as a mixture of *Rp* and *Sp* diastereoisomers as α and β anomers. ^{1}H NMR (CD_3OD 500 MHz): δ 7.38 (2H, d J = 7.4 Hz, $-CH\underline{Ph}$), 7.19 (8H, m, $-CH\underline{Ph}$ and $-CH_2\underline{Ph}$), 6.95 (0.2H, d J = 8.7 Hz, $-OPh$), 6.81 (1.8H, d J = 8.7 Hz, $-OPh$), 6.75 (0.2H, d J = 9.2 Hz, $-OPh$), 6.52 (1.8H, d J = 8.75 Hz, $-OPh$), 5.47 (1H, s, $-C\underline{H}Ph$), 4.76 (1H, d J = 3.6 Hz $-CH$-1, α), 4.61 (3H, m, $-CH$-3, $-OC\underline{H}CH_2$, and $C\underline{H}_{2a}Ph$), 4.43 (1H, d J = 10.3 Hz, $C\underline{H}_{2b}Ph$), 4.21 (1H, m, $-CH$-2), 4.07 (1H, dd J = 10.18 and 4.7 Hz, $-C\underline{H}_{2a}6$), 4.02 (1H, m, $-CHCO_2$), 3.77 (1H, m, $-CH$-5), 3.66 (2H, m, $-CH$-4 and $-CH_{2b}6$), 3.56 (2.5H, s, $-OCH_3$), 3.55 (0.5H, s, $-OCH_3$), 3.14 (2H, m, $-C\underline{H}_2N$), 1.87 (3H, s, $-NHCOC\underline{H}_3$), 1.63 (8H, $-C\underline{H}_2CH_2N$, $-C\underline{H}_2$ (cyclohex)), 1.30 (6H, m, $-C\underline{H}_2$, (cyclohex)). ^{13}C NMR (CD_3OD 125 MHz): δ 22.95 ($-NHCOC\underline{H}_3$), 23.00 ($-NHCOC\underline{H}_3$), 24.62 ($-CH_2$ (cyclohex), 24.67 ($-CH_2$ (cyclohex), 26.10 (d $J_{(C-P)}$ = 8.2 Hz, $-C\underline{H}_2CH_2N$), 26.48 ($-C\underline{H}_2$ (cyclohex)), 32.46 (m, $-C\underline{H}_2CHCO_2$, $-C\underline{H}_2$ (cyclohex)), 48.80 ($C\underline{H}_2N$, under the residual solvent peak), 54.43 (d $J_{(C-P)}$ = 3.8 Hz, C-2), 54.35 (d $J_{(C-P)}$ = 3.8 Hz, C-2), 56.12 ($-OCH_3$), 56.17 ($-OCH_3$), 61.62 (d $J_{(C-P)}$ = 7.3 Hz, ($-C\underline{H}CO_2$), 64.42 (C-5), 69.75 ($-CH_2$-6), 70.83 ($-OC\underline{H}_2Ph$), 74.63 ($-O\underline{C}HCH_2$), 76.64 (d $J_{(C-P)}$ = 7.6 Hz C-3), 76.77 (d $J_{(C-P)}$ = 7.6 Hz, C-3), 81.61 (d $J_{(C-P)}$ = 2.0 Hz, C-4), 98.59 (C-1), 102.18 (C-1), 102.93 ($-\underline{C}HPh$), 115.49 ($-\underline{Ph}OCH_3$), 115.75 ($-\underline{Ph}OCH_3$), 122.10 (d $J_{(C-P)}$ = 5.3 Hz, $-\underline{Ph}OCH_3$), 122.29 (d $J_{(C-P)}$ = 5.3 Hz, $-\underline{Ph}OCH_3$), 127.77 ($-CH\underline{Ph}$), 129.20 ($-CH\underline{Ph}$), 129.22 ($-CH_2\underline{Ph}$), 129.57 ($-CH_2\underline{Ph}$), 129.62 ($-CH_2\underline{Ph}$), 130.11($-CH\underline{Ph}$), 138.51 ("*ipso*" $-CH\underline{Ph}$) 138.95 ("*ipso*" $-CH_2\underline{Ph}$), 145.7 (d $J_{(C-P)}$ = 7.6 Hz "*ipso*" $-POPh$), 158.05 ("*ipso*" $-\underline{Ph}OCH_3$), 173.38, ($-NH\underline{C}OCH_3$), 173.47 ($-NH\underline{C}OCH_3$), 174.30 ($-\underline{C}O_2$(cyclohexyl)). ^{31}P NMR (CD_3OD 202 MHz): 0.96, 0.58 (ratio 10:1).

*Standard Procedure 4: Synthesis of Phosphoramidates **9t–y** by Hydrogenation of **14t** and **15u–y**.* A solution of 2-acetamido-2-deoxy-4,6-*O*-benzylidene-3-*O*-substituted-phosphate-(D)-glucopyranose **14t** or 1-*O*-benzyl-2-acetamido-2-deoxy-4,6-*O*-benzylidene-3-*O*-substituted-phosphate-(D)-glucopyranose **15u–y** (0.28 mmol) was hydrogenated in MeOH or ethanol (5 mL) under pressure of 54 psi for 6 h in presence of a catalyst (10% Pd/C or 10% $Pd(OH)_2$/C). The catalyst was removed by filtration through a Celite pad. and the solvent was removed under reduced pressure to obtain pure compounds unless specified otherwise.

*2-Acetamido-2-deoxy-3-O-[4-methoxyphenyl-(cyclohexyloxy-(L)-prolinyl)]-phosphate-(D)-glucopyranose (**9v**).* The compound was prepared as described in standard procedure 4 (EtOH, 10% $Pd(OH)_2$/C) and obtained in 12% yield as a mixture of *Rp* and *Sp* diastereoisomers as α and β anomers, after purification by preparative TLC using CH_2Cl_2/MeOH 92/8 (V/V). ^{1}H NMR (CD_3OD 500 MHz): δ 7.15 (1.5H, m, $-OPh$), 7.01 (0.5H, d J = 9.2 Hz, $-OPh$), 6.78 (1.5H, d J = 9.2 Hz, $-OPh$), 6.70 (0.5H, d J = 9.2 Hz, $-OPh$) 4.98 (0.6H, d J = 3.9 Hz $-CH$-1α), 4.62 (1.3H, m, CH-1β and $-OC\underline{H}CH_2$), 4.39 (0.6H, m, $-CH$-3), 4.22 (0.6H, m, $-CH$-3), 4.11 (0.76H, m, $-CHCO_2$) 3.99 (0.9H, m, $-CH$-2), 3.75 (0.8H, m, $-CH$-5), 3.67 (4.8H, m, $-CH_2$-6 and $PhOC\underline{H}_3$), 3.51 (1H, t J = 9.62 Hz, $-CH$-4), 3.44 (0.5H, t J = 9.62, $-CH$-4), 3.30 (1H, m, $-C\underline{H}_{2a}N$), 3.20 (1H, m, $-C\underline{H}_{2b}N$, overlap with the solvent), 2.10 (1H, m, $C\underline{H}_{2a}CHCO_2$), 1.91 (2H, s, $-NHCOC\underline{H}_3$), 1.90 (1H, s, $-NHCOC\underline{H}_3$), 1.81 (3H, $C\underline{H}_{2b}CHCO_2$, $-C\underline{H}_2CH_2N$), 1.63 (4H, m, $-CH_2$ (cyclohex)), 1.28 (6H, m, $-CH_2$ (cyclohex)). ^{13}C NMR (CD_3OD 125 MHz): δ 23.01 ($-NHCOC\underline{H}_3$), 23.33 ($-NHCOC\underline{H}_3$), 24.59 ($-C\underline{H}_2$ (cyclohex)), 24.62 ($-CH_2$ (cyclohex)), 26.10 (d $J_{(C-P)}$ = 8.2 Hz, $-C\underline{H}_2CH_2N$), 26.25 (d $J_{(C-P)}$ = 8.2 Hz, $-C\underline{H}_2CH_2N$), 26.43 ($-CH_2$ (cyclohex)), 26.46 ($-CH_2$ (cyclohex)), 32.46 (m, $-C\underline{H}_2CHCO_2$, $-CH_2$ (cyclohex), 48.80 (d J = 3.7 Hz, $-CH_2N$, overlap with the solvent), 54.59 (d $J_{(C-P)}$ = 3.8, C-2), 56.05 ($-OCH_3$), 56.09 ($-OCH_3$), 61.96 (d $J_{(C-P)}$ = 7.3 Hz, ($-C\underline{H}CO_2$), 66.46 ($-CH_2$-6), 62.610 (CH_2-6), 70.80 (d $J_{(C-P)}$ = 3.1 Hz, C-4), 70.90 ((d $J_{(C-P)}$ = 3.1 Hz, C-4), 72.95 (C-5), 74.44 ($-OC\underline{H}CH_2$), 74.74 ($-OC\underline{H}CH_2$), 77.52 (C-5), 81.05 (d $J_{(C-P)}$ = 7.0 Hz, C-3), 82.87 (d $J_{(C-P)}$ = 8.7 Hz, C-3), 92.83 (C-1α), 96.29 (C-1β), 115.19 ($-\underline{Ph}OCH_3$), 115.58 ($-\underline{Ph}OCH_3$), 122.32 (d $J_{(C-P)}$ = 4.7 Hz, $-\underline{Ph}OCH_3$), 122.56 (d $J_{(C-P)}$ = 4.3 Hz, $-\underline{Ph}OCH_3$), 145.94 (d $J_{(C-P)}$ = 7.1 Hz "*ipso*" $-POPh$), 158.28 ("*ipso*" $-\underline{Ph}OCH_3$), 173.46, ($-NH\underline{C}OCH_3$), 173.78, ($-NH\underline{C}OCH_3$), 174.50 (d $J_{(C-P)}$ = 1.5 Hz, $-\underline{C}O_2$cyclohex), 175.06 (d $J_{(C-P)}$ = 1.5 Hz, $-\underline{C}O_2$cyclohexyl). ^{31}P NMR (CD_3OD 202 MHz): 2.42, 1.80 (ratio 10:4). MS (ES+) 609.2 (MNa^+). HPLC: (gradient H_2O/CH_3CN from 100/0 to 0/100 in 15 min, flow = 1 mL/min, λ = 275 nm): t_R 13.75 min, 14.19 min.

^{31}P NMR Stability Assay in Human and Guinea Pig Serum. The phosphoramidate (10 mg) is dissolved in DMSO (50 μL) and D_2O (150 μL), into an NMR tube. The NMR tube is warmed in the NMR chamber at 37 °C. Then either the human serum or the guinea pig serum (300 μL) was added to the NMR tube quickly. NMR experiments were programmed into the computer to record data every 15 min usually for 12 h (overnight) or longer if needed. The data was

then processed via Fourier transform and then deconvoluted to further clarify the product signals and integrate them. The pH of the samples was measured before and after these experiments, which remained at 7.0.

^{31}P NMR Stability Assay in Chondrocyte Cell Lysate. ^{31}P NMR experiments were conducted using chondrocyte cell lysate obtained from Prof. Catersons group (1 mL of the cell lysate solution contained the contents of 10 million chondrocyte cells) to which was added deuterated water (D_2O) and the compound of interest. An NMR tube containing 250 μL of lysate (equivalent to 2.5 million cells) in 0.5 mL of D_2O was warmed in the NMR chamber until a stable temperature of 37 °C was met and then a blank ^{31}P NMR was taken. Compound was added to the lysate solution to give a 15 mM concentration, the contents were shaken, and the tube returned to the NMR from which the first recording was taken (ca. 3 min). The NMR experiments were then set up to record data every half hour for up to 12 h overnight, with a final experiment taken in the morning prior to removing the sample (13 h 42 min).

Glycosaminoglycan (GAG) Release Assay. Full-depth articular cartilage were dissected from the metacarpophalangeal joints of calves of approximately 6 months of age or human cartilage from joint replacement surgery (approximately 4 mm diameter punches for bovine cartilage and 2 mm for human cartilage). After an equilibration period of 24 h, explants were cultured for one day in 200 μL of DMEM + hydrolyzed lactalbumin + 50 μg/mL vitamin C + penicillin/streptomycin + ITS (insulin−transferrin−selenium) in the presence or absence of the compounds. After one day, GAG release was induced by addition of 10 ng of IL-1. Six days after IL-1 induction, the cartilage and culture medium were collected for analysis. During this six-day period, the culture medium was replaced every two days.

GAG release was analyzed using the Blyscan colorimetric assay (Biocolor, Belfast, UK). To measure GAG content of the cartilage explants, the tissue was digested with papain. The culture medium of day 4, 6, and 8 was pooled before GAG analysis. GAG release was expressed as percentage of the total GAG content of the explants (i.e., GAG medium + GAG in papain digest).

A control condition with 0.25% DMSO was included because the test compounds were dissolved in DMSO. A condition with glucosamine hydrochloride was added for comparison. DMSO + IL1 control value for human explant experiment was 18.4%, whereas the control GAG-fold increase values for bovine explant experiments at 10, 1, and 0.1 mM were respectively 2.6, 4.9, and 5.1 fold.

Each culture condition was performed with cartilage of five different paws/patients to account for biological variation. ANOVA followed by LSD posthoc tests were used for determining differences between groups ($p \leq 0.05$ was considered significant).

Compounds that showed a nonstatistically significant reduction of the GAG release compared to IL-1-stimulated in the presence of 0.25% DMSO condition were considered inactive.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis and spectroscopic data of all compounds: ^{1}H, ^{13}C NMR, ^{31}P NMR, HPLC, MS. Purity data of phosphate prodrugs of *N*-acetyl-(D)-glucosamine. HPLC traces of the prodrugs **8a**/**9a**, **8b**/**9b**, **8c**/**9c**, **8c**, **9c**, **8e**/**9e**, **8f**/**9f**, **8g**/**9g**, **8h**/**9h**, **8i**/**9i**, **8j**/**9j**, **8l**, **9l**, **8m**/**9m**, **8o**, **8p**, **9p**, **8t**, **9t**, **8u**, **9u**, **8v**, **9v**, **9x**, **10a**, **10b**, **10c**, and **10o**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

GPI, glycosylphosphatidylinositol; GAGs, glycosaminoglycan; PGs, proteoglycans; OA, osteoarthritis; UDP, uridine diphosphate; NMI, *N*-methyl imidazole; IL-1, interleukin 1

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