Phosphate Prodrugs Derived from *N*-Acetylglucosamine Have Enhanced Chondroprotective Activity in Explant Cultures and Represent a New Lead in Antiosteoarthritis Drug Discovery

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We report the application of the phosphoramidate ProTide approach, developed by us for antiviral nucleosides, to sugar derivatives with potential chondroprotection against osteoarthritis. In particular, *N*-acetylglucosamine was converted to a series of 06 arylaminoacyl phosphoramidates with ester and amino acid variation. Compounds were prepared by two routes, with or without sugar protection, and were isolated as phosphate diastereoisomers. The compounds were assayed for cellular toxicity and for inhibition of IL-1 induced glycosaminoglycan (GAG) release (i.e., proteoglycan degradation) from bovine articular cartilage in vitro explant cultures. By comparison to the *N*-acetyl glucosamine parent, some of the analogues show a significant enhancement in efficacy in the inhibition of inflammatory cytokine-induced proteoglycan degradation.

Introduction

Osteoarthritis (OA[a]) is a common muscoskeletal disease leading to pain, loss of mobility, and significant reduction in the quality of life of those affected. At present no chemotherapeutic intervention is proven to be fully effective at ameliorating the progression of the disease. A number of glucosamine-containing nutraceutical preparations are widely used for both prophylaxis of and therapy in OA.[1] Some studies have indicated a positive impact of glucosamine in both laboratory models and human disease.[2] Other clinical trials have revealed little or no therapeutic benefit,[3−5] leaving glucosamine as an unproven therapy at present.

A number of biochemical mechanisms of action of glucosamine have been suggested in vitro.[6−10] None has been convincingly accepted as relevant in the clinic, although action of the O-6 phosphate on cartilage degradation appears to be at least one favored putative mechanism.[11]

Glucosamine is a highly polar molecule (ClogP = −2.4)[12] and indeed will be very largely protonated at physiological pH, making it poorly membrane permeable by passive diffusion. Active transport is possible via the glucose transporters, but administered drug will then compete with endogenous glucose.[13] Bearing in mind the issues of transport and phosphorylation, we began to wonder whether a preformed glucosamine O-6 phosphate may have any therapeutic benefit. However, on account of its charge, high polarity, and probable instability to dephosphorylation in vivo, the free phosphate may be of limited direct utility. Given our longstanding expertise and interest in the use of phosphate prodrugs (ProTides) derived from antiviral and anticancer nucleosides,[14] we wondered if similar methods may be effective here. We herein report our first efforts in this regard.

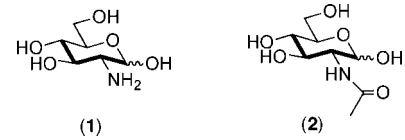

Figure 1. Glucosamine (**1**) and *N*-acetyl glucosamine (**2**).

Chemistry

Rather than apply phosphate prodrug methods directly to glucosamine (**1**, Figure 1), we considered *N*-acetylglucosamine (**2**) a more appropriate target for several reasons. Thus, N-acetylation is linked to the putative mechanism of action of the glucosamine phosphate,[11] N-acetylation satisfactorily protects the amine function in the phosphorylation reaction, it further enhances the lipophilicity of the prodrug and removes the potential for protonation in vitro, and there is also the possibility of in vivo cleavage (thus acting as a dual prodrug). Thus, we commenced our studies on **2** (Figure 1). Early application of our conventional phenylaminoacyl phosphoramidate chemistry[14] here indicated a degree of ProTide instability, with phenol liberation on chromatography, that we had not previously encountered with very extensive (>2000 analogues) experience of nucleoside O5′-phosphate analogues. This may indicate some neighboring group participation, leading to phenyl loss, in the products in this case, not possible for stereoelectronic reactions in the ribose phosphates of nucleoside. In order to stabilize the phenyl phosphate, we investigated electron-donating substituents and found *p*-methoxy to be suitable. All further examples were thus *p*-methoxyphenyl analogues.

Thus, *p*-methoxyphenol was reacted with $POCl_3$ in diethyl ether at low temperature to afford the aryl phosphorodichloridate in quantitative yield (Scheme 1). This was reacted with a series of amino acid ester hydrochlorides in dichloromethane in the presence of triethylamine to give a family of phosphorochloridates. The amino acids utilized were L-Ala, Phe, Pro, Val,

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[a] Abbreviations: OA, osteoarthritis; IL-1, interleukin1; GAG, glycosaminoglycan; THF, tetrahydrofuran.

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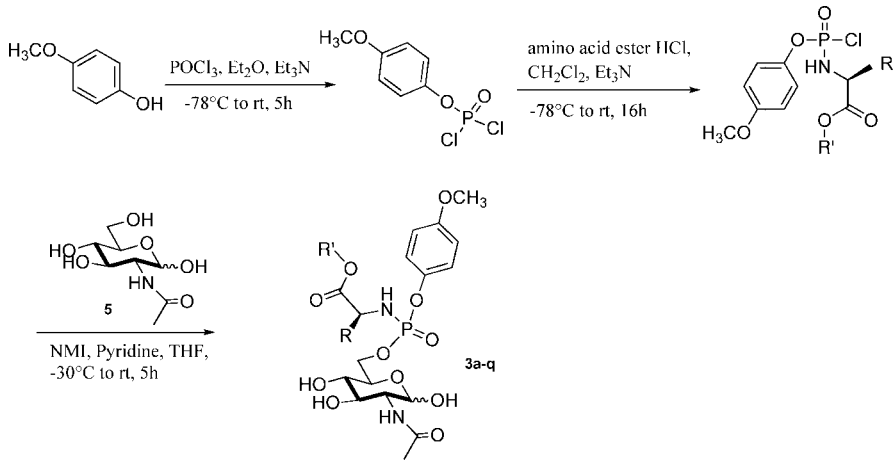
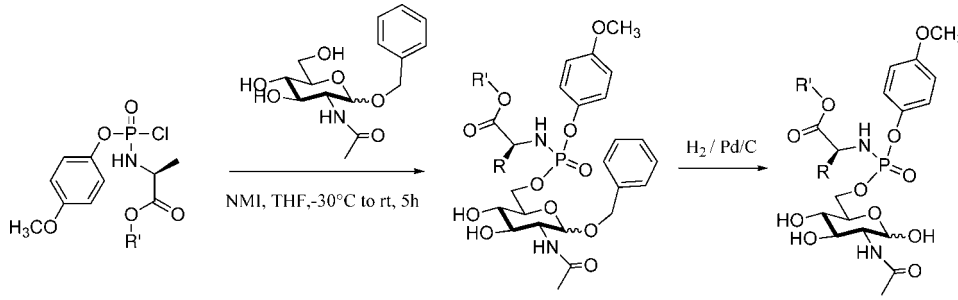
Scheme 1. General Synthetic Pathway for the Synthesis of *N*-Acetylglucosamine Aryloxy Phosphoramidates



Scheme 2. Alternative Synthetic Pathway for the Synthesis of the Aryloxy Phosphoramidates Using O-1 Benzyl Protected *N*-Acetylglucosamine



Leu, Ile, Gly, Met, D-Ala, and α,α-dimethylglycine, each as their benzyl ester. Ethyl esters were prepared of Ala, Gly, Pro, Val, and *N*-methylglycine (sarcosine), and other examples were MeAla, iPrVal, cyclohexylVal, and *n*-butylPro. In most cases, and in particular when the amino acid was chiral, the phosphorochloridates appeared as two closely spaced ^{31}P NMR signals, roughly equal in intensity, corresponding to the two phosphate diastereoisomers. Proton NMR data also confirmed their structures. In most cases they were used crude in the next step; in some cases they were purified by quick flash column chromatography on silica.

Two alternative routes were explored to gain access to the desired target compounds. In the first case, **2** was allowed to react with the appropriate phosphorochloridate in THF/pyridine in the presence of *N*-methylimidazole at −30 °C to room temperature (Scheme 1). In every case complex mixtures of regio- and stereoisomeric monophosphorylated products were formed along with minor quantities of polyphosphates. Extensive and repeated chromatographic purification gave the desired O-6 phosphates in every case, but yields were low and variable (in the range of approximately ≤1−21%). Bearing the yield in mind, an alternative route was investigated. Thus, **2** was converted into its *O*-1-benzyl derivative using benzyl alcohol and HCl gas. The product was isolated in ∼80% yield in an α/β ratio of 2:1 as judged by NMR. This protected sugar was reacted with several phosphorochloridate reagents (Scheme 2) to give the O-1-blocked O-6-phosphates in 3−8% yield. This yield was not sufficiently improved by comparison to reaction with free **2** to warrant further exploration, but catalytic hydrogenation was found to yield the desired O-1 free analogues in good yield. It may well be that alternative protection of the O-3 and/or O-4 sugar hydroxyl groups, with or without O-1 protection, would further improve the yield of O-6 phosphates, but the routes herein described gave sufficient stocks of products for initial biological evaluation.

In each case the target compounds were isolated as four stereoisomers, α/β at C1 and *R*/*S* at the phosphorus. The α forms clearly predominated by ^{1}H NMR, while the phosphate stereoisomers were often roughly 1:1. ^{31}P NMR in general showed four peaks in the range δ 4.0−4.5. The nature of the O-6-phosphate link was proven by ^{13}C NMR where a 6 Hz P−O−CH_2 coupling was observed. Phosphorus coupling to (only) the C6 of the sugar confirmed the location of the phosphate as did downfield shifts of C6 relative to (**2**). Mass spectral, HPLC, and/or microanalytical data (Supporting Information) further confirmed the structure and purity of the target compounds.

Discussion

To examine the affect 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), a model in vitro culture system was established whereby 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. 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 (Table 1, control GAG-fold), with the mean value (n = 2−5) reported. A fold-increase was also calculated for cartilage explants cultured in the presence of glucosamine

Table 1. Efficacy and Toxicity Data of Glucosamine (**1**), *N*-Acetylglucosamine (**2**), and *N*-Acetylglucosamine Phosphoramidate Derivatives (**3a**−**r**)[a]

			10 mM				1 mM					0.1 mM				
				control GAG	GAG	% reduction GAG	MTT		control GAG	GAG	% reduction GAG	MTT		control	GAG	% reduction GAG	MTT
	AA	ester	*n*	fold	fold	fold (±SE)	(±SE)	*n*	fold	fold	fold (±SE)	(±SE)	*n*	GAG fold	fold	fold (±SE)	(±SE)
1			2	2.6	2.0	19.0 (30.42)	56 (0.5)	5	4.9	4.7	3.4 (4.52)	88 (2.51)	4	5.1	5.1	0.8 (5.93)	106 (4.18)
2			1	3.2	2.9	9.3	61 (1.59)	3	4.4	4.5	−1.0 (7.57)	70 (1.68)	2	4.5	4.3	5.4 (0.41)	107 (2.03)
3a	Ala	Bn	2	2.6	2.0	24.2 (36.29)	85 (3.28)	3	4.4	4.2	5.6 (3.88)	81 (2.72)	2	4.5	4.6	−2.2 (4.44)	104 (1.2)
3b	Phe	Bn	1	2.1	2.1	2.3	49 tox(2.23)										
3c	Pro	Bn	2	2.6	0.8	65.6 (11.12)	63 (0.36)	1	4.2	4.1	2.2	85 (0.75)	2	3.3	4.0	−21.9 (41.32)	78 (1.45)
3d	Val	Bn	1	3.2	0.5	85.3	40 tox(1.38)	1	4.2	0.9	77.6	100 (3.7)	9	4.1	4.0	2.5 (9.50)	104 (0.88)
3e	Leu	Bn	2	2.6	0.9	64.0 (15.93)	39 tox(1.48)	2	4.7	2.6	44.7 (8.58)	20tox(0.02)	2	4.7	4.1	12.2 (30.42)	87 (2.98)
3f	Ile	Bn	1	2.1	0.5	74.6	56 (1.92)	1	4.2	1.2	70.8	118 (6.15)	2	3.9	3.9	5.2 (6.46)	110 (3.12)
3g	Me_2Gly	Bn						1	4.2	4.2	−0.3	95 (1.11)	2	3.3	3.4	−2.2 (1.00)	99 (1.56)
3h	Gly	Bn						1	4.2	1.2	73.6	100 (1.85)	5	3.9	4.2	−12.2 (15.42)	99 (1.92)
3i	Met	Bn						1	4.2	1.6	62.2	49tox(2.45)	2	4.7	3.8	18.0 (3.21)	103 (5.46)
3j	D-Ala	Bn						2	4.0	2.8	31.0 (6.38)	66 (1.92)	4	4.2	3.4	24.3 (16.28)	96 (2.91)
3k	Ala	Et										76 (2.34)	4	4.2	4.4	−1.9 (10.19)	89 (1.29)
3l	Gly	Et										37tox(1.98)	4	3.0	3.6	−19.3 (13.90)	78 (2.05)
3m	Sar	Et										100 (0.02)	2	4.7	5.4	−16.3 (11.23)	100 (0.9)
3n	Val	Et											2	3.8	4.4	−21.1 (25.80)	94 (3.19)
3o	Val	iPr						2	4.0	3.6	12.1 (29.04)	56 (1.74)	4	3.7	3.2	14.0 (19.01)	93 (1.24)
3p	Pro	nBu											2	3.8	4.1	−17.5 (36.25)	98 (2.95)
3q	Val	Chex										30 (2.91)	4	4.9	4.8	−6.3 (24.31)	92 (1.9)
3r	Ala	Me										37 (3.52)	2	4.7	3.9	15.8 (0.75)	97 (2.23)

[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; values indicated are the average of those experiments. 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.

derivatives with or without IL-1. These values have been tabulated as GAG-fold enumeration in Table 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. 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 (Table 1). Drug efficacy was measured as the reduction in this fold-increase in GAG release into the culture media. Thus, glucosamine (**1**) treatment at 10 mM led to a reduction in the increased in GAG (before/after exposure) to 2-fold, representing a 19% reduction relative to the control (IL-1 only treated explant culture), while *N*-acetylglucosamine (**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).

The first phosphate prodrug prepared was the *p*-methoxyphenylbenzylalanine compound (**3a**). This was designed on the basis of the efficacy of this ProTide motif on a range of antiviral and anticancer nucleosides.[14] Thus, it is notable that **3a** is significantly more effective than **1** and **2** at reducing the level of GAG released into the media after IL-1 exposure, from 2.6-fold (control) to 2.0-fold (**3a** at 10 mM). Moreover, **3a** is significantly less toxic than **1** and **2** at 10 mM with 85% cell viability by the MTT assay. However, on further dilution, **3a** loses activity, being inactive at 1 and 0.1 mM. Thus, parent **3a** appears more active than both glucosamine and *N*-acetylglucosamine, but it is only active at approximatey 10 mM and not 1 mM. If this in vitro assay were reflected in vivo, such levels of drug would most likely not be feasible, and thus, we sought to enhance the potency of **3a** by structural modification of the prodrug.

In the first instance the benzyl ester was retained and the amino acid varied. The amino acid unit in ProTides has been extensively studied by us for antiviral nucleosides, and alanine has emerged as the most effective motif.[14] Notably D-alanine has in general been observed to be poorly effective.[15] However, given the very different target cell type in the present assay, it remained possible that other (amino acid) structure−activity relationships would operate in the present case. Thus, we first prepared the phenylalanine analogue **3b**, designed to increase the side chain bulk of **3a** and also its lipophilicity. However as the data in Table 1 reveal, **3b** is less active than **3a**; indeed, it is notably cytotoxic. Particularly given the toxicity profile of **3b**, it was not pursued at higher dilutions.

Other amino acid variations in this first batch were proline (**3c**), valine (**3d**), leucine (**3e**), and isoleucine (**3f**). All of these analogues showed some degree of cytotoxicity, but some also appeared to show enhanced potency over **3a** in the GAG release assay. Thus, each was studied at higher dilutions (1 and 0.1 mM). In general, toxicity was lost (with the exception of the leucine compound **3e** at 1 mM) and they became less active, in a dose-dependent manner. Thus, **3d**−**f** were all more active than **1**, **2**, and **3a** at 1 mM, although **3e** was toxic at this concentration. At 0.1 mM all of the analogues were nontoxic but all were poorly active at best. Although differences did not reach statistical significance, data implied that **3e** (Leu) was the most potent analogue to this point.

The next batch of compounds (**3g**−**j**) featured other amino acid variations again with benzyl ester retention. Because activity had now been observed at 1 mM and given the low yield of compounds and to preserve sample, subsequent batches were not tested at 10 mM. Compound **3g** contains the achiral usual amino acid α,α-dimethylglycine, which we have found to be highly effective in some nucleoside examples, particularly hepatitis assays in hepatoma cells.[16] However, here it was inactive (and nontoxic) at 1 mM. However, the glycine (**3h**), methionine (**3i**), and D-alanine (**3j**) compound all appeared active at 1 mM, although the Met compound was also toxic. The D-alanine case (**3j**) is particularly interesting given our prior experience as noted above. Thus, **3j** is more active than **3a** (L-Ala) at 1 mM, although with some toxicity, but the D-compound retains activity at 0.1 mM, unlike the L-isomer, and is then nontoxic. At 0.1 mM **3j** gives 96% cell viability and reduces the GAG increase from 4.2-fold in control to 3.4-fold (treated), representing a 24% reduction in GAG increase relative to control. It thus becomes the most active compound to date.

We finally examined ester variation, replacing the benzyl ester by ethyl for a series of amino acids: Ala (**3k**), Gly (**3l**), *N*-methylglycine (**3m**), and valine (**3n**). No clear trends were observed here, but in general the ethyl esters were less active than their benzyl analogues. Other esters were also briefly investigated in a small number of cases. The isopropylvaline case (**3o**) emerged as of potential interest here, being rather similar to **3i** described above. The cyclohexyl ester on valine was poorly active in this case (**3q**).

Conclusion

A family of 18 phosphate prodrugs of *N*-acetylglucosamine is herein reported. Parent *N*-acetylglucosamine is inactive in the bioassay used at 10 mM and also toxic. Glucosamine has some chondroprotective activity at 10 mM, but not 1 mM, but is also toxic at 10 mM. By contrast, several of the phosphate prodrugs are active at concentrations as low as 0.1 mM and are noncytotoxic. They are thus approximately ≥100-fold more active than glucosamine in this assay. Unusual and novel SARs have also emerged, with prior amino acid SARs in particular being greatly revised. Of particular note is the emergence of D-alanine as an amino acid of choice and also the relatively poor activity of L-alanine, the previously preferred amino acid. It is notable that the amino acid structure−activity relationships we observe in this series, and in particular the relatively poor performance of L-alanine, are markedly contrasting to those we have extensively observed for phosphoramidate ProTides of nucleosides.[14] The reasons for these differences may arise from structural (nucleoside versus sugar) or metabolic (cell to cell) origins and are the subject of active current investigation in our laboratories.

Several additional hits have herein emerged that now form the basis of a hit to lead optimization in our laboratories. If the apparent potency enhancements over glucosamine that we herein report can be translated to an in vivo efficacy boost, this family could have the potential to represent a paradigm shift in the glucosamine-based therapy of osteoarthritis treatments.

General Experimental Details

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

Thin Layer Chromatography (TLC). Precoated, aluminum backed plates (60 F_{254}, 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 in H_2SO_4 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 are quoted in parts per million (ppm) downfield from tetramethylsilane, and coupling constants (*J*) are in hertz. Spectra were calibrated to the residual signal of the deuterated solvent used. 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, and in some cases the diastereoisomers of particular compounds can be identified and are arbitrarily assigned A and B.

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.

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.

Standard Procedure 1: Synthesis of Phosphorodichloridate Species. Phosphorus oxychloride (1.0 mol equiv) and the appropriate substituted phenol (1.0 mol equiv) were stirred with anhydrous diethyl ether (30 mol equiv) under argon. Anhydrous triethylamine (2.0 mol equiv) was added at −78 °C and allowed to warm to room temperature for 4 h with stirring while under an anhydrous atmosphere of argon. The triethylamine salt was removed by filtration under nitrogen and the filtrate evaporated to give the product as a clear liquid.

Standard Procedure 2: Synthesis of Phosphorochloridate Species. Phosphorodichloridate (1.0 mol equiv) and the appropriate amino ester hydrochloride salt (1.0 mol equiv) were suspended in anhydrous DCM (61.6 mol equiv) under argon. Anhydrous triethylamine (2.0 mol equiv) was added dropwise at −78 °C, and after 15 min the mixture was left to rise to room temperature and stirred for 2 h while under an anhydrous atmosphere of argon. The formation of phosphorochloridate was monitored by ^{31}P NMR. The solvent was removed under reduced pressure, after which the resulting triethylamine salt was removed by resuspending the crude product in anhydrous diethyl ether and the salt removed by filtration under nitrogen or was purified by fast flash chromatography [hexane/ethyl acetate 1/1 (v/v)].

Standard Procedure 3: Synthesis of Phosphoramidate Species. *N*-Acetyl-D-glucosamine was coevaporated with pyridine three times (17 mol equiv) and dried under vacuum overnight. To a

stirring solution/suspension of dry *N*-acetyl-D-glucosamine (1.0 mol equiv) in anhydrous pyridine (105 mol equiv) and NMI (5.0 mol equiv) the appropriate phosphorochloridate (1.2 mol equiv) in anhydrous THF (1 M solution) was added, at − 40 °C, dropwise over 15 min. After 15 min the mixture was left to rise to room temperature and stirred at room temperature for 2−5 h. The reaction was quenched with methanol, and the solvent was removed under reduced pressure. The crude obtained was dissolved in DCM and purified by flash chromatography [using a gradient elution method of 100% DCM (v/v) to DCM/MeOH 85:15 (v/v)].

4-Methoxyphenyl Phosphorodichloridate. The compound was prepared as described in standard procedure 1, using 4-methoxyphenol (4.2 g, 33.83 mmol), phosphorus oxychloride (5.19 g, 33.83 mmol), triethylamine (4.75 mL, 33.83 mmol) in anhydrous diethyl ether (100 mL). The product was obtained as a clear oil (8.1 g, 99% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.70 (3H, s, −OC$\underline{H}_3$), 6.80 (2H, d J = 9.15 Hz, −O$\underline{Ph}$), 7.12 (2H, d J = 9.15 Hz, −O$\underline{Ph}$). ^{31}P NMR ($CDCl_3$, 202 MHz): δ 4.31.

1-*O*-Benzyl-*N*-acetyl-D-glucosamine. The compound was synthesized by the addition of *N*-acetyl-D-glucosamine (2.0 g, 9.0 mmol) into a solution of protonated benzyl alcohol (20 mL, 59.1 mmol), which was prepared by purging HCl gas into benzyl alcohol (20 mL, 59.1 mmol) for approximately 15 min. The solution was left stirring at room temperature and monitored by TLC; after 12 h no starting material was seen. The solution was evaporated to an emulsion and then dissolved in a small quantity of DCM and precipitated out by addition of diethyl ether. The solid was collected by filtration, and triturated using chloroform. This resulted in a white solid (2.2 g, 7.0 mmol, 78%). Ratio of α to β sugar was found to be 2:1. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.45−7.25 (5H, m, −$\underline{Ph}$), 4.92 (1H, d, J = 12.0 Hz, −C($\underline{H}$)HPh β), 4.87 (1H, d, J = 3.9 Hz, H-1α), 4.88 (1H, d, J = 12.0 Hz, −C($\underline{H}$)HPh α), 4.62 (1H, d, J = 12.0 Hz, −CH($\underline{H}$)Ph β), 4.52 (1H, d, J = 12.0 Hz, −CH($\underline{H}$)Ph α), 4.49 (1H, d, J = 8.5 Hz, H-1β), 3.92 (1H, d, J = 3.4 Hz, H-2α), 3.92 (1H, d, J = 3.4 Hz, H-2α), 3.90 (1H, d, J = 3.6 Hz, H-2 β), 3.86 (1H, d, J = 11.6 Hz, ($\underline{H}$)H-6β), 3.85 (1H, d, J = 11.6 Hz, ($\underline{H}$)H-6α), 3.77−3.68 (2H, m, H($\underline{H}$)-6 α/β, H-3), 3.41−3.33 (2H, m, H-4, H-5), 1.98 (3H, s, −NCOC$\underline{H}_3$). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 173.43, 173.38 (−NC$\underline{O}$CH$_3$), 139.45, 139.23 ("*ipso*"-CH$_2$$\underline{Ph}$), 129.56, 129.50, 129.35, 128.97, 128.90, 128.80 (−$\underline{Ph}$), 102.07 (C-1β minor isomer), 97.64 (C-1α), 78.22 (C-5 α), 76.03 (C-5 β), 74.28 (C-3), 72.65, 72.51 (C-4), 72.65 (1-O$\underline{C}$H$_2$Ph β), 72.51 (1-O$\underline{C}$H$_2$Ph α), 62.94 (CH$_2$-6β), 62.80 (CH$_2$-6α), 57.40 (C-2β), 55.52 (C-2α), 23.30 (−NCO$\underline{C}$H$_3$ β), 22.84 (−NCO$\underline{C}$H$_3$ α). MS (ES+) *m*/*z*: 335.1 (MNa$^+$H$^+$).

4-Methoxyphenyl(benzoxy-L-alaninyl) Phosphorochloridate. The compound was prepared as described in standard procedure 2, using methoxyphenyl phosphorodichloridate (8.2 g, 34.0 2mmol), L-alanine benzyl ester hydrochloride (7.34 g, 34.02 mmol), triethylamine (9.4 mL, 68.04 mmol), anhydrous dichloromethane (100 mL) under argon. The solvent was removed under reduced pressure, the crude residue was resuspended in anhydrous ether, filtered, and the filtrate was reduced to give the product as a clear oil (12.07 g, 92.5% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.20 (5H, m, −CH$_2$$\underline{Ph}$), 7.10 (2H, m, −O$\underline{Ph}$), 6.77 (2H, m, −O$\underline{Ph}$), 55.14 (2H, s, −OC$\underline{H}_2$Ph A), 5.12 (2H, s, −OC$\underline{H}_2$Ph B), 4.20 (2H, m, −C$\underline{H}$NH and −N$\underline{H}$), 3.71 (3H, s, −PhOC$\underline{H}_3$), 1.44 (3H, d, J = 6.90 Hz, −CHC$\underline{H}_3$, A), 1.43 (3H, d J = 6.70 Hz, −CHC$\underline{H}_3$ B). ^{31}P NMR ($CDCl_3$, 202 MHz): δ 8.54, 8.21, (ratio 1: 1).

2-Deoxy-2-(acetylamino)-6-[4-methoxyphenyl(benzoyl-L-alanine)]phosphate-D-glucopyranoside (3a). The compound was prepared as described in standard procedure 3, using *N*-acetyl-D-glucosamine (3 g. 13.56 mmol), NMI (6.2 mL, 78.15 mmol), a solution of 4-methoxyphenyl(benzoxy-L-alaninyl) phosphorochloridate (6 g, 15.63 mmol) in anhydrous THF (15 mL), and pyridine (100 mL). After 15 min the mixture was allowed to slowly warm to room temperature and stirred at room temperature for 3 h. The crude residue was purified by flash chromatography using a gradient elution solvent system of DCM/MeOH (98:2 to 95:5, then 9:1 v/v). This gave the pure product **3a** as a white solid (0.6 g, 7.8% yield). ^{1}H NMR (CD_3OD, 500 MHz, mixture of four diastereoisomers, two major in α form and two minor in β form): δ 7.36 (5H, m, −OCH$_2$$\underline{Ph}$), 7.13 (2H, m, −O$\underline{Ph}$), 6.87 (2H, m, −O$\underline{Ph}$), 5.6 (3H, m, −C$\underline{H}$-1α and −OC$\underline{H}_2$Ph), 4.63 (1H, m, −C$\underline{H}-1\beta$), 4.31 (2H, m, −C$\underline{H}_2$-6), 4.04 (1H, m, −C$\underline{H}CH_3$), 3.98 (1H, m, −C$\underline{H}$-5), 3.88 (1H, m, −C$\underline{H}$-2α), 3.77 (4H, m, −PhOC$\underline{H}_3$ and −C$\underline{H}$-3α), 3.63 (1H, m, −C$\underline{H}$-2β), 3.46 (1H, m, −CH-3β), 3.45 (1H, m, −C$\underline{H}$-4), 2.02 (3H, s, −NHCOC$\underline{H}_3$), 1.41 (3H, m, −CHC$\underline{H}_3$). ^{13}C NMR (CD_3OD, 125 MHz): δ 20.54 (d, $J_{(C-P)}$= 7.5 Hz, −CH$\underline{C}$H$_3$ A), 20.59 (d, $J_{(C-P)}$= 7.5 Hz, −CH$\underline{C}$H$_3$ B), 22.67 (−NHCO$\underline{C}$H$_3$, α-isomer), 22.95 (−NHCO$\underline{C}$H$_3$, β-isomer), 51.66 (−$\underline{C}$HCH$_3$ A), 51.58 (−$\underline{C}$HCH$_3$ B), 55.85 ($\underline{C}$-2 α-isomer), 56.12 (−OCH$_3$, α-isomer), 56.22 (−OCH$_3$ β-isomer), 67.43 (d, $J_{(C-P)}$= 5 Hz, −$\underline{C}$H$_2$-6 A), 67.72 (d, ($J_{(C-P)}$= 5 Hz, $\underline{C}$H$_2$-6, B), 67.96 (−O$\underline{C}$H$_2$Ph A), 68.03 (−O$\underline{C}$H$_2$Ph B), 71.63 (d, $J_{(C-P)}$= 7.5 Hz, C-5), 71.83 (C-4 A), 72.08 (C-4 B), 72.54 (C-3 A), 72.57 (C-3 B), 92.64 (C-1α A), 92.69 (C-1α B), 97.14 (C-1β), 115.63 (−$\underline{Ph}$OCH$_3$ A), 115.65 (−$\underline{Ph}$OCH$_3$ B), 122.34 (d, $J_{(C-P)}$ = 4.2 Hz, −$\underline{Ph}$OCH$_3$ A), 122.43 (d, $J_{(C-P)}$ = 4.2 Hz, −$\underline{Ph}$OCH$_3$ B), 129.33 (−CH$_2$$\underline{Ph}$), 129.40 (−CH$_2$$\underline{Ph}$), 129.61 (−CH$_2$$\underline{Ph}$), 137.30 ("*ipso*"-CH$_2$$\underline{Ph}$), 145.68 (d, $J_{(C-P)}$ = 6.85 Hz, "*ipso*"-PO$\underline{Ph}$ A), 145.77 (d, $J_{(C-P)}$ = 7.0 Hz, "*ipso*"-PO$\underline{Ph}$ B), 158.26 ("*ipso*"-$\underline{Ph}$OCH$_3$ A), 158.32 ("*ipso*"-$\underline{Ph}$OCH$_3$ B), 173.72, (−$\underline{C}$OCH$_3$), 175.0 (d, J = 5.75 Hz, −$\underline{C}$O$_2$CH$_2$Ph). ^{31}P NMR (CD_3OD, 202 MHz): 4.41 (α-isomer A), 4.39 (β-isomer A), 4.25 (α-isomer B), 4.14 (β-isomer B). MS (E/I) 591.1716 (MNa$^+$). HPLC: H_2O 80% to CH_3CN 20%, λ = 275 nm, flow 1 mL/min, t_R = 4.40 min.

Biological Assay. Materials. All reagents were obtained from Sigma (Poole, U.K.) and tissue culture plastics and reagents from Invitrogen (Karlsruhe, Germany), unless otherwise stated.

Isolation and Culture of Bovine Chondrocytes and MTT Cytotoxicity Assay. Monolayer chondrocyte cultures were obtained from the metacarpophalangeal joints of mature cattle (>18 months old). Full-depth articular cartilage tissue slices were dissected under sterile conditions and subjected to standard pronase and collagenase digestion to isolate the chondrocytes using well established procedures.[17−19] In brief, the tissue was digested in 0.1% (w/v) pronase (*Streptomyces griseus*; Boehringer Mannheim, Lewes, U.K.) in Dulbecco's modified Eagle's medium (DMEM) containing 5% FCS (v/v) and gentamicin (50 μg/mL) for 1 h at 37 °C, 5% CO_2 with agitation, washed, and then further digested with 0.04% (w/v) collagenase (*Clostridium histolyticum*; Worthington, Freehold, NJ) in the same above media and incubated overnight with agitation as before. Cells were filtered through a 40 μm Nitex filter (Falcon; BD Biosciences, Bedford, MA) and washed before cell numbers were established. Isolated cells were cultured in 1 mL of serum-free DMEM media containing gentamicin (50 μg/mL), 1% (v/v) HEPES buffer and supplemented with or without the appropriate glucosamine compound (10−0.1 mM). After 96 h of culture, the cell viability of the chondrocytes in the presence or absence of each compound was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay.[20] This assay is a colorimetric toxicity assay based on the detection of intracellular mitochondrial activity within viable cells. MTT (a water soluble tetrazolium salt) is converted to an insoluble purple formazan product (impermeable to cell membranes of viable cells) by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria of viable cells only. In brief, 200 μL of the media was removed from each well and replaced with 200 μL of 5 mg/mL thiazolyl blue tetrazolium bromide to make a final 1 mg/mL concentration and cultured for 3 h. The 1 mL was then removed, and the formed intracellular MTT formazan crystals were dissolved with the addition of 200 μL of DMSO; 100 μL from each well was transferred to a 96-well plate, and the optical density was measured at 570 nm. Chondrocytes cultured in the absence of any glucosamine compound were taken as 100% viable, and the percentage toxicity of experimental cultures was determined as [(OD at 540 nm treated cells)/(OD at 540 nm untreated cells)] × 100.

Articular Cartilage Explant Cultures and Analysis of Glycosaminoglycan Concentration in Media. Full-depth articular cartilage explants were dissected from mature bovine metacarpophalangeal joints. The explants (50−100 mg/explant) underwent

an equilibraion period by culture in DMEM media containing 10% FCS (v/v), gentamicin (50 μg/mL), and 1% (v/v) HEPES buffer for 48 h, after which explants were washed (3 × 10 min) in the same media minus FCS. Explants were then cultured in triplicate in individual wells of a 24-well tissue culture plate containing 1 mL of DMEM media (as previously described minus FCS) in the presence or absence of the appropriate glucosamine compound (10−0.1 mM), after which 10 ng/mL recombinant human IL-1α was added to half of the wells. After a further 96 h culture period, the sulfated GAG (representative of the proteoglycan content of the media or explants following papain digestion) was determined using the dimethyl-methylene blue (DMMB) dye binding assay as previously reported[21] with chondroitin sulfate (CS) from shark chondroitin sulfate C used as a standard. The DMMB assay is a strong metachromatic dye that binds to the negatively charged glycosaminoglycan chains attached to the core protein of aggrecan. This causes a change in the absorption spectrum of the dye. In order to solubilize the cartilage explants the cysteine proteinases, papain was used to degrade and solubilize the extracellular matrix. Cartilage explants were digested with 1 μg/mL papain suspension (papaya latex) in 0.05 M sodium acetate, 0.025 M EDTA, pH 5.6, containing 5 mM cysteine HCl. The papain and cysteine were added just prior to use. Following the appropriate dilutions of the samples in a final 40 μL volume, 200 μL of DMMB solution was added and the absorbance read at 525 nm. From these data a percentage fold-increase in GAG release generated by the addition of IL-1 was calculated relative to the appropriate control culture (i.e., with [GAG-fold, Table 1] or without [control GAG-fold, Table 1] the addition of glucosamine, *N*-acetylglucosamine, or the phosphoramidate dervivatives) as % GAG release from appropriate control culture divided by the % GAG release from IL-1 treated culture, where % GAG release was calculated as

Total GAG released into culture media × 100.

$$\{(\text{total GAG released into culture media})/[(\text{total GAG released into culture media}) + (\text{total GAG from papain digested explant})]\} \times 100$$

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Supporting Information Available: Microanalytical data of target compounds and HPLC data and spectra of target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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