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Novel Biologically Active Series of N-Acetylglucosamine Derivatives for the Suppressive Activities on GAG Release

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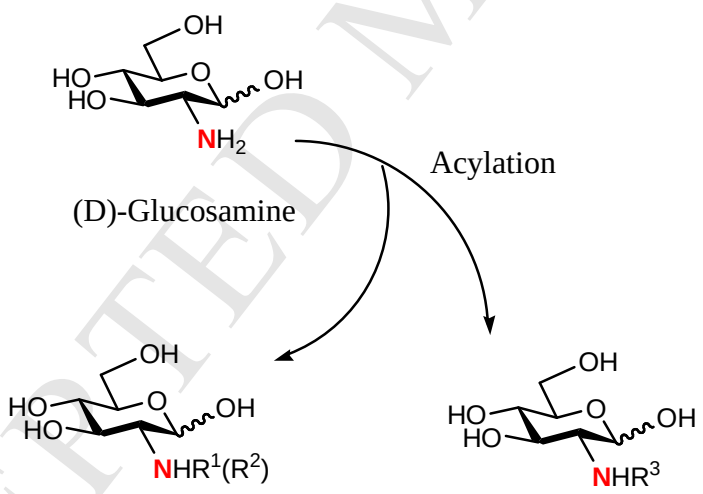
ABSTRACT

(D)-Glucosamine and other nutritional supplements have emerged as safe alternative therapies for osteoarthritis, a chronic and degenerative articular joint disease. N-acetyl-(D)-glucosamine, a compound that can be modified at the N position, is considered to improve the oral bioavailability of (D)-glucosamine and has been proven to possess greater in vitro chondroprotective activity compared with the parent agent. In this study, to further utilize these properties, we focus on the modification of the N position with a benzenesulfonyl and different isoxazole formyl groups. Among these compounds, the 3-(2-chlorobenzene)-5-methyl-isoxazole formyl chloride and p-methoxybenzenesulfonyl chloride modifying structures proved to be the most active of the series and efficiently processed the chondrocytes in vitro. These novel N-position substitution compounds may represent promising leads for osteoarthritis drug development.



KEYWORDS. Biological Evaluation, Glucosamine, N-Acetylglucosamine Derivatives

1. Introduction

2-Amino-2-deoxy-(D)-glucose ((D)-glucosamine, **Figure 1, (1)**), an amino monosaccharide derivative of (D)-glucose, is found in

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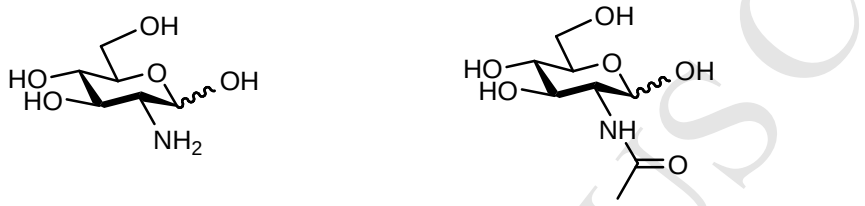
numerous biologically potent molecules such as cell surface N-glycoproteins, hyaluronic acid, glycosphingolipids, glycosylphosphatidylinositol anchors, blood group antigens, bacterial cell wall, lipopolysaccharides, chitin/chitosan, and glycosaminoglycan (GAG) chains.[1] (D)-glucosamine and its acetylated derivative, *N*-acetylglucosamine (GlcNAc), have been widely used in food, cosmetics, pharmaceutical industries, and biomedical applications[2] and are currently produced by acid hydrolysis of chitin (a linear polymer of GlcNAc) extracted from crab and shrimp shells.[3] Glucosamine (GlcN) is a precursor of the glycosaminoglycans and proteoglycans that make up articular cartilage[3] and it plays an important role in the biosynthesis of GAGs,[4] which form proteoglycans (PGs) when covalently bonded to a protein core.[1,5] PGs are major components of chondrocytes, cellular components that play a pivotal role in joint function.[6] Failure of chondrocytes to maintain the balance between synthesis and degradation of the extracellular matrix may lead to osteoarthritis.[1,7]



1 **2**

Figure 1. Structure of (**1**) (D)-glucosamine and (**2**) N-acetyl-(D)-glucosamine.

Osteoarthritis is the most common form of arthritis and a degenerative and progressive joint disorder characterized by joint pain, tenderness, limitation of movement, crepitus, occasional effusion, and variable degrees of joint inflammation, generally causing significant disability.[8-10] The etiology of osteoarthritis is multifactorial. It can be treated using analgesics and nonsteroidal anti-inflammatory drugs, but these drugs can cause serious adverse gastrointestinal and cardiovascular effects, especially with long-term use; moreover, they do not address the underlying physiology of the disease.[1] Disease-modifying drugs that interfere with the progression of the condition are vital for effective treatment.

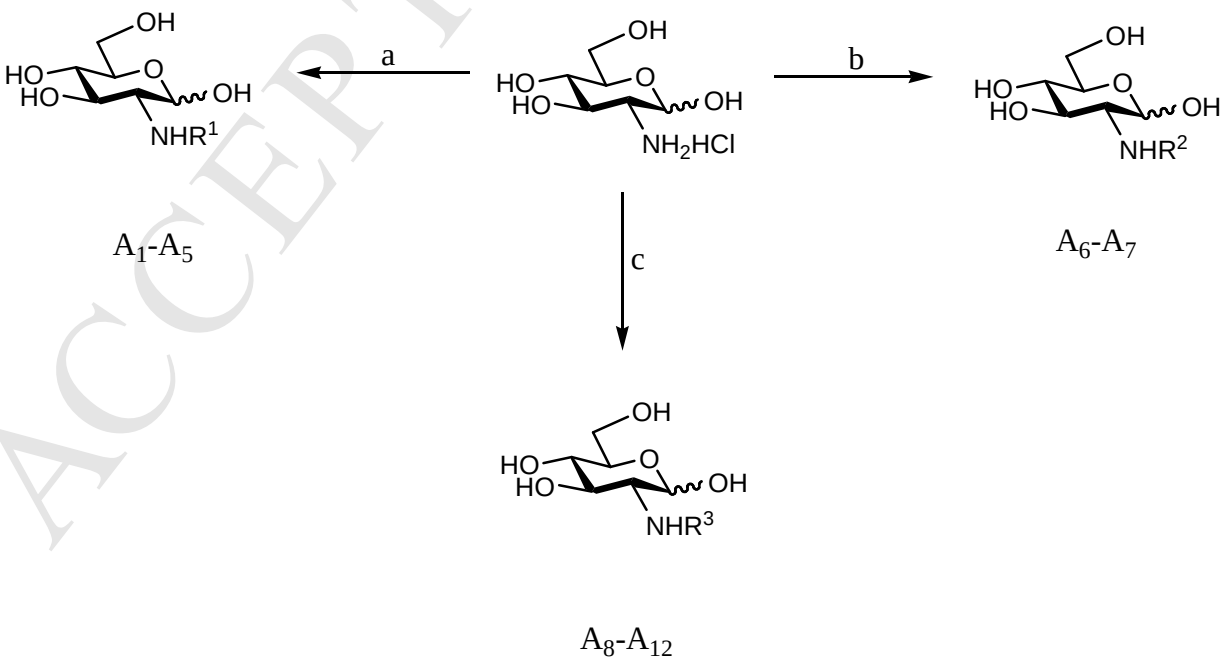
Over past several years, products containing chondroitin (GAGs) and **1** have been increasingly recommended by general practitioners and rheumatologists worldwide, and nowadays, **1** (either as sulfate or chloride salts) represents one of the most common agents used to treat osteoarthritis with no major known side effects.[11-14] Acting as a substrate for cartilage repair by directly stimulating PG synthesis by chondrocytes, glucosamine is an essential component of PG in normal cartilage.[11,15,16] Various studies have presented experimental evidence that **1**, and to a higher degree, N-acetyl-(D)-glucosamine (**Figure 1, (2)**) possess a unique range of anti-inflammatory activities, inhibit NO and cyclooxygenase (COX-2), and sequentially inhibit IL-1β.[17] N-acetyl-(D)-glucosamine typically includes a single glucosamine unit and **2** is one of the building blocks for glycoproteins, formed from (D)-glucose and/or **1**

during the in vivo biosynthesis of PGs.[1,18] Moreover, 2 is widely used as a supplement for treating osteoarthritis and is reported to show no adverse effect.[19-21] However, an updated report from clinical trials of the drug in the treatment of osteoarthritis indicated that the single agent **1** or **2** shows limited efficacy for osteoarthritis. Reasons for this are complicated but should be mainly due to the refractory of osteoarthritis and certain unknown imperfect properties of these compounds. Therefore, the discovery of a new generation of an inhibitor of GAG release with a potent anti-osteoarthritis activity that work against the degradation of used GAG by cartilage cells is now required for drug research and development. (D)-Glucosamine itself is a pharmacologically active, efficient anti-osteoarthritis compound. Osteoarthritis is actually connected with the function mechanism of drugs such as COX-2 inhibitors, which can significantly improve and effectively treat osteoarthritis by acting on COX-2 receptors. The structure of isoxazole can be observed in some COX-2 inhibitor drugs such as valdecoxib. Studies have also shown that sulfonyl or sulfonamide groups are effective specific COX-2 inhibitors, and a typical medicine called leflunomide tablets, which is used to treat rheumatoid arthritis, is an isoxazole derivative. To conduct further research on and modification of (D)-glucosamine, the conjugation of two bioactive fragments, now accepted as an effective approach for drug design, is applied here. In addition, isoxazole has anti-inflammatory properties and is biologically active; thus, it may perform more effectively when it or sulfonyl is combined with (D)-glucosamine. Therefore, in an effort to discover an inhibitor of GAG release with a high potency against osteoarthritis, we performed a rational drug design in which a new series of N-acetyl-(D)-glucosamine derivatives were synthesized.

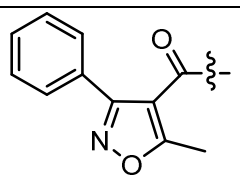
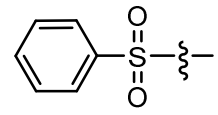
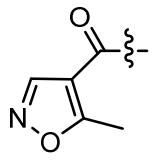
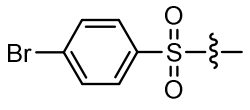
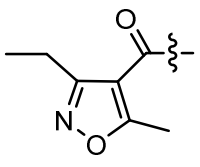
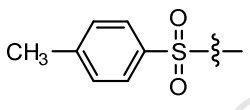
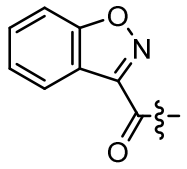
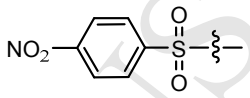
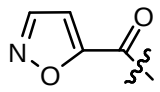
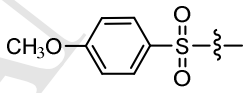
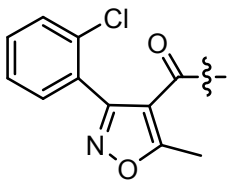
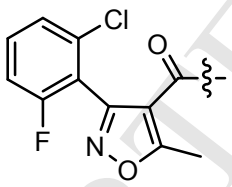
2. Results and discussion

2.1 Chemistry.

All target compounds were easily prepared using standard methods. Synthetic approaches used to prepare the new compounds **A_1–A_5** are depicted in **Scheme 1**.



A_1-A_5 A_6-A_7 A_8-A_{12}

compd	R^1 or R^2	yield(%)	compd	R^3	Yield(%)
A_1		70	**A_8**		87
A_2		60	**A_9**		88
A_3		65	**A_{10}**		85
A_4		68	**A_{11}**		80
A_5		60	**A_{12}**		90
A_6		90			
A_7		90			

Scheme 1. Reagents and conditions: (a) R^1-OH, DMF, EDCI, HOBt, TEA, −5 ℃to rt, 24 h; (b) TEA, DMF, R^2-Cl, −5 ℃, 4 h; (c) TEA, DMF, R^3-Cl, −5 ℃, 4 h.

An activated ester prepared by the reaction of commercially available EDCI (1-(3-dimethylaminopropyl)-3-2 ethylcarbodiimide hydrochloride) together with 3-phenyl-5-methyl-4-isoxazole acid and commercially available HOBt (N- hydroxybenzotrizole) was reacted with (D)-glucosamine hydrochloride to produce **A_1**. Similarly, the target compounds **A_2–A_5** were prepared by the same method using different acids as reactants in substitution. The compounds were obtained as mixtures with a range of yields between 60% and 70% **(Scheme 1)**.

The synthetic routes for compounds **A_6–A_7** are outlined in **Scheme 1**. It is relatively simple to obtain **A_6** and **A_7** through the reaction of acyl chloride and amide. Because the reaction is exothermic, we performed it in an ice bath, added the right amount of alkali to catalyze the reaction, and then neutralized it by releasing hydrogen chloride to protect the glucosamine from becoming an ammonium salt. To make the reaction continue smoothly, it was best to use excess glucosamine as a reactant. After successfully optimizing the reaction conditions, we attempted the synthesis of glucosamine analogues **A_6** and **A_7** using (D)-glucosamine hydrochloride with different acyl chlorides. Results indicate that the reaction proceeded smoothly, yielding the target products **A_6** and **A_7**.

In the synthesis of the series of glucosamine derivatives **A_8–A_{12}**, the mechanism and the reaction conditions were similar to the synthesis of **A_6** and **A_7**. Hence, sulfuryl chloride with (D)-glucosamine hydrochloride under ice bath conditions and with TEA/pyridine resulted in the compounds **A_8–A_{12} (Scheme 1)**.

2.2 Biological activity.

Regarding the pathogenesis of osteoarthritis, primary influencing factors have been identified as interleukin 1 (IL-1), the tumor necrosis factor,[22] and the hypoxia-inducible factor.[23] In particular, IL-1 is a more important factor as research indicates that IL-1 in the synovial fluid and serum of patients with osteoarthritis was higher than that in normal people.[24]

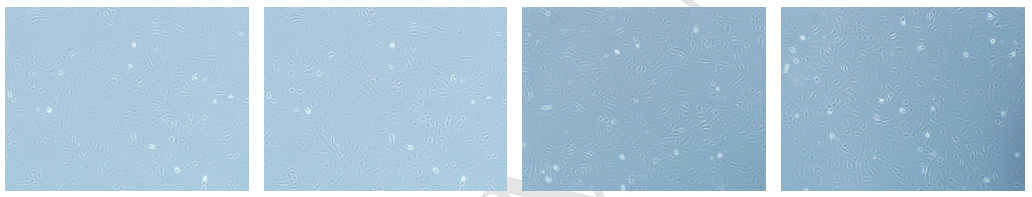


Figure 2. Primary rabbit articular cartilage cells

To examine the effect of a series of new glucosamine derivatives on the loss of aggrecan (cartilage PG) from articular cartilage (an early event in the development of matrix degradation in osteoarthritis), we cultivated primary rabbit articular cartilage cells **(Figure 2)**. A model in vitro culture system was established whereby cartilage explants were exposed to IL-1 (to induce the loss of aggrecan from a tissue) in the presence or absence of glucosamine derivatives. This exposure led to an increase in levels of GAG released compared with the untreated controls; this represents a loss of aggrecan from the tissue due to degradation by matrix proteases.[25] In each experiment, a control level of the increasing GAG released from the explant as a result of exposure to IL-1 was determined. An examination of cartilage explants cultured in the presence of glucosamine derivatives with IL-1 was also performed. The percentage of reduction in GAG release, calculated through measuring the successive GAG content using an ELISA-kit, is shown in **Table 1**. Thus, in the first set of experiments, where drug dose was 10 mM, the mean increase in GAG release in the control (i.e., IL-1 alone, no glucosamine derivatives added) was higher than those groups treated by glucosamine derivatives. Drug efficacy was measured as the reduction in the increase of GAG released into the culture media. Thus, treatment with **1** at 10 mM led to an approximately 20%

reduction relative to the control (IL-1 only treated explant culture) in GAG released (before/after exposure) into the culture media, whereas that with **2** led to an approximately 39% reduction relative to the control. In parallel cytotoxicity assays conducted using the CKK 8 assay on a chondrocyte monolayer culture, both **1** and **2** were cytotoxic at 10 mM with 70%–80% cell viability relative to the control. At present, whether the apparent inhibition of GAG released by these agents was in part attributable to cytotoxicity is unclear. It was ascertained that these compounds were considerably less cytotoxic at 1 and 0.1 mM. However, it was difficult to inhibit IL-1-induced aggrecan release from the tissue (percentage (%) of GAG reduction) for both compounds at lower concentrations. Therefore, it is not impossible to deduce that the chondroprotective effect of **1** and **2** at 10 mM is simply due to cytotoxicity.

Almost all new compounds were more active than **1** at 10 mM, representing a 20% GAG reduction relative to **1**. Mostly, the new compounds showed a lower percentage of GAG reduction at lower dilutions.

The first new compound series was $\mathbf{A_1}$–$\mathbf{A_7}$. This series was designed on the basis of the drug efficacy of the isoxazole group and the potency of glucosamine over a range of anti-osteoarthritis conditions. $\mathbf{A_1}$–$\mathbf{A_7}$ significantly reduced the level of GAG released into the media after IL-1 exposure ($\mathbf{A_1}$–$\mathbf{A_7}$ at 10 mM). Nonetheless, compared with **2**, $\mathbf{A_1}$, $\mathbf{A_2}$, $\mathbf{A_4}$, and $\mathbf{A_7}$ were less effective ($\mathbf{A_1}$–$\mathbf{A_7}$ at 10 mM). Surprisingly, $\mathbf{A_6}$ showed better effectiveness at reducing the level of GAG released into the media at 10 mM. Moreover, $\mathbf{A_6}$ was more effective than the other new compounds at 1 mM with 39% GAG reduction. On further dilution, $\mathbf{A_1}$–$\mathbf{A_7}$ lost activity, becoming inactive at 0.1 mM, although the potency of $\mathbf{A_7}$ was still better than that of $\mathbf{A_6}$. In addition, the percentage of GAG reduction changed from 39% to 37%, i.e., a smaller range. $\mathbf{A_6}$ was more cytotoxic at 10–0.1 mM than most of the other compounds; however, it is highly likely that its efficacy is connected to its cytotoxicity. Even so, $\mathbf{A_6}$ was significantly more active, led to a reduction in GAG released into the culture media, and was less cytotoxic with a 64%–85% cell viability at 10–0.1 mM.

Table 1

Efficacy and Toxicity Data of Glucosamine **1**, N-Acetylglucosamine **2**, and Glucosamine Derivatives ($\mathbf{A_1}$–$\mathbf{A_{12}}$)

	10 mM			1 mM			0.1 mM		
	GAG content μg/L (±SD)	GAG reduction (%)	CCK 8 (±SD)	GAG content μg/L (±SD)	GAG reduction (%)	CCK 8 (±SD)	GAG content μg/L (±SD)	GAG reduction (%)	CCK 8 (±SD)
1	1616.25 (0.04)	20.41	80.07 (0.92)	1212.81 (0.05)	40.28	101.72 (1.58)	1070.00 (0.04)	47.31	96.64 (4.65)
2	1240.83	38.90	73.26	1511.88	25.55	68.74	1597.50	21.34	90.81

	(0.02)		(1.05)	(0.04)		(1.34)	(0.01)		(2.04)
A_1	1357.50	33.15	70.47	1605.06	20.96	94.26	2159.77	-6.35	107.38
	(0.01)		(1.54)	(0.05)		(5.13)	(0.04)		(4.55)
A_2	1285.91	36.68	70.71	1447.84	28.71	70.71	1818.86	10.44	90.40
	(0.04)		(1.72)	(0.04)		(1.72)	(0.00)		(1.94)
A_3	1091.54	46.25	64.97	1303.52	35.81	83.68	1530.23	24.65	83.68
	(0.02)		(1.03)	(0.04)		(2.39)	(0.02)		(2.39)
A_4	1245.57	38.67	68.91	1301.25	35.92	85.89	1279.09	37.02	85.89
	(0.03)		(1.53)	(0.03)		(2.80)	(0.01)		(2.80)
A_5	1114.32	45.13	78.26	1284.77	36.73	96.72	1326.83	34.66	95.00
	(0.05)		(2.13)	(0.07)		(4.21)	(0.03)		(1.08)
A_6	1080.80	46.78	64.40	1233.07	39.28	74.57	1280.23	36.96	85.48
	(0.04)		(1.00)	(0.01)		(1.64)	(0.05)		(0.97)
A_7	1512.05	25.54	71.29	1247.27	38.58	88.52	1173.41	42.22	92.86
	(0.02)		(1.30)	(0.06)		(2.61)	(0.01)		(2.50)
A_8	1337.50	34.14	69.57	1346.25	33.71	87.28	1335.00	34.26	95.32
	(0.03)		(1.06)	(0.03)		(2.58)	(0.00)		(4.41)
A_9	1507.50	25.77	74.98	1690.63	16.75	84.17	1887.50	7.06	102.71
	(0.04)		(1.15)	(0.03)		(0.82)	(0.03)		(5.74)
A_{10}	1048.75	48.36	72.44	1501.88	26.04	94.01	1403.21	30.90	95.65
	(0.04)		(1.51)	(0.05)		(2.16)	(0.02)		(1.89)
A_{11}	855.00	57.90	69.24	1299.64	36.00	89.75	1313.75	35.31	95.90
	(0.06)		(4.38)	(0.03)		(14.34)	(0.04)		(3.43)
A_{12}	1021.25	49.71	70.22	995.63	50.97	90.73	936.88	53.87	102.91
	(0.02)		(2.30)	(0.03)		(3.00)	(0.01)		(2.52)
control									
GAG content µg/L				2030.80					

The average increase in GAG released into the culture media in IL-1. Treated culture is calculated using the appropriate control (minus IL-1) for explants cultured in the absence (control GAG content) and presence (GAG content) of glucosamine compounds at concentrations ranging from 10 to 0.1 mM. The percentage of GAG reduction was calculated using {[control GAG − sample GAG]/(control GAG)} × 100. The effects of different concentrations of glucosamine compounds on chondrocyte viability were assessed using the CKK 8 assay. The percentage of cell viability was calculated relative to the control cells (absence of glucosamine compounds, taken as 100%). As shown in the table, some of the high standard deviation (SD) readings are due to the biological variability of the cartilage obtained.

When the next batch of compounds **A_8–A_{12}** was examined, it was clear that these compounds were more active than **1** and **2** at 10 mM. **A_{12}** showed good biological activity at 10–0.1 mM and led to a reduction in GAG released into the culture media, resulting in an approximately 50% reduction relative to the control (IL-1 only treated explant culture) at 10mM and above 50% reduction at 1–0.1 mM. In addition, **A_{11}** was highly effective at 10 mM; however, it was only active at approximately 10 mM and not at 1 mM. If this in vitro assay was reflected in vivo, such levels of drugs would most likely not be feasible. **A_{12}** was quite effective at 1 and 0.1 mM dilutions. Surprisingly, at 1 mM, **A_{12}** was less toxic than the other compounds and showed 90% cell viability, and at 0.1 mM, it exhibited no cytotoxicity and showed 102% cell viability.

3. Conclusion

In this study, a series of new glucosamine derivatives was designed and synthesized. The biological activity of these compounds is discussed based on cellular activity. Numerous compounds showed high potency in both biochemical and cell function assays. Among these, two most active compounds were selected to conduct a preliminary in vitro anti-osteoarthritis assay. In conclusion, cell viability shows a decreasing trend with increasing concentrations of glucosamine compounds. The results of the GAG content indicate that most of the compounds showed an obvious biological activity. In particular, **A_{11}** showed a strong biological activity at 10 mM, whereas **A_6** and **A_{12}** showed a good biological activity at three different concentrations. When considering cell viability, **A_6** and **A_{12}** are presented as new viable compounds.

4. Experimental section

4.1 General

All reagents and solvents were obtained from commercial suppliers and used without further purification unless otherwise indicated. Anhydrous solvents were dried and purified by conventional methods prior to use. Column chromatography was performed on silica

gel (200–300 mesh). All reactions were monitored by thin-layer chromatography (TLC) and silica gel plates, which were made by 0.3% sodium carboxymethyl cellulose solution and visualized under UV light. ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker AV□-400 spectrometer at 400 and 100 MHz, respectively. Coupling constants (J) are expressed in hertz. Spin multiplicities are described as singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). Chemical shifts (δ) are listed in parts per million relative to tetramethylsilane as an internal standard. Mass spectral data were acquired using a Water Q-TOF Premier mass spectrometer.

4.2 Chemistry

4.2.1. Synthesis of N-(3-phenyl-5-methyl-4-isoxazole)-benzoylamino-2-deoxyglucose (A_1).

A solution of 3-phenyl-5-methyl-4-isoxazole formic acid (0.2 g, 1 mmol) in DMF (5 mL) was stirred. EDCI (1-(3-dimethylamino propyl)-3-2 ethyl carbon imine hydrochloride) (0.22 g, 1.2 mmol) and HOBt (N-hydroxybenzotrizole) (0.16 g, 1.2mmol) were added sequentially and activated for approximately 1 h. Glucosamine hydrochloride (0.43 g, 2 mmol) and triethylamine (0.4 g, 4 mmol) were then added, and the solution was stirred for an additional 24 h at room temperature. The reaction was monitored by TLC. The mixture was extracted with ethyl acetate (EA) after the end of the reaction. The organic layer was separated, washed with a saturated sodium chloride solution, dried over $MgSO_4$, and concentrated in vacuo. The residue was washed with ethanol/dichloromethane (v/v = 2:5), and the title compound **A_1** was obtained as a white solid (70% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.27 (m, 2H), 7.65 (m, 3H), 6.60 (s, 1H, NH-CO), 5.04 (d, 1H) , 3.58 (m, 2H), 3.30 (d, *J* = 25.2 Hz, 2H), 3.05 (q, *J* = 7.3 Hz, 1H), 2.53 (d, *J* = 3.6 Hz, 3H,CH_3), 1.18 (s, 4H, OH). ^{13}C NMR (100 MHz, DMSO-d6) δ169.75, 161.44, 159.95, 128.71, 127.83, 112.85, 90.29, 72.10, 71.12, 70.12, 61.01, 54.80, 45.26, 10.93, 8.37. HRMS (ESI) calcd for $C_{17}H_{20}N_2O_7$ $[M+Na]^+$: 387.1179, found 387.1166.

4.2.2. Synthesis of N-(5-methyl-4-isoxazole)-formylamino-2-deoxyglucose (A_2).

A_2 was prepared in a same manner as **A_1**. ^{1}H NMR (400 MHz, DMSO) δ 6.28 (s,1H), 5.42 (d, 1H), 4.53 (m, H), 3.79 (m, 1H), 3.72 (d, *J* = 33.6 Hz, 2H), 3.45 (d, *J* = 13.7 Hz, 1H), 3.29 (m, H), 3.27 (s, 1H), 2.43 (s, 3H), 1.67 (s, 1H), 1.35 (s, 1H), 1.23 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d6) δ170.11, 165.57, 152.96, 118.38, 93.90, 75.23, 71.19, 69.81, 61.95, 56.05, 13.27. HRMS (ESI) calcd for $C_{11}H_{16}N_2O_7$ $[M+H]^+$: 289.1044, found 289.1050.

4.2.3. Synthesis of N-(3-ethyl-5-methyl-4-isoxazole)-formylamino-2-deoxyglucose (A_3).

A_3 was prepared in a similar way to **A_1**. ^{1}H NMR (400 MHz, DMSO) δ 6.77 (s, 1H), 6.12 (s, 1H), 5.27 (d, 1H), 4.13 (m, 1H), 3.81 (m, 1H), 3.69 (s, 1H), 3.45 (d, *J* = 12.0 Hz, 2H), 3.22 (m, 1H), 3.07 (q, *J* = 7.8 Hz, 2H), 2.43 (s, 3H), 1.76 (s, 1H), 1.44 (s, 1H), 1.32 (t, 3H), 1.24 (s, 1H). ^{13}C NMR (100MHz, DMSO-d6) δ 169.67, 165.81, 165.46, 114.72, 93.90, 75.23, 71.19, 69.81, 61.95, 56.05, 20.54, 14.04, 11.54. HRMS (ESI) calcd for $C_{13}H_{20}N_2O_7$ $[M+H]^+$: 317.1360, found 317.1365.

4.2.4. Synthesis of N-(3-benzisoxazole)-formylamino-2-deoxyglucose (A_4).

A_4 was prepared by the same method as **A_1**. ^{1}H NMR (400 MHz, DMSO) δ 8.42-7.72 (m, 4H), 6.57 (s, 1H), 5.58 (d, 1H), 4.76 (q, 1H), 3.73 (q, *J* = 37.5 Hz, 1H), 3.55 (m 1H), 3.44 (d, 1H), 3.39 (d, 2H), 1.65 (s, 1H), 1.29 (s, 1H), 1.24 (s, 1H), 1.05 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d6) δ 162.52, 161.57, 150.60, 133.55, 124.14, 121.90, 117.28, 111.20, 93.90, 75.23, 71.19, 69.81, 61.95, 56.05. HRMS (ESI) calcd for $C_{14}H_{16}N_2O_7$ $[M+H]^+$: 325. 1037, found 325. 1045.

4.2.5. Synthesis of N-(5-isoxazole)-formylamino-2-deoxyglucose (A_5).

A_5 was prepared by the same method as **A_1**. ^{1}H NMR (400 MHz, DMSO) δ 6.13 (s, 1H), 5.19 (d, 1H), 3.84 (m, 1H), 3.75 (m, 1H), 3.65 (d, *J* = 37.4 Hz, 2H), 3.42 (m, 1H), 2.02 (s,1H), 1.85 (s, 1H), 1.51 (s, 1H), 1.24 (s, 4H). ^{13}C NMR (100 MHz, DMSO-d6) δ 161.12, 158.08, 153.55, 106.44, 93.90, 75.23, 71.19, 69.81, 61.95, 56.05. HRMS (ESI) calcd for $C_{10}H_{14}N_2O_7$ $[M+H]^+$: 275.0880, found 275.0888.

4.2.6. Synthesis of N-(3-(2-chlorophenyl)-5-methyl-4-isoxazole)-formylamino-2-deoxyglucose (A_6).

Glucosamine hydrochloride (0.43 g, 2 mmol) and triethylamine (0.4 g, 4 mmol) were dissolved in DMF (5 mL) and cooled to −5℃. Then, 3-(2-chlorophenyl)-5-methyl-4-isoxazole formyl chloride (0.23 g, 1 mmol) was added and stirred at −5 ℃ for 4 h. The product was extracted with EA. Extracts were washed with saturated sodium chloride solution, dried over $MgSO_4$, and evaporated to yield a crude product. The crude product was then purified using silica gel chromatography, which resulted in a white solid product (90% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.15 (m, H), 7.55 (m, 3H), 6.53 (s, 1H, NH-CO), 5.04 (m, 1H) , 3.58 (m, 2H), 3.30 (d, 2H), 3.11 (q, *J* = 7.3 Hz, H), 2.53 (d, *J* = 3.6 Hz, 3H, CH_3), 1.36 (s, 1H), 1.29 (s, 1H), 1.21 (s, 1H), 1.18 (s, 4H, OH). ^{13}C NMR (100 MHz, DMSO-d6) δ 170.36, 161.13, 160.35, 133.02, 131.68, 130.40, 127.39, 114.06, 96.05, 90.04, 77.09, 72.71, 71.06, 70.37, 61.08, 54.72, 12.81. HRMS (ESI) calcd for $C_{17}H_{19}ClN_2O_7$ $[M+Na]^+$: 421.0779, found 421.0730.

4.2.7. Synthesis of N-(3-(2-chloro-5-fluorophenyl)-5-methyl-4-isoxazole)-formylamino-2-deoxyglucose (A_7).

A_7 was prepared by the same method as **A_6**. ^{1}H NMR (400 MHz, DMSO) δ 7.98 (s, 1H), 7.64 (m, 2H), 7.35 (s, 1H), 6.52 (d, *J* = 20.0 Hz, 1H), 5.10 (m, 1H), 4.70 (s, 1H), 4.65 (m, 2H), 4.43 (m, 1H), 3.60 (m, 1H), 3.44 (d, 1H), 2.69 (s, 3H), 2.54 (s, 3H). ^{13}C NMR (100MHz, DMSO-d6) δ 169.86, 159.47, 153.52, 130.87, 126.21, 114.53, 95.56, 90.53, 76.20, 74.25, 72.21, 70.86, 69.59, 60.54, 57.24, 53.93, 11.62. HRMS (ESI) calcd for $C_{17}H_{18}ClFN_2O_7$ $[M+Na]^+$: 439.0685, found 439.0670.

4.2.8. Synthesis of N-benzenesulfonamido-2-deoxyglucose (A_8).

Glucosamine hydrochloride (0.43 g, 2 mmol) was charged in a 25 mL round-bottom flask together with TEA (0.4 g, 4 mmol). Dry DMF (5 mL) was then added; thereafter, benzenesulfonyl chloride (0.18 g, 1 mmol) at −5 ℃ was added. After addition, the resulting

solution was stirred for 4 h. After the reaction was complete, ethyl acetate was added for extraction. The combined organic phase was washed with saturated sodium chloride solution, dried over $MgSO_4$, and concentrated under vacuum. Chromatographic purification with an EtOH/dichloromethane (v/v = 4:1) gradient yielded white solid **A_8** (87% yield). ^{1}H NMR (400 MHz, DMSO) δ 7.79 (m, 2H), 7.71 (m, 3H), 4.91 (d, 1H), 4.72 (m, 1H), 4.62 (s, 1H), 4.44 (m, 1H), 3.83 (m, 1H), 3.76 (m, 1H), 3.44 (d, 2H), 3.06 (m, 1H), 1.34 (s, 1H), 1.44 (s, 1H), 1.16 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d6) δ 140.66, 133.99, 128.96, 128.02, 93.28, 75.23, 70.85, 69.81, 61.95, 58.91. HRMS (ESI) calcd for $C_{12}H_{17}NO_7S$ $[M+H]^+$: 320.0805, found 320.0770.

4.2.9. Synthesis of N-(4-bromo)-benzenesulfonylamino-2-deoxyglucose (A_9).

A_9 was prepared in a similar manner as **A_8**. ^{1}H NMR (400 MHz, DMSO) δ 7.79 (m,4H), 4.91 (d, *J* = 6.8 Hz, 1H), 4.72 (m,1H), 4.62 (s, 1H), 4.44 (m, 1H), 3.39 (d, 2H), 3.06 (m,1H), 2.97 (m, 1H), 2.51 (s, 1H), 1.44 (s, 1H), 1.34 (s, 1H), 1.16 (s,1H). ^{13}C NMR (100 MHz, DMSO-d6) δ 131.56, 130.49, 128.54, 127.19, 124.84, 120.86, 90.51, 69.20, 58.21, 45.57, 38.94, 8.60. HRMS (ESI) calcd for $C_{12}H_{16}BrNO_7S$ $[M+H]^+$: 397.9910, found 397.9913.

4.2.10. Synthesis of N-(4-methyl)-benzenesulfonylamino-2-deoxyglucose (A_{10}).

A_{10} was prepared in a similar manner as **A_8**. ^{1}H NMR (400 MHz, DMSO) δ 7.48 (d, 2H), 7.11 (d, *J* = 18.3 Hz, 2H), 4.91 (d, 1H), 4.61 (s, 1H), 4.02 (m, 1H), 3.65 (m, 1H), 3.37 (d, 2H), 3.14 (m,1H),3.02 (m, 1H), 2.51 (s,3H), 1.80 (s,1H), 1.73 (s,1H), 1.30 (s,1H), 1.01 (s,1H). ^{13}C NMR (100 MHz, DMSO-d6) δ 144.06, 138.77, 129.53, 126.91, 93.28, 75.23, 70.85, 69.81, 61.95, 58.91, 21.15. HRMS (ESI) calcd for $C_{13}H_{19}NO_7S$ $[M+H]^+$: 334.0961, found 334.0967.

4.2.11. Synthesis of N-(4-nitro)-benzenesulfonylamino-2-deoxyglucose (A_{11}).

A_{11} prepared in the same manner as **A_8**. ^{1}H NMR (400 MHz, DMSO) δ 8.30 (d, *J* = 8.2 Hz, 2H), 8.05 (d, 2H), 5.92 (s, 1H), 4.92 (d, 1H), 4.57 (s,1H), 3.20-2.90 (m, 4H), 3.42 (d, 2H), 1.30 (s,1H), 1.21 (s, 1H), 1.01 (s, 1H). ^{13}C NMR (100MHz, DMSO-d6) δ 151.91, 149.96, 129.99, 122.99, 93.28, 75.23, 70.85, 69.81, 61.95, 58.91. HRMS (ESI) calcd for $C_{12}H_{16}N_2O_9S$ $[M+H]^+$: 365.0656, found 365.0663.

4.2.12. Synthesis of N-(4-methoxyl)-benzenesulfonylamino-2-deoxyglucose (A_{12}).

A_{12} was prepared in the same manner as **A_8**. ^{1}H NMR (400 MHz, DMSO) δ 7.73 (d, *J* = 8.6 Hz, 2H), 7.05 (d, *J* = 8.6 Hz, 1H), 6.85 (s, 1H), 4.92 (d, *J* = 6.1 Hz, 1H), 4.57 (s, 1H), 3.75 (s, 3H), 3.20-2.90 (m, 4H), 3.42 (d, 2H), 2.5 (s, 1H), 1.30 (s, 1H),1.21 (s, 1H), 1.01 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d6) δ 162.20, 132.82, 128.54, 113.80, 91.19, 71.25, 60.56, 58.22, 54.91, 44.59, 8.31. HRMS (ESI) calcd for $C_{13}H_{19}NO_8S$ $[M+H]^+$: 350.0900, found 350.0906.

4.3 Biological activity assay

4.3.1. Isolation and culture of rabbit chondrocytes and CCK-8 cytotoxicity assay.

Monolayer chondrocyte cultures were obtained form the metacarpophalangeal joints of mature rabbits. Full-depth articular cartilage tissue slices were dissected under sterile conditions and subjected to standard methods to isolate the chondrocytes using well established procedures.[22] In brief, the tissue was digested for 1h at 37 ℃, 5% CO_2 with agitation, washed, and then further digested in the same above media and incubated overnight with agitation as before. Cells were filtered through a 0.22 μm Nitex filter and washed before cell numbers were established. Isolated rabbit chondrocytes were cultured in 1 mL of serum-free media containing gentamicin (50μg/mL), 1% (V/V) HEPES buffer and supplemented with or without the appropriate glucosamine compound (10-0.1 mM). After 96h of culture, the cell viability of the chondrocytes in the presence or absence of each compound was determined using the Cell Counting Kit (CCK-8) cell viability assay. This assay is a colorimetric toxicity assay based on the detection of intracellular mitochondrial activity within viable cells. CCK-8 is converted to a an soluble orange-yellow formazan product by succinate dehydrogenase within the mitochondria of viable cells only. 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.

4.3.2. Articular cartilage explant cultures and analysis of glycosaminoglycan concentration in media.

Full-depth articular cartilage explants were dissected from mature rabbits metacarpophalangeal joints. The explants underwent an equilibration period by culture in media, after which explants were washed (3× 10 min). Explants were then cultured in triplicate in individual wells of a 24-well tissue culture plate containing 1 mL of media 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 supernatant liquid was treated according to methods of the rabbit GAG ELISA Kit (ELISA Kit measured the expression of GAG), the value of OD determined by ELISA combined with the standard curve of GAG to obtained a value of GAG content. From these data a increasing content in GAG release generated by the addition of IL-1 was calculated relative to the appropriate control culture (i.e., with [GAG content, Table 1] or without [control GAG content, Table 1] the addition of glucosamine, N-acetylglucosamine, or the amidate dervivatives) as increasing GAG content, and % GAG reduction was calculated by a formula.

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Abbreviations

GAGs, glycosaminoglycan;

PGs, proteoglycans;

IL-1, interleukin 1.

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Highlights:

1. New N-acetylglucosamine derivatives are synthesized.

2.The suppressive activities on GAG release of the derivatives rises as different substituents.

3. The synthesized new derivatives posses excellent suppressive activities on GAG release.