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Molecular docking-guided synthesis of NSAID–glucosamine bioconjugates and their evaluation as COX-1/COX-2 inhibitors with potentially reduced gastric toxicity

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are a powerful class of inhibitors targeting two isoforms of the family of cyclooxygenase enzymes (COX-1 and COX-2). While NSAIDs are widely used in the management of pain, in particular as a treatment for osteo- and rheumatoid arthritis, their long-term use has been associated with numerous on- and off-target effects. As the carboxylic acid moiety present in common NSAIDs is responsible for some of their adverse effects, but is not required for their anti-inflammatory activity, we sought to mask this group through direct coupling to glucosamine, which is thought to prevent cartilage degradation. We report herein the conjugation of commonly prescribed NSAIDs to glucosamine hydrochloride and the use of molecular docking to show that addition of the carbohydrate moiety to the parent NSAID can enhance binding in the active site of COX-2. In a preliminary, in vitro screening assay, the diclofenac-glucosamine bioconjugate exhibited 10-fold greater activity toward COX-2, making it an ideal candidate for future in vivo studies. Furthermore, in an intriguing result, we observed that the mefenamic acid-glucosamine bioconjugate displayed enhanced activity toward COX-1 rather than COX-2.

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AUTHOR CONTRIBUTIONS

RAJL conceived the idea, synthesized compounds, performed the molecular modeling, and wrote the manuscript. YT synthesized compounds and co-wrote the manuscript. CM conducted the biological screening of the synthesized compounds. BCS supervised the molecular docking and edited the manuscript. CSS synthesized compounds. CDH supervised the study. All authors approved the manuscript.

[†]Professor Alan R. Katritzky passed away 10th February 2014.

Keywords

COX-1/COX-2; glucosamine bioconjugates; molecular docking; NSAIDs

1 | INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed for the management of short- and long-term pain, especially that associated with arthritis (Zhang et al., 2019). More than 40 million Americans are affected by arthritis and arthritis is the leading cause of work disability, costing American society an estimated $400 billion per year (Murphy et al., 2018). Aspirin was first marketed 150 years ago, and at least 20 different NSAIDs are currently used as treatments for pain and inflammation. Despite their widespread use and therapeutic efficacy, the long-term use or chronic administration of NSAIDs is associated with numerous on- and off-target effects, including gastrointestinal (GI) mucosa, ulceration and hemorrhage, as well as renal dysfunction, hepatotoxicity, platelet inhibition and aspirin-induced asthma.

Although NSAIDs have diverse chemical structures (Figure 1), they share the same mode of action; that is the prevention of the biosynthesis of pro-inflammatory prostaglandins from arachidonic acid, *via* the inhibition of the enzyme *Cyclooxygenase* (COX). In humans, two COX isoforms exist: COX-1 and COX-2, which are regulated differently. COX-1 is constitutively expressed, especially in the GI tract, whereas COX-2 expression is mostly induced in inflamed regions (Mitchell & Kirkby, 2019). Different NSAIDs will inhibit COX-1 and COX-2 to varying degrees (Cryer & Feldman, 1998) but in spite of these differences, the GI side effects can typically be attributed to two causes: (a) inhibition of the COX-1 isoform and its protective effect on GI mucosa, and (b) an increase of local irritation due to the carboxylic acid function present in the scaffold of most NSAIDs. Current clinical methods to reduce the side effects of NSAIDs include prescribing the lowest possible dose for the shortest time necessary to control symptoms (Hart & Huskisson, 1984; Scheiman & Hindley, 2010), and co-administering gastro-protective drugs or synthetic prostaglandins (Kourounakis et al., 2000). These methods, however, have limited effectiveness.

Since the carboxylic acid moiety of NSAIDs is partly responsible for the GI toxicity (Hawkey et al., 2000) and is not required for the anti-inflammatory activity (Ullah et al., 2015), efforts are currently in hand to develop new NSAIDs without these deleterious functions. Two strategies have been investigated: (a) the synthesis of NSAID pro-drugs where the carboxylic acid is masked as an ester or amide and (b) the development of COX-2-specific inhibitors. The latter molecules have the advantage of avoiding the GI-tract side effects associated with the inhibition of COX-1 and these strategies have resulted in highly selective COX-2 inhibitors, including celecoxib, rofecoxib, and etoricoxib. However, COX-2 inhibitors are associated with an increased risk of adverse cardiovascular events because these molecules are unable to discriminate COX-2 associated with inflammation from COX-2 constitutively present outside the GI tract in the cardiovascular system. Therefore, while it was initially thought that only the newer COX-2 selective NSAIDs were associated with cardiovascular risks, it is now emerging that more traditional NSAIDs,

including ibuprofen and naproxen, can also lead to an increased risk of adverse cardiovascular events (MacDonald et al., 2017; Nissen et al., 2016). Thus, there is a need for new and improved treatments for the management of pain and inflammation.

A hallmark of arthritis is the progressive wearing down of cartilage connective tissue composed of glycosylaminoglycans (GAGs) including chondroitin sulfate and keratin. Since glucosamine (GlcN) is a key component of these GAGs, GlcN may act as a chondroprotective agent to prevent or delay cartilage degradation. While the use of GlcN as a supplement is not without controversy (Dahmer & Schiller, 2008; Vangsness et al., 2009; Zhang et al., 2019), and the mode of action of GlcN is unclear (Kang et al., 2015), GlcN may be beneficial in a subset of patients with moderate to severe osteoarthritis (Clegg et al., 2006). In addition, there are currently no known adverse side effects to taking GlcN (Huskisson, 2008), and concomitant administration of GlcN with NSAIDs may lead to a lower required dose and potentially fewer adverse effects (Altman et al., 2005). Moreover, glucosamine hydrochloride is an inhibitor of COX-2 activity by preventing COX-2 co-translation and facilitating COX-2 protein turnover (Jang et al., 2007). Therefore, we hypothesized that the direct conjugation of GlcN to an NSAID would result in a series of NSAID pro-drugs with the possibility that these compounds would retain their COX-1/2 activity, while releasing in situ the cartilage protecting GlcN. Furthermore, there is growing interest in glycosylated drugs as a tool for targeted drug delivery (Chen & Huang, 2019). We report herein, the synthesis of these compounds, molecular docking studies to determine whether appending a carbohydrate to an NSAID would be predicted to disrupt binding in the active site of COX-2 and their in vitro potency against COX-1 and COX-2.

2 | EXPERIMENTAL SECTION

2.1 | General methods and materials

Chemicals were purchased from SigmaAldrich, Tokyo Chemical Industries and ChemImpex and were used without further purification. THF and CH_2Cl_2 were distilled prior to use, and all reactions were performed under an inert atmosphere. Melting points were determined on a capillary point apparatus equipped with a digital thermometer. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ using 300 or 500 MHz spectrometers, and assignments were made using 2D NMR experiments. The spectra were referenced to the residual solvent peak ($CDCl_3$: ^{1}H = 7.27 ppm, ^{13}C = 77.0 ppm). The following abbreviations are used to describe spin multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br s = broad singlet, dd =doublet of doublets, ddd =double of doublet of doubles, dt =double of triplets and app =appears. Mass spectrometry was performed with electrospray ionization (ESI). Ether refers to diethyl ether.

2.2 | Synthesis of GlcN–NSAID conjugates—Route B

The NSAID (2.6 mmol) was dissolved in anhydrous THF (10 ml) and cooled to 0°C. Hydroxybenzotriazole (HOBt), (3.6 mmol) and *N*-(3-dimethylaminpropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI) (3.1 mmol) were added and the reaction mixture was stirred at 0°C for 60 min. Peracetylated GlcN **1** (2.6 mmol) was dissolved in anhydrous CH_2Cl_2 (20 ml), and Et_3N (6.5 mmol) was added. After 60 min, this solution was added

slowly to the reaction vessel containing the NSAID. The GlcN flask was rinsed with anhydrous THF (2 × 5 ml) and added to the reaction mixture. The reaction was allowed to warm to r.t. and stirred for a further 16 hr until complete consumption of the starting material was observed by TLC (CH_2Cl_2:MeOH, 9:1, v/v). The reaction mixture was concentrated under reduced pressure, and the residue was dissolved in EtOAc (50 ml). The organic layer was washed sequentially with 2 M HCl (3 × 30 ml), $NaHCO_{3\ (satd.\ aq)}$ (2 × 30 ml), H_2O (30 ml), and brine (30 ml). It was then dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Flash chromatography (CH_2Cl_2 → CH_2Cl_2:MeOH, 95:5, v/v) gave the desired product.

2.2.1 | 1,3,4,6-Tetra-O-acetyl-2-deoxy-2-(2-((2,6-dichlorophenyl)amino)phenyl)acetamido)-β-D-glucopyranose, 3—

Off-white solid; Yield 27%; m.p. 175–177°C; ^{1}H NMR (500 MHz) δ_H 7.56 (s, 1H, NH), 7.36 (d, J = 8.0 Hz, 2H, ArH), 7.09 (t, J = 6.4 Hz, 2H, ArH), 7.01 (t, J = 8.1 Hz, 1H, ArH), 6.91 (t, J = 7.4 Hz, 1H, ArH), 6.48 (d, J = 8.2 Hz, 1H, ArH), 6.05 (d, $J_{NH,2}$ = 9.6 Hz, 1H, NH), 5.71 (d, $J_{1,2}$ = 8.8 Hz, 1H, H-1), 5.18 (t, J = 9.9 Hz, 1H, H-3), 5.13 (t, J = 9.5 Hz, 1H, H-4), 4.34 (q, J = 9.6 Hz, 1H, H-2), 4.26 (dd, $J_{6a,6b}$ = 12.6 Hz, $J_{6a,5}$ = 4.5 Hz, 1H, H-6a), 4.12 (dd, $J_{6b,6a}$ = 12.5 Hz, $J_{6b,5}$ = 2.5 Hz, 1H, H-6b), 3.81 (ddd, $J_{5,4}$ = 9.5 Hz, $J_{5,6a}$ = 4.5 Hz, $J_{5,6b}$, = 2.2 Hz, 1H, H-5), 3.63 (d, J = 13.9 Hz, 1H, C*H*HAr), 3.60 (d, J = 14.0 Hz, 1H, CH*H*Ar), 2.09 (s, 3H, CH_3, *OAc*), 2.03 (s, 3H, CH_3, *OAc*), 1.85 (s, 3H, CH_3, *OAc*), 1.83 (s, 3H, CH_3, *OAc*); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 172.0, 171.3, 170.7, 169.5, 169.2 (5 × C=O), 142.9, 137.5, 130.3, 130.1, 128.8, 128.0, 124.4, 124.1, 121.5, 117.4 (10 × C, *aromatics*), 92.4 (C-1), 73.0 (C-5), 72.2 (C-3), 67.6 (C-4), 61.2 (C-6), 52.3 (C-2), 41.1 (CH_2Ar), 20.7, 20.6, 20.4, 20.3 (4 × CH_3, *OAc*); HRMS (ESI-TOF) *m/z* for $C_{28}H_{30}{}^{35}Cl_2N_2O_{10}Na^+$ $[M + Na]^+$ calcd 647.1170, found 647.1179.

2.2.2 | 1,3,4,6-Tetra-O-acetyl-2-deoxy-2-(2-((2,3-dimethylphenyl)amino)benzamido)-β-D-glucopyranose, 5—

Pale yellow foam; Yield 63%; m.p. 192–193°C; ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 9.00 (s, 1H, NH), 7.27 (app s, 1H, ArH, *COSY and HSQC peak*), 7.21 (t, J = 7.9 Hz, 1H, ArH), 7.12 (d, J = 8.0 Hz, 1H, ArH), 7.07 (t, J = 7.7 Hz, 1H, ArH), 6.97 (d, J = 7.3 Hz, 1H, ArH), 6.85 (d, J = 8.4 Hz, 1H, ArH), 6.66 (t, J = 7.5 Hz, 1H, ArH), 6.35 (d, $J_{NH,2}$ = 9.6 Hz, 1H, NH), 5.82 (d, $J_{1,2}$ = 8.9 Hz, 1H, H-1), 5.32 (t, J = 10.2 Hz, 1H, H-3), 5.23 (t, J = 9.7 Hz, 1H, H-4), 4.58 (app q, J = 9.8 Hz, 1H, H-2), 4.30 (dd, $J_{6a,6b}$ = 12.5 Hz, $J_{6a,5}$ = 4.6 Hz, 1H, H-6a), 4.18 (dd, $J_{6b,6a}$ = 12.6 Hz, $J_{6b,5}$ = 2.4 Hz, 1H, H-6b), 3.88 (ddd, $J_{5,4}$ = 10.1 Hz, $J_{5,6a}$ = 4.6 Hz, $J_{5,6b}$ = 2.2 Hz, 1H, H-5), 2.33 (s, 3H, CH_3), 2.17 (s, 3H, CH_3), 2.11 (s, 3H, CH_3), 2.08 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 2.02 (s, 3H, CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 171.4, 170.7, 169.6, 169.5, 169.3 (5 × C=O), 147.4, 139.2, 138.2, 132.8, 131.2, 127.1, 126.0, 125.9, 121.5, 117.0, 115.8, 114.9 (12 × C, *aromatics*), 92.8 (C-1), 73.2 (C-5), 72.6 (C-3), 67.8 (C-4), 61.7 (C-6), 52.9 (C-2), 20.8 (CH_3), 20.7 (CH_3), 20.61 (2 × CH_3), 20.59 (CH_3), 13.8 (CH_3); HRMS (ESI-TOF) *m/z* for $C_{29}H_{34}N_2O_{10}Na^+$ $[M + Na]^+$ calcd 593.2106, found 593.2109.

2.3 | Synthesis of GlcN–NSAID conjugates—Route C

Peracetylated GlcN **1** (1.3 mmol), NSAID–Cl (1 mmol), and DIPEA (0.45 ml, 2.6 mmol) were dissolved in anhydrous CH_2Cl_2 (10 ml) at 0°C. The reaction mixture was allowed to

warm to r.t. and stirred for a further 16 hr until complete consumption of the starting material was observed by TLC (CH_2Cl_2:MeOH, 9:1, v/v). The reaction mixture was concentrated under reduced pressure, and the residue was dissolved in EtOAc (50 ml). The organic layer was washed sequentially with 2 M HCl (3 × 30 ml), NaHCO3 (satd. aq) (2 × 30 ml), H_2O (30 ml), and brine (30 ml). It was then dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Flash chromatography (CH_2Cl_2 → CH_2Cl_2:MeOH, 95:5, v/v) gave the desired product.

2.3.1 | 1,3,4,6-Tetra-O-acetyl-2-deoxy-2-(2-(1-(4-chlorobenzoyl)-6-methoxy-2-methyl-1H-indol-3-yl)acetyl)acetamido)-β-D-glucopyranose, 4—Off-white solid; Yield 82%; m.p. 200–202°C; ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.75 (d, J = 8.4 Hz, 2H, *p*Cl-ArH), 7.50 (d, J = 8.4 Hz, 2H, *p*Cl-ArH), 6.86 (d, J = 9.1 Hz, 1H, ArH, *indole*), 6.84 (d, J = 2.4 Hz, 1H, ArH, *indole*), 6.70 (dd, J = 9.0 Hz, J = 2.5 Hz, 1H, ArH, *indole*), 5.67 (d, J = 8.7 Hz, 1H), 5.61 (d, J = 8.9 Hz, 1H, NH), 5.08 (*app* p, J = 9.4 Hz, 2H, H-3 and H-4), 4.24 (dd, $J_{6a,6b}$ = 12.5 Hz, $J_{6a,5}$ = 4.6 Hz, 1H, H-6a), 4.18 – 4.12 (m, 1H), 4.09 (dd, $J_{6b,6a}$ = 12.6 Hz, $J_{6b,5}$ = 2.3 Hz, 1H, H-6b), 3.84 (s, 3H, CH_3 *OMe*), 3.72 (ddd, $J_{5,4}$ = 9.5 Hz, $J_{5,6a}$ = 4.4 Hz, $J_{5,6b}$ = 2.3 Hz, 1H, H-5), 3.57 (s, 2H, C$\underline{H}_2$Ar), 2.37 (s, 3H, CH_3, *OMe*), 2.08 (s, 3H, CH_3, *OAc*), 2.01 (s, 3H, CH_3, *OAc*), 2.00 (s, 3H, CH_3, *OAc*), 1.79 (s, 3H, CH_3, *OAc*); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 170.7, 170.6, 170.1, 169.20, 169.15, 168.3 (6 × C=O), 156.3 (C_q, *ArOMe*), 139.5, 136.4, 133.6, 131.3, 131.0, 130.0, 129.2, 115.2, 112.0, 111.9, 100.9 (11 × C, *aromatics*), 92.5 (C-1), 73.0 (C-5), 71.9 (C-3/4), 67.5 (C-3/4), 61.6 (C-6), 55.7 (CH_3, *OMe*), 53.7, 32.3 (CH_2, *indole*), 20.7, 20.6, 20.5, 20.2 (4 × CH_3, *OAc*), 13.0 (CH_3, *indole*); HRMS (ESI-TOF) *m/z* for $C_{33}H_{35}{}^{35}ClN_2O_{12}Na^+$ $[M + Na]^+$ calcd 709.1771, found 709.1786.

2.3.2 | 1,3,4,6-Tetra-O-acetyl-2-deoxy-2-(((2-((R/S)-2,4(2-methylpropyl)phenyl)propanoyl) acetamido)-β-D-glucopyranose, 6—Off-white solid, a mixture of diastereomers; Yield 42%; ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.12 (dd, J = 8.1 Hz, J = 3.3 Hz, 2H, ArH), 7.05 (d, J = 7.7 Hz, 2H, ArH), 5.91 (*app* t, $J_{NH,2}$ = 10.6 Hz, 1H, NH), 5.66 (d, $J_{1,2}$ = 8.8 Hz, 0.5H, H-1), 5.60 (d, J = 8.8 Hz, 0.5H, H-1), 5.17 (q, J = 10.6 Hz, 1H, H-3), 5.07 (2 × t, J = 9.7 Hz, 1H, H-4), 4.24 (*app* tq, J = 12.7, 5.3, 4.3 Hz, 2H, H-2 and H-6a), 4.09 (d, $J_{6b,6a}$ = 12.4 Hz, 1H, H-6b), 3.79 (ddt, $J_{5,4}$ = 10.3 Hz, $J_{5,6a}$ = 5.3 Hz, $J_{5,6b}$ = 2.6 Hz, 1H, H-5), 3.42 (p, J = 6.9 Hz, 1H, C$\underline{H}$$CH_3$), 2.40 (2 × t, J = 7.1 Hz, 2H, C$\underline{H}_2$$CH(CH_3)_2$), 2.04 (s, 3H, CH_3, *OAc*), 2.02 (s, 1.5H, ½ CH_3, *OAc*), 2.01 (s, 1.5H, ½ CH_3, *OAc*), 1.98 (s, 3H, CH_3, *OAc*), 1.79 (s, 2.5H, ½ CH_3, *OAc* and $CH_2C\underline{H}(CH_3)_2$), 1.71 (s, 1.5H, ½ CH_3, *OAc*), 1.42 (2 × t, J = 6.9 Hz, 3H, $CHC\underline{H}_3$), 0.86 (2 × t, J = 6.7 Hz, 6H, $CH(C\underline{H}_3)_2$); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 174.44, 174.36 (2 × C=O, *diastereomers*), 171.0, 170.8 (2 × C=O, *diastereomers*), 170.6 (C = O), 169.24 (C = O), 169.20, 169.06 (2 × C=O, *diastereomers*), 140.7 (C_q, *Ar*), 138.0, 137.9 (2 × C_q, *Ar, diastereomers*), 129.4 (ArH), 126.88, 126.86 (2 × ArH, *diastereomers*), 92.3, 92.1 (2 × C-1, *diastereomers*), 72.80, 72.76 (2 × C-5, *diastereomers*), 72.3, 71.9 (2 × C-3, *diastereomers*), 68.0, 67.9 (2 × C-4, *diastereomers*), 61.7 (C-6), 52.6, 52.5 (2 × C-2, *diastereomers*), 46.8, 46.6 (2 × $\underline{C}HCH_3$, *diastereomers*), 44.8 ($\underline{C}H_2CH(CH_3)_2$), 30.1 ($CH_2\underline{C}H(CH_3)_2$), 22.2 ($CH_2CH(\underline{C}H_3)_2$), 20.63, 20.58, 20.48, 20.43, 20.40, 20.3, 20.1 (CH_3, *OAc*), 18.2, 18.0 (2 × $CH\underline{C}H_3$, *diastereomers*).

2.3.3 | 1,3,4,6-Tetra-O-acetyl-2-deoxy-2-(((R)-2-(6-methoxynaphthalen-2-yl)propanoyl) acetamido)-β-D-glucopyranose, 7—Off-white solid; Yield 58%; m.p. 207–209°C; ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.69 (t, J = 8.1 Hz, 2H, ArH), 7.61 (s, 1H, ArH), 7.30 (d, J = 8.5 Hz, 1H, ArH), 7.15 (d, J = 8.6 Hz, 1H, ArH), 7.10 (s, 1H, ArH), 5.67 (d, $J_{1,2}$ = 8.6 Hz, 1H, H-1), 5.61 (*br* s, 1H, NH), 5.07 (*app* p, J = 9.4 Hz, 2H, H-3 and H-4), 4.23 (dd, $J_{6a,6b}$ = 12.8 Hz, $J_{6a,5}$ = 4.2 Hz, 1H, H-6a), 4.19 (*app* q, $J_{2,3}$ = 9.8 Hz, $J_{2,1}$ = 8.9 Hz, 1H, H-2), 4.08 (d, $J_{6b,6a}$ = 12.6 Hz, 1H, H-6b), 3.91 (s, 3H, CH_3, *OMe*), 3.80–3.70 (m, 1H, H-5), 3.59 (q, J = 7.1 Hz, 1H, C$\underline{H}$$CH_3$), 2.06 (s, 3H, CH_3, *OAc*), 2.01 (s, 3H, CH_3, *OAc*), 1.96 (s, 3H, CH_3, *OAc*), 1.55 (s, 3H, CH_3, *OAc*), 1.53 (d, J = 7.1 Hz, 3H, CHC$\underline{H}_3$); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 174.3, 170.7, 170.6, 169.3, 169.2 (5 × C=O), 157.8, 135.6, 133.8, 129.2, 128.9, 127.5, 125.83, 125.82, 119.2, 105.5 (10 × C, *aromatics*), 92.5 (C-1), 72.9 (C-5), 71.8, 67.7 (2 × CH, C-3 and C-4), 61.6 (C-6), 55.3 (CH_3, *OMe*), 53.0 (C-2), 47.2 ($\underline{C}$$HCH_3$), 20.69, 20.66, 20.5, 20.0 (4 × CH_3, *OAc*), 18.1 (CH$\underline{C}$$H_3$); HRMS (ESI-TOF) *m/z* for $C_{28}H_{33}NO_{11}Na^+$ $[M + Na]^+$ calcd 582.1946, found 582.1962.

2.3.4 | 1,3,4,6-Tetra-O-acetyl-2-deoxy-2-(2-hydroxybenzamido)-β-D-glucopyranose, 8—Off-white solid; Yield 69%; m.p. 196–198°C; ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.36 (t, J = 7.7 Hz, 1H, ArH), 7.28 (d, J = 7.9 Hz, 1H, ArH), 7.11–7.02 (m, 1H, NH), 6.97 (d, J = 8.3 Hz, 1H, ArH), 6.70 (t, J = 7.6 Hz, 1H, ArH), 5.80 (d, $J_{1,2}$ = 8.9 Hz, 1H, H-1), 5.43 (t, J = 10.1 Hz, 1H, H-3), 5.22 (t, J = 9.8 Hz, 1H, H-4), 4.61 (q, J = 9.6 Hz, 1H, H-2), 4.30 (dd, $J_{6a,6b}$ = 12.5 Hz, $J_{6a,5}$ = 4.8 Hz, 1H, H-6a), 4.18 (d, $J_{6b,6a}$ = 12.3 Hz, 1H, H-6b), 3.94 (dd, $J_{5,4}$ = 10.0 Hz, $J_{5,6a}$ = 4.3 Hz, 1H, H-5), 2.10 (s, 3H, CH_3, *OAc*), 2.09 (s, 3H, CH_3, *OAc*), 2.07 (s, 3H, CH_3, *OAc*), 2.03 (s, 3H, CH_3, *OAc*); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 1712.0, 170.7, 170.2, 169.5, 169.3 (5 × C=O), 161.5, 134.7, 125.5, 118.9, 118.5, 113.6 (6 × C, *aromatics*), 92.4 (C-1), 73.0 (C-5), 72.7 (C-3), 68.0 (C-4), 61.8 (C-6), 52.3 (C-2), 20.8, 20.7, 20.6, 20.5 (4 × CH_3, *OAc*); HRMS (ESI-TOF) *m/z* for $C_{21}H_{25}NO_{11}Na^+$ $[M + Na]^+$ calcd 490.1320, found 490.1343.

2.3.5 | 1,3,4,6-Tetra-O-acetyl-2-deoxy-2-(2-acetoxybenzamido)-β-D-glucopyranose, 9—Compound **8** was dissolved in pyridine (2 ml) and Ac_2O (1 ml) and stirred at r.t. for 20 hr. The mixture was diluted with CH_2Cl_2 (20 ml) and washed sequentially with HCl (4 M, 3 × 20 ml), $NaHCO_{3\ (satd.\ aq)}$ (1 × 20 ml), and brine (1 × 20 ml). The chlorinated layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure to afford the title compound (52 mg, 90%).

Off-white amphorous solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.58 (dd, J = 7.6, 1.8 Hz, 1H, ArH), 7.48 (td, J = 7.8, 1.6 Hz, 1H, ArH), 7.30–7.24 (m, 1H, ArH), 7.09 (d, J = 8.2 Hz, 1H, ArH), 6.41 (d, $J_{NH,2}$ = 9.3 Hz, 1H, NH), 5.83 (d, $J_{1,2}$ = 8.8 Hz, 1H, H-1), 5.30 (dd, $J_{3,2}$ = 10.7 Hz, $J_{3,4}$ = 9.3 Hz, 1H, H-3), 5.18 (t, $J_{4,3}$ = $J_{4,5}$ = 9.6 Hz, 1H, H-4), 4.46 (dt, $J_{2,3}$ = 10.6 Hz, J = 9.0 Hz, 1H, H-2), 4.29 (dd, $J_{6a,6b}$ = 12.5 Hz, $J_{6a,5}$ = 4.5 Hz, 1H, H-6a), 4.15 (dd, $J_{6b,6a}$ = 12.5 Hz, $J_{6b,5}$ = 2.2 Hz, 1H, H-6b), 3.86 (ddd, $J_{5,4}$ = 10.0 Hz, $J_{5,6a}$ = 4.5 Hz, $J_{5,6b}$ = 2.2 Hz, 1H, H-5), 2.31 (s, 3H, CH_3, *OAc*), 2.11 (s, 3H, CH_3, *OAc*), 2.10 (s, 3H, CH_3, *OAc*), 2.05 (s, 3H, CH_3, *OAc*), 2.03 (s, 3H, CH_3, *OAc*); ^{13}C NMR (126 MHz, $CDCl_3$) δ_C 171.2, 170.7, 169.7, 169.3, 169.0, 165.8 (6 × C=O), 148.5, 132.5, 128.8, 127.3, 126.2, 123.3 (6 × C, *aromatics*), 92.4 (C-1), 72.9 (C-5), 72.2 (C-3), 67.8 (C-4), 61.6 (C-6), 53.0 (C-2), 21.2,

21.0, 20.8, 20.6, 20.5 (5 × CH_3, *OAc*), HRMS (ESI) *m/z* for $C_{23}H_{27}NO_{12}Na^+$ $[M + Na]^+$ calcd 532.1425, found 532.1425.

2.4 | Molecular modeling

The crystal structure of 4COX (PDB) was imported into the Schrödinger Maestro suite (Version 12.4.079, Release 2020–2), processed using the Protein Preparation Wizard (Madhavi Sastry et al., 2013) and subjected to restrained minimization by using the OPLS3e force field. SMILES (Table S2) for the NSAIDs, NSAID–glyconjugates and the deacetylated NSAID–glycoconjugates were imported into Maestro to generate three-dimensional structures which were prepared for docking using the LigPrep application and ligand ionization states at pH 7.0 ± 2.0 were generated using Epik (v.5.2079) (Shelley et al., 2007). Receptor grids were generated using the Receptor Grid Generation Wizard, and Glide (v.87079) (Friesner et al., 2004; Halgren et al., 2004) was used to screen the prepared ligands and generate docked molecules.

2.5 | Biological activity

The potency (IC_{50} values in μM) of the synthesized compounds to inhibit ovine COX-1 and human recombinant COX-2 was determined using a commercial COX Fluorescent Inhibitor Screening Assay Kit (catalog number 700,100, Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Stock solutions of test compounds were made at 10 mM in DMSO. In black 96-well plates, 2 μl samples of various concentrations of 100× inhibitor solutions ($[I]_{final}$ between 2.5 and 100 μM) were added to a series of buffer solutions (160 μl, 100 mM Tris-HCl, pH 8.0) with either COX-1 or COX-2 (10 μl) enzyme in the presence of Heme (10 μl) and fluorometric substrate (ADHP, 10-acetyl-3,7-dihydroxyphenoxazine, 10 μl). The reactions were initiated by adding 10 μl of arachidonic acid solution and then incubated for two minutes at room temperature. The fluorescence of resorufin that is produced by the reaction between PGG2 and the ADPH substrate was measured at an excitation wavelength of 535 nm and an emission wavelength of 590 nm. The intensity of this fluorescence is proportional to the activity of the COX enzymes. Percent inhibition was calculated by comparison with the 100% initial activity sample value (no inhibitor). The concentration of the test compound causing 50% inhibition of the COX activity (IC_{50}, μM) was calculated from the concentration–inhibition response curve (determinations in triplicate). Indomethacin and celecoxib were used as the positive drug controls for the COX-1 and COX-2 assays, respectively.

3 | RESULTS AND DISCUSSION

3.1 | Design and synthesis

We have shown previously that conjugation of NSAIDs to paracetamol and anti-ulcer drugs can be achieved through an amino acid linker to afford potential pro-drugs. In addition, certain of these paracetamol–NSAID conjugates showed improved activity relative to the parent NSAID (Katritzky, Jishkariani, et al., 2009; Sahu et al., 2013; Tiwari et al., 2014). We therefore extended this chemistry to the conjugation of glucosamine with various NSAIDs. To that end, a series of NSAID–amino acid derivatives were synthesized *via* NSAID–benzotriazolide methodology, and the resulting conjugates were coupled with 2-deoxy-2-

amino-1,3,4,6-tetra acetate glucosamine hydrochloride [$(AcO)_4$GlcN] **1** by the in situ preparation of the corresponding hydroxybenzotriazole (HOBt) derivative (Jones et al., 2014). When indomethacin-based NSAID–amino acids were conjugated to compound **1**, the presence of a bulky amino acid side chain resulted in the formation of rotamers. An alternative strategy was then developed by coupling GlcN-amino acid conjugates to different NSAIDs. In selected cases, *N*-Cbz deprotection by catalytic hydrogenation resulted in an unanticipated, although previously observed, concomitant loss of the anomeric acetate to form the alpha-hemiacetal (Liefländer, 1967a, 1967b). To circumvent these issues, we removed the amino acid linker and reacted the NSAID of interest directly with compound **1** (Scheme 1) (Silva et al., 1999). Compound **1**, a peracetylated derivative of GlcN, was chosen, since the esterification of carbohydrates increases their membrane permeability, a concept that has been exploited for delivering pharmacologically active agents to a target cell-type or tissue (Jones et al., 2004) or for improving the pharmacokinetic properties of the compound (Carroux et al., 2013).

The use of *N*-acyl benzotriazoles is a rapid, cheap, and safe method for the coupling of carboxylic acids to primary and secondary amines (Katritzky et al., 2007), and this chemistry has been used previously for the synthesis of NSAID–amino acid conjugates (Katritzky, Jishkariani, et al., 2009; Sahu et al., 2013; Tiwari et al., 2014). Benzotriazole chemistry has also been used to prepare either anomeric or C-6 carbohydrate-derived bioconjugates (Bajaj et al., 2012; Katritzky et al., 2007; Katritzky, Cusido, et al., 2008; Katritzky, Narindoshvili, et al., 2008). Therefore, we reasoned that it would also be applicable to derivative **1** (Route A). Despite the previous successes in applying benzotriazole chemistry to the synthesis of carbohydrate–bioconjugates, all our efforts to react NSAID–benzotriazolides with **1** at the 2-amino position resulted in hydrolysis of benzotriazole to generate the parent NSAID. This was attributed to the relatively low reactivity of GlcN (Zhang et al., 1999), which was further decreased due to the presence of the electron-withdrawing acetate groups. Thus, we prepared alternative glucosamine derivate 2 (Aly & Schmidt, 2005), in which the acetate groups were substituted by electron-donating benzyl ether groups [$(BnO)_4$GlcN]. However, our attempts to couple benzotriazole derivatives of NSAIDs to compound **2** were also unsuccessful.

Since benzotriazole is considered a mild method of activating esters for peptide synthesis (Katritzky, Angrish, et al., 2009), more stringent methods of ester activation were used. In our efforts to synthesize NSAID–amino acid–GlcN compounds, coupling agents were used for the conjugation of NSAIDs to **1** (Scheme 1, route B) (Jones et al., 2014). In the majority of cases, the peptide-coupling agents hydroxybenzotriazole (HOBt) and *N*-(3-dimethylaminpropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI) were suitable for the synthesis of the target compounds (Table 1). For NSAIDs containing the propionic acid motif, such as ibuprofen and naproxen, this strategy proved ineffectual and required the preparation of the corresponding acid chloride derivatives of the NSAIDs (Scheme 1, route C). Using this method, we were able to isolate our target compounds (**6** and **7**) in reasonable yields. Although sufficient quantities of bioconjugates **3**–**5** were obtained using HOBt and EDCI (Scheme 1, route B), the acid chloride route (C) was also attempted, and in the case of indomethacin resulted in an improved yield. However, we were unable to isolate the acid

chloride derivative of mefenamic acid; this may be due to the usage of oxalyl chloride for the acid chloride synthesis as opposed to alternative reagents such as thionyl chloride. The low yield of diclofenac–GlcN when using coupling agents was attributed to the competing, more rapid cyclization of diclofenac (Baldwin, 1976). The cyclized version of diclofenac has indeed been prepared previously *via* the formation of the acid chloride (Moser et al., 1990). Preparation of the acid chloride derivative of salicylic acid was not attempted due to the presence of the phenol group. Aspirin–GlcN derivative **9** was prepared by the acetylation of salicyclic acid–GlcN **8** with acetic anhydride in the presence of pyridine (Scheme 2).

3.2 | Molecular docking

To investigate the ability of the GlcN–NSAIDs to maintain binding within the COX-2 binding pocket, we performed molecular docking studies of the deacetylated form of the NSAID–GlcNs, the parent NSAIDs and celecoxib, a COX-2 selective inhibitor, to the deposited COX-2 crystal structure, 4COX (Kurumbail et al., 1996). The top hit from our docking experiment was the beta anomer of indomethacin–GlcN. As observed from the ligand alignment, appending the carbohydrate moiety to indomethacin does not prevent it from adopting the same pose as the parent NSAID (Figure 2). To validate the docking parameters, we included the cognate ligand (indomethacin), in our ligand library, and this was the second hit (Table S1). Gratifyingly, the third and fourth hits, respectively, were the alpha anomer of indomethacin–GlcN and celecoxib, which has the same binding site as indomethacin. Consistent with the reported COX-2 crystal structure, 4COX, the docked indomethacin displays interactions between its benzoyl oxygen and S530, as well as between the carboxylate and R120 (Figure 3b and Figure S2b). The presence of the sulfonamide in celecoxib facilitates a pi–pi stacking interaction with the phenol ring of Y355 (Figure 3d and Figure S2d) (Wang et al., 2010) an interaction that is maintained in both indomethacin and indomethacin–GlcN (Figure 3b,c and Figure S2b,c). The conjugation of GlcN to NSAIDs results in additional contacts between the glycoconjugate and the COX-2 binding site. In the case of indomethacin, the conjugation of GlcN facilitates three additional hydrogen bonds between the carbohydrate moiety and E524, in the case of the β-anomer (Figure 3a and Figure S2a) and a single hydrogen bond to E524 and an aromatic hydrogen bond (Levitt & Perutz, 1988) to Y115 in the case of the α-anomer. Thus, it appears that the conjugation of GlcN can form additional, potentially stabilizing interactions between the NSAID–glycoconjugate and the COX-2 active site.

3.3 | Biological evaluation

Since the carboxylic acid moiety is not required for the biological activity of NSAIDs, (Ullah et al., 2016) we hypothesized that the GlcN derivatives would still inhibit COX-1 and COX-2 and we tested this in vitro. In the initial screen (Figure S3), ibuprofen (**6**) and naproxen (7), which have propanoic acid backbones, as well as salicyclic acid-derived bioconjugates (**8**–**9**) showed limited activity against both COX-1 and COX-2. On the other hand, the acetic acid motif-containing diclofenac and indomethacin, and fenamate NSAID–GlcN conjugates (**3**–**5**) showed comparable activity to the reference drugs. It should be noted that mefenamic acid–GlcN **5** showed an unexpected, greater inhibitory activity toward COX-1 than COX-2 that was not observed in the parent NSAID (Table S3; Cryer & Feldman, 1998).

Based on the results of the preliminary screen, IC_{50} values were determined for NSAID–GlcN conjugates **3**–**5** (Table 2). Consistent with the parent indomethacin (Table 2; J. A. Mitchell et al., 1993), and Table S3), indomethacin–GlcN **4** showed similar inhibitory activity toward COX-1 and COX-2, while mefenamic acid–GlcN **5** maintained an unexpected, enhanced activity toward COX-1 versus COX-2. Diclofenac–GlcN **3** demonstrated a 10-fold greater activity toward COX-2 than COX-1, making it an interesting candidate for in vivo studies to confirm that the conjugate preserves its selectivity. Importantly, this experiment did not take into account the benefit of the prodrug approach, as no esterase was used to remove the acetate groups to restore the native GlcN residue.

4 | CONCLUSIONS

A series of NSAID–GlcN bioconjugates were designed and synthesized, and their in vitro biological activity was assessed. Molecular modeling and docking studies of the target compounds suggested that the presence of a carbohydrate moiety appended to the NSAID would not disrupt binding in the COX-2 active site. Due to the structural diversity of the NSAIDs used in this study, different synthetic strategies were required to access the target derivatives. Coupling of GlcN to NSAIDs was achieved using either a combination of HOBt with EDCI or the preparation of the acid chloride derivative of the NSAID. These strategies allowed the synthesis of sufficient material to probe the activity of the NSAID–GlcN bioconjugates in COX-1/COX-2 inhibition assays. Various inhibitory effects of the NSAID–GlcN derivatives against COX-1/COX-2 were observed. In the majority of cases, conjugation of $(AcO)_4$GlcN did not improve the inhibitory activity relative to the corresponding NSAID. However, diclofenac, indomethacin, and mefenamic acid all showed increased COX-1/COX-2 inhibition when conjugated to $(AcO)_4$GlcN. In the case of diclofenac–GlcN **3**, selectivity toward COX-2 was observed whereas with mefenamic acid–GlcN **5**, an unexpected selectivity toward COX-1 was observed. Compared to COX-2, COX-1 selective inhibitors have been less thoroughly investigated; nevertheless, they have been implicated in several conditions including atherosclerosis, endothelial dysfunction, and pre-term labor (Perrone et al., 2010). Whether or not this in vitro selectivity is retained in vivo would be worth investigating in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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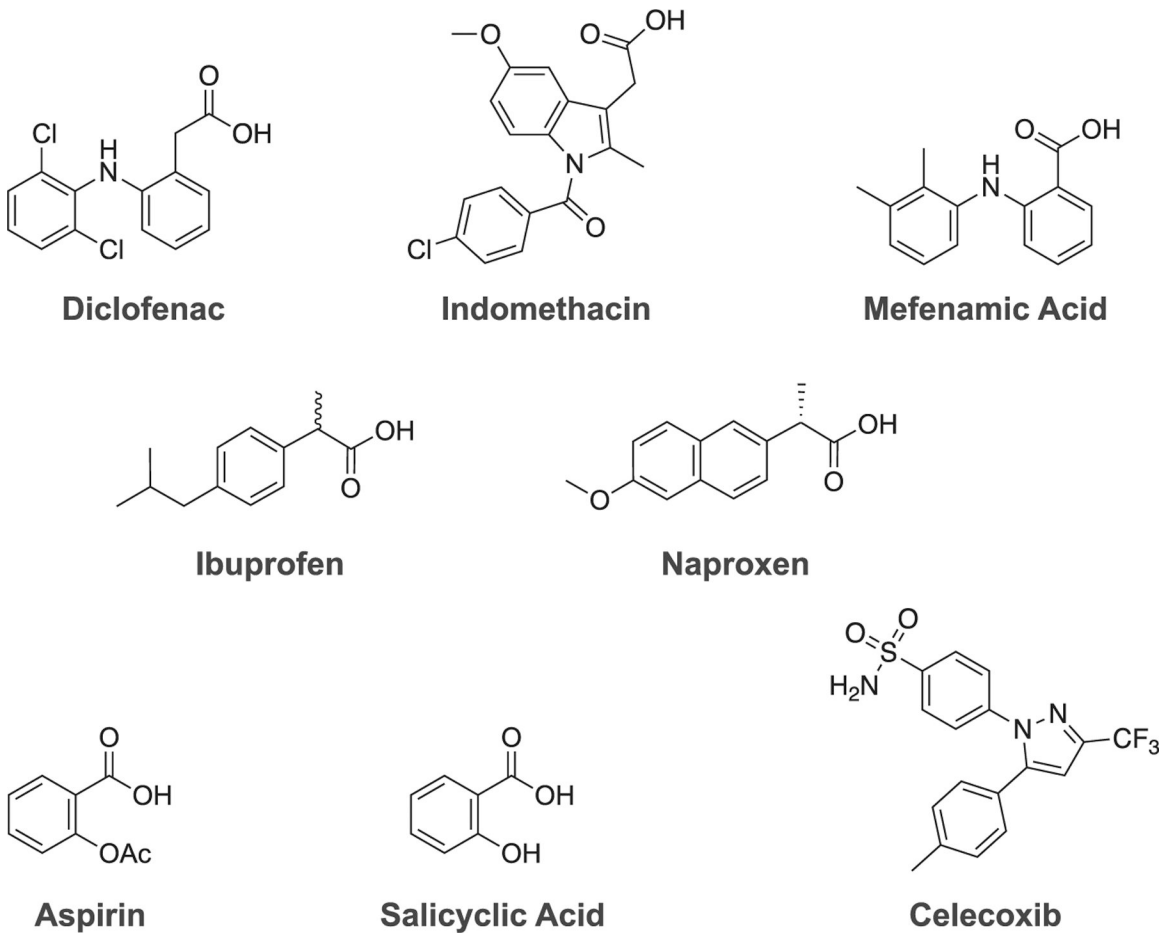


FIGURE 1.

NSAIDs used in this study. The NSAIDs used in this study may be divided into four classes based on their structural features. Acetic acids: diclofenac and indomethacin; Fenamates: mefenamic acid; Propionic acids: ibuprofen and naproxen; Salicylates: aspirin and salicyclic acid. Celecoxib, a COX-2 selective inhibitor used as a reference compound for the molecular docking and in the inhibition assay is also shown

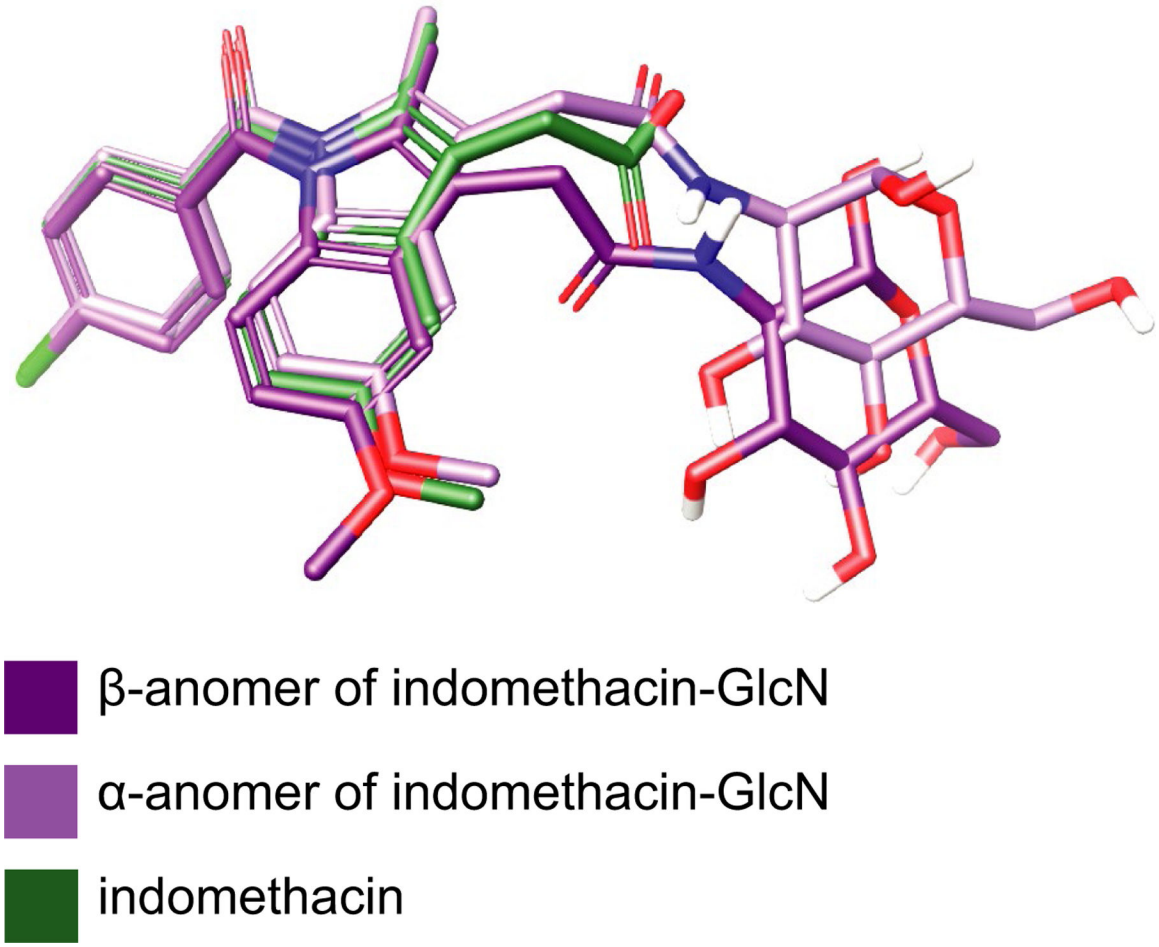


FIGURE 2.

Overlay of the 3D structure of indomethacin, and the α- and β-anomers of indomethacin–GlcN, showing that the indomethacin portion of each molecule adopts the same position

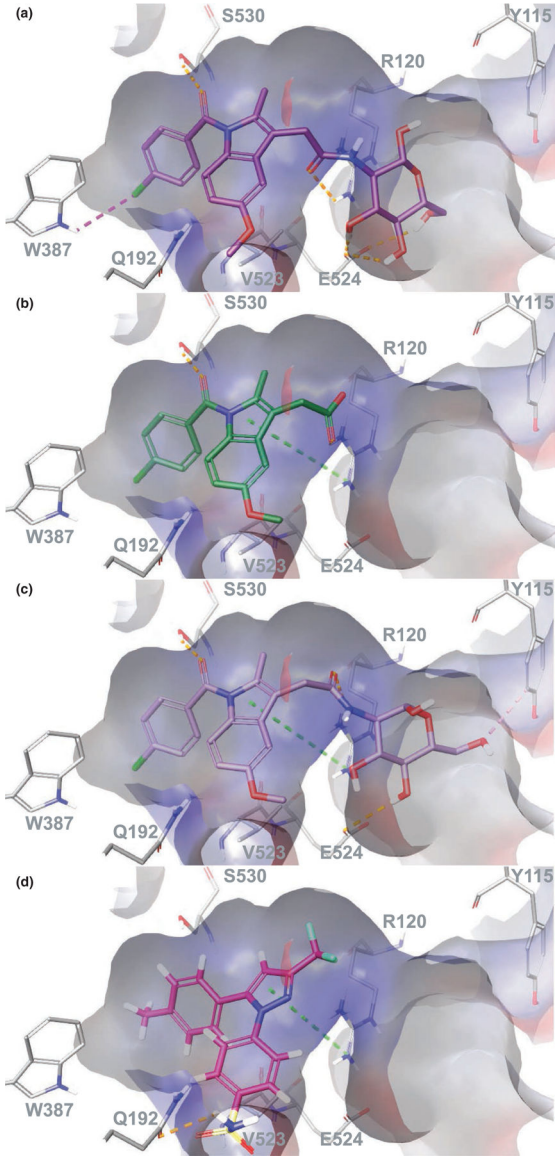
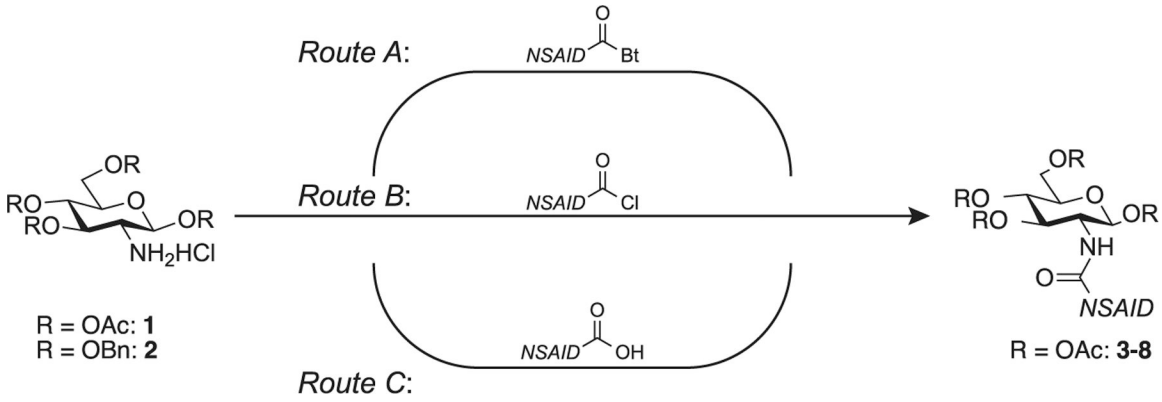


FIGURE 3.

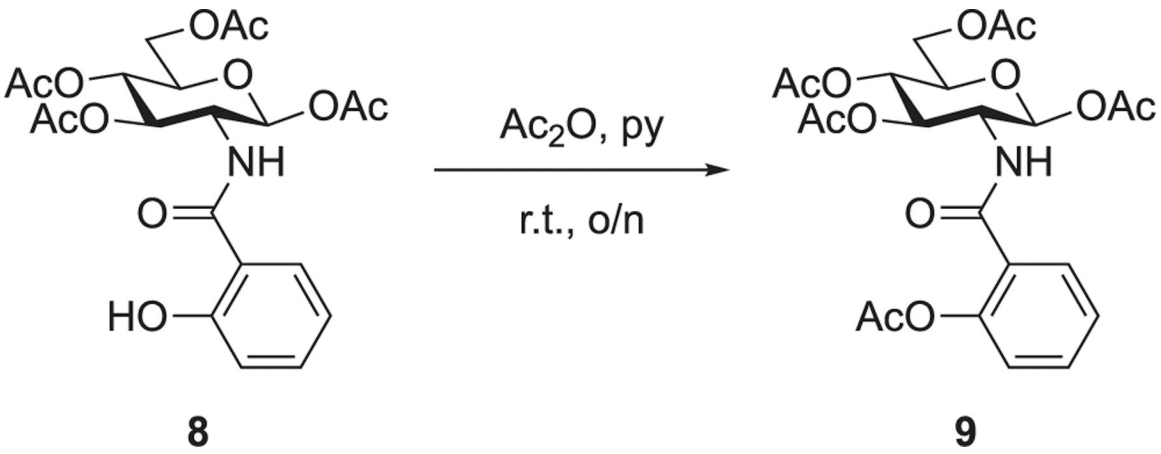
Docking of small molecule inhibitors into the cyclooxygenase active site of COX-2. (a) Deprotected indomethacin–GlcN (β-anomers); (b) indomethacin; (c) Deprotected indomethacin–GlcN (α-anomers); (d) Celecoxib. (a) Docking shows hydrogen bonding interactions between S530 and the benzoyl oxygen, R120 and the carbonyl in the amide bond; in addition, there is a halogen bond between W387 and the indomethacin chlorine. The presence of the carbohydrate affords three additional hydrogen bonds to E524. (b) Docking shows the hydrogen bond between S530 and the benzoyl oxygen as well as the pi-cation interaction between R120 and the indole ring shown in the original crystal structure. (c) Docking shows hydrogen bonding interactions between S530 and the benzoyl oxygen, R120 and the carbonyl in the amide bond; in addition, there is a pi-cation interaction to the indole ring. The presence of the carbohydrate affords one additional hydrogen bond to E524 as well as an aromatic H-bond to Y115. (d) Docking shows a pi-cation interaction between

the pyrazole ring and R120 and hydrogen bonding between the sulfonamide and E192. Some of the interactions have been removed for clarity, see Figure S2 for the full ligand interaction diagrams



SCHEME 1.

Synthesis of glucosamine–NSAID bioconjugates. Route A: Et_3N, H_2O-MeCN or DIPEA, DMF; Route B: DIPEA, CH_2Cl_2; Route C: Et_3N, HOBt, EDCI, CH_2Cl_2



8 **9**

SCHEME 2.
Acetylation of salicylic acid–GlcN **8** to afford aspirin–GlcN **9**

TABLE 1

NSAID–GlcN Bioconjugates

		Yield (%)	
Entry	**Product**	**Route B**	**Route C**
1	Diclofenac–GlcN(OAc)$_4$, **3**	27[a]	*n*/a[a]
2	Indomethacin–GlcN(OAc)$_4$, **4**	43	82
3	Mefenamic acid–GlcN(OAc)$_4$, **5**	63	*b*
4	Ibuprofen–GlcN(OAc)$_4$, **6**	No reaction	42
5	Naproxen–GlcN(OAc)$_4$, **7**	No reaction	58
6	Salicylic Acid–GlcN(OAc)$_4$, **8**	69	*n*/a[c]

[a]Cyclization of diclofenac occurred.

[b]Acid chloride not formed.

[c]Due to the presence of the free hydroxyl group, synthesis of the acid chloride derivative was not attempted [c] due to the presence of the free hydroxyl group, synthesis of the acid chloride derivative was not attempted.

TABLE 2

COX-1 and COX-2 IC_{50} values were calculated for compounds **3**–**5**. The % inhibition for the reference compounds, indomethacin and celecoxib, was measured at 100 μM

		COX-1	COX-2
Entry	**Product**	**IC_{50} (μM)**	
1	Diclofenac–GlcN, **3**	25.5	<2.5
2	Indomethacin–GlcN, **4**	24.9	33.6
3	Mefenamic acid–GlcN, **5**	<2.5	>100
4[a]	Diclofenac (sodium salt)	3.8	0.84
5[a]	Indomethacin	0.28	14
6[a]	Mefenamic acid	–	5.34
Entry	Reference Compound	% Inhibition at 100 μM	
7	Indomethacin[b]	64.7	95.3
8	Celecoxib[c]	20.1	96.1

[a] IC_{50} values given for the parent NSAIDs are literature values for in vitro assays to assess COX-1 and/or COX-2 inhibition; diclofenac (Abdu-Allah et al., 2020), indomethacin (Mitchell et al., 1993), and mefenamic acid (Savjani et al., 2017). In the case of mefenamic acid, only the IC_{50} value for COX 2 was reported.

[b] Reference compound for COX-1 inhibition.

[c] Reference compound for COX-2 inhibition.