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Note

Synthesis and evaluation of an *N*-acetylglucosamine biosynthesis inhibitor

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ABSTRACT

The structural rationale, synthesis and evaluation of an inhibitor designed to block glucosamine synthesis by competitively inhibiting the action of glutamine: fructose-6-phosphate amidotransferase and subsequently reducing the transformation of any glucosamine-6-phosphate formed to UDP-*N*-acetylglucosamine are described. The inhibitor 2-acetamido-2,6-dideoxy-6-sulfo-D-glucose (D-glucosamine-6-sulfonate) is an analog of glucosamine-6-phosphate in which the phosphate group in the latter is replaced with a sulfonic acid group. The inhibitor is designed to function by three different modes which together reduce UDP-*N*-acetylglucosamine synthesis. This reduction was confirmed by evaluating the effect of the inhibitor on bacterial cell-wall synthesis and by demonstrating that it inhibits acetylation of glucosamine-6-phosphate competitively and by acting as a surrogate substrate. Inhibition of glucosamine production or suitably activated glucosamine in bacteria leads to disruption of the peptidoglycan structure, which results in softening, bulging, deformation, fragility and lysis of the cells. These modifications were documented by scanning electron microscopy for bacteria treated with the inhibitor. They were observed for inhibitor concentrations in the 20 mg/mL range for *Escherichia coli* and *Bacillus subtilis* and the 5 mg/mL range for *Rhizobium trifolii*.

Amino sugars such as D-glucosamine (2-amino-2-deoxy-D-glucose), galactosamine and mannosamine (the *galacto*- and *manno*-isomers, respectively) are characterized by a nitrogen atom being attached directly to the carbon chain of a carbohydrate molecule. These are all hexose sugars and are members of the hexosamine group. D-Glucosamine is the most common amino sugar, and it is an integral part of all living systems. It is now known that amino sugars, make up the most abundant form of organic matter in the oceans.[1] Many bacteria can utilize glucosamine as the only source of carbon and are able to transform it to fructose-6-phosphate and initiate the glycolytic pathway.[2] Amino sugars and their derivatives are present in many glycolipids and most glycoproteins, showing the crucial importance of hexosamine biosynthesis for cell survival.[3–8] They are also major components of bacterial and fungal cell walls.[9]

The conversion of glucose to glucosamine is a critical biochemical pathway. Fructose-6-phosphate is the species that primes glucosamine biosynthesis. The main limiting step of this pathway is catalyzed by the enzyme glucosamine synthase, also known as glutamine: fructose-6-phosphate amidotransferase or GFAT (EC 2.6.1.16) in eukaryotes. This enzyme catalyzes the conversion of fructose-6-phosphate in the presence of glutamine into glucosamine-6-phosphate.

It is of universal importance to prokaryotes and eukaryotes. Because of its wide occurrence in bacteria and fungi, the production of inhibitors for this enzyme is an appealing strategy in the development of new antibiotics and antifungals.

Hexosamines in mammals have great clinical significance. These compounds are now known to be involved in the establishment and development of many diseases, such as type II diabetes, Alzheimer's disease, rheumatoid arthritis and cancer.[10–14] Although the molecular basis of these diseases is well appreciated, the development of cures is still a significant challenge. A limited availability of therapies with a low percentage of cures is currently available. The development of new drugs that function by interfering with the hexosamine pathway is an active strategy. The main enzyme of the hexosamine biosynthetic pathway (HBP) responsible for glucosamine biosynthesis is called glutamine fructose-6-phosphate amidotransferase (GFAT), and it has two structural domains. One of these is for binding glutamine (N-terminal or glutaminase domain) and another for binding fructose-6-phosphate (C-terminal or isomerase domain). There is also a hinge region that allows the interaction of the two substrates. In prokaryotes, GFAT consists of four polypeptide chains, and it seems to be active as a homodimer,[15,16] while in eukaryotes it is thought to be active as an heterodimer. This enzyme catalyzes the transfer of the amino group from glutamine to fructose-6-phosphate producing glutamic acid and glucosamine-6-phosphate. This enzyme is a thiol protease, and the proposed mechanism of action is through the formation of an acyl

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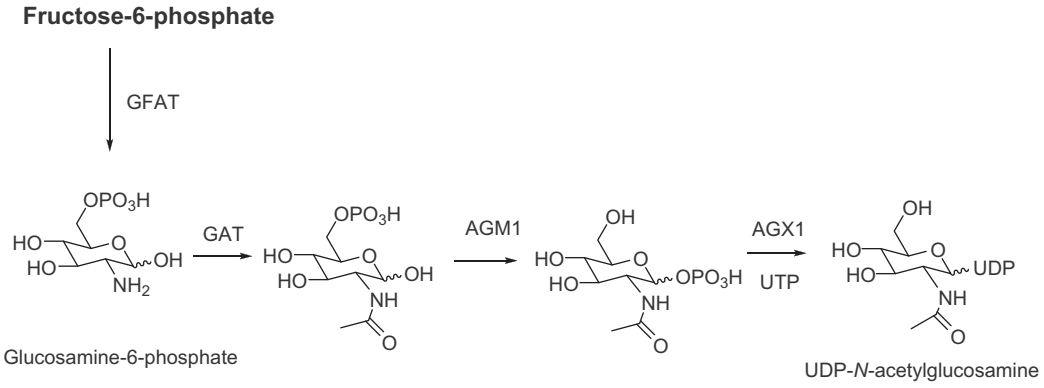


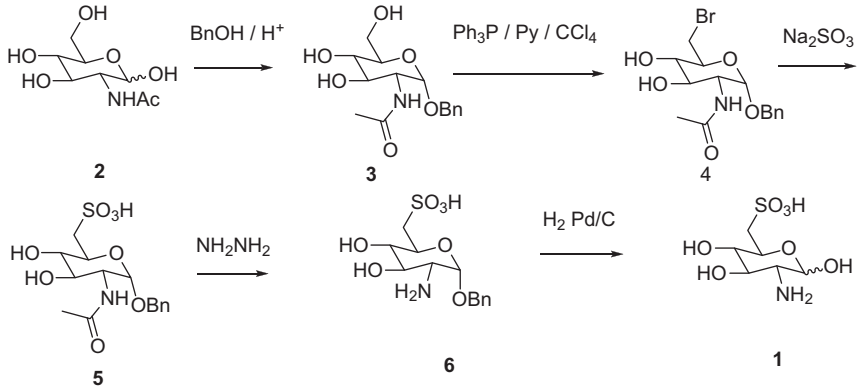
GFAT Glutamine:fructose-6-phosphate amidotransferase

GAT Acetyl-CoA:D-glucosamine-6-phosphate *N*-acetyltransferase

AGM1 Phospho-*N*-acetylglucosamine mutase

AGX1 UDP-GlcNAc Pyrophosphorylase

Scheme 1. Biosynthetic steps in the conversion of fructose-6-phosphate to UDP-*N*-acetylglucosamine.

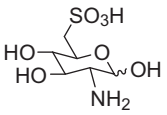


Scheme 2. Synthetic route to 2-amino-2,6-dideoxy-6-*O*-sulfo-D-glucose.

intermediate between the cysteine residue (Cys1) and the glutamine in the glutaminase domain, releasing ammonia; the intermediate is hydrolyzed forming glutamic acid and regenerating the free enzyme. In the isomerase domain, the carbonyl of fructose-6-phosphate reacts with a lysine residue (Lys603) to form a Schiff base. This is attacked by ammonia produced in the glutaminase domain, liberating the free lysine and forming a 2-imine derivative. Through the action of a glutamic acid residue (Glu488), the substrate is then isomerized to glucosamine-6-phosphate, the product of the overall reaction.[15–17]

Glucosamine is incorporated into other biomolecules through its activated form, UDP-*N*-acetylglucosamine. This is formed by the successive action of three enzymes on glucosamine-6-phosphate (Scheme 1). The first is acetyl-CoA: D-glucosamine-6-phosphate *N*-acetyltransferase (GAT) which converts the amino group to an acetamido group using acetyl-CoA as the acyl donor. The second step is the transfer of the phosphate group from the 6-position to the 1-position by phospho-*N*-acetylglucosamine mutase (AGM1) to form *N*-acetylglucosamine-1-phosphate. The third and last step is by the action of the enzyme UDP-GlcNAc pyrophosphorylase (AGX1), which catalyzes the process in which uridine triphosphate (UTP) reacts with *N*-acetylglucosamine-1-phosphate to form UDP-GlcNAc and pyrophosphate.

A structural analog of glucosamine-6-phosphate in which the phosphate group is replaced with a similar charged group presents many modes of inhibiting UDP-*N*-acetylglucosamine synthesis. It would bind to GFAT inhibiting formation of the glucosamine-6-phosphate. It would also inhibit GAT by again competing with glucosamine-6-phosphate binding. If the similarity is close it would be converted to an acyl derivative that could bind to AGM1 but not lead to the formation of *N*-acetylglucosamine-1-phosphate. Such an inhibitor could function by three effectively different modes. To this end, 2-amino-2,6-dideoxy-6-sulfo-D-glucose (D-glucosamine-6-sulfonate, compound **1**), an analog of D-glucosamine-6-phosphate in which the phosphate group is replaced with a sulfonate group, was synthesized and evaluated for its ability to inhibit glucosamine synthesis.



1

The synthetic method used in the synthesis of **1** is illustrated in Scheme 2.

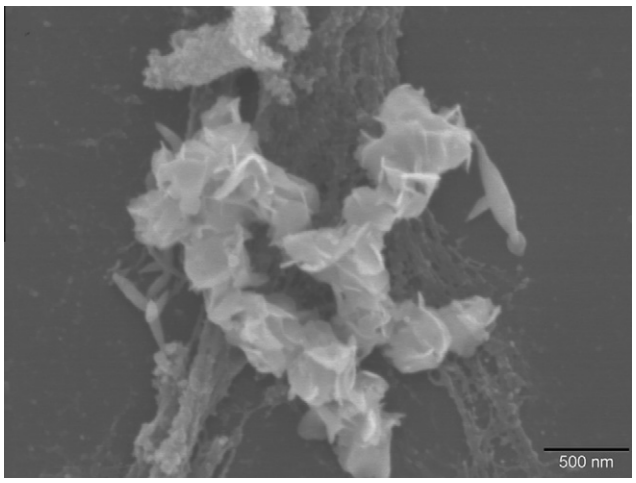


Figure 1. Representative Scanning Electron Microscopy image of the bacterial strains. *R. trifolli*: 1.5 mg/mL of inhibitor.

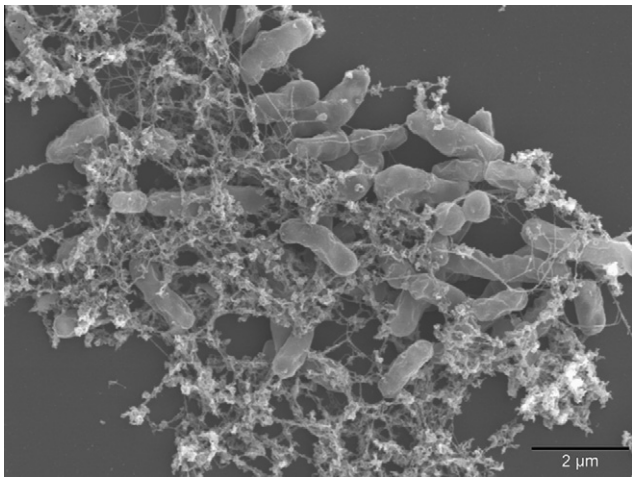


Figure 2. Representative Scanning Electron Microscopy image of the *R. trifolli*: control cells.

The D-glucosamine-6-sulfonate concentration that resulted in a 50% reduction in growth of the bacterial cells (IC_{50}) was obtained by optical density measurement at 600 nm. The results are as follows: *Bacillus subtilis* PY74, 2.88 mg/mL; *Rhizobium trifolii* ANU843, 0.56 mg/mL; *Escherichia coli* DH5α, 4.72 mg/mL. Complete inhibition of cell growth was observed for *R. trifolii, B. subtilis* and *E. coli* by the inhibitor at concentrations 5, 20, and 20 mg/mL, respectively.

In order to evaluate whether the bacterial growth inhibition observed was connected to the disruption of cell-wall synthesis, the ultra structures of the cell walls were assessed by scanning electron microscopy. Glucosamine is the primary building block of peptidoglycan in the cell wall. This is a complex macromolecule that gives bacteria cells their strength and rigidity and that results in the characteristic cigar shapes of many bacteria. Inhibition of peptidoglycan synthesis results in a loss of shape in cells with fragile walls that might be enlarged or those with spherical or irregular shapes.[18,19] Cells treated with glucosamine-6-sulfonate show a high frequency of lysis and many have bloated irregular shapes (Fig. 1) when compared to control cells (Fig. 2). More examples of these morphological changes can be found in Supplementary data. These results are consistent with the inhibition of peptidoglycan synthesis. This is accord with the expectation that enough glucosamine would not be available for this because of the several modes by which glucosamine-6-sulfonate would inhibit *N*-acetylglucosamine synthesis.

As mentioned earlier the glucosamine-6-phosphate analog can potentially bind to several enzymes on the hexosamine pathway. It could inhibit GFAT as a competitive inhibitor and also act as a competitive and allosteric inhibitor of GAT. It is also possible for **1** to act as a substrate for GAT thus diverting acyl equivalents away from the synthesis of *N*-acetylglucosamine. The sulfonated *N*-acetylglucosamine thus formed cannot be converted to UDP-*N*-acetylglucosamine. To evaluate the latter possibilities, GAT activity was measured in an assay in which the transfer of acetyl groups from acetyl-CoA to glucosamine-6-phosphate was evaluated by measuring coenzyme A formation during the process using 5,5′-dithio-*bis*(2-nitrobenzoic acid). This forms the highly colored 5-mercapto-2-nitro-benzoic acid on reaction with coenzyme A by disulfide exchange. This colored substance can be readily monitored at 412 nm. An extract of lysed yeast cells was used as the source of the enzyme. The results are presented in Figure 3. Compound **1** inhibits the acetylation of glucosamine-6-phosphate by about 60% at two and four times the substrate concentration and almost completely at eight times the substrate concentration. The very small difference observed between the two lower inhibitor concentrations hints that there is another aspect to the interactions besides a simple competition. This was made clear when the natural substrate was removed and compound **1** was used as a substrate. It was a very effective acceptor for acetyl groups from acetyl-CoA under catalysis by GAT at a level of 40% of the native substrate. The GAT enzyme also presents an allosteric site for

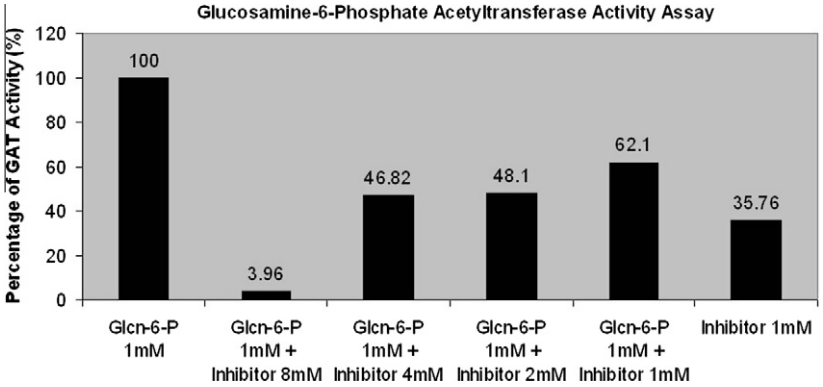


Figure 3. Glucosamine-6-phosphate acetyltransferase (GAT) activity in presence of 1 mM of glucosamine-6-phosphate and 8, 4, 2 and 1 mM of D-glucosamine-6-sulfonate inhibitor. Controls: glucosamine-6-phosphate (Glcn-6-P): 1 mM; inhibitor: 1 mM.

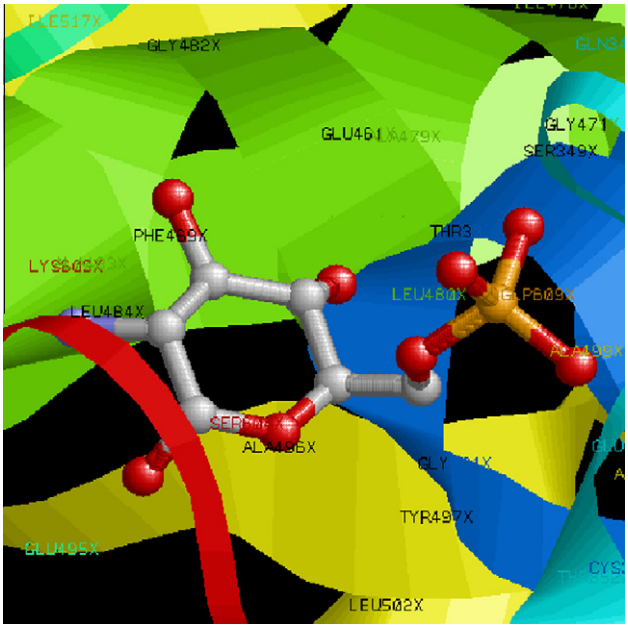


Figure 4. View of the active site of the isomerase domain of *E. coli* in presence of the fructose-6-phosphate substrate (in red).

binding *N*-acetylglucosamine-6-phosphate, which is its natural inhibitor.[20,21] This could explain the complete inhibition by **1** at very high concentrations. The glucosamine-6-sulfonate is not capable of being transformed to UDP-*N*-acetylglucosamine-6-phosphate because the sulfonate group cannot be removed by the same mechanism that removes the phosphate group.

The glucosamine-6-phosphate analog was designed to act as a competitive inhibitor for the isomerase active site and for the enzymes GAT and AGM1, leading to a decrease in the production of *N*-acetylglucosamine-6-phosphate within the cell. The fragility of cell walls resulting in cell deformation, and (at higher concentrations) cell lysis, was expected and detected in all strains incubated with the inhibitor (Fig. 1 and Figs. S1–S7 in the Supplementary data). Gram-negative strains should be even more susceptible to the inhibitory effects of these inhibitors since they also depend on *N*-acetylglucosamine production for the synthesis of lipid A, a main constituent of the lipopolysaccharide (LPS). Lipopolysaccharides form regular crystalline arrays in the outer membrane.[22] These features make bacteria cells good systems for the evaluation of the efficacy of these inhibitors. In summary, 2-amino-2,6-dideoxy-6-sulfo-D-glucose-inhibited bacterial cell wall biosynthesis is consistent with expectations. This compound should be a valuable tool in elucidating the contribution of amino sugars to biochemical processes and in developing antimicrobial and therapeutic agents.

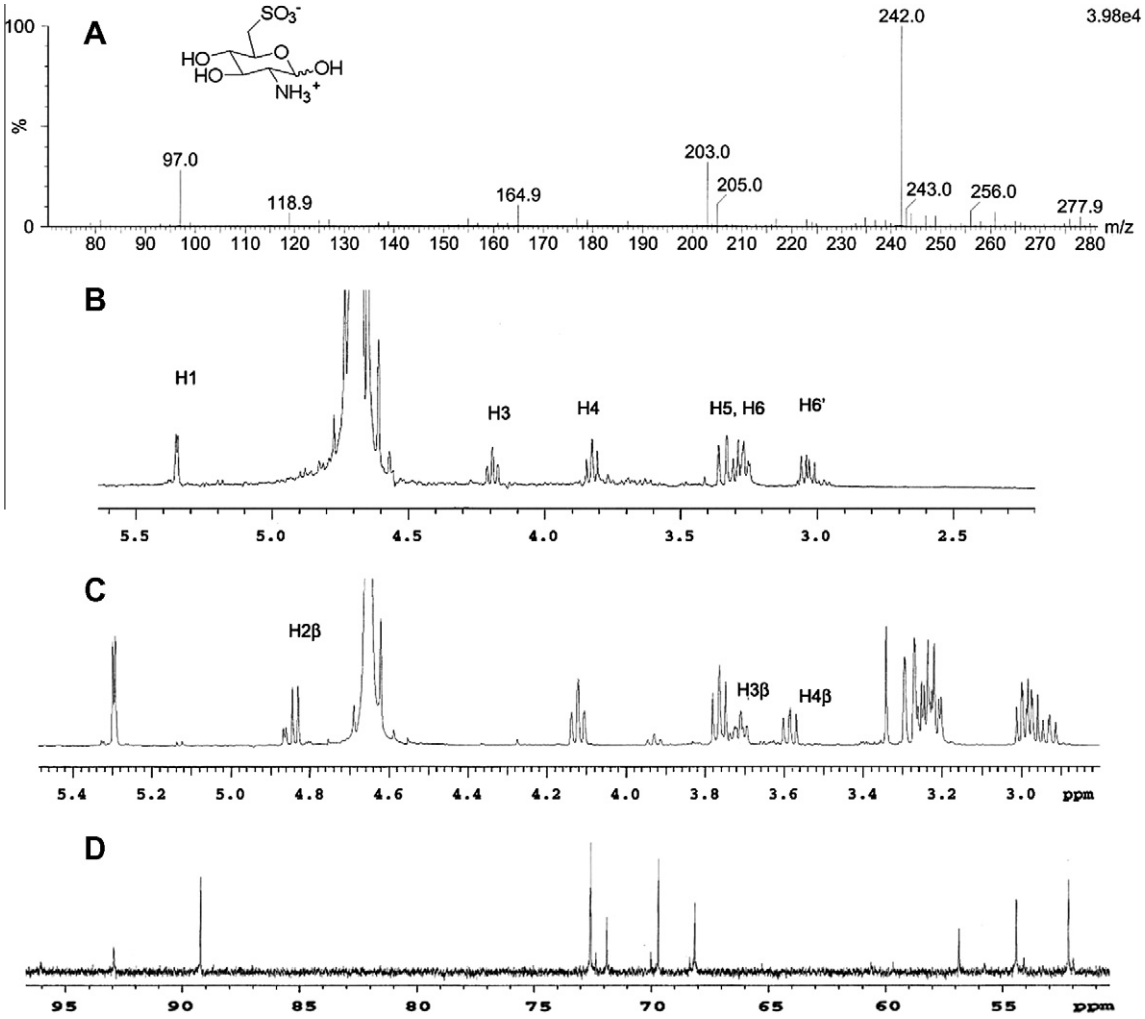


Figure 5. (A) ^{1}H NMR spectrum of the pure α anomeric form of compound **1** obtained directly after heating in acid and cooling. (B) ^{1}H NMR spectrum after allowing the product to mutarotate to a 2:1 mixture of α and β anomers. (C) ^{13}C NMR spectrum of the mixture of anomers. (D) Negative-ion electrospray-ionization mass spectrum of **1** showing a strong molecular ion.

1. Experimental

1.1. Structural comparison of D-glucosamine-6-sulfonate (1) and D-glucosamine-6-phosphate

D-Glucosamine-6-phosphate contains a phosphate group ($-O-PO_3^{2-}$) attached to the carbon 6 of the sugar structure. The glucosamine-6-phosphate analog contains a sulfonic acid group ($-SO_3^{2-}$) replacing this $-O-PO_3^{2-}$ group, with the sulfur atom being directly attached to the C6 of the aminosugar. In its mono-ionized form the phosphate group is the same in charge and similar in shape and size to the sulfonic acid group but with an extra atom connecting the carbon. The analog should easily fit the active site.

The structure of the binding site of glucosamine-6-phosphate synthase of *E. coli* (PDB ID: 2vf5[23]) is shown in Figure 4. The active site of the isomerase domain contains a P-loop, which is composed by residues 347–352. This loop is responsible for stabilizing the phosphate group in the correct orientation to allow the transfer of ammonia to the C2 of fructose phosphate. The P-loop residues interact with the oxygen atoms of the phosphate group through hydrogen bonds between the hydroxyl groups of the Ser347, Ser349 and Thr352 and the amino group of the main chain of Ser349 and Gln348.[16] These interactions do not require the presence of any metals or other components that would make these strong interactions; therefore, the replacement of the phosphate group by a sulfonate group should give a molecular species that binds comparably well compared to the phosphorylated species.

1.1.1. Benzyl 2-acetamido-6-bromo-2,6-dideoxy-α-D-glucopyranoside

2-Acetamido-2-deoxy-D-glucopyranose was converted to a mixture of the α- and β-benzyl glycosides as described by Kushida and Ichiro.[24] This consisted of heating 10 g of 2-acetamido-2-deoxy-D-glucopyranose in 200 g of dry benzyl alcohol in the presence of 2 g of Amberlite IR120 (H^+) at 60 °C for 3 h. The resin was filtered off and the excess benzyl alcohol was removed under reduced pressure at 60 °C. One gram of the 5:1 α:β mixture of benzyl glycosides so formed was converted to a corresponding mixture of benzyl 2-acetamido-6-bromo-2,6-dideoxy-D-glucopyranosides without further purification using triphenylphosphine and pyridine as described by Galemmo and Horton.[25] The product was purified by chromatography on silica using 2:1 acetone–dichloromethane. Yield 0.42 g (40%) of the pure α anomer: mp 183–184 (^{1}H and ^{13}C NMR data match that reported).[24]

1.1.2. 2-Amino-2,6-dideoxy-6-sulfo-D-glucopyranose

Benzyl 2-acetamido-6-bromo-2,6-dideoxy-α-D-glucopyranoside (0.4 g) was dissolved in 5 mL of water. Sodium sulfite (0.5 g) was added and the mixture was heated at 80 °C for 3 h. The solution was cooled and poured down a column (20 g) of strong base anion-exchange resin (Dowex Monosphere 550A, OH form). The resin bed was washed with water (200 mL) and then with 5% NaCl (100 mL). The NaCl wash was concentrated almost to dryness and then treated with 200 mL MeOH at room temperature. The mixture was stirred for 10 min and filtered. The filtrate was concentrated to a syrup that was dissolved in 0.5 mL of hydrazine and heated at 90 °C to remove the acetyl group. Excess hydrazine was removed on a rotary evaporator at 60 °C under high vacuum, followed by successive evaporation of several 10 mL volumes of water at the same temperature. The resulting solid was dissolved in 1 mL of water and a few drops of HCl added to adjust the pH to between 5 and 6. MeOH (10 mL) was then added followed by 0.4 g of 10% palladium-on-carbon. The mixture was hydrogenolyzed at 30 psi pressure for 5 h to remove the benzyl group yielding the desired product (110 mg) after filtration and evaporation of solvent. This was purified on an XAD-7 column (10 × 1 cm) using 70% EtOH in water as the eluent. Evaluation of the product by ^{1}H NMR spectroscopy (D_2O) at this stage revealed the expected loss of the signal at ~2.1 ppm for the *N*-acetyl group as well as the signals between 7.3 and 7.5 ppm for the benzyl group and the appearance of multiplets at 2.9–3.1 ppm characteristic of the protons on the sulfonated C6 position. The product existed almost exclusively as the α anomer when heated in acid and cooled, but this mutarotated to a 2:1 mixture of α and β anomers after standing for a while. IR [CaF_2 film, cm^{-1}] 3408, 2911, 2592, 1873, 1734 ^{1}H NMR: 5.29, d, J = 3 Hz, H1α; 4.84, d, J = 8 Hz, H1β; 4.12, t, J = 8 Hz, H3α; 3.76, t, J = 8 Hz, H4α; 3.71, t, J = 8 Hz, H3β; 3.58, t, J = 8 Hz, H3β; 3.2–3.5, m, H5 and H6α and β; 2.90–3.01, m, H6′α and β. ^{13}C NMR (mixture of anomers): 89.2, 93.0, 72.4, 72.0, 70.0, 69.7, 68.2, 68.1, 56.8, 54.4, 52.2. HRMS: Calcd for $C_6H_{12}NO_7S$, m/z 242.0334; found m/z 242.0345. A composite presentation of the spectral analyses of **1** is shown in Figure 5.

1.2. Evaluation of glucosamine-6-sulfonate activity in bacterial systems

1.2.1. Activity assay

D-Glucosamine-6-sulfonate was evaluated for its ability to inhibit cell wall formation in three bacteria strains (*E. coli* DH5α, *R. trifolii* ANU843 and *B. subtilis* PY74). The first two strains are Gram negative and the last one is Gram positive. This allowed the efficacy of the compound as an *N*-acetyl-D-glucosamine synthesis inhibitor over a broad spectrum of bacteria to be evaluated. The strains cultured in liquid media were treated in the log phase with the concentrations of the analog varying from 0 to 20 mg/mL. Their growth rates were evaluated after 6 h, 18 h and 24 h of incubation by monitoring the optical density at 600 nm. The results are expressed as the concentration that inhibits 50% of the bacteria growth (IC_{50}) in mg/mL after 24 h of treatment.

1.2.2. Scanning electron microscopy

The bacteria after 24 h of treatment with the inhibitor were shadowed with gold and their morphology was visualized by scanning electron microscopy (JEOL 6300F with field emission (Oxford EDS).

1.2.3. Glucosamine-6-phosphate acetyl transferase activity assay

The assay to verify if the analog has inhibitory activity over that of the GAT enzyme we performed the assay as previously described.[26] Yeast cells were lysed in 0.6 M sorbitol, 0.02 M HEPES–KOH buffer and used as source of enzyme. Tris–HCl 50 mM, pH 7.4, 1 mM EDTA, 1 mM glucosamine-6-phosphate, 0.5 mM acetyl-CoA, 0.5 mM of 5,5′-dithio-bis(2-dinitrobenzoic acid), 75 μL of yeast lysate and 20, 10, 5, 2.5 mg/mL of inhibitor were mixed up to 250 μL per well to observe if it has any inhibitory activity over the GAT enzyme. The possibility of the inhibitor acting as a substrate of GAT was also investigated by evaluating the result without glucosamine-6-phosphate and with the same concentration of inhibitor as was used for glucosamine-6-phosphate.

Supplementary data

Supplementary data (scanning electron micrographs) associated with this article can be found, in the online version, at doi:10.1016/j.carres.2011.07.004.

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