Synthesis, structure and bioactivity of primary sulfamate-containing natural products

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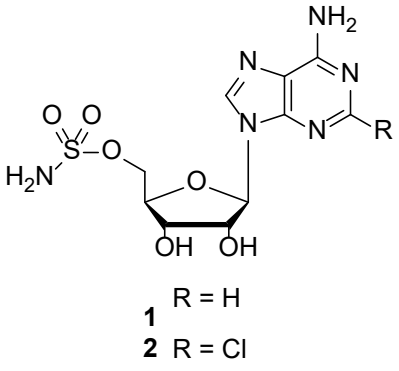
Abstract

Here we report the synthesis of natural products (NPs) 5'-*O*-sulfamoyl adenosine **1** and 5'-*O*-sulfamoyl-2-chloroadenosine **2**. As primary sulfamates these compounds represent an uncommon class of NPs, furthermore there are few NPs known that contain a N-S bond. Compounds **1** and **2** were evaluated for inhibition of carbonic anhydrases (CA), a metalloenzyme family where the primary sulfamate is known to coordinate to the active site zinc and form key hydrogen bonds with adjacent CA active site residues. Both NPs were good to moderate CA inhibitors, with compound **2** a 20 to 50-fold stronger CA inhibitor (K_i values 65 – 234 nM) than compound **1**. The protein X-ray crystal structures of **1** and **2** in complex with CA II show that it is not the halogen-hydrophobic interactions that give compound **2** a greater binding energy but a slight movement in orientation of the ribose ring that allows better hydrogen bonds to CA residues. Compounds **1** and **2** were further investigated for antimicrobial activity against a panel of microbes relevant to human health, including Gram-negative bacteria (4 strains), Gram-positive bacteria (1 strain) and yeast (2 strains). Antimicrobial activity and selectivity was observed. The minimum inhibitory concentration (MIC) of NP **1** was 10 μM against Gram-positive *Staphylococcus aureus* and NP **2** was 5 μM against Gram-negative *Escherichia coli*. This is the first time that NP primary sulfamates have been assessed for inhibition and binding to CAs, with systematic antimicrobial activity studies also reported.

Keywords: carbonic anhydrase, inhibitor, natural product, sulfamate, nucleoside, antimicrobial

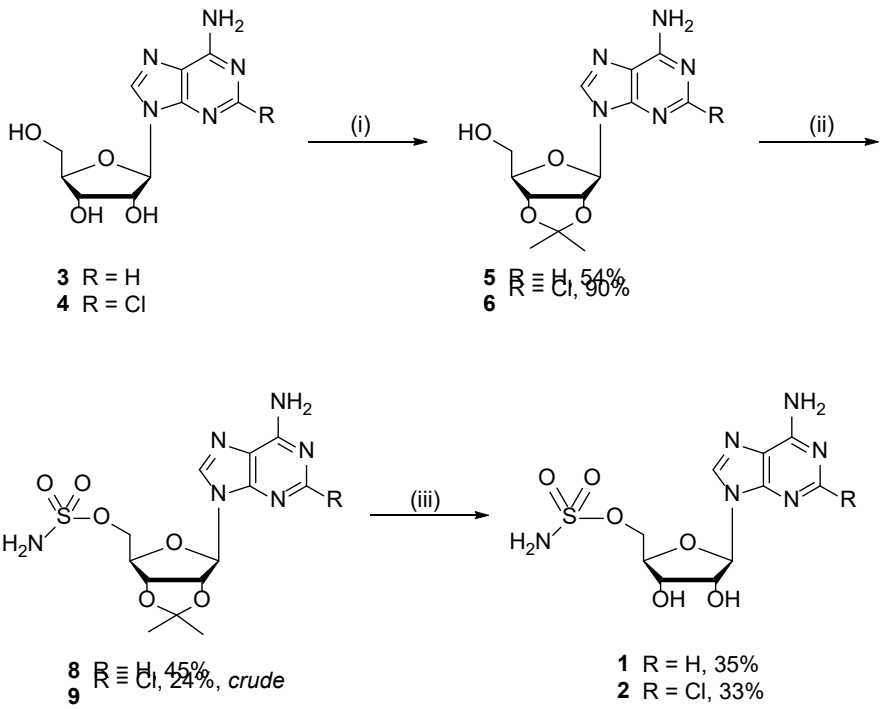
Carbonic anhydrases (CA, EC 4.2.1.1) are zinc metalloenzymes that catalyse the reversible hydration of carbon dioxide to bicarbonate and a proton: $CO_2 + H_2O \leftrightarrows HCO_3^- + H^+$. This equilibrium is critical for human health, and blocking the endogenous chemistry catalysed by CAs is a current target for therapeutic intervention.[1] Most reported small molecule CA inhibitors incorporate a primary sulfonamide ($-SO_2NH_2$) or primary sulfamate ($-O-SO_2NH_2$) as a zinc binding functional group that blocks the enzyme activity by coordination to the catalytic zinc.[2] Natural products (NPs) offer extraordinary chemical diversity, and in recent times have been explored as a source of novel CA inhibitors. Given the prominence of primary sulfonamides and primary sulfamates as CA inhibitors we conducted a literature search of the *Dictionary of Natural Products* (DNP) database against these functional groups.[3] We identified just seven compounds, consistent with the known low representation of N–S bonds in nature.[4] We recently reviewed the discovery, total synthesis, and known bioactivity of the seven NPs[5] and have reported extensive CA inhibition studies of a representative NP primary sulfonamide, Psammaplin C.[6] Here we sought to further build our knowledge of the properties of NP primary sulfamates, 5'-*O*-sulfamoyl adenosine **1** and 5'-*O*-sulfamoyl-2-chloroadenosine **2** (Figure 1).[5] These 5'-sulfamate substituted adenosine nucleosides have not before been investigated for interaction with CA enzymes so the aim of this study was to synthesise **1** and **2** then evaluate the enzyme inhibition properties against a comprehensive panel of human CA isozymes. We were also keen to determine the binding pose of the NPs in the CA active site using protein X-ray crystallography. Lastly, as there is sporadic literature available that indicates some antimicrobial potential of 5'-sulfamate substituted adenosine nucleosides, we were interested to assess the antibacterial and antifungal activity of these compounds.



1 R = H
2 R = Cl

Figure 1: Natural product primary sulfamates 5'-*O*-sulfamoyl adenosine **1** and 5'-*O*-sulfamoyl-2-chloroadenosine **2**.

Compounds **1** and **2** are 5'-*O*-sulfamoyl adenosine compounds that differ at the C-2 substituent. Compound **1** is unsubstituted at C-2, while compound **2** has a chlorine at C-2 (Figure 1). To synthesise **1** and **2** the required 2',3'-*O*-isopropylidene protected adenosine precursors **5** and **6** were prepared from commercially available adenosine **3** and 2-chloroadenosine **4** using standard conditions of acetone and tosylic acid (Scheme 1). Compounds **3** and **4** were treated with chlorosulfonyl amine **7** (prepared from chlorosulfonyl isocyanate and formic acid) in the presence of NaH[7], DMAP[8] or DMA as base to give a mixture of desired product (sulfamates **8** and **9**) and uncharacterised products. We propose that unreacted formic acid carried forward from the preparation of **7** and/or the strong base promoted cyclonucleoside formation as a side reaction.[7,8] The successful sulfamoylation of 2',3'-*O*-isopropylidene compounds **5** and **6** has been described using **7** prepared from chlorosulfonyl isocyanate and water in acetonitrile as solvent.[9] Compound **7** is formed instantaneously under these conditions, the reaction mixture is concentrated by azeotroping with toluene under reduced pressure and the crude **7** used immediately. Sulfamoylation with crude **7** in the presence of DBU as base gave the corresponding 5'-*O*-sulfamoyl adenosine compounds **8** and **9** in moderate yields without cyclonucleoside byproducts. The 2',3'-*O*-isopropylidene protecting group of compounds **8** and **9** was removed using TFA and water solution (4:2) to afford the target NPs **1** and **2** (Scheme 1).



Scheme 1: Synthesis of NP sulfamates **1** and **2**. Reagents and conditions: (i) *p*-TsOH, acetone, rt, 4 h; (ii) $ClSO_2NH_2$ **7**, DBU, DCM, rt, 15 h; (iii) TFA:H_2O (4:2), 0 °C–rt, 4 h.

Four different hCA isozymes were selected to investigate the inhibition characteristics of the NP sulfamates **1** and **2**, CA I, II, IV and CA IX, Table 1. CA I is an abundant enzyme and typically considered an off-target for CA inhibitor applications in clinical use. CA II is also abundant and is a well-established biological target for anti-glaucoma and anti-edema drugs.[10] Membrane-anchored isozyme CA IV has recently been implicated in wound healing[11] while CA IX is implicated in the invasion and metastasis of tumours through a pH modulating role.[12] The CA isozymes have a similar funnel shaped active site architecture such that isozyme selective small molecule inhibitors are challenging to develop.[2] The NPs were moderate to good CA inhibitors, with the chlorine substituent at C-2 of **2** leading to a consistent increase (20 to 50-fold) in CA inhibition across the CA enzyme panel (K_i values 65.2 - 234 nM) compared to the unsubstituted analogue **1** (K_i values 3100 - 6350 nM). This data indicates that the chlorine of **2** may occupy a site in the enzyme cavity that is not occupied by the corresponding aryl hydrogen of **1**.

Table 1: CA inhibition for NPs **1** and **2** at hCA I, II, IV and IX.

Compound	K_i (nM)[a,b]			
	hCA I	hCA II	hCA IV	hCA IX
1	6350	3410	4450	3100
2	167	65.2	234	143

[a]Errors in the range of ± 5% of the reported value, from three determinations. [b]Obtained using a stopped flow assay that monitors the physiological reaction (CA catalysed hydration of CO_2).

Having established that NP sulfamates were indeed inhibitors of CA enzymes we were keen to discern the detailed structural parameters underlying interactions of the sulfamate with the CA active site. Here we have utilised protein X-ray crystallography to determine the crystal structures of sulfamates **1** and **2** in complex with CA II. The crystal structures were determined to 1.28 Å (CA II:**1**) and 1.50 Å (CA II:**2**) resolution, respectively (Figure 2 and 3). There was clear electron density for the sulfamate moiety of compounds **1** and **2** directly adjacent to the zinc atom in the CA II active site. The two protein-compound structures can be superposed with an rmsd of 0.11 Å, with the residues within the active site being essentially identical in

position and orientation (His94, His96, His119, Leu198 to Pro202), Figure 2. The density seen for compound **1** is clearer than the density for compound **2** as compound **2** may have a second (minor) pose in the active site (and has been refined with an occupancy of 90%, unlike compound **1** which was refined at full occupancy), Figure 3. The direct sulfamate-zinc interaction is strong evidence in support of our original hypothesis that NP sulfamates should inhibit CA activity. In addition to the sulfamate-zinc interaction several other interactions of NPs **1** and **2** with the CA II residues are observed as the ligand extends beyond the catalytic pocket. The Gln92 side chain is slightly rotated between the two structures (Figure 2) and makes a distinctly tighter interaction with compound **2** (the side chain nitrogen has bond distances of 2.5 and 2.9 Å to the ribose hydroxyl residues whereas the distances are 3.2 and 3.7 Å to the same atom in the complex with compound **1**). This side chain may also have a minor alternate conformation that puts it in the same orientation as seen with the compound **1** structure. It seems that the chlorine in the purine ring interacts sterically with two hydrophobic residues near the top of the funnel-like active site, residues Phe131 (3.8 Å) and Val135 (3.4 Å), and this interaction may push the ribose ring slightly over to give the tighter hydrogen bonding interactions to Gln92. The purine ring of compound **1** sits slightly deeper in the hydrophobic pocket and makes interactions with Phe131 (3.5-3.7 Å) but is 4 Å or more away from Val135. We suggest that it is not the halogen-hydrophobic interactions that give compound **2** a greater binding energy but the slight movement in orientation which allows much better hydrogen bonds between the ribose ring hydroxyls and the Gln92 side chain.

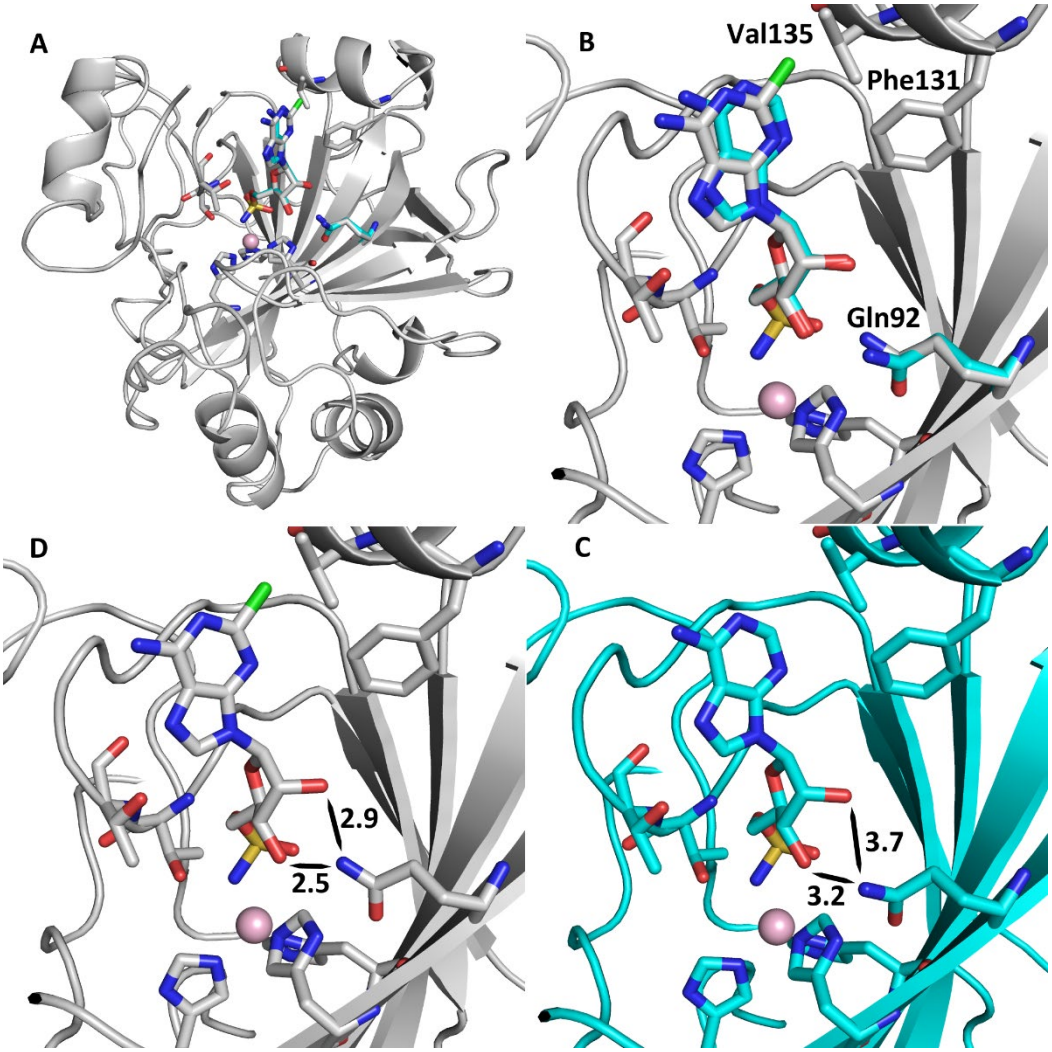


Figure 2: X-ray crystal structures. **A**: overview of the CAII structures with **1** and **2** superposed. **B**: a zoomed in view of panel A with carbons in **1** coloured cyan and carbons in **2** coloured grey. Important residues mentioned in the text that are involved in compound binding are highlighted as sticks and labelled. **C**: 5'-*O*-sulfamoyl adenosine **1** in complex with hCA II with distances from the Gln92 side chain to the ribose sugar hydroxyl atoms shown. **D**: 5'-*O*-sulfamoyl-2-chloroadenosine **2** in complex with hCA II with distances from the Gln92 side chain to the ribose sugar hydroxyl atoms shown. The small pink sphere represents the zinc atom in the active site of the hCAII protein in all panels.

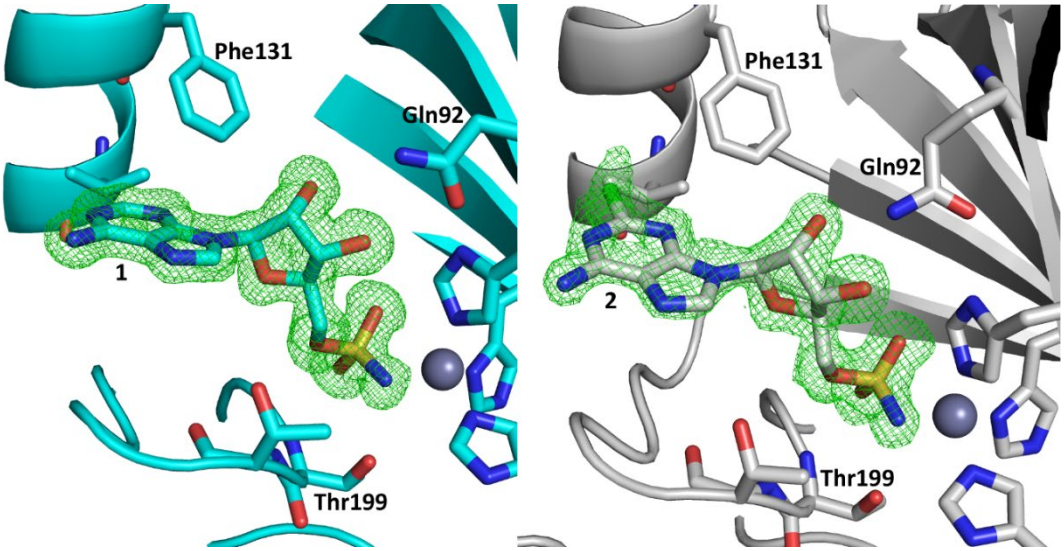


Figure 3: Difference electron density shown for the compounds **1** and **2** in the active site of CAII. The difference density is shown at a 3σ level and coloured as green wireframe, important residues for binding of the compounds are labelled (except Val135 which is partially blocked from view in this orientation).

Although NPs **1** and **2** have closely related chemical structures, they possess differing but broad spectrum antimicrobial[4,13,14], antitubercular[15-17], and herbicidal[18] bioactivity. Furthermore, these compounds are inhibitors of serotonin-induced platelet aggregation and have potential to aid development of antithrombotic therapeutics.[19,20] Recently[21], the gene cluster that encodes the biosynthesis of **2** was discovered leading to a proposed biosynthesis for S-N bond formation to incorporate a sulfamate group onto the ribose moiety of adenosine.

As drug resistant bacteria and fungi are prevalent concerns for human health we screened NPs **1** and **2** against a panel of five bacteria strains (Gram-positive and Gram-negative) and two fungi strains in collaboration with the Community for Open Antimicrobial Drug Discovery, CO-ADD. Compounds with activity >50% growth inhibition at 20 μM were classified as hits, Table 2. NP **1** was a hit against Gram-positive *Staphylococcus aureus* and displayed low activity against *Klebsiella pneumoniae* (~50% growth inhibition at 20 μM). The chlorinated NP **2** was a hit only against Gram-negative *Escherichia coli*. Hits were followed up to determine a minimum inhibitory concentration (MIC) against the microbe, with **1** displaying a value of 10 μM and **2** a value of 5 μM against *S. aureus* and *E. coli*, respectively, Table 2. A

counter-screen to assess mammalian cell cytotoxicity results showed that **1** and **2** had CC_{50} (concentration with 50% cytotoxicity) values 0.8 μM and <0.15 μM, respectively, against eukaryotic human embryonic kidney cells (HEK-293), Table 3. Although there is good selectivity with respect to antibacterial activity the safety window for mammalian cell cytotoxicity was considered insufficient for follow up hit validation studies of the NPs.

Table 2: Primary screening of antimicrobial activity of NPs **1** and **2** at 20 μM.[a]

Name	**Strain**	**Organsim**	**Type**	**1**	**2**
Staphylococcus aureus	ATCC 43300	Bacteria	G+ve		
Escherichia coli	ATCC 25922	Bacteria	G-ve		
Klebsiella pneumoniae	ATCC 700603	Bacteria	G-ve		
Acinetobacter baumannii	ATCC 19606	Bacteria	G-ve		
Pseudomonas aeruginosa	ATCC 27853	Bacteria	G-ve		
Candida albicans	ATCC 90028	Fungi	Yeast		
Cryptococcus neoformans var. grubii	H99; ATCC 208821	Fungi	Yeast		

Compounds with activity >50% growth inhibition at 20 μM were classified as hits (n=2), green shading. Non-hits, red shading. G+ve: Gram-positive, G-ve: Gram-negative.

Table 3: Secondary screening of antimicrobial hit activity and cytotoxicity of NPs **1** and **2**.

	1	**2**
	MIC (μM)	
S. aureus ATCC 43300	10	>20
E. coli ATCC 25922	>20	5
	CC_{50} (μM)	
Human embryonic kidney cells HEK-293 ATCC CRL-1573	0.8	<0.15

[a]Bacterial strains and cell line sources are detailed in Supplementary Information.

This is the first time NP sulfamates have been evaluated for in vitro inhibition of CA enzymes for which sulfamates are well known as inhibitors. Herein we have synthesised 5'-*O*-sulfamoyl

adenosine **1** and its chlorinated derivative 5'-*O*-sulfamoyl-2-chloroadenosine **2**, as representative compounds of the few NP sulfamates that are known. Both **1** and **2** inhibited CAs at moderate to good levels, with the chlorinated derivative **2** having 20 to 50-fold greater CA inhibition compared to **1**. Using protein X-ray crystallography we confirmed that the sulfamate group of **1** and **2** coordinate to the CA active site zinc in a manner consistent with CA:sulfamate structures previously reported. A different orientation of the ribose ring of NP **2** allows shorter hydrogen bond distances to CA residues compared to NP **1**. The NPs displayed good selectivity across a panel of microbes, with **1** a hit (MIC 10 μM) only against Gram-positive *S. aureus* and NP **2** a hit (MIC 5 μM) only against Gram-negative *E. coli*. Both compounds displayed mammalian cell cytotoxicity, precluding further development of this compound class for human drug development, although compounds may be useful for other antimicrobial applications. The NPs have been submitted to the Compounds Australia Compound Collection (Academic) (www.compoundsaustralia.com).[22]

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Supplementary Data

Supplementary data associated with this article contains experimental procedures, ^{1}H, ^{13}C NMR and HRMS spectra of compounds, X-ray crystallography statistics and CA inhibition and activity assay information.

All crystallography data were deposited into the PDB with accession codes 6C7W (**1**) and 6C7X (**2**).

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