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Inhibition of the β-carbonic anhydrases from *Mycobacterium tuberculosis* with *C*-cinnamoyl glycosides: Identification of the first inhibitor with anti-mycobacterial activity

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ABSTRACT

A small series of *C*-cinnamoyl glycoside containing the phenol moiety was tested for the inhibition of the three *Mycobacterium tuberculosis* β-carbonic anhydrases (CAs, EC 4.2.1.1) with activities in the low micromolar range detected. The compounds were also tested for the inhibition of growth of *M. tuberculosis* H_{37}Rv strain, leading to the identification of (*E*)-1-(2′,3′,4′,6′-tetra-*O*-acetyl-β-D-glucopyranosyl)-4-(3-hydroxyphenyl)but-3-en-2-one (**1**) as the first carbonic anhydrase inhibitor with anti-tubercular activity.

Infection with *Mycobacterium tuberculosis* and related mycobacteria affects a large fraction of the world population, with an estimated 9.4 million new cases and 1.7 million deaths annually.[1] Furthermore, tuberculosis (TB) infection including both multidrug-resistant tuberculosis (MDR-TB) and drug-resistant tuberculosis (XDR-TB) is a leading cause of death worldwide. The development of novel anti-TB drugs that do not possess cross-resistance with current anti-mycobacterial drugs and have minimal toxicity, is urgently needed due to the increasing incidence of MDR-TB, XDR-TB, and TB–HIV co-infection. In the last 10 years the research on *M. tuberculosis* has made much progress with the genome unrevealed[2] and the discovery of possible new drug targets which may lead to the development of compounds possessing a novel mechanism of action and thus resolve the drug resistance problem mentioned above. Among the new such proteins identified after the *M. tuberculosis* genome was published, there are also three β-carbonic anhydrases (CAs, EC 4.2.1.1) which have been cloned, purified and characterized by Jones' and Supuran's groups.[3,4]

CAs are a superfamily of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide to give bicarbonate and a proton.[5] Whereas the α-CA family, mainly present in mammals has been thoroughly investigated from the drug design viewpoint,[6] only recently CAs belonging to the β- and γ-CA families, which are widespread in bacteria and fungi (the β-CAs) and *Archaea* (the β- and γ-CAs), respectively, started to be considered for such a purpose. Two β-CAs from *M. tuberculosis* were successfully cloned and crystallized by Jones' group: Rv1284, also denominated mtCA 1, and Rv3588c, also designated as mtCA 2.[3] Supuran's group then identified, cloned and characterized the third such enzyme, encoded by the gene Rv3273, and denominated mtCA 3.[4] The precise physiological function of the three β-CAs from this pathogen is not known for the moment. Rv1284 is generally believed to be essential for survival of *Mycobacterium* and was found to be highly up-regulated under starvation conditions. In contrast, there is no evidence suggesting that Rv3273 is essential for mycobacterial growth.[7,8] Several sulfonamides and sulfamates inhibitors of the three mycobacterial carbonic anhydrases has been reported.[9] Such studies shows mtCAs to be druggable targets for developing anti-mycobacterial agents with a diverse mechanism of action compared to the clinically used drugs for which many strains led to the development of MDR-TB and XDR-TB. However the in vivo proof of concept study showing that inhibition of these enzymes leads to mycobacterial death or growth inhibition is still missing.[9]

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Phenol was reported to act as an inhibitor of the zinc enzyme carbonic anhydrase.[10] Indeed, phenol binds to CA in a diverse manner compared to classical inhibitors of the sulfonamide type, by interacting with a zinc-bound water molecule through hydrogen bonding and with no direct interaction between the inhibitor and the zinc.[11] Supuran's group has recently investigated the interaction of phenol and some derivatives with α- and β-carbonic anhydrases enzymes displaying low micromolar/submicromolar inhibition.[10,12] Thus, by binding in non-classical ways to CAs, phenols as well their derivatives constitute interesting leads to design novel CA inhibitors with selectivity and/or specificity for some of the medicinal targets belonging to this enzyme family.

The use of glycomimetics in the design of CA inhibitors has proven to be a successful approach and now constitutes one of the most attractive ways to develop new generations of effective and selective inhibitors.[13,14] The stereochemical diversity across the carbohydrate tails provides the opportunity for interrogation of subtle differences in active site topology of CA isozymes. On the last years one of our groups has developed several methodologies for the synthesis of *N*-glycosyl sulfonamides by sulfonamidoglycosylation of carbohydrate derivatives. We reported the synthesis of a series of α-D-hex-2-enopyranosyl sulfamides, which was evaluated against CA isozymes; one *erythro* compound was found to be a very effective CA IX inhibitor.[15] Recently we have prepared novel *N*-β-glycosyl sulfamides and shown that they selectively target cancer-associated CAs (IX and XII) with K_is in the low nanomolar range.[16] In the search of non-sulfonamide CAIs belonging to different classes of compounds, we have very recently developed the synthesis of *C*-cinnamoyl glycosides, where the carbohydrate moiety is tethered to a phenol CA pharmacophore through a carbon chain.[17] The *C*-glycosides were generally effective mammalian carbonic anhydrase inhibitors, with inhibition constants in the low micromolar range.

Very recently galactose-derived aryl enones were shown to be effective as antimycobacterial agents.[18] Likewise alkanoyl glycosides derived from β-*C*-glycosidic ketones were shown to be inhibitors of α-glucosidase.[19]

In order to discover novel probes that may help in the investigation and control of tuberculosis through a new mechanism of action, we evaluated the *M. tuberculosis* CAs inhibition and the antitubercular activity of a series of *C*-cinnamoyl glycosides containing the phenol moiety.

A set of new *C*-cinnamoyl glycosides (Fig. 1) was synthesized as outlined in Scheme 1 and described previously by us.[17] *C*-Cinnamoyl glycosides **1**–**4** have been prepared by aldol condensation of β-*C*-glucosyl and β-*C*-galactosyl ketones with 3-hydroxy or 4-hydroxybenzaldehyde at room temperature in the presence of pyrrolidine as catalyst. The *O*-acetate protecting groups of the carbohydrate moiety were next removed using triethylamine in methanol/water to afford the deprotected *C*-glycosides **5**–**8**.

The inhibitory activity of the *C*-glycosides **1**–**8** and phenol against human CA II and the purified *M. tuberculosis* β-CAs encoded by genes Rv1284, Rv3273 and Rv3588c are presented in Table 1. These inhibition data were acquired using a stopped flow assay that monitors the physiological reaction, that is, the CA catalyzed hidration of CO_2.[20]

A number of structure–activity relationships (SARs) were identified in this study and are summarized as follows:

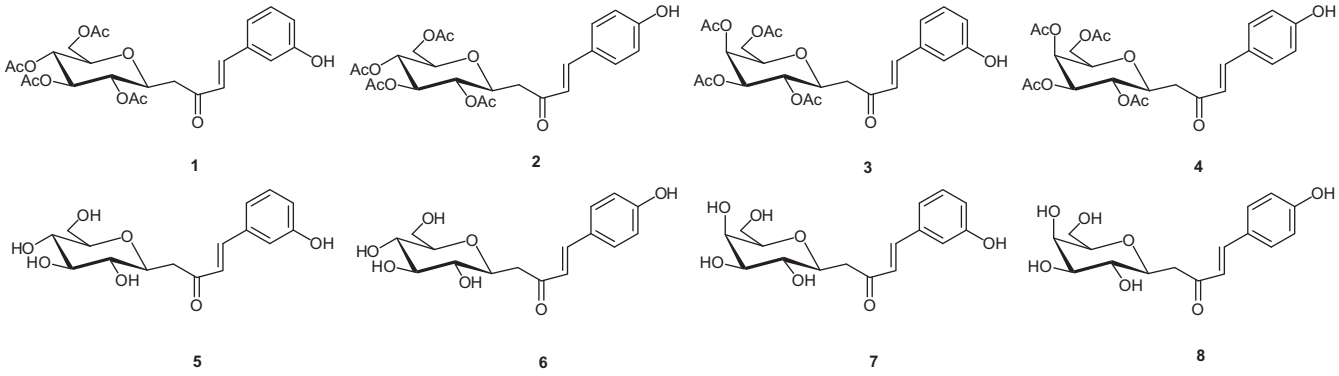
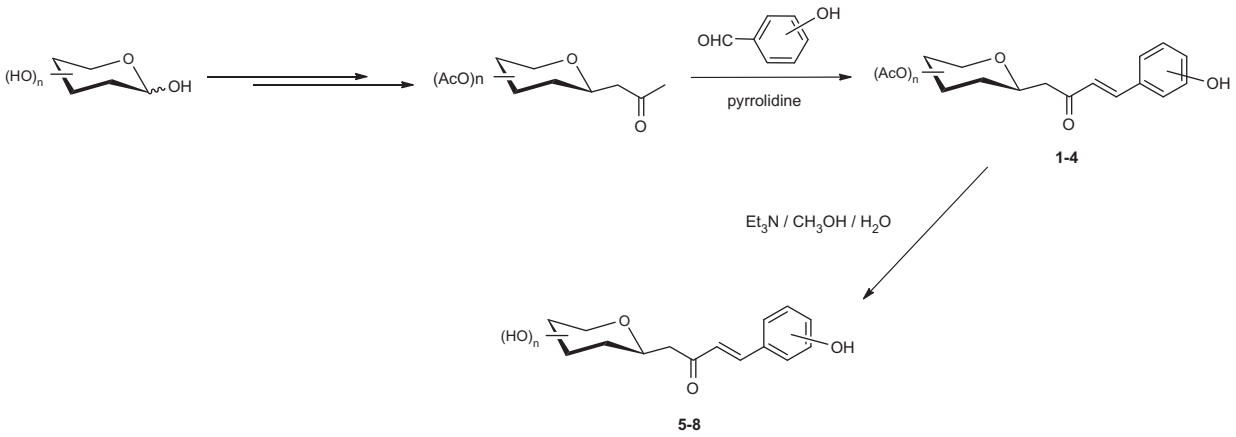


Figure 1. Peracetylated C-glycosides (**1**–**4**) and fully deprotected derivatives (**5**–**8**).



Scheme 1. Preparation of *C*-cinnamoyl glycosides **1**–**8**.

Table 1
Inhibition of hCA II and the β-CAs from *Mycobacterium tuberculosis* with the *C*-cinnamoyl glycosides **1**–**8** and phenol (as standard)[a]

Compound	K_i (μM)[b]			
	hCA II[c]	mt Rv1284	mt Rv3273	mt Rv3588c
1	7.0	2.1	19.0	0.64
2	3.9	2.9	13.1	0.35
3	7.1	3.8	15.6	0.87
4	5.5	4.5	12.0	1.15
5	7.8	0.14	6.21	0.24
6	8.8	0.93	4.13	0.13
7	3.1	1.16	3.25	0.51
8	6.8	4.5	4.13	0.94
Phenol	5.5	64.0	79.0	ND

[a] All CAs are recombinant enzymes obtained in the authors' laboratory as reported earlier.[10]
[b] Errors in the range of 5–10% of the reported value, from three different determinations.
[c] From Ref. 17.

Table 2
Anti-tubercular activity of *C*-cinnamoyl glycosides

Compound	Concentration		
	100 μg/ml	50 μg/ml	⩽25 μg/ml
1	MIC[a] (+)[b]	(+/−)[c]	No[d]
2	MIC (+)	(+/−)	No
3	No	No	No
4	No	No	No
5	No	No	No
6	(+/−)	(+/−)	No
7	MIC (+)	(+/−)	No
8	No	No	No

[a] MIC (Minimum Inhibitory Concentration), minimum drug concentration leading to growth inhibition.
[b] (+) indicates complete growth inhibition.
[c] (+/−) indicates partial inhibition.
[d] No indicates no inhibition. Growth/no growth was assessed in all cases by naked eye observation of turbidity.

(i) Isoform hCA II was inhibited by compounds **1**–**8** with K_is in the range 3.1–8.8 μM, showing a flat SAR with less variation of the inhibitory power, similar to that of phenol (K_i of 5.5 μM).
(ii) All investigated *C*-cinnamoyl glycosides were efficient, micromolar inhibitors of Rv1284 mtCA with inhibition constants in the range of 0.14–4.5 μM. This inhibition is greater than that for phenol (K_i of 64.0 μM), this confirms that attaching carbohydrate moieties to the CA phenol pharmacophore could improve its inhibitory activity.
(iii) The Rv3273 mtCA was inhibited very weakly by phenol (K_i of 79.0) while compounds **1**–**8** exhibited enhanced enzyme inhibition for this isozyme compared to phenol. Peracetylated *C*-glycosides **1**–**4** exhibited moderate Rv3273 inhibitory activity with inhibition constants in the range 12.0–19.0 μM. Deprotected compounds **5**–**8** were more effective inhibitors with K_is in the range 4.13–6.21 μM.
(iv) The *C*-glycosides investigated all showed an efficient low micromolar or submicromolar inhibitory activity on the mtCA Rv3588c, with K_is in the range 0.13–1.15 μM.

The anti-tubercular activity of compounds **1**–**8** was investigated by using fresh cultures of *M. tuberculosis* H_{37}Rv grown and tested as previously described.[21,22] Tests were carried on by duplicate. Our preliminary results clearly demonstrated that compounds **1**, **2**, **6** and **7** displayed growth inhibition activity at concentrations between 50 and 100 μg/ml (Table 2).

Table 3
Evaluation of antimycobacterial activity on solid medium

Compound	MIC (μg/ml)
1	3.125–6.25
2	>100
7	100[a]

[a] Pinpoint colonies.

This quick method is based on the final turbidity of the microtiter well plate and is not quantitative. It is usually taken as a 'yes/no' evaluation of activity, although compounds with weak activities may cause various degrees of inhibition of growth, indicated as (+/−) in Table 2. A more precise method is the evaluation of growth inhibition on solid medium that also allows for the observation of colony morphology and/or size alterations. Thus, we repeated the tests for the compounds exhibiting the most promising activity on solid medium containing different concentrations of compounds. To this end, we plated small aliquots of fresh cultures of *M. tuberculosis* H_{37}Rv containing a low CFU amount (10^3) in solid Middlebrook ADS glycerol medium supplemented with varying amounts of the selected compounds (**1**, **2** and **7**). The plates were sealed and incubated at 37 °C for 30 days followed by visual inspection (Table 3). In this case, compound **1** was again the most active, causing a 99.9% reduction in colony counts ($MIC_{99.9\%}$) at concentration values as low as 3125–625 μg/ml. Compounds **2** and **7** demonstrated some activity at high concentrations (compound **2** $MIC_{99.9\%}$ >100 μg/ml, compound **7** $MIC_{99.9\%}$ = 100 μg/ml) although in the case of **2** an effect on the size of the colonies growing at sub-inhibitory concentrations was seen. This was taken as proof of some activity on the mycobacterial metabolism capable of restricting growth but not large enough to prevent it. This result is compatible with the fact that there are three mtCAs, that may be inhibited at different degrees, thus yielding colonies with restricted size.

In conclusion, we have investigated the enzyme inhibition profile of a small series of *C*-cinnamoyl glycosides containing the phenol moiety, against purified *M. tuberculosis* β-CAs. The *C*-glycosides were generally effective CA inhibitors, with inhibition constants in the low micromolar. Also the glycosides were screened against *M. tuberculosis* H_{37}Rv and (*E*)-1-(2′,3′,4′,6′-tetra-*O*-acetyl-β-D-glucopyranosyl)-4-(3-hydroxyphenyl)but-3-en-2-one (**1**) was identified as the first mtCA inhibitor with antimycobacterial activity. Although the *C*-glycoside **1** showed no selectivity for the mtCAs over the ubiquitous isozyme CA II, its physicochemical properties would lead to a decreased affinity for cytosolic CAs,[17] however further in vivo studies are necessary to confirm this hypothesis. Thus **1** may be useful as lead compound for the development of novel chemotherapeutic agents against *Mycobacterium tuberculosis*.

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20. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity as reported by Khalifah (Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561). Phenol red (at a concentration of 0.02 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, and the Cheng–Prussoff equation (Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099) as reported earlier and represent the mean from at least three different determinations.
21. Bacterial strains and growth media: *Mycobacterium tuberculosis* strain $H_{37}Rv$ was grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 1/10 v/v of ADS (a solution containing 50 g/L BSA fraction V, 20 g/L dextrose and 8.1 g/L NaCl), glycerol (1% w/v) herein designated 7H9-ADS-G for short. Tween 80 was added to prevent clumping (0.05% w/v). When needed, solid media (Middlebrook 7H11 supplemented with ADS (1/10 v/v) and glycerol (1% v/v) was used. Cultures were grown at 37 °C under gentle agitation.
22. Anti-mycobacterial activity assay. Stock solutions for all the tested compounds were made in DMSO at 10 mg/ml. Working solutions were made by dilution in the above described 7H9-ADS-G medium at a final concentration of 400 μg/ml. Antimycobacterial activity was determined by a two-fold dilution of the compounds in Middlebrook 7H9-ADS-G medium. For this purpose 96-well plates (Falcon, Cat number 3072, BectonDickinson, Lincoln Park, NJ) were used. The 96-well plates received 100 μL of Middlebrook 7H9 broth and a serial twofold dilution of the compounds was made directly on the plate. The initial and final drug concentrations tested were 100 and 0.78 μg/ml, respectively. Four compounds were tested in duplicate in each microtiter plate, Rifampicin (from 2 to 0.16 μg/ml; stock solution prepared as a 10 mg/ml solution in methanol) was used as control drug. Two rows were used for growth control (medium and inoculum alone) and sterility control (medium alone). The inoculum was prepared as a 1/25 dilution of a fresh mid-log *M. tuberculosis* $H_{37}Rv$ suspensión (O.D. equivalent to Mc Farland 1.0 scale value) made in Middlebrook 7H9-ADS-G. A 100-μL aliquot (containing approximately 10^6 Colony Forming Units) was used to inoculate the wells except for the row used for sterility testing. Plates were sealed with Parafilm and incubated at 37 °C for 7 days. Minimum inhibitory concentration (MIC) was defined as the lowest drug concentration preventing mycobacterial growth as judged by visual inspection. Evaluation of activity on solid medium was performed by adding the chosen amounts of each compound to 3 ml of molten medium, pouring the mixture on 24 wells microtiter plates, and after hardening, plating aliquots ($\approx 1 \times 10^3$ CFU) of fresh *M. tuberculosis* $H_{37}Rv$ cultures onto the surface of the medium. Incubation proceeded for 30 days at 37 °C before visual inspection. $MIC_{99.9\%}$ was taken as the concentration that caused a 99.9% reduction in cell counts.