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SHORT COMMUNICATION

C-glycosides incorporating the 6-methoxy-2-naphthyl moiety are selective inhibitors of fungal and bacterial carbonic anhydrases

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Abstract

A small series of C-glycosides containing the methoxyaryl moieties was tested for the inhibition of the β -class carbonic anhydrases (CAs, EC 4.2.1.1) from *Cryptococcus neoformans* and *Brucella suis*. Many compounds showed activities in the micromolar or submicromolar range and excellent selectivity for pathogen CAs over human isozymes. The deprotected glycosides incorporating the 6-methoxy-2-naphthyl moiety showed the best inhibition profile and therefore represent leads for the development of novel anti-infectives with a new mechanism of action.

Keywords

Carbohydrate, carbonic anhydrase, pathogen

History

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Introduction

Carbonic anhydrases (CAs) are a superfamily of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide to give bicarbonate and a proton¹. Whereas the α -CA family, mainly present in mammals has been thoroughly investigated from the drug design viewpoint², 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.

Cryptococcus neoformans is a basidiomycetes naturally found in soil contaminated with pigeon guano. Healthy individuals do not normally suffer from *C. neoformans* infections. However, upon inhalation into the alveoli of immunosuppressed individuals, for example AIDS patients and people undergoing chemotherapy, *C. neoformans* is able to colonize deep tissues and transverse into the nervous system where it can cause cryptococcal meningitis³. The fungus experiences a dramatic raise in CO₂ concentration during transitions from the natural environment to its mammalian host. This increase in CO₂ promotes biosynthesis of a polysaccharide capsule, an important *C. neoformans* virulence factor⁴. The CO₂-sensing system of *C. neoformans* includes two prominent enzymes, the carbonic anhydrase Can2 and the fungal adenylyl cyclase Cac1. Can2, a member of β -CA family,

is essential for survival of *C. neoformans* in its natural environment. Consistently, cultivation of *C. neoformans* on medium supplemented with CA inhibitors (CAIs) prevented growth of *C. neoformans* in low CO₂ conditions typical for natural environments⁵. Thus, this β -CA constitute attractive targets for development of antifungals that target horizontal transmission. *Brucella* sp. is a facultative intracellular *Coccobacillus* responsible for brucellosis, the major bacterial zoonosis worldwide, in a variety of mammals including ruminants and human. Human brucellosis may become chronic, eventually causing death. Virulence is linked to the capacity of the bacteria to replicate inside the macrophage host cell and to escape from the host immune system. The genome of the bacterial pathogen *Brucella suis* contains two CAs belonging to the β -class: bsCA I and bsCA II^{6,7}. These two CAs were shown to be catalytically efficient, with activity for the CO₂ hydration reaction similar to that of the human (h) isoform hCA II, and are inhibited by many sulfonamides/sulfamates⁸. Furthermore, certain sulfonamide CAIs were shown to inhibit the bacterial growth in cell cultures⁸. Clearly parasite CAs may lead to anti-infectives with a new mechanism of action which bypasses the drug-resistance problems of clinically used agents^{9–11}.

The use of glycomimetics in the design of CAIs 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^{12,13}. Recently our group has applied the ‘‘sugar approach’’ to the preparation of C-cinnamoyl phenols, where the carbohydrate moiety is tethered to a phenol CA pharmacophore through a carbon chain¹⁴. These compounds have been tested as inhibitors of the *Mycobacterium tuberculosis* β -CAs and have shown better inhibitory activity against mCAs than phenol. Also the antitubercular activity of the C-glycosyl

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phenols was investigated, allowing us to identify the first mtCAs inhibitor with antimycobacterial activity¹⁵.

Very recently we developed a novel series of *C*-glycosides containing the methoxyaryl scaffold and investigated them as inhibitors against human isozymes of carbonic anhydrase, allowing us to identify four potent and highly selective inhibitors of hCA IX and XII¹⁶. These results confirm that attaching carbohydrate moieties to CA methoxyaryl pharmacophore improves and enhances its inhibitory activity. It should be noted that only very-recently methoxyphenyl derivatives have been investigated as CAIs because it was considered that they do not bear any moiety normally associated with CA inhibition in their molecules.

Thus, in the search of non-sulfonamide CAIs belonging to different classes of compounds, we report here the synthesis of a series of *C*-glycosides incorporating the methoxyaryl moiety, and their inhibitory activity against the off-target hCA I and II, and *C. neoformans* β -CA encoded by the gene Can2, and *Brucella suis* β -CAs. This current study provides further grounding for the discovery of novel β -CA inhibitors which virtually can lead to new antibacterial or antifungal agents.

Materials and methods

C-glycosides **1–12** were previously described, and have been prepared by aldol reaction of aryl aldehydes with per-*O*-acetylated *C*-glucosyl or *C*-galactosyl ketones and subsequent deprotection using triethylamine in methanol/water¹⁶.

An Applied Photophysics stopped-flow instrument had been used for assaying the CA catalyzed CO₂ hydration activity¹⁷. Phenol red (at a concentration of 0.02 mM) had been used as

indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na₂SO₄ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO₂ hydration reaction for a period of 10–100 s. The CO₂ 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 upto 0.01 nM was 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-Prusoff equation¹⁸ as reported earlier and represent the mean from at least three different determinations.

Results and discussion

A set of *C*-cinnamoyl glycosides (Figure 1) was synthesized as outlined in Scheme 1 and described previously by us¹⁴. *C*-cinnamoyl glycosides **1–6** have been prepared by aldol condensation of β -*C*-glucosyl and β -*C*-galactosyl ketones with 3-methoxybenzaldehyde, veratraldehyde or 6-methoxy-2-naphthaldehyde at room temperature in the presence of pyrrolidine as catalyst. The *O*-acetate protecting groups of the carbohydrate

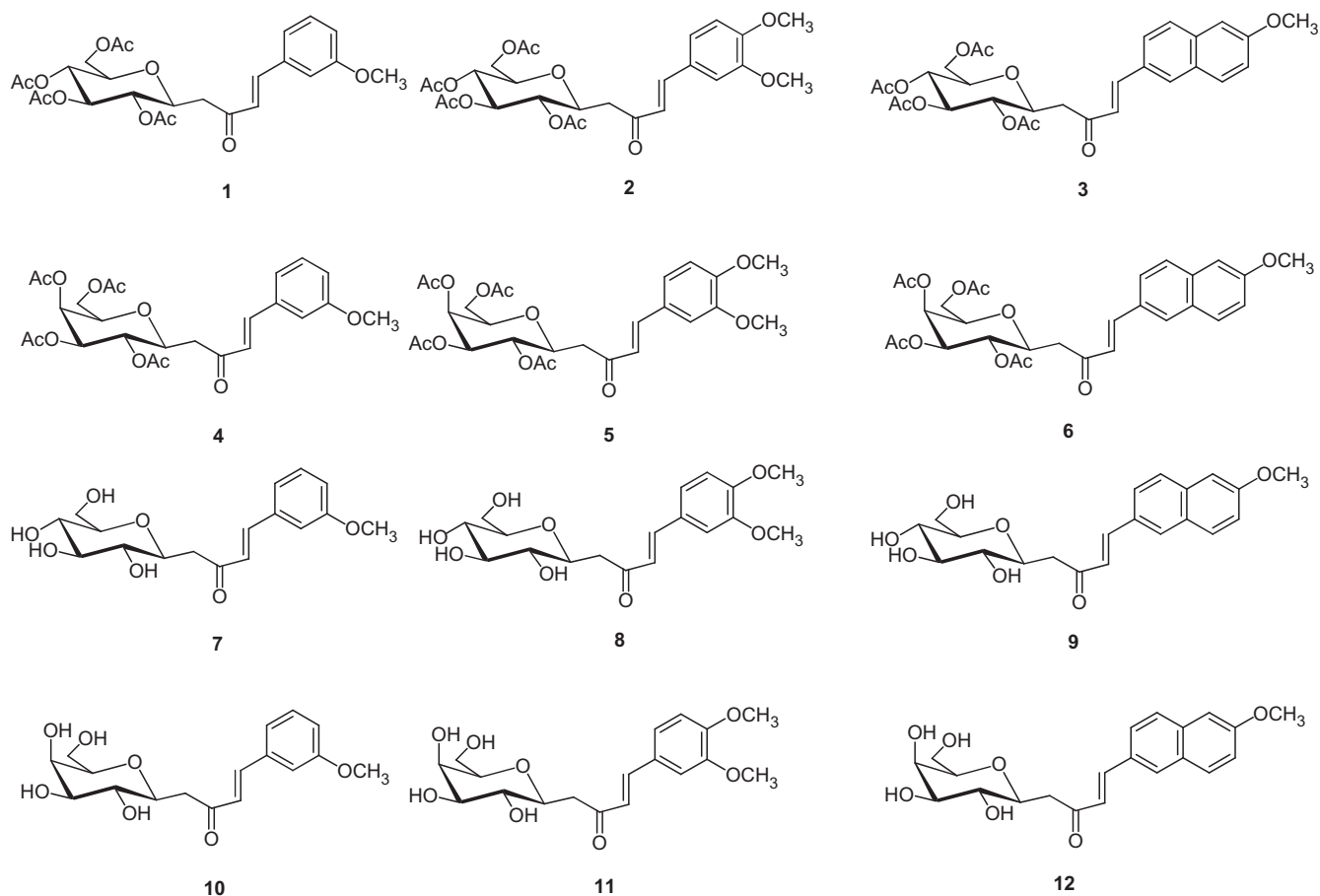
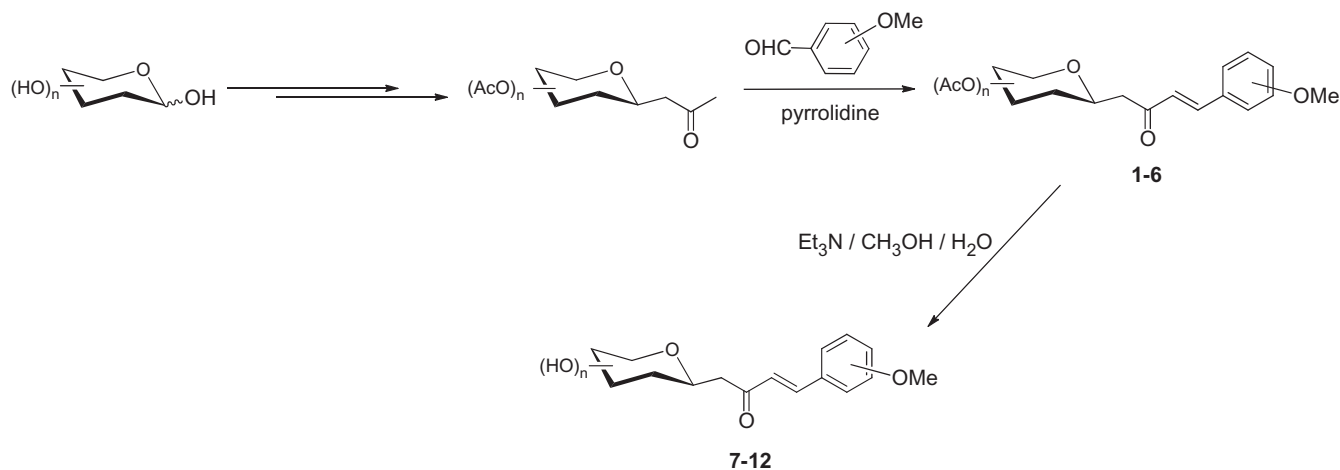


Figure 1. Per-*O*-acetylated *C*-glycosides (**1–6**) and fully deprotected derivatives (**7–12**).



Scheme 1. Preparation of C-cinnamoyl glycosides 1–12.

Table 1. Inhibition of mammalian α -CAs and pathogen β -CAs with the C-glycosides 1–12.^a

| C-glycoside | K_i (μM) ^b | | | | |
|-------------|--------------------------------------|--------|------|--------|---------|
| | hCA I | hCA II | Can2 | bsCA I | bsCA II |
| 1 | 5.67 | 0.42 | 8.00 | 0.89 | 0.49 |
| 2 | 8.36 | 0.54 | 1.59 | 1.76 | 0.36 |
| 3 | >50 | 0.41 | 8.10 | 3.92 | 0.70 |
| 4 | 8.69 | >50 | >50 | >50 | 4.02 |
| 5 | 4.67 | 0.63 | 0.22 | 4.36 | 0.37 |
| 6 | 6.65 | >50 | 7.82 | >50 | 8.05 |
| 7 | 7.44 | >50 | 7.81 | 7.56 | 8.01 |
| 8 | 6.51 | 0.44 | 0.42 | 2.58 | 0.31 |
| 9 | >50 | >50 | 3.13 | 0.42 | 0.22 |
| 10 | 8.67 | >50 | >50 | >50 | 8.69 |
| 11 | 7.48 | 0.55 | 0.24 | 4.49 | >50 |
| 12 | 7.21 | >50 | 2.37 | 0.43 | 0.21 |
| AAZ | 0.25 | 0.012 | 0.01 | 0.063 | 0.303 |

^aAll CAs are recombinant enzymes obtained in the authors' laboratory as reported earlier⁵.

^bErrors in the range of 5–10% of the reported value, from 3 different determinations.

moiety were next removed using triethylamine in methanol/water to afford the deprotected C-glycosides 7–12.

The inhibitory activity of the C-glycosides 1–12 against human CA I and CA II and the purified pathogen β -CAs, Can2, bsCA I and bsCA II are presented in Table 1. These inhibition data were acquired using a stopped flow assay that monitors the physiological reaction, namely, the CA catalyzed hydration of CO₂.

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

- The cytosolic isoform hCA I was inhibited by most of the C-glycosides investigated here (i.e. 1–2, 4–8 and 10–12) in the micromolar range, with K_i values in the range of 4.67–8.69 μM . 6-Methoxy-2-naphthyl glucoside 3 and its deprotected derivative 9 are ineffective as hCA I inhibitors.
- The C-glycosides showed a very interesting inhibition profile against hCA II. It should be noted that all glycosyl derivatives of veratraldehyde (i.e. 2, 5, 8 and 11) showed to be good inhibitors in the low micromolar range with K_i values in the range of 0.44–0.63 μM . Also compounds 1 and 3 are good hCA II inhibitors in the micromolar range. However being a ubiquitous, housekeeping isoform, this may not be a valuable property in another context if

compounds targeting other isoforms should also possess activity against hCA II. Thus it is interesting to note that some of the novel compounds showed poor inhibition against hCA II while retaining good inhibition against pathogen CAs. C-galactosyl derivatives 4 and 6 and their deprotected analogues 10 and 12 were ineffective as inhibitors of hCA II. Also C-glucosides 7 and 9 showed a highly reduced activity against this isozyme.

- The activity of the C-glycosides against *C. neoformans* β -CA (Can2) comprised compounds with three distinct inhibition profiles. The first group includes the compounds containing the veratrole moiety (i.e. 2, 5, 8 and 11) and exhibited very good Can2 inhibitory activity with inhibition constants in the range 0.22–1.59 μM . The second group (compounds 1, 3, 6, 7, 9 and 12) showed weaker Can2 inhibitory activity, with K_i s in the range 2.37–8.10 μM . The third group includes the galactosyl derivatives of 3-methoxybenzaldehyde (4 and 10) which showed to be ineffective as inhibitors of Can2.
 - Brucella suis* enzyme, bsCA I was inhibited by most of the C-glycosides investigated here (i.e. 1–3, 5, 7–9, 11 and 12) in micromolar or submicromolar range, with K_i values in the range of 4.56–0.42 μM . The per-*O*-acetylated C-galactosides 4 and 6, and the deprotected derivative 10 were ineffective as inhibitors of this pathogen isozyme. It is significant to note that the deprotected C-glycosides containing the 6-methoxy-2-naphthyl moiety (9 and 12) showed to be the most efficient inhibitors of bsCA I.
 - The inhibition profile for bsCA II lays in two distinct groups. The first group includes compounds 1–3, 5, 8, 9, 12 with K_i s of 0.31–0.70 μM while glycosides 4, 6, 7 and 10 (second group) were less effective bsCA II inhibitors with K_i s in the range 4.02–8.69 μM . The deprotected galactoside containing the veratrole moiety 11 showed to be ineffective as inhibitor of bsCA II. As for bsCA I, the glycosides 9 and 12 were the best inhibitors of the *Brucella suis* isozyme II.
- Selectivity for β -CAs against human CAs is an important consideration for downstream use of these C-glycosides novel probes that may help in the investigation and control of infectious diseases. The selectivity ratios of β -CA inhibition versus hCA I and hCA II inhibition are presented in Table 2. The clinically used CA inhibitor acetazolamide (AAZ) was less effective inhibitor for the bacterial and fungal β -CA enzymes. The reverse trend was observed for several of the glycosides reported here, with some compounds exhibiting a marked selectivity for the inhibition of the β -class CAs over the α -CAs. The selectivity ratios listed in

Table 2. Selectivity ratios of K_i for β -CAs compared to human α -CA isozymes I and II for the *C*-glycosides **1–12**.^a

| C-glycoside | Selectivity | | | | | |
|-------------|-------------|--------------|---------------|---------------|---------------|----------------|
| | hCA I/Can2 | hCA I/bsCA I | hCA I/bsCA II | hCA II/Can II | hCA II/bsCA I | hCA II/bsCA II |
| 1 | 0.71 | 6.37 | 11.61 | 0.05 | 0.47 | 0.86 |
| 2 | 5.26 | 4.75 | 23.22 | 0.34 | 0.31 | 0.67 |
| 3 | >6 | >13 | >71 | 0.05 | 0.94 | 0.59 |
| 4 | <0.1 | <0.1 | <0.1 | – | – | >12 |
| 5 | 21.23 | 1.07 | 11.78 | 2.86 | 0.14 | 1.70 |
| 6 | 0.85 | <0.1 | 0.83 | >6 | – | >6 |
| 7 | 0.95 | 0.98 | 0.93 | >6 | >6 | >6 |
| 8 | 15.50 | 2.52 | 21 | 0.96 | 0.17 | 0.71 |
| 9 | >16 | >119 | >227 | >16 | >119 | >227 |
| 10 | <0.1 | <0.1 | 1 | – | – | 5.75 |
| 11 | 31.17 | 1.66 | <0.1 | 2.30 | 0.12 | <0.01 |
| 12 | 3.04 | 16.77 | 34.34 | >21 | >116 | >238 |
| AAZ | 25 | 3.96 | 0.83 | 1.2 | 0.19 | 0.04 |

^aThe K_i ratios are indicative of isozyme selectivity for pathogen CAs *in vitro* and are calculated as K_i (human CA)/ K_i (β -CA).

Table 2 show that *C*-glycosides **9** and **12** were up to several hundred-fold selective for pathogen CAs over human CA I and CA II and thus may represent leads for better discriminating the inhibition of β -CAs from pathogen CAs. This observation provides a compelling opportunity to explore the 6-methoxy-2-naphthyl moiety scaffold in the development of potent and selective glycosyl inhibitors for the β -family of CAs. Clearly, the per-*O*-acetylated glycosides containing this scaffold (**3** and **6**) were less effective in this respect. *C*-glycoside **6** showed a maximum of 6-fold selectivity for pathogen CAs over human CA II and no selectivity for β -CAs over human CA I. On the other hand, *C*-glycoside **3** showed an excellent selectivity for the inhibition of β -CA enzymes over hCA I, but no selectivity for pathogen CAs over hCA II.

In conclusion, we have investigated the enzyme inhibition profile of a series of *C*-glycosides incorporating the methoxyaryl moiety (compounds **1–12**) against a panel of CAs encompassing the human α -CAs I and II, the fungal *C. neoformans* β -CAS and the pathogenic *Brucella suis* enzymes. The two best performing compounds, **9** and **12**, preferentially inhibited pathogen CAs over human CAs and therefore represent leads for the development of novel anti-infectives with a new mechanism of action.

Declaration of interest

The authors report no declarations of interest. This work was financed in part by an EU grant (Dynano) to CTS, and by UNLP and CONICET (Argentina). P.A.C is member of the Scientific Research Career of CONICET.

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