

RESEARCH ARTICLE

Synthesis of 3,4-dihydropyrrolidine-2,5-dione and 3,5-dihydroxybenzoic acid derivatives and evaluation of the carbonic anhydrase I and II inhibition

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Abstract

The inhibition of two human cytosolic carbonic anhydrase (hCA, EC 4.2.1.1) isozymes I and II, with some 3,4-dihydropyrrolidine-2,5-dione and 3,5-dihydroxybenzoic acid derivatives, were investigated by using the esterase assay, with 4-nitrophenyl acetate (4-NPA) as substrate. Compounds **10–13** showed K_i values in the range of 112.7–441.5 μM for hCA I and of 3.5–10.76 μM against hCA II, respectively. These hydroxyl group containing compounds generally were competitive inhibitors. Some hydroxyl group containing compounds investigated here showed effective hCA II inhibitory effects, in the same range as the clinically used sulfonamide acetazolamide, and might be used as leads for generating enzyme inhibitors possibly targeting other CA isoforms which have not been yet assayed for their interactions with such agents.

Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) enzymes are involved in important physiological and pathological functions, such as pH and CO_2 homeostasis, respiration and transport of $\text{CO}_2/\text{HCO}_3^-$ between metabolizing tissues and the lungs, ion secretion in different tissues/organs and biosynthetic reactions (e.g. gluconeogenesis, lipogenesis and ureagenesis)¹. CA isoforms are found in a variety of tissues where they participate in several important biological processes such as acid–base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and electrolyte secretion. Many CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited/activated for the treatment of a range of disorders such as edema, glaucoma, obesity, cancer, epilepsy and osteoporosis^{1–5}.

Pyrrolidine derivatives are widely used as prodrugs. Notably, 5-lactams such as pyrrol-2-one and its derivatives have illustrated

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anti-tumor activity. Recently, N-substituted maleimides and 5-ylidene pyrrol-2(5H)-ones have received growing attention^{6–8}, due to their biological characteristics and potentials for organic synthesis as key compounds for synthesis of bio-active molecules, which are Oteromycin, Talaroconvolutin A, Azaspirene, Fusarin, Clausenamide, and so on^{9,10}.

Our groups recently investigated the interaction of 12 mammalian CA isozymes with several types of phenolic compounds, such as catechol, resorcinol, a series of phenols, a series of phenolic acids and some other derivatives^{11–14}. Here, we extend these earlier investigations to series of phenolic compounds, some of which are widely used as antioxidant food additives or as drugs. Among the various natural or unnatural phenolic compounds with antioxidant properties, these compounds are very active in quenching reactive oxygen species¹³. They are reported to possess anti-cancer, anti-carcinogenic, anti-mutagenic, anti-bacterial, anti-viral or anti-inflammatory activities^{12,13}. Phenol, phenolic compounds and hydroxybenzoic acid derivatives are widely used as prodrugs or drugs. Salicylic acid is known for its ability to ease aches, pains, and reduce fevers. These medicinal properties, particularly fever relief, have been known since ancient times, and it was used as an anti-inflammatory drug^{12,13}.

In the present study, we have purified CA I and II (hCA I and hCA II) from human erythrocytes and examined the *in vitro* inhibition effects of above mentioned hydroxyl group containing compounds on these enzymes, using the esterase activity of hCA I and II, with 4-NPA as substrate.

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Materials and methods

Chemicals

The CNBr-activated Sepharose 4B, protein assay reagents, *p*-aminobenzene sulphonamide, L-tyrosine, 4-nitrophenylacetate (4-NPA) and chemicals for electrophoresis were purchased from Sigma-Aldrich Co. (St Louis, MO). All other chemicals were of analytical grade and obtained from either Sigma or Merck (Darmstadt, Germany). Reagents for syntheses of compounds **10–13**; L-(+)-tartaric acid, benzylamine, aniline, cyclohexylamine, 3,5-dihydroxybenzoic acid and triethylamine were purchased from Aldrich and (benzotriazol-1-yloxy) tris (dimethylamino) phosphonium hexafluorophosphate (BOP reagent) from Alfa Aesar (Ward Hill, MA). Other common chemicals and solvents are commercially available and were used after distillation or treatment with drying agents. Progress of reactions was monitored by thin layer chromatography (Merck, TLC Silica gel 60 F₂₅₄) in a suitable solvent system. Column chromatography was performed on Merck Silica Gel 60 (70–230 mesh). Melting points were determined with a Reichert thermovar micro melting point apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were taken on a Bruker Ultra Shield Plus 400 operating at 400 MHz for ¹H- and 100 MHz for ¹³C-nuclei. NMR spectra were recorded in deuterated dimethyl sulfoxide (DMSO-*d*₆) solvent using TMS as internal standard and chemical shifts referred to δ were expressed in parts per million, ppm. FT-IR spectra were measured with a Perkin-Elmer BXII ATR spectrometer. Elemental analyses were carried out by a Carlo Erba 1106 instrument.

Synthesis of (3*R*, 4*R*)-1-benzyl-3,4-dihydroxy-2,5-dioxopyrrolidine (**10**)

Benzylamine (5.35 g, 0.05 mol) and L-(+)-tartaric acid (7.5 g, 0.05 mol) were refluxed in xylene overnight with a Dean-Stark apparatus. The reaction mixture was cooled to ambient temperature and resulting crystalline product was filtered off. After successive washing with portions of hexane, crude product was recrystallized from ethanol to give product as pale yellow solid, 83%, m.p. 198–200 °C (200–201 °C)¹⁵. FT-IR (cm⁻¹): 3189 br, 2885, 1710, 1452, 1390, 1155, 1099, 1003. ¹H-NMR (400 MHz, DMSO-*d*₆): δ 7.35–7.23 (m, 5H, ArH), 6.30 (d, *J* = 5.24 Hz, 2H, -OH), 4.55 (d, *J* = 7.22 Hz, 2H, -CH, H3 and H4), 4.38 (d, *J* = 4.62 Hz, 2H, -PhCH₂). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 174.6, 135.9, 128.5, 127.5, 127.5, 74.5, 41.1. Anal. found: C, 59.51; H, 5.07; N, 6.27. Calcd for C₁₁H₁₁NO₄: C, 59.73; H, 5.01; N, 6.33.

Synthesis of (3*R*, 4*R*)-1-phenyl-3,4-dihydroxy-2,5-dioxopyrrolidine (**11**)

Freshly distilled aniline (4.65 g, 0.05 mol) and L-(+)-tartaric acid (7.5 g, 0.05 mol) were refluxed in xylene with a Dean-Stark apparatus. After overnight reaction, the resulting crystalline product was filtered off and washed several times with hexane. Recrystallization from ethanol provided white crystalline product with 87% yield, m.p. 248–250 °C (249–250 °C)¹⁶. FT-IR (cm⁻¹): 3338 br, 3064, 1710, 1497, 1393, 1182, 1103, 999. ¹H-NMR (400 MHz, DMSO-*d*₆): δ 7.51–7.39 (m, 3H, ArH), 7.32–7.30 (m, 2H, ArH), 6.39 (dd, *J* = 4.64 Hz and *J* = 1.78 Hz, 2H, -OH), 4.57 (dd, *J* = 4.69 Hz and *J* = 1.65 Hz, 2H, -CH, H3 and H4). ¹³C-NMR (100 MHz, DMSO-*d*₆): δ 174.0, 132.0, 128.9, 128.3, 126.9, 74.4. Anal. found: C, 58.11; H, 5.51; N, 6.54. Calcd for C₁₀H₉NO₄: C, 57.97; H, 4.38; N, 6.76.

Synthesis of 3,5-dihydroxy-N-(benzyl)benzamide (**12**)

3,5-dihydroxybenzoic acid (1.54 g, 10 mmol, 1 eq.) was dissolved in 20 ml of acetonitrile. To solution, triethylamine (4.18 ml,

30 mmol, 3 eq.), (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (4.42 g, 10 mmol, 1 eq.) and benzylamine (1.07 g, 10 mmol, 1 eq.) were added. The mixture was stirred at room temperature for 6 h. The precipitate was filtered off and then washed with acetonitrile. The filtrate was evaporated under vacuum, taken up with ethyl acetate then washed with 20 ml of 2N HCl solution, 20 ml of saturated NaHCO₃ solution and with 20 ml of a saturated solution of NaCl. The product was purified by column chromatography on silica gel eluting with mixture of ethyl acetate-hexane (2:1) to yield a white solid (65%), m.p. 213–215 °C. FT-IR (cm⁻¹): 3380, 3301, 3035, 1655, 1536, 1154, 997. ¹H-NMR (400 MHz, DMSO-*d*₆): δ 9.48 (s, 2H, -OH), 8.83 (t, *J* = 6.03 Hz, 1H, -NH), 7.33–7.19 (m, 5H, ArH), 6.70 (d, *J* = 2.15 Hz, 2H, H2 and H6), 6.35 t, *J* = 2.15 Hz, 1H, H4), 4.40 (d, *J* = 6.03 Hz, 2H, PhCH₂). ¹³C-NMR (100 MHz, DMSO-*d*₆): δ 166.5, 158.3, 139.9, 136.6, 128.2, 127.1, 126.6, 105.4, 105.1, 42.5. ANAL. FOUND: C, 68.87; H, 5.64; N, 5.60. Calcd for C₁₄H₁₃NO₃: C, 69.12; H, 5.39; N, 5.76.

Synthesis of 3,5-dihydroxy-N-(cyclohexyl)benzamide (**13**)

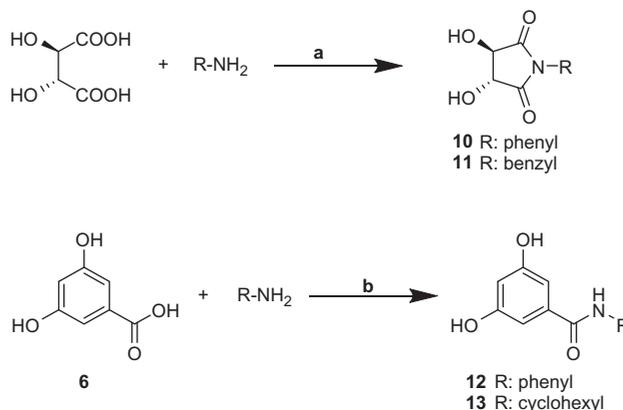
In a flask, 3,5-dihydroxybenzoic acid (1.54 g, 10 mmol, 1 eq.) was dissolved in 20 ml of acetonitrile, and triethylamine (4.18 ml, 30 mmol, 3 eq.), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (4.42 g, 10 mmol, 1 eq.) and cyclohexylamine (0.99 g, 10 mmol, 1 eq.) were added. The mixture was stirred at room temperature for 6 h and then the precipitate was filtered off by washing with acetonitrile. The filtrate was evaporated under vacuum and taken up with ethyl acetate. Successive washings with 20 ml of a solution of 2N HCl, 20 ml of saturated NaHCO₃ solution and 20 ml of a saturated solution NaCl provided the crude. The product was purified by chromatography on silica (eluent: EtOAc/hexane: 2/1) to yield a white solid (69%). Melting point 203–205 °C. FT-IR (cm⁻¹): 3382, 3281, 1636, 1553, 1149, 858. ¹H-NMR (400 MHz, DMSO-*d*₆): δ 9.39 (s, 2H, -OH), 7.97 (t, *J* = 8.02 Hz, 1H, -NH), 6.64 (d, *J* = 2.14 Hz, 2H, H2 and H6), 6.32 (t, *J* = 2.10 Hz, 1H, H4), 1.76–1.04 (m, 11H, aliphatic hydrogens). ¹³C-NMR (100 MHz, DMSO-*d*₆): δ 165.6, 158.1, 137.1, 105.4, 104.8, 48.1, 32.3, 25.2, 24.9. Anal. found: C, 66.81; H, 7.45; N, 5.87. Calcd for C₁₃H₁₇NO₃: C, 66.36; H, 7.28; N, 5.95.

CA purification assay

Purification of two human CA isozymes (hCA I and hCA II) were previously described with a simple one-step method by a Sepharose-4B aniline-sulfanilamide affinity column chromatography^{17,18}.

CA activity assay and kinetic studies

CA activity was assayed by following the change in absorbance at 348 nm of 4-NPA to 4-nitrophenolate ion over a period of 3 min at 25 °C using a spectrophotometer (Shimadzu UV-VIS, Kyoto, Japan) according to the method described by Verpoorte et al.¹⁹ The inhibitory effects of compounds **1–13** and AZA were examined. All compounds were tested in triplicate at each concentration used. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor an Activity %-[Inhibitor] graph was drawn. To determine K_i values, three different inhibitor concentrations were tested; In these experiments, 4-NPA was used as substrate at five different concentrations (0.15–0.75 mM). The Lineweaver-Burk curves were drawn²⁰. Regression analysis graphs were drawn for IC₅₀ using inhibition % values by a statistical package (SPSS for windows, version 10.0; SPSS Inc., Chicago, IL) on a computer (Student's *t*-test; *n*: 3).



Scheme 1. Reagents and conditions. (a) xylene reflux; (b) acetonitrile, rt, TEA, BOP reagent.

Results and discussion

The rationale of investigating phenols as CA inhibitors (CAIs) is due to the fact that the simple phenol (PhOH) has been shown to be the only competitive inhibitor with CO₂ as substrate for the main isoform of CA, i.e. human CA II (hCA II)¹⁴. The X-ray crystal structure for the adduct of hCA II with phenol¹⁴. The phenyl moiety of phenol was found to lay in the hydrophobic part of the hCA II active site, where CO₂, the physiologic substrate of the CAs, binds in the precatalytic complex, explaining thus the behavior of phenol as a unique CO₂ competitive inhibitor. Only recently, our groups investigated the interactions of some simple phenols, salicylic acid derivatives, some antioxidant phenolic compounds, some natural product polyphenols and phenolic acids with all mammalian isozymes, CA I–XV^{11–14}, evidencing some low micromolar/submicromolar inhibitors as well as the possibility to design isozyme selective CAIs. Indeed, the inhibition profile of various isozymes with this class of agents is very variable, with inhibition constants ranging from the millimolar to the submicromolar range for many simple phenols^{11–13}.

(3R, 4R)-3,4-dihydroxy-2,5-dioxypyrrolidine derivatives (**10** and **11**) were synthesized^{15,16} by the reaction of L-(+)-tartaric acid with primary amines. Benzamide structures (**12** and **13**) were established (Scheme 1) by coupling 3,5-dihydroxybenzoic acid with primary amines using BOP (benzotriazol-1-yloxy-tris(dimethylamino)-phosphoniumhexafluorophosphate) as coupling reagent. Hydroxyl groups of benzoic acid were not protected since BOP coupling is safely mediated in the presence of unprotected alkyl and phenolic hydroxyl groups in many examples^{21–23}.

We report here the first study on the inhibitory effects of compounds **10–13** on the esterase activity of hCA I and II. The previous reports by Innocenti et al.¹¹ investigated other phenol derivatives (including catechol and resorcinol) by using a stopped flow, CO₂ hydration assay for monitoring CA inhibition. Data of Table 1 show the following regarding inhibition of hCA I and II with compounds **10–13**, by an esterase assay¹⁹, with 4-NPA as substrate:

(i) Against the slow cytosolic isozyme hCA I, compounds **10–13** behave as good inhibitors (Figure 1), with K_i values in the range of 112.7–441.5 μM similarly to the structurally related compounds **1–8** and acetazolamide (AZA) (K_is of 0.55–4003 μM). It is interesting to note that the compounds **10** and **11** were much better hCA I inhibitors as compared to the corresponding compounds **12** and **13** from which they were prepared. Kinetic investigations (Lineweaver–Burk plots, data not shown) indicate that similarly to sulfonamides and inorganic anions^{3–5,17–20}, all the investigated natural compounds act as

Table 1. hCA I and II inhibition data some compounds, by an esterase assay with 4-NPA as substrate¹⁹.

Compound	K _i (μM)*	
	hCA I	hCA II
Phenol (1)†	10.2	5.5
Catechol (2)†	4003	9.91
Resorcinol (3)†	795	7.7
Hydroquinone (4)†	10.7	0.09
Pyrogallol (5)†	7.41	0.54
3,5-Dihydroxybenzoic acid (6)†	0.55	0.51
1-Tosyl-1H-pyrrole-2(5H)-one (7)‡	37.3	23.1
5-Hydroxy-1-tosyl-1H-pyrrole-2(5H)-one (8)‡	14.6	0.51
Acetazolamide (9)	36.2	0.37
(3R, 4R)-1-Benzyl-3,4-dihydroxy-2,5-dioxypyrrolidine (10)	133	8.0
(3R, 4R)-1-Phenyl-3,4-dihydroxy-2,5-dioxypyrrolidine (11)	112.7	3.5
3,5-Dihydroxy-N-(benzyl)benzamide (12)	441.5	10.76
3,5-Dihydroxy-N-(cyclohexyl)benzamide (13)	392.2	9.48

*Mean from at least three determinations. Errors in the range of 3–8% of the reported value (data not shown).

†From Ref 24.

‡From Ref 25.

competitive inhibitors with 4-NPA as substrate, i.e. they bind in different regions of the active site cavity as compared to the substrate. However, the binding site of 4-NPA itself is unknown, but it is presumed to be in the same region as that of CO₂, the physiological substrate of this enzyme^{11–14}.

(ii) A better inhibitory activity has been observed with compounds **10–13** investigated here for the inhibition of the rapid cytosolic isozyme hCA II (Table 1). Structure–activity relationship (SAR) is thus quite sharp for this small series of hydroxylic compounds: the 2,5-dioxypyrrolidine containing compounds **10** and **11** are ineffective leads, with two 3, 5 dihydroxy moieties is already a submicromolar hCA II inhibitor. This trend is maintained when different groups are present in the *meta* position to the phenol OH moiety, such as in resorcinol. The best hCA II inhibitor in this series of derivatives were compounds **10** and **11**, which with a K_i of 8.0–3.5 μM. It must be stressed that K_is measured with the esterase method are always in the micromolar range because hCA I and II are weak esterases.^{24–27}

In a recent study, it was reported that catechol and resorcinol²⁶ a simple compound lack of the sulfonamide, sulfamate, or related functional groups which are typically found in all known CAIs – act as a CAI inhibitor, and could represent the starting point for a new class of inhibitors that may have advantages for patients with sulfonamide allergies^{24–30}. The sulfonamide zinc-binding group is thus superior to the thiol one (from the thiooxone hydrolysis

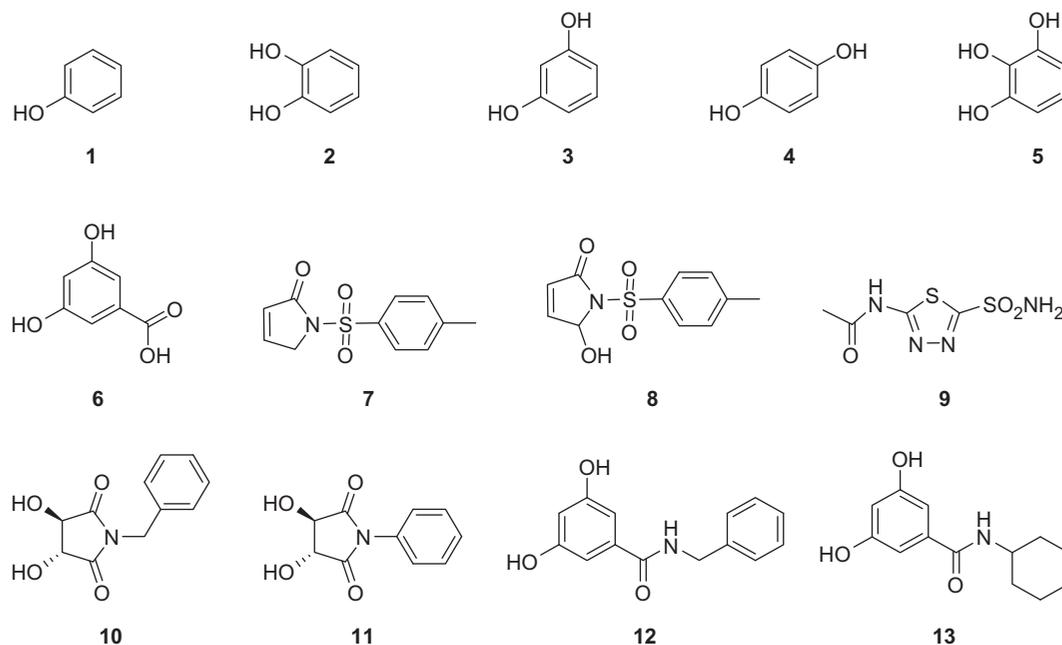


Figure 1. Structure of tested compounds.

product) for generating CAIs with a varied and sometimes isozyme-selective inhibition profile against the mammalian enzymes. However, it is critically important to explore further classes of potent CAIs in order to detect compounds with a different inhibition profile as compared to the sulfonamides and their bioisosteres and to find novel applications for the inhibitors of these widespread enzymes.

Conclusion

Compounds **10–13** used in this study affect the activity of CA isozymes due to the presence of the different functional groups (OH, phenyl, benzyl, and cyclohexyl) present in their aromatic scaffold. Our findings here indicate thus another class of possible CAIs of interest, in addition to the well-known sulfonamides/sulfamates/sulfamides, the phenols/biphenyl diphenols bearing bulky *ortho* moieties in their molecules. Indeed, some hydroxylic compounds investigated here showed effective hCA I and II inhibitory activity, in the low micromolar range, by the esterase method which usually gives K_I -s an order of magnitude higher as compared to the CO_2 hydrase assay^{30–45}. These findings point out that substituted hydroxylic compounds may be used as leads for generating potent CAIs eventually targeting other isoforms which have not been assayed yet for their interactions with such agents.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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