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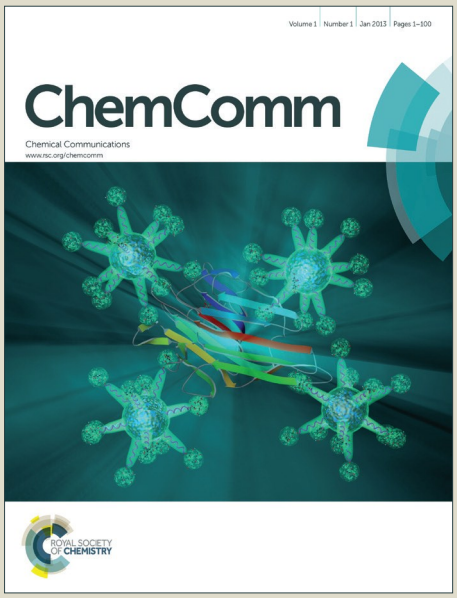

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Benzoxaborole as a New Chemotype for Carbonic Anhydrase Inhibition

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In this paper we report the synthesis of a series of benzoxaborole derivatives, their inhibition properties against some Carbonic Anhydrases (CAs), recognized as important drug targets, and the characterization of the binding mode of these molecules to the CA active site. Our data provide the first experimental evidence that benzoxaboroles can be efficiently used as CA inhibitors.

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metallo-enzymes, present in most living organisms, which catalyze the reversible hydration of carbon dioxide to bicarbonate and proton ($CO_2 + H_2O \leftrightarrows HCO_3^- + H^+$).[1] In humans 15 different isoforms have been so far described, which show variable catalytic properties, cellular localization and tissue distribution. These enzymes are involved in a large number of physiological processes and their abnormal levels and/or activities have been often associated with different human diseases. Consequently, many human CAs (hCAs) are interesting targets for the design of inhibitors or activators with biomedical applications.[1]

CA inhibitors (CAIs), which have initially found applications as diuretics, antiglaucoma agents and antiepileptics, are now emerging as pharmacological agents against cancer and obesity.[1] However, their use as drugs is limited by the lack of selectivity against the different CA isoforms. In order to improve the selectivity profiles, recent years saw an increasing interest for the development of new CAIs, alternative to the first generation inhibitors such as sulfonamides and sulfamates. Thus, novel chemotypes, among which the coumarins, thiocoumarins, sulfocoumarins, polyamines, dithiocarbamates, hydroxamates and carboxylates, were reported as CAIs and some of them were shown to possess very interesting selectivity properties.[1] For example, some coumarins were demonstrated to inhibit specifically the tumor associated isoforms hCA IX and hCA XII over the cytosolic, more widespread hCA I and hCA II (off-target enzymes for many pharmacological applications). These compounds were also shown to significantly inhibit the growth of primary tumors and metastases *in vivo*.[2] Thus, research in this field is still very active, with an increasing number of novel chemotypes examined for their interaction with this superfamily of enzymes.

To date, boron-containing compounds have been only scarcely investigated as CAIs. An inhibition activity in the micromolar range against some hCAs has been shown by simple boronic acids, incorporating aromatic, arylalkyl and arylalkenyl moieties.[3, 4] Although some of these compounds have been proposed as proteasome inhibitors,[5] with bortezomib A quite effective for the treatment of multiple myelomas and other malignancies,[6] many concerns, mainly related to the high reactivity and metabolic instability, have been raised on the use of such derivatives as drugs.[7, 8] Thus, alternative boron-containing moieties to be incorporated in enzyme inhibitors started to be ultimately considered, with benzoxaboroles constituting an interesting such class of molecules. Indeed, these compounds were found to have a very stable oxaborole ring and a higher hydrolytic resistance of the boron–carbon bond in comparison with the boronic acids.[9] Many such molecules do also possess strong anti-inflammatory, antifungal and antibacterial activity.[10-13]

Considering our interest for alternative chemotypes (to the sulfonamides) for the development of novel classes of CAIs, we have investigated the capability of benzoxaboroles to inhibit some CAs present in humans and recognized as potential drug targets, such as isoforms hCA I, II, IX and XII. In particular, starting from benzoxaborole **1** (Scheme 1) as lead molecule, we synthesized a small library of urea- and thiourea-benzoxaborole derivatives, tested their inhibition properties against the above mentioned isoforms and elucidated their interaction mode with the CA active site by means of X-ray crystallographic studies. Our data provide the first experimental evidence that benzoxaboroles can be efficiently used as CAIs, thus representing an interesting scaffold for developing new molecules with a therapeutic potential for the treatment of various diseases.

A straightforward strategy was used to synthesize benzoxaborole derivatives starting from 6-

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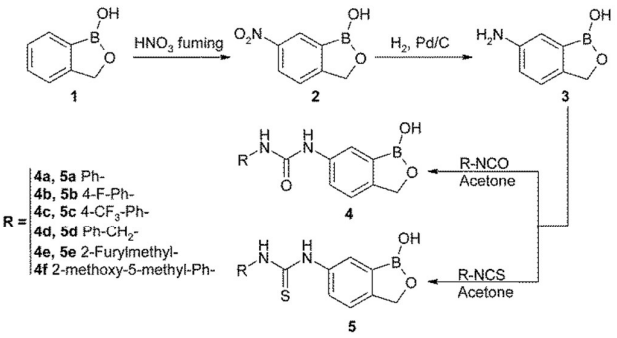
aminobenzo[c][1,2]oxaborol-1(3H)-ol **3** (Scheme 1). The latter was prepared as described in the literature[14-16] starting with the nitration in position 6 of commercially available 1,3-dihydro-1-hydroxy-2,1-benzoxaborole **1** with fuming nitric acid at –45 °C, followed by hydrogenation over palladium on carbon at room temperature. Compound **3** was then reacted in acetone with either isocyanates or isothiocyanates to obtain the ureas **4** or thioureas **5**, respectively (Scheme 1).
Compounds were then assayed for their inhibitory properties against hCA I, II, IX and XII (Table 1).



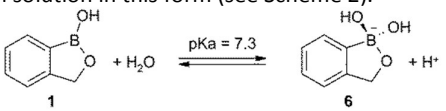
Scheme 1. Synthesis of benzoxaborole derivatives

Table 1. Inhibitory activity of compounds **1-3**, **4a-4f** and **5a-5e** against the hCA isoforms I, II, IX and XII, determined by a Stopped-Flow, CO_2 hydration assay. Data relative to the standard CAI acetazolamide (**AAZ**) are also shown for comparison.

	K_I (nM)[a]			
	hCA I[b]	**hCA II**[b]	**hCA IX**[c]	**hCA XII**[c]
1	5690	8180	>50000	>50000
2	6352	504	94	92
3	9434	590	813	640
4a	654	730	1060	240
4b	235	480	843	175
4c	487	456	845	93
4d	557	439	925	184
4e	613	841	1230	663
4f	98	89	414	69
5a	548	1148	436	76
5b	355	1500	336	89
5c	417	1838	93	71
5d	380	1305	610	42
5e	258	1500	336	89
AAZ	250	12	25	5.7

[a] Errors in the range of ±5–10% of the reported value from three different determinations. [b] Full length. [c] Catalytic domain.

Among the twelve catalytically active hCAs, these isoforms were chosen for our studies since two of them, the cytosolic hCA I and II, are widespread house-keeping enzymes involved in a host of physiologic processes,[17] whereas the transmembrane hCA IX and XII are tumor-associated enzymes, abundant in hypoxic tumors and recently validated as antitumor targets.[18] Indeed, one CA IX/XII inhibitor, belonging to the sulfonamide series, is currently in Phase I clinical trials for the management of hypoxic, metastatic tumors.[2] The data of Table 1 show that benzoxaborole **1** is a weak, micromolar hCA I and II inhibitor, and does not significantly inhibit the transmembrane isoforms hCA IX and XII (K_I > 50 μM). However, the introduction of simple substituents of the NO_2 or NH_2 type on the 6 position of the benzoxaborole ring, as in **2** and **3**, leads to a dramatic change of the CA inhibitory properties. Indeed, these compounds inhibit hCA IX and XII with K_Is ranging between 92-94 nM and 640-813 nM respectively, whereas their efficacy against hCA I is substantially unaltered. hCA II inhibition by these compounds is also an order of magnitude more efficient compared to the parent compound **1**. Ureas **4** and thioureas **5** show increased hCA I inhibition compared to the simple derivatives **1-3**, with K_Is in the range of 98-654 nM. The hCA II inhibitory properties are even more interesting, with ureas **4** behaving as medium potency inhibitors and thioureas **5** showing a weak activity (K_Is > 1 μM). hCA IX is inhibited efficiently by two benzoxaborole derivatives, the nitro compound **2** and thiourea **5c**, which have K_Is of 94 and 93 nM, respectively. The remaining ureas/thioureas are much less effective hCA IX inhibitors, with K_Is of 336-1230 nM. The strongest inhibition is observed for isoform hCA XII, for which **2**, **4c**, **4f** and all thioureas **5** show K_Is in the range of 42-93 nM. The remaining compounds are less effective, with K_Is of 184-663 nM. Data show that even subtle structural changes induce great alterations of inhibitory properties against all investigated CA isoforms, with some compounds able to reach nanomolar inhibition constants against the studied isoforms (see for example **4f** against hCA I, hCA II and hCA XII). It is thus highly probable that much more effective CAIs belonging to this new chemotype may be designed by tailoring the nature and substitution pattern at the heterocyclic ring system. These data prove the capability of the newly developed molecules to act as CAIs, but do not provide any information regarding their mechanism of action. To clarify this important aspect, detailed X-ray crystallographic studies were undertaken. In particular, the high-resolution crystal structure of hCA II in complex with benzoxaborole **1**, the lead compound of the series here investigated, was solved. hCA II was chosen as model isoform for crystallization, since it readily forms crystals and many studies have been reported on its adducts with different classes of inhibitors.[1] Crystals of the adduct were obtained following a procedure well described in literature.[19] In particular, native hCA II was crystallized by the hanging drop vapor diffusion method at pH 8.7, and the obtained crystals were soaked in the precipitant solution containing the inhibitor at a concentration of 100 mM (see ESI for details). The structure was refined by using REFMAC[20] of the CCP4 suite[21] and the native hCA II (PDB code 1CA2)[22] as an initial model. Inspection of the electron density maps at various stages of the crystallographic refinement revealed the binding of the inhibitor to the enzyme active site in its tetrahedral anionic form **6** (Scheme 2). This finding is in agreement with the observation that at the pH used for crystallization experiments (i.e., 8.7), compound **1** is mainly present in solution in this form (see Scheme 2).[10]



Scheme 2. Lewis/Brønsted acidic properties of benzoxaborole.

Interestingly, two different binding modes, both characterized by the coordination of the tetrahedral derivative to the catalytic zinc ion, were observed (Fig. 1A and S1). In the first one, hereafter indicated as **binding mode A**, one of the hydroxyl groups of the benzoxaborole was anchored to the catalytic zinc ion, completing its tetrahedral coordination (Fig. 1B), whereas, in the second one, hereafter indicated as **binding mode B**, the inhibitor was bound to the zinc ion with

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two of its oxygen atoms, generating a trigonal bipyramidal coordination geometry of zinc (Fig. 1C). In both binding modes, additional polar and van der Waals interactions contributed to the stabilization of the adduct (Fig. 1B and 1C).

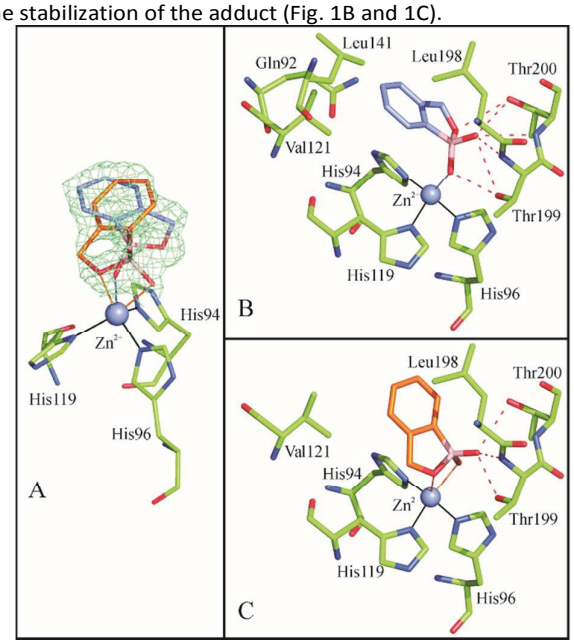

Fig. 1 (A) σA-weighted |2Fo-Fc| map (contoured at 1.0 σ) relative to the inhibitor molecule in the hCA II/**1** adduct crystallized at pH 8.7. The two possible coordination modes of the inhibitor are depicted. The zinc ion coordination is drawn in black for the histidines, in blue for **binding mode A** and in orange for **binding mode B**. (B) Detail of the interactions of inhibitor **1** with enzyme active site in **binding mode A**. (C) Detail of the interactions of inhibitor **1** with enzyme active site **in binding mode B**. In (B) and (C) residues involved in polar and van der Waals (distance < 4 Å) interactions are shown. Continuous lines indicate zinc ion coordination, whereas dashed lines indicate hydrogen bond distances.

The observation that in both cases the inhibitor binding to the enzyme was mediated by the tetrahedral anionic form **6**, prompted us to investigate which was the binding mode of **1** at physiological pH, where, on the basis of the equilibrium reported in Scheme 2, the trigonal planar form of the inhibitor was also present. To clarify this point, we set up new crystallization conditions of the hCA II/**1** adduct at pH 7.4 (see the ESI for details). Interestingly, the analysis of the obtained structure revealed that also in this case the inhibitor bound the enzyme in its anionic tetrahedral form **6**, adopting two binding modes identical to those observed in the structure crystallized at pH 8.7 (Fig. S2 and S3). Based on this finding we can clearly conclude that the binding mode of benzoxaborole **1** to the enzyme active site is independent on pH and is always associated to the anionic tetrahedral form **6**, probably because in this way the electron avid boron atom has eight electrons in its last shell. However, the question whether only the anionic tetrahedral form **6** can bind the active site or the trigonal planar form **1** can bind and then is transformed in the tetrahedral one **6,** by reaction with the hydroxide anion bound to the zinc ion, remains unanswered. In any case, the observation that the tetrahedral form is involved in the mechanism of action of the benzoxaboroles is in agreement with previously reported data on benzoxaboroles as inhibitors of Leucyl-tRNA synthetase.[12, 23]

Interestingly, a careful analysis of the electron density maps in other regions of the protein showed that in the structure obtained at pH 7.4, benzoxaborole **1** bound other sites on the protein surface, beyond the active site. In particular, two molecules of **1** in the trigonal planar form were present in a cleft on the protein surface, delimited by residues Ser2, His3, His4, Trp5, His10, Asn11, His15, Trp16, Lys18 and Asp19 (Fig. 2), whereas three other molecules formed adducts with three histidines, highly exposed to the solvent (His3, His4 and His10), at the amino-terminal part of the enzyme (Fig. 2). The formation of such kind of dative adducts between boron containing derivatives and His residues has been already described for some protease inhibitors belonging to the boronic acid class.[24]

The additional binding sites, observed in the structure at pH 7.4, were not present in that obtained at pH 8.7, and were never reported for other classes of CAIs investigated through X-ray crystallography. In such derivatives, one nitrogen atom from the imidazole ring coordinates the boron atom of the inhibitor as the fourth, tetrahedral ligand (Fig. 2). These additional interactions could represent a limit in the use of benzoxaboroles as drugs, indicating their propensity to react in a non-specific way not only with the target protein but also with other protein systems possibly present *in vivo* and possessing accessible nucleophile residues. We should however remark that the very large excess of **1** used in the crystallization experiments, is likely unpractical *in vivo*, suggesting that the occurrence of such unwished chemical bonds and non covalent interactions is only governed by the *in vitro* experimental conditions and might never take place at the concentrations expected for a therapeutic use. Moreover, the occurrence of such adducts only in the structure at pH 7.4 strongly suggests that their formation is strictly related to the presence of the neutral trigonal form of the inhibitor, which is nearly absent at pH 8.7. Thus, it can be hypothesized that, lowering the pKa of benzoxaborole derivative with proper substituents of the aromatic ring,[25] and consequently reducing the amount of the trigonal form of **1** present at physiological pH, the formation of unspecific adducts as those shown in Fig. 2 may be suppressed.

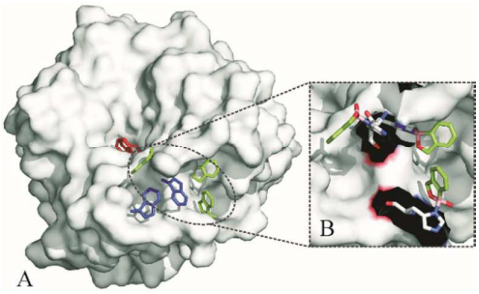

Fig. 2 Solvent accessible surface of hCA II showing the binding sites of inhibitor **1** (A). The tetrahedral form **6** coordinating the catalytic zinc ion is colored in red, the trigonal form **1** is drawn in blu, whereas the inhibitor molecules involved into dative adducts with exposed histidine residues are shown in green (B).

These findings may also constitute the basis for mapping exposed His residues from proteins with an unresolved structure, by means of benzoxaborole derivatives, which are inexpensive, stable and easily identifiable via mass spectrometric experiments.

To verify if the substitution pattern on the benzoxaborole ring could influence the binding mode of the inhibitors to the enzyme active site, we also crystallized the best hCA II inhibitor of the series,

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namely compound **4f**, in complex with its target enzyme (see ESI for experimental details). Interestingly, analysis of the structure of the hCA II/**4f** adduct showed that the bulky substituent of the benzoxaborole ring has a great effect in influencing the interaction of the inhibitor with the enzyme active site. Indeed, in this case only one binding mode is observed (Fig. 3A and S4), which is completely different from **binding mode A** of compound **1** (Fig. 3B) and rather similar to **binding mode B** (Fig. 3C), even if some differences in the orientation of the benzoxaborole ring can be observed also in this case.

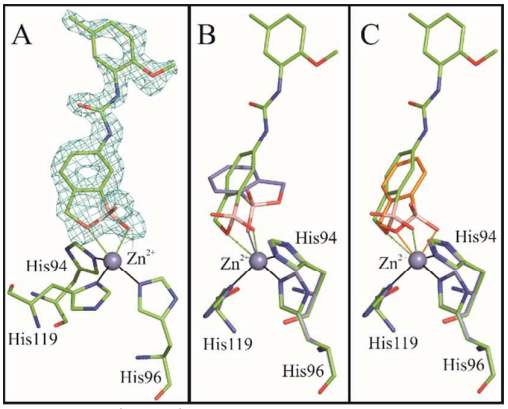

Fig. 3 (A) σA-weighted |2Fo-Fc| map (contoured at 1.0 σ) relative to the inhibitor molecule in the hCA II/**4f** adduct. (B) Structural superposition of the hCA II/**4f** adduct with hCA II/**1** adduct in **binding mode A.** (C) Structural superposition of the hCA II/**4f** adduct with hCA II/**1** adduct in **binding mode B**.

It is worth noting that the inhibitor **4f** cannot assume the **binding mode A** when it interacts with hCA II active site, since in such case the bulky tail in position 6 would clash with the hydrophobic pocket defined by residues Phe131, Leu141, Val121 and Val143. The stabilization of only one binding mode and the formation of a higher number of polar and van der Waals interactions with enzyme active site (see Fig. S4) can be considered as the main factors responsible with the improved inhibition properties against hCA II of compound **4f** with respect to **1**.

In conclusion, altogether data here reported provide evidence that benzoxaboroles can be efficiently used as a new class of CAIs, presenting interesting inhibition properties and a completely new binding mode to the CA active site. Moreover, the substitution pattern at the benzoxaborole ring can be used to finely modulate the affinity and the binding mode toward the different CA isoforms. Finally, our data also suggest that the high reactivity of benzoxaborole **1** towards His residues may be useful to map the exposition of such amino acids in other poorly investigated proteins.

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