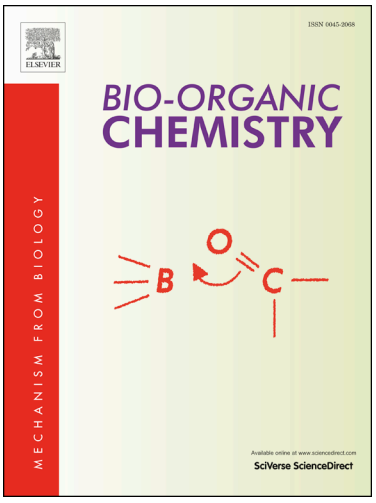

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PII: S0045-2068(18)31115-5
DOI: https://doi.org/10.1016/j.bioorg.2019.01.030
Reference: YBIOO 2734

To appear in: *Bioorganic Chemistry*

Received Date: 1 October 2018
Revised Date: 12 December 2018
Accepted Date: 17 January 2019

Please cite this article as: S. Bua, S.M. Osman, Z. AlOthman, C.T. Supuran, A. Nocentini, Benzenesulfonamides incorporating nitrogenous bases show effective inhibition of β-carbonic anhydrases from the pathogenic fungi *Cryptococcus neoformans*, *Candida glabrata* and *Malassezia globosa*, *Bioorganic Chemistry* (2019), doi: https://doi.org/10.1016/j.bioorg.2019.01.030

Benzenesulfonamides incorporating nitrogenous bases show effective inhibition of β-carbonic anhydrases from the pathogenic fungi *Cryptococcus neoformans, Candida glabrata* and *Malassezia globosa*

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Abstract

There is an urgent need for new chemotherapic agents to treat human fungal infections due to emerging and spreading globally resistance mechanisms. Among the new targets that have been recently investigated for the development of antifungal drugs there are the metallo-enzymes Carbonic Anhydrases (CAs, EC 4.2.1.1). The inhibition of the β-CAs identified in many pathogenic fungi leads to an impairment of parasite growth and virulence, which in turn leads to a significant anti-infective effect. Based on antifungal nucleoside antibiotics, the inhibition of the β-CAs from the resistance-showing fungi *Candida glabrata* (CgNce103), *Cryptococcus neoformans* (Can2) and *Malasszia globosa* (MgCA) with a series of benzenesulfonamides bearing nitrogenous bases, such as uracil and adenine, is here reported. Many such compounds display low nanomolar (< 100 nM) inhibitory potency against Can2 and CgNce103, whereas the activity of MgCA is considerably less affected (inhibition constants in the range 138.8-5601.5 nM). The β-CAs inhibitory data were compared with those against α-class human ubiquitous isoforms. Interesting selective inhibitory activities for the target fungal CAs over hCA I and II were reported, which make nitrogenous base benzenesulfonamides interesting tools and leads for further investigations in search of new antifungal with innovative mechanisms of action.

Keywords: Carbonic anhydrase, inhibitor, metalloenzymes, adenine, uracil, antifungal.

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Introduction

Anti-microbial resistance is one of the biggest threats to global health and a major concern in clinical practice because it impairs the efficacy of common therapies for infectious diseases, among which fungal infections.[1-4] Invasive fungal infections can cause disability and death. Some species of fungi are naturally resistant to treatment with some antifungal medications. Other species can develop resistance due to improper antifungal use, such as too low dosages or treatment courses that are not long enough.[5] Antifungal resistance is a noteworthy problem with *Candida* infections. Some types of *Candida* are increasingly resistant to the first-line and second-line antifungal medications, such as fluconazole and the echinocandins [6]. Treatment failures against *Cryptococcus neoformans* also continue to occur for a variety of reasons including direct antifungal drug resistance. Cryptococcosis has become a relatively common infection worldwide mainly affecting the lungs, but also responsible for meningitis and encephalitis [7]. *Malassezia globosa* is considered as the most probable causative agent for the onset of dandruff. Many drug-resistant fungal strains of *M. globosa* to azole antifungals have been reported and impair the efficacy of common anti-dandruff cosmetic treatments.[8].

A main strategy to overtake the spread of antimicrobial resistance consists in the identification of novel therapeutic targets and thus anti-infectives with alternative mechanisms of action. The metalloenzymes carbonic anhydrases (CAs, EC 4.2.1.1) are essential in the life cycle (pH homeostasis and biosynthetic reactions) as well as in the virulence of many bacterial, fungal and protozoan pathogens.[9] Many data exist in the literature, which show that interference with CA activity in various parasites leads to an impairment of parasite growth and virulence.[9] CA enzymes are classified into seven structurally unrelated groups, α-, β-, γ-, δ-, ζ- η- and θ-CAs.[10-13] In particular, β-CAs isoforms were identified in many pathogenic fungi, among which *C. glabrata* (CgNce103), *C. neoformans* (Can2) and *M. globosa* (MgCA).[9-11]. Several works demonstrated that these CAs play an important role in the CO_2 sensing of the pathogens and in the regulation of sexual development [9,14-16]. For instance, physiological concentrations of CO_2/HCO_3^- induce prominent virulence attributes in *C. glabrata* or *C. neoformans* through direct activation of the fungal adenylyl cyclase [14,15].

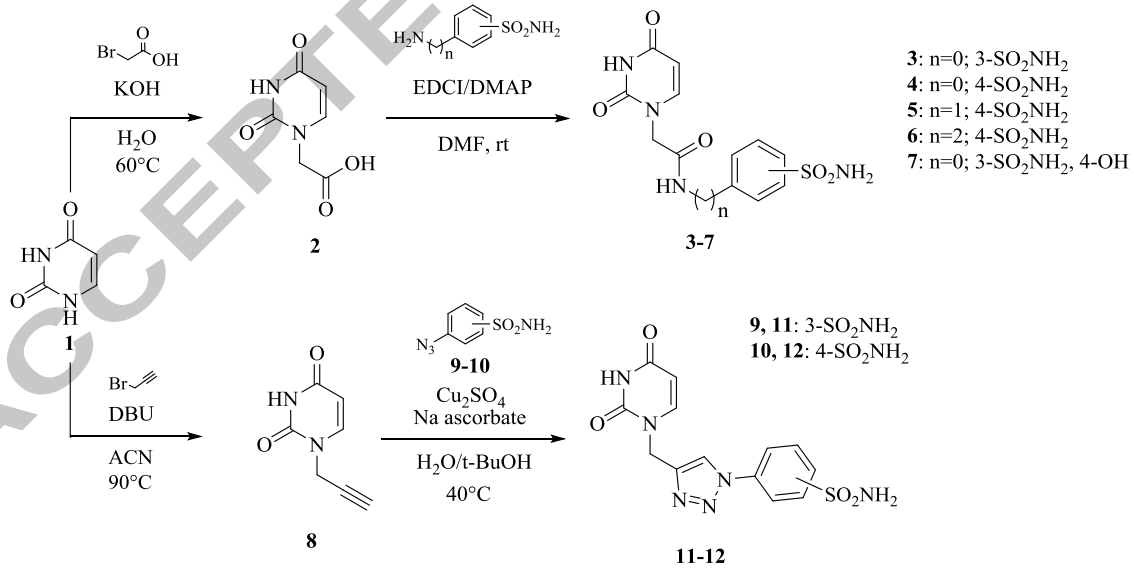
The inhibitory profile against β-CAs of most important classes of CA inhibitors, such as sulfonamides and bioisosteres, inorganic anions, and carboxylate, have been investigated in detail.[16-18] Recently, new chemotypes, such as phenols, dithiocarbamates, and benzoxaboroles were also reported as efficient fungal β-CA inhibitors.[19-25] MgCA inhibition by sulfonamides led to growth defects of the fungus *in vivo*, exhibiting antidandruff effects equivalent to those of the standard azole drug ketoconazole.[16]

Nucleoside antibiotics represent a unique class of antimicrobial products derived from nucleosides and nucleotides.[26] They display diverse biological activities including antibacterial, antifungal, antiviral, insecticidal, immunostimulative, immunosuppressive, and antitumor activities. For all these reasons, we investigate here nitrogenous bases-bearing benzenesulfonamides as inhibitors of CgNce103, Can2 and MgCA. The compounds were designed according to the molecular hybridization approach to strengthen the anti-fungal effect of the pathogen CA inhibition with the anti-microbial activity of nitrogenous bases, such as uracil and adenine, derivatives.

Chemistry

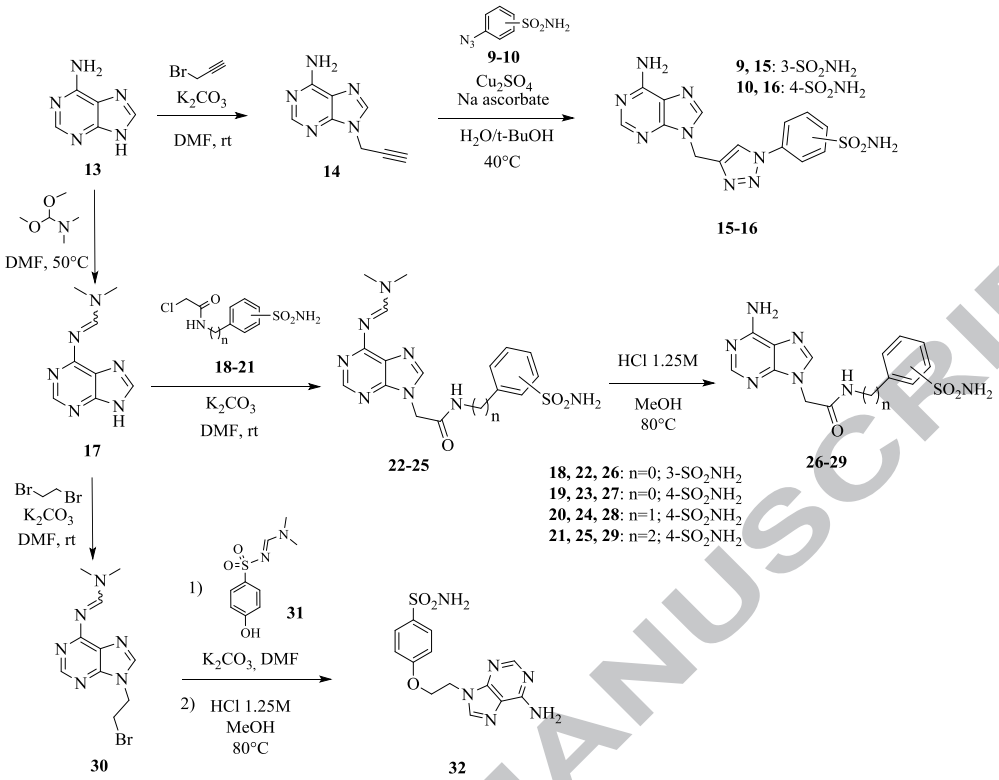
A benzenesulfonamide scaffold was variably appended with uracil and adenine moieties by means of different length spacer of the ether, amide and its bioisoster triazole types, as previously reported by us.[27] The bioisosteric amide/triazole substitution was pursued due to the anti-fungal activity that triazoles and their derivatives were shown to possess.[28] Uracil and adenine were linked to the main scaffold through the N1 and N9 moieties, respectively, to keep the connection by which nitrogenous bases are incorporated in nucleotides and nucleic acids.

The triazole derivatives (**11-12**, **15-16**) were prepared by copper-catalyzed azide−alkyne cycloadditions (CuAAC), using N1-propargyluracil (**8**) or the N9-propargyladenine (**14**) (Schemes 1 and 2).[27]



Scheme 1. General synthetic procedure for uracil derivatives **2-12**.

The amide derivatives (**3-7**, **22-29**) were prepared according to two diverse synthetic pathways depending on the considered nitrogenous base (Schemes 1 and 2).[27]



Scheme 2. General synthetic procedure for adenine derivatives **15-32.**

Carbonic anhydrase inhibition

The CA inhibitory activities of compounds **3-7**, **11-12**, **15-16**, **22-29**, **32**, in addition to acetazolamide (AAZ) as standard inhibitor, were measured against MgCA, Can2, and CgNce103 by a stopped flow CO_2 hydrase assay.[29] The displayed inhibitory activities were compared to those against the off-target widely-distributed hCA I and hCA II. The following structure–activity relationship (SAR) were obtained from the data reported in Table 1.

Table 1. Inhibition data of hCA I, hCA II, and β-CA isoforms MgCA, Can2, and CgNce103, with sulfonamides **3−7**, **11−12**, **15−16**, **22−29**, and **32** and the standard inhibitor acetazolamide (**AAZ**) by a Stopped Flow CO_2 hydrase assay.

Cmpd	K_I^* (nM)				
	MgCA	Can2	CgNce103	hCA I	hCA II
3	1238.6	309.5	383.1	7966.7	704.3
4	391.2	53.2	83.1	645.5	17.7
5	620.3	87.1	146.6	1565.7	495.4
6	349.3	19.3	46.2	658.9	42.1
7	981.8	182.7	304.4	9840.2	4357.7
11	5012.8	750.7	1010.2	4871	388.1
12	718.9	50.4	149.6	256.2	0.85

15	5601.5	1146.8	1126.4	>10000	655.3
16	578.4	124.1	124.6	410.9	5.6
22	1861.3	843.9	950.1	>10000	756.2
23	617.7	143.2	75.3	966.3	30.7
24	862.2	108.6	180.6	4721.4	87
25	153.9	16.8	31.9	1962.6	57.8
26	1474.6	660.2	460.5	>10000	889.2
27	699.3	49.8	94.6	666.6	8.4
28	908.7	117.5	174.7	977.1	59.2
29	190.2	9.1	56.9	811.8	8.2
32	138.8	27.5	87.3	552.6	78
AAZ	74000	10	11	250	12

* Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5-10 % of the reported values).

i. The inhibitory capability of derivatives **3-7**, **11-12**, **15-16**, **22-29**, **32** strongly depends on the length and kind of the spacer and the type of nitrogenous base for both the three fungal isozymes and human isoforms. Most of the adenine- and uracil-bearing compounds reported a moderate inhibitory potency against the fungal isoform MgCA, with K_Is ranging between 138.8 and 5012.8 nM. Adenine compounds **32**, **25** and **29** reported the best inhibitory activity with K_Is value of 138.8, 153.9 and 190.2 nM, respectively. These compounds share the longest linkers between the inhibitory scaffold and the amide portion or the adenine core. The free NH_2 confers to **29** better inhibition activity than the protected-amine **25**. The length of the linker between main scaffold and nitrogenous base has minor influence on the inhibition in case of uracile compounds. For instance, the uracile derivatives **4** and **6** report very similar K_Is (391.2 and 349.3 nM, respectively), although **4** lacks the ethyl spacer present in compound **6.** The minor steric hindrance represented by uracil compared to adenine likely justifies this effect.

   A remarkable fall of efficacy is observed by switching any substituent on the benzenesulfonamide from the *para* to the *meta* position. Compound **3** is 3.2 time less potent (K_I value 1238.6 nM) than its analog **4**. Similar trends are evident from data in Table 1 for the remaining *meta*-derivatives **7**, **11**, **15**, **22** and **26** which report the worst inhibitory activities, with K_Is ranging between 981.8 and 5012.8 nM.

ii. Can2 is the most inhibited isoform by the sulfonamides herein reported. Compounds **4**, **5**, **6**, **12**, **25**, **27**, **29** and **32** report K_I values even below 100 nM (9.1–87.1 nM). In this case the length of the linker has a major influence on the inhibitory efficacy compared to the type of nitrogenous base and elicits analog trends for both uracil and adenine compounds. In detail, a methylene linker between the amide and benzenesulfonamide ring (**5**, **24**, **28**) ascribes to the

derivative a worse Can2 inhibition efficacy than the absence of a linker (**4**, **23**, **27**), that is in turn less effective than a two-carbon atoms spacer (**6**, **25**, **29**).

Likewise, against MgCA, *meta*-substituted compounds (**3**, **7**, **11**, **15**, **22**, **26**) reported the worst K_I compared to *para*-substituted ones. The bioisosteric substitution of the amide linker with a bulkier triazole one lowers the inhibitory efficacy of both *meta*- (**11** and **15**) and *para*-substituted (**12** and **16**, K_Is of 50.4 and 124.1 nM) derivatives compared to analog amides devoid of the alkyl spacer. As a result, uracil **6** and adenine **25** and **29** stand out as the best Can2 inhibitors herein reported (K_Is of 19.3, 16.8, 9.1 nM, respectively). The introduction of *p*-OH group on the benzenesulfonamide ring positively impacts the inhibitory activity of the compound **7** (K_I value of 182.7 nM) with respect to derivative **3** (K_I of 309.5 nM).

iii. The isoform CgNce103 is intermediately inhibited by the reported sulfonamides with K_Is in the range 31.9-1126.4 nM. The general tendency described for Can2 can be applied for this isoform too. The *meta*-substituted derivatives are again the worst inhibitors with K_I values in the range 304.4-1126.4 nM. Uracil **6** (K_I of 46.2 nM) and adenine **25** (K_I of 31.90 nM), both appending the ethylene linker, showed the best CgNce103 inhibitory potencies herein reported. Conversely, the introduction of methylene spacer between main scaffold and connection reduces the inhibitory potency of **5** (K_I = 146.6 nM), **24** (K_I = 180.6 nM) and **28** (K_I = 174.7 nM) in comparison to both aliphatic linker-devoid and and ethylene bearing derivatives.

iv. The data in Table 1 outline interesting target/off-target CAs selectivity profiles. First, MgCA is inhibited in a comparable range with the ubiquitous isoform hCA I, but rather less than the other human isoform hCA II. Conversely, all sulfonamides more effectively inhibit the β-CAs Can2 and CgNce103 than hCA I, and some such compounds even than hCA II, though to a minor extent. In detail, the *m*-substituted uracil compounds (amides) **3** and **7** show selectivity index (SI) of two to twenty for the target Can2/CgNce103 over hCA II. Compounds **5** and **6** inhibit Can2 two- to five-fold better than the human isozyme. Adenines **25** and **32** show a Can2/hCA II SI of 3. All triazole derivatives more efficiently inhibit hCA II than all β-CAs herein evaluated.

Conclusions

A main strategy to overtake the spread of antifungal resistance consists in the identification of novel therapeutic targets and anti-infectives with alternative mechanisms of action. Carbonic anhydrases were shown to be essential in the life cycle (pH homeostasis and biosynthetic reactions) as well as in the virulence of many bacterial, fungal and protozoan pathogens. In particular, the inhibition of

the β-CAs identified in many pathogenic fungi leads to an impairment of parasite growth and virulence, which in turn leads to a significant anti-infective effect. Based on antifungal antibiotics incorporating nucleosides, we reported here the inhibition study of β-CAs from the resistance-showing fungi *Candida glabrata* (CgNce103), *Cryptococcus neoformans* (Can2) and *Malasszia globosa* (MgCA) with a series of benzenesulfonamides bearing nitrogenous bases. Many such compounds display excellent low nanomolar inhibitory potency against Can2 and CgNce103, whereas the activity of MgCA is considerably less affected (inhibition constants in the range 138.8-5601.5 nM). The inhibition profiles were shown to depend on the length and positioning of the spacer which connects the two pharmacophores. The β-CAs inhibitory data were compared with those against the off-target hCA I and II. Interesting target/off-target selective efficacies were reported, which make nitrogenous base benzenesulfonamides interesting leads for further studies in search of new antifungal with innovative mechanisms of action.

Experimental sections

Chemistry

The synthesis of sulfonamides **3−7**, **11−12**, **15−16**, **22−29**, and **32** was reported earlier by our group.[27]

Carbonic Anhydrase Inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalysed CO_2 hydration activity.[29] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20mM Hepes (pH 7.5) as buffer, and 20mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalysed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17mM 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-deionised water and dilutions up to 0.01nM were done thereafter with the assay buffer. 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 nonlinear least-squares methods using PRISM 3 and the Cheng-Prusoff equation, as reported earlier, and represent the

mean from at least three different determinations.[30-33] All CA isoforms were recombinant ones obtained in-house as reported earlier.[34-36].

Acknowledgments. This work was financed in part by a DSFP project to Profs. Z. AlOthman and CT Supuran.

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Silvia Bua, Sameh M. Osman, Zeid AlOthman, Claudiu T. Supuran*, Alessio Nocentini*

- There is an great need for new antifungal agents to treat resistant infections.
- Inhibition of β-CAs from resistance-showing fungi was assessed with sulfonamides.
- Comparison was made with α-class human ubiquitous isoforms.
- Benzenesulfonamides bearing nitrogenous bases showed potency and selectivity.

Benzenesulfonamides incorporating nitrogenous bases show effective inhibition of β-carbonic anhydrases from the pathogenic fungi *Cryptococcus neoformans*, *Candida glabrata* and *Malassezia globosa*

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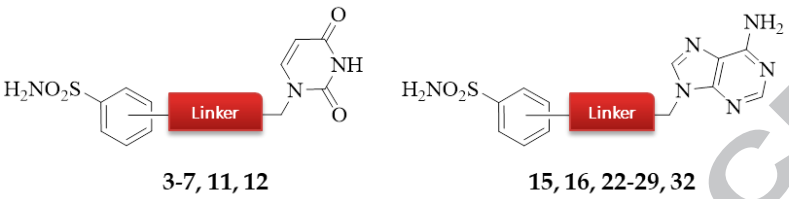

3-7, 11, 12 **15, 16, 22-29, 32**

Fungal β-CAs Inhibition

K_I MgCA 138.8-5601.5 nM
K_I Can2 9.1-1146.8 nM
K_I CgNce103 31.9-1126.4 nM