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Synthesis and biological evaluation of cyclic imides incorporating benzenesulfonamide moieties as carbonic anhydrase I, II, IV and IX inhibitors

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ABSTRACT

A group of cyclic imides was synthesized by reaction of amino-substituted benzenesulfonamides with a series of acid anhydrides such as succinic, maleic, tetrahydrophthalic, pyrazine-2,3-dicarboxylic acid anhydride, and substituted phthalic anhydrides. The synthesized sulfonamides were evaluated as carbonic anhydrase (CA, EC 4.2.1.1) inhibitors against the human (h) isoforms hCA I, II, IV and IX, involved in a variety of diseases among which glaucoma, retinitis pigmentosa, etc. Some of these sulfonamides showed effective inhibitory action (in the nanomolar range) against the cytosolic isoform hCA II and the transmembrane, tumor-associated one hCA IX, making them interesting candidates for preclinical evaluation in glaucoma or various tumors in which the two enzymes are involved. hCA I and IV were on the other hand less inhibited by these sulfonamides, with inhibition constants in the micromolar range.

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1. Introduction

In previous works from these and other groups,¹⁻¹¹ the preparation and biological activity of cyclic imides incorporating primary sulfonamide moieties were investigated. Phthalimides were definitely the privileged such scaffolds, being shown that they may lead to effective inhibitors of the metallo-enzyme carbonic anhydrase (CA, EC 4.2.1.1).^{2,8-11} Furthermore, some substituted-phthalimides derived from aromatic sulfonamides such as sulfanilamide, metanilamide or aminoalkyl-benzenesulfonamides, not only showed low nanomolar activity as CA inhibitors (CAIs), but some of them were also highly isoform-selective inhibitors for several CAs involved in relevant pathologies such as glaucoma,¹² epilepsy,¹³ obesity,¹⁴ or cancer.¹⁵ Indeed, of the 15 different human(h) CA isoforms known to date, many are involved in such and other pathologies,¹⁶ and finding isoform-selective inhibitors

is rather challenging, although many such compounds belonging to various classes of inhibitors were reported by now to possess such properties.¹⁷ Sulfonamides are in clinical use as CAIs for more than 70 years, primarily as antiglaucoma agents or diuretics.¹²⁻¹⁷

Continuing our interest in sulfonamide CAIs, in this work we report the synthesis and human (h) hCA I, II, IV and IX inhibitory activity of cyclic imides obtained from amino-substituted benzenesulfonamides and a series of acid anhydrides such as succinic, maleic, tetrahydrophthalic, pyrazine-2,3-dicarboxylic acid anhydride, and substituted phthalic anhydrides.

2. Results and discussion

2.1. Drug design and chemistry

The lead molecules for designing the CAIs synthesized here were the sulfanilamides incorporating phthalimides moieties reported earlier by one of these groups.^{2,9-11} We have shown that reaction of amino-substituted benzenesulfonamides, such as sulfanilamide, 4-aminomethylbenzenesulfonamide or 4-aminoethylbenzenesulfonamide with phthalic anhydride or substituted-phthalic anhydrides leads to the corresponding phthalimides derivatives in

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high yields.^{2,9-11} Furthermore, the new sulfonamides showed effective inhibitory action against human CA isoforms I, II, IV and IX.^{2,9-11}

In the present work we have included diverse anhydrides for the preparation of the imides, among which succinic, maleic, tetrahydrophthalic, pyrazine-2,3-dicarboxylic acid anhydride, substituted phthalic anhydrides and norbornene dicarboxylic anhydride. In this way, a wider range of chemical space is being explored in order to investigate the effects of the cyclic imide scaffold on the biological activity of these new sulfonamides.

The preparation of designed imides analogues **1-15** is shown in Schemes 1 and 2. Condensation of acid anhydrides with 4-(2-aminoethyl)benzenesulfonamide in refluxing acetic acid afforded imides **1-12** in good yields (Scheme 1).¹⁻⁶

The remaining compounds, **13-15** (Scheme 2) were prepared in a similar manner, but in this case the norbornene dicarboxylic anhydride has been employed, leading to the tricyclic sulfonamides **13-15**. Such scaffolds were not employed earlier for obtaining CAIs.

Compounds **1-15** have been characterized in detail by physico-chemical methods which confirmed their structures (see Experimental for details).

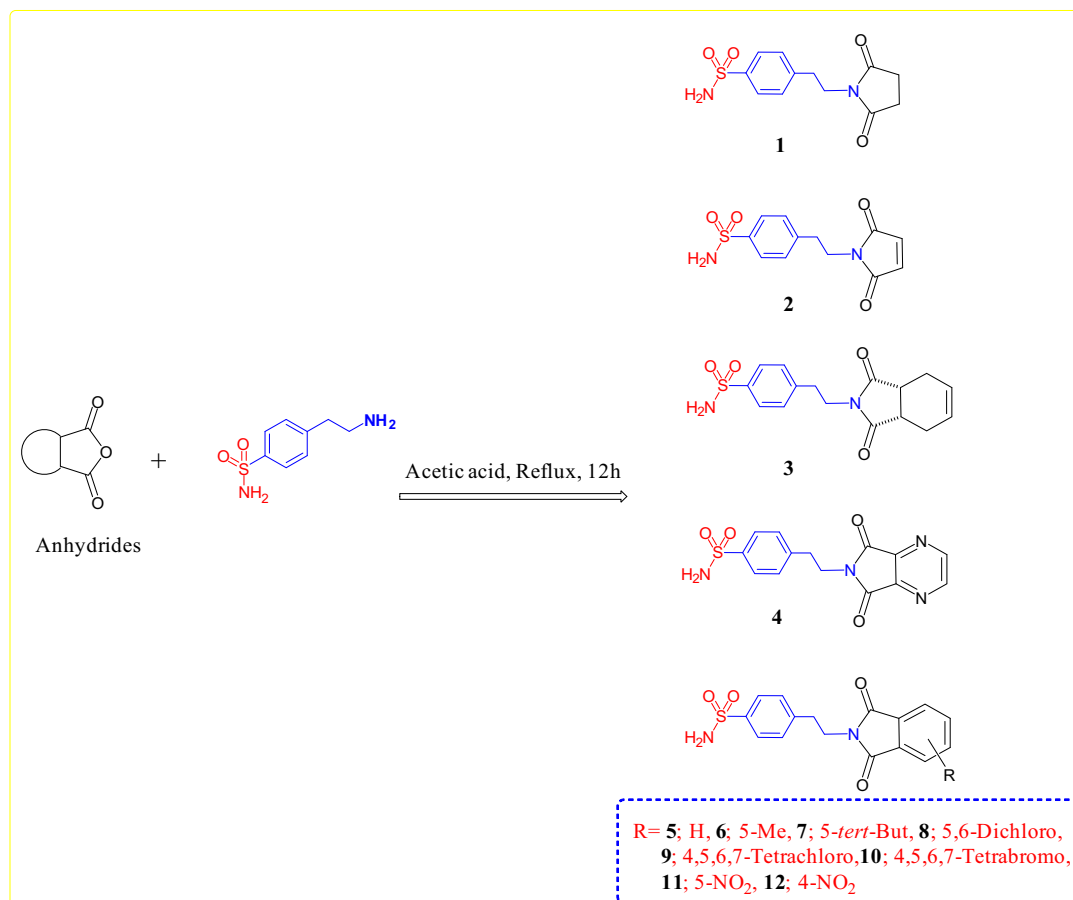
2.2. Carbonic anhydrase inhibition

The newly synthesized compounds **1-15** were screened for their CA inhibitory activity by a stopped-flow CO₂ hydrase assay¹⁸ against the following isoforms: hCA I, II (cytosolic isoforms involved in glaucoma¹³ and possibly other pathologic states)¹⁹ and hCA IV (an isoform involved in glaucoma and other eye

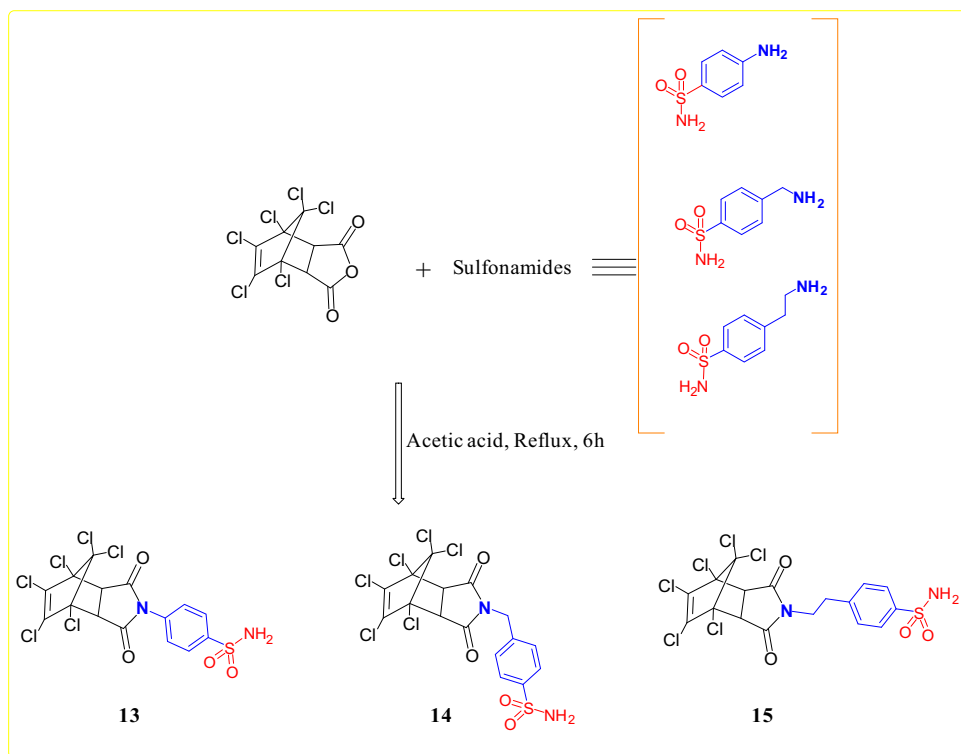
diseases, such as retinitis pigmentosa),²⁰ as well as the transmembrane, tumor-associated hCA IX^{15,21} see Table 1.

The following structure-activity relationships (SARs) are observed and reported for these series of compounds:

- (i) The slow cytosolic isoform hCA I was inhibited by all the investigated compounds with inhibition constants ranging between 49.2 and 5121 nM. Sulfonamides incorporating tetrahydrophthalimido and *tert*-butyl phthalimido moieties **3** and **7** showed the best inhibitory activity (K_i of 49.2 and 95.7 nM, respectively). The other scaffolds were less potent and inhibited this isoform in the high nanomolar range (K_i ranging between 179 and 865 nM). An interesting case was constituted by the isosteric substitution of the chlorine atoms in the phthalimido compounds **9** with bromine, in **10**, which led to a significant decrease of the inhibitory potency. Considering the monocyclic imides **1, 2** and the bicyclic scaffold **3-15**, the inhibition potency against the hCA I did not vary significantly, except for compound **3**, which was the best inhibitor in the subseries. The linker between the hexachloronorbornene dicarboxylic scaffold and the sulfonamide part of the molecules present in **13, 14** and **15** also modulated the inhibition profile. The introduction of a methylene chain, as in **14**, increased the inhibition potency from 390 nM (**13**) to 201 nM (**14**), but a further chain elongation, as in **15**, decreased two times the K_i for the last compound.
- (ii) hCA II, the dominant physiologic isoform, was inhibited by all compounds in the low to high nanomolar range, with K_i s of 5-693 nM. The tetrahydrophthalimido derivative **3**



Scheme 1. Synthesis of the designed cyclic imide derivatives **1-12**.



Scheme 2. Synthesis of the designed hexachloro norbornene dicarboxylic imides derivatives **13–15**.

Table 1

In vitro CA I, II, IV and IX inhibition with compounds **1–15** and acetazolamide as standard, by a stopped-flow, CO₂ hydrase assay.¹⁸

Compound	K _i (nM) ^a			
	hCA I	hCA II	hCA IV	hCA IX
1	446	73.6	9578	296
2	617	84.5	4849	274
3	49.2	5.0	973	296
4	441	426	9380	4188
5	491	49.4	5461	296
6	425	60.5	2925	390
7	95.7	89.9	9391	459
8	435	79.0	9358	428
9	562	68.1	9232	284
10	5121	693	8839	2389
11	865	140	8175	1636
12	179	85.4	8517	2667
13	390	41.5	83.0	13.7
14	201	54.6	912	16.9
15	484	23.2	6764	113.6
AAZ	250	12.1	74	25.2

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

showed a very good inhibition activity against this isoform, with a K_i of 5 nM. The replacement of the monocyclic imido scaffold with a bicyclic one did not have a significant effect on the inhibition profiles of these sulfonamides (Table 1). Only in the case of the pyrazine containing sulfonamide **4**, a decreased inhibitory potency was observed compared to the other members of the series, this compound being one of the weakest hCA II inhibitor (K_i of 426 nM). The substitution on the phthalimido scaffold in compounds **5–12** did not lead to significant changes in the inhibition potency, except for the tetrabromo-substituted compound **10**, which was a medium potency – weak hCA II inhibitor (K_i 693 nM). Furthermore, the substitution of the nitro group from position 5 of compound **11** to position 4 (**12**), increased nearly two

times the potency of the corresponding sulfonamide against this. Unlike the other cytosolic isoform, the introduction of a linker in the norborneneimido derivatives **13–15** had diverse effects on the inhibition profile: a decrease of the potency was observed for a methylene linker (**13**, a K_i of 41.5 nM whereas **14** had a K_i of 54.6 nM), whereas a further chain elongation, as in **15**, led to an increase of the potency with a K_i of 23.2 nM for the last compound.

(iii) The membrane-bound hCA IV was inhibited by most of compounds investigated here in the micromolar range. The monocyclic imido derivatives **1** and **2** showed K_is in the range of 4.85–9.57 μM. The maleimido scaffold present in **3** led to a compound which was two times more potent than the succinimido analog **1**. Also for this isoform, the groups

present on the phthalimido scaffold did not influence significantly the inhibition profile. The compounds incorporating hexachloronorbornene dicarboxylic scaffolds, **13–15**, showed a very interesting inhibition profile. The linker between the imido scaffold and the sulfonamide head was crucial for the inhibition potency. A direct correlation between the length of the linker and the inhibition constant was observed: **13** showed a nanomolar inhibition constant (K_i 83 nM), whereas an increase in the linker length to a methylene or an ethylene group led to a drastic decrease of potency, to the micromolar range (K_i of 912 nM for compound **14** and K_i of 6764 nM for **15**).

- (iv) hCA IX, the tumor-associated isoform, was effectively inhibited by many of the compounds reported here, in high nanomolar range except for compounds **13** and **14** which were active in the low nanomolar range. The monocyclic imido derivatives **1** and **2** had nearly the same inhibition profile with a K_i of 296 nM and 274 nM, respectively. The phthalimido scaffold present in **5–12** led to high nanomolar hCA IX inhibitors, except for nitro-substituted compounds **11** and **12**. For these compounds the potency has decreased to the micromolar range, with K_i of 1636 nM and 2667 nM, respectively. Moreover, the hexachloro-norbornene scaffold present in **13–15**, led to an interesting SAR for the inhibition of this isoform. Compounds **13** and **14** incorporating no linker or a methylene linker, exhibited low nanomolar K_i s whereas the derivative with a longer chain **15**, as for isoform hCA IV, led to a marked decreased in the inhibition potency (Table 1).

3. Conclusions

We investigated a series of cyclic imides as CA inhibitors, continuing our research in this field of enzyme modulators. The compounds were synthesized by reaction of amino-substituted benzenesulfonamides with a series of dicarboxylic acid anhydrides such as succinic, maleic, tetrahydrophthalic, pyrazine-2,3-dicarboxylic acid anhydride, as well as substituted phthalic anhydrides. The synthesized sulfonamides were evaluated for the inhibition of isoforms hCA I, II, IV and IX, involved in a variety of diseases among which glaucoma, retinitis pigmentosa, tumors, etc. They showed effective inhibitory action against the cytosolic isoform hCA II and the transmembrane, tumor-associated one hCA IX, making them interesting candidates for preclinical evaluation in glaucoma or various tumors in which the two enzymes are involved. hCA I and IV were on the other hand less inhibited by these sulfonamides.

4. Experimental part

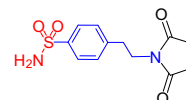
4.1. Chemistry

Melting points (uncorrected) were recorded on Barnstead 9100 Electrothermal melting apparatus. IR spectra were recorded on a FT-IR Perkin-Elmer spectrometer. ^1H NMR and ^{13}C NMR were recorded in DMSO- d_6 on Bruker 500 and 125 MHz instrument, respectively, using TMS as internal standard (chemical shifts in δ ppm). Mass spectra were recorded on a Agilent 6320 Ion Trap mass spectrometers. Elemental analysis was carried out for C, H and N at the Research Centre of College of Pharmacy, King Saud University and the results are within $\pm 0.4\%$ of the theoretical values. Compounds **2**, **5**, **9**, **10** and **11** were prepared according to their reported procedure.^{7–11}

4.1.1. General procedure for the synthesis of cyclic imides **1**, **3**, **4**, **6–8**, **12**

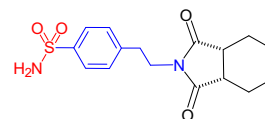
A mixture of 4-(2-aminoethyl)benzenesulfonamide (0.5 g, 2.5 mmol), anhydrous sodium acetate (0.41 g, 5.0 mmol) and an acid anhydride (2.5 mmol) was stirred in glacial acetic acid (15 mL). The mixture was heated under reflux for 12 h and the reaction was monitored with the help of TLC. After completion of reaction the solvent was removed under reduced pressure and the crude solid obtained was washed with water, dried and re-crystallised from an appropriate solvent.

4.1.1.1. 4-(2-(2,5-Dioxopyrrolidin-1-yl)ethyl)benzenesulfonamide (**1**).



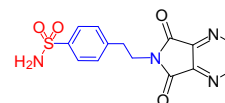
White crystals, M.p. 243–245°, 95% yield (CH₃OH); IR (KBr, cm^{-1}): 3496 (NH), 1795, 1725 (C=O), 1382, 1194 (O=S=O); ^1H NMR (500 MHz, CDCl₃/DMSO- d_6): δ 2.60 (s, 4H), 2.85–2.88 (t, 2H, $J = 7.5$ Hz), 3.60–3.63 (t, 2H, $J = 7.5$ Hz), 7.28 (s, 1H), 7.35–7.37 (d, 2H, $J = 8.0$ Hz), 7.74–7.76 (d, 2H, $J = 8.0$ Hz), 8.22–8.23 (t, 1H, $J = 1.5$ Hz); ^{13}C NMR (125 MHz, CDCl₃/DMSO- d_6): δ 28.37, 33.15, 39.21, 126.28, 129.47, 142.76, 142.78, 177.74; C₁₂H₁₄N₂O₄S: m/z (281.99).

4.1.1.2. 4-(2-(1,3-Dioxo-1,3,3a,4,7,7a-hexahydro-2H-isoindol-2-yl)ethyl)benzenesulfonamide (**3**).



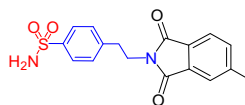
White crystals, M.p. 196–198°, 91% yield (CH₃OH); IR (KBr, cm^{-1}): 3230 (NH), 1767, 1699 (C=O), 1333, 1179 (O=S=O); ^1H NMR (500 MHz, CDCl₃/DMSO- d_6): δ 2.11–2.15 (m, 2H), 2.34–2.37 (d, 2H, $J = 13.5$ Hz), 2.83–2.86 (t, 2H, $J = 7.5$ Hz), 3.04–3.05 (t, 2H, $J = 3.0$ Hz), 3.61–3.64 (t, 2H, $J = 7.0$ Hz), 5.73–5.77 (m, 2H), 7.21 (s, 2H), 7.27–7.29 (d, 2H, $J = 8.0$ Hz), 7.72–7.74 (d, 2H, $J = 8.5$ Hz); ^{13}C NMR (125 MHz, CDCl₃/DMSO- d_6): δ 25.33, 33.09, 38.86, 42.74, 127.08, 129.33, 142.30, 142.42, 142.78, 179.94; C₁₆H₁₈N₂O₄S: m/z (333.70).

4.1.1.3. 4-(2-(5,7-Dioxo-5,7-dihydro-6H-pyrrolo[3,4-b]pyrazin-6-yl)ethyl)benzenesulfonamide (**4**).



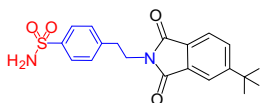
Gray powder, M.p. 120–122°, 76% yield (CH₃OH/CH₂Cl₂); IR (KBr, cm^{-1}): 3430 (NH), 1782, 1707 (C=O), 1318, 1129 (O=S=O); ^1H NMR (500 MHz, DMSO- d_6): δ 2.76–2.79 (t, 2H, $J = 7.0$ Hz), 3.27–3.03 (t, 2H, $J = 7.0$ Hz), 7.38–7.41 (t, 3H, $J = 8.0$ Hz), 7.74–7.75 (d, 3H, $J = 8.5$ Hz), 8.01–8.03 (t, 1H, $J = 5.0$ Hz), 9.11 (s, 1H); ^{13}C NMR (125 MHz, DMSO- d_6): δ 35.28, 38.65, 126.13, 129.54, 142.47, 144.22, 149.01, 169.77; C₁₄H₁₂N₄O₄S: m/z (331.76).

4.1.1.4. 4-(2-(5-Methyl-1,3-dioxoisindolin-2-yl)ethyl)benzenesulfonamide (6).



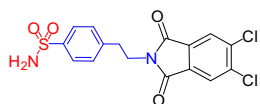
White crystals, Mp 221–222°, 93% yield (CH₃OH/CH₂Cl₂); IR (KBr, cm⁻¹): 3466 (NH), 1767, 1722 (C=O), 1300, 1131 (O=S=O); ¹H NMR (500 MHz, CDCl₃/DMSO-*d*₆): δ 2.43–2.45 (d, 3H, *J* = 9.0 Hz), 2.96–2.99 (t, 2H, *J* = 7.0 Hz), 3.79–3.84 (q, 2H, *J* = 8.0, 7.0 Hz), 6.97 (s, 2H), 7.26–7.00 (t, 2H, *J* = 8.5 Hz), 7.46–7.49 (t, 1H, *J* = 7.5 Hz), 7.53–7.54 (d, 1H, *J* = 7.5 Hz), 7.60–7.63 (t, 1H, *J* = 7.5 Hz), 7.71–7.74 (t, 2H, *J* = 7.5 Hz); ¹³C NMR (125 MHz, CDCl₃/DMSO-*d*₆): δ 22.00, 34.19, 38.62, 123.17, 123.80, 126.30, 129.19, 132.20, 134.76, 142.41, 142.50, 145.43, 167.91, 168.02; C₁₇H₁₆N₂O₄S; *m/z* (344.07).

4.1.1.5. 4-(2-(5-(*tert*-Butyl)-1,3-dioxoisindolin-2-yl)ethyl)benzenesulfonamide (7).



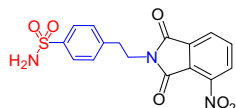
White powder, M.p. 160–162°, 89% yield (CH₃OH/CH₂Cl₂); IR (KBr, cm⁻¹): 3493, 3095 (NH), 1780, 1727 (C=O), 1360, 1236 (O=S=O); ¹H NMR (500 MHz, DMSO-*d*₆): δ 1.32 (s, 9H), 2.96–2.99 (t, 2H, *J* = 7.0 Hz), 3.82–3.85 (t, 2H, *J* = 7.5 Hz), 6.94 (s, 2H), 7.28–7.30 (d, 2H, *J* = 8.0 Hz), 7.69–7.70 (t, 2H, *J* = 7.0 Hz), 7.73–7.55 (t, 3H, *J* = 8.5 Hz); ¹³C NMR (125 MHz, DMSO-*d*₆): δ 31.17, 34.22, 35.73, 38.61, 120.30, 123.13, 126.32, 129.18, 131.22, 132.07, 142.39, 142.46, 158.56, 167.88, 168.26; C₂₀H₂₂N₂O₄S; *m/z* (387.15).

4.1.1.6. 4-(2-(5,6-Dichloro-1,3-dioxoisindolin-2-yl)ethyl)benzenesulfonamide (8).



White crystals, Mp 295–296°, 91% yield (CH₃OH); IR (KBr, cm⁻¹): 3469, 3369 (NH), 1727, 1670 (C=O), 1366, 1167 (O=S=O); ¹H NMR (500 MHz, DMSO-*d*₆): δ 2.99–3.02 (t, 2H, *J* = 7.5 Hz), 3.83–3.86 (t, 2H, *J* = 7.5 Hz), 7.31 (s, 2H), 7.40–7.42 (d, 2H, *J* = 8.0 Hz), 7.71–7.73 (t, 2H, *J* = 8.5 Hz), 8.15–8.16 (t, 2H, *J* = 8.5 Hz); ¹³C NMR (125 MHz, DMSO-*d*₆): δ 33.78, 39.34, 125.74, 126.26, 129.67, 131.88, 137.81, 142.80, 166.32; C₁₆H₁₂Cl₂N₂O₄S; *m/z* (398.60).

4.1.1.7. 4-(2-(4-Nitro-1,3-dioxoisindolin-2-yl)ethyl)benzenesulfonamide (12).

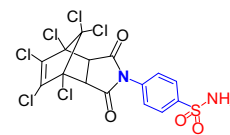


Yellow powder, Mp 238–240°, 86% yield (CH₃OH/CH₂Cl₂); IR (KBr, cm⁻¹): 3485 (NH), 1779, 1732 (C=O), 1362, 1228 (O=S=O); ¹H NMR (500 MHz, DMSO-*d*₆): δ 2.99–3.02 (t, 2H, *J* = 6.0 Hz), 3.83–3.86 (t, 2H, *J* = 7.5 Hz), 7.32 (s, 2H), 7.44–7.45 (d, 2H, *J* = 8.5 Hz), 7.73–7.75 (t, 2H, *J* = 6.0 Hz), 8.05–8.07 (t, 1H, *J* = 5.0 Hz), 8.15–8.16 (t, 1H, *J* = 5.5 Hz), 8.28–8.29 (t, 1H, *J* = 5.0 Hz); ¹³C NMR (125 MHz, DMSO-*d*₆): δ 33.72, 39.44, 123.44, 126.29, 127.32, 128.80, 129.65, 133.92, 136.70, 142.82, 142.89, 144.76, 163.65, 166.19; C₁₆H₁₃N₃O₆S; *m/z* (374.54).

4.1.2. General procedure for the synthesis of cyclic imides 13–15

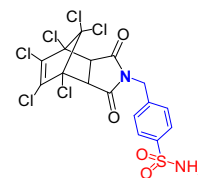
A mixture of benzenesulfonamide (2.5 mmol), anhydrous sodium acetate (0.41 g, 5.0 mmol) and 4,5,6,7,8,8-hexachloro-3a,4,7,7a-tetrahydro-4,7-methanoisobenzofuran-1,3-dione (0.927 g, 2.5 mmol) were stirred in glacial acetic acid (15 mL) and heated under reflux for 6 h. After the evaporation of the solvent under reduced pressure, the precipitate obtained was washed with water, dried and re-crystallised from an appropriate solvent.

4.1.2.1. 4-(4,5,6,7,8,8-Hexachloro-1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2H-4,7-methanoisindol-2-yl)benzenesulfonamide (13).



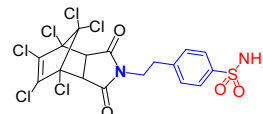
White powder, M.p. > 350°, 95% yield (CH₃OH); IR (KBr, cm⁻¹): 3370, 3250 (NH), 1769, 1708 (C=O), 1388, 1144 (O=S=O); ¹H NMR (500 MHz, DMSO-*d*₆): δ 4.28 (s, 2H), 7.34–7.35 (d, 2H, *J* = 8.5 Hz), 7.52 (s, 2H), 8.00–8.01 (d, 2H, *J* = 8.5 Hz); ¹³C NMR (125 MHz, DMSO-*d*₆): δ 52.70, 79.44, 104.15, 127.52, 131.12, 134.07, 145.07, 169.85; C₁₅H₈Cl₆N₂O₄S; *m/z* (526.43).

4.1.2.2. 4-((4,5,6,7,8,8-Hexachloro-1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2H-4,7-methanoisindol-2-yl)methyl)benzenesulfonamide (14).



White powder, M.p. 201–203°, 87% yield (CH₃OH/CH₂Cl₂); IR (KBr, cm⁻¹): 3307, 3229 (NH), 1759, 1711 (C=O), 1369, 1180 (O=S=O); ¹H NMR (500 MHz, CDCl₃/DMSO-*d*₆): δ 3.95 (s, 2H), 4.52 (s, 2H), 6.89 (s, 2H), 7.36–7.38 (d, 2H, *J* = 8.5 Hz), 7.77–7.78 (d, 2H, *J* = 8.0 Hz); ¹³C NMR (125 MHz, CDCl₃/DMSO-*d*₆): δ 42.49, 52.09, 79.43, 104.37, 126.39, 129.74, 130.58, 138.23, 143.87, 170.05; C₁₆H₁₀Cl₆N₂O₄S; *m/z* (539.50).

4.1.2.3. 4-(2-(4,5,6,7,8,8-Hexachloro-1,3-dioxo-1,3,3a,4,7,7a-hexahydro-2H-4,7-methanoisindol-2-yl)ethyl)benzenesulfonamide (15).



White powder, M.p. 226–228°, 83% yield (CH₃OH/CH₂Cl₂); IR (KBr, cm⁻¹) ν : 3323, 3213 (NH), 1751, 1714 (C=O), 1373, 1165 (O=S=O); ¹H NMR (500 MHz, CDCl₃/DMSO-*d*₆): δ 2.71–2.74 (t, 2H, *J* = 10.0 Hz), 3.55–3.58 (q, 2H, *J* = 10.0, 5.5 Hz), 4.01–4.02 (d, 2H, *J* = 3.5 Hz), 7.17 (s, 2H), 7.33–7.34 (t, 2H, *J* = 4.0 Hz), 7.76–7.78 (q, 2H, *J* = 8.5, 4.5 Hz); ¹³C NMR (125 MHz, CDCl₃/DMSO-*d*₆): δ 33.42, 39.60, 52.17, 79.26, 104.36, 126.41, 129.20, 130.71, 141.39, 143.04, 170.38; C₁₇H₁₂Cl₆N₂O₄S: *m/z* (552.19).

4.2. Carbonic anhydrase inhibition

An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic/inhibition of various CA isozymes.¹⁸ Phenol Red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.4) as buffer, 0.1 M Na₂SO₄ or NaClO₄ (for maintaining constant the ionic strength; these anions are not inhibitory in the used concentration),²² following the CA-catalyzed CO₂ hydration reaction for a period of 5–10 s. Saturated CO₂ solutions in water at 25 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10 mM (in DMSO-water 1:1, v/v) and dilutions up to 0.01 nM done with the assay buffer mentioned above. At least 7 different inhibitor concentrations have been used for measuring the inhibition constant. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. The inhibition constants were obtained by non-linear least-squares methods using the Cheng-Prusoff equation, as reported earlier,¹¹ and represent the mean from at least three different determinations. All CA isozymes used here were recombinant proteins obtained as reported earlier by our group.^{23–26}

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