A Class of 4-Sulfamoylphenyl-ω-aminoalkyl Ethers with Effective Carbonic Anhydrase Inhibitory Action and Antiglaucoma Effects

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ABSTRACT: We report a series of 4-sulfamoylphenyl-ω-aminoalkyl ethers as carbonic anhydrase (CA, EC 4.2.1.1) inhibitors. The structure–activity relationship was drawn for the inhibition of four physiologically relevant isoforms: hCA I, II, IX, and XII. Many of these compounds were highly effective, low nanomolar inhibitors of all CA isoforms, whereas several isoform-selective were also identified. X-ray crystal structures of two new sulfonamides bound to the physiologically dominant CA II isoform showed the tails of these derivatives bound within the hydrophobic half of the enzyme active site through van der Waals contacts with Val135, Leu198, Leu204, Trp209, Pro201, and Pro202 amino acids. One of the highly water-soluble compound (as trifluoroacetate salt) showed effective IOP lowering properties in an animal model of glaucoma. Several fluorescent sulfonamides incorporating either the fluorescein-thiourea (7a–c) or tetrathiomylrhodamine-thiourea (9a,b) moieties were also obtained and showed interesting CA inhibitory properties for the tumor-associated isoforms CA IX and XII.

INTRODUCTION

Carbon dioxide (CO₂) is a very stable form of carbon, the central element of life on this planet and one of the simplest molecules that was probably highly abundant in the primordial earth atmosphere. This gas reacts with water, leading to H₂CO₃, which is an unstable compound that is spontaneously transformed into bicarbonate and protons. However, the reaction between CO₂ and water is particularly slow at pH values of 7.5 or lower, which is usually the physiologic pH value in many tissues and organisms. Carbon dioxide hydration becomes, on the other hand, very effective at higher pH values, being instantaneous at pH > 12. Moreover CO₂ is an important molecule in all life processes, being generated in high amounts in most organisms. To catalyze its rapid transformation into bicarbonate, catalysts evolved in all life kingdoms, that is, the enzymes known as carbonic anhydrases (CAs, EC 4.2.1.1). Six genetically diverse such enzyme families are presently known—the α, β, γ, δ, ε, and η-CAs—with the last class discovered quite recently. CAs not only face the conversion of the high amounts of CO₂ formed in the metabolic processes, transforming it in bicarbonate and protons, but they also manage the acid–base equilibria connected to this reaction. In fact, the products formed in the catalyzed reaction are either ions with strong buffering activity (bicarbonate) or hydrated protons (H⁺ ions).

The regulation of pH is a highly important process in all life forms, since many biochemical reactions are tightly regulated by it. This is probably the reason why so many genetic CA families are presently known so that in some organisms, a multitude of different CA families with many isoforms have been described, each with specialized functions. The necessity of a tight/precise pH regulation may thus explain why most organisms investigated so far contain multiple CA isoforms, although they differ significantly by their catalytic activity, susceptibility to various classes of inhibitors, subcellular localization, and many other such features. For example, in humans, 15 different CA isoforms, all belonging to the α-class, have been described.

Most mammals (including humans) possess two blood isoforms, denominated CA I and CA II, with a total concentration of these proteins as high as 0.2 mM. However, the catalytic activity of the human (h) isoform hCA I is much lower compared with that of hCA II, and in addition, hCA I is also inhibited by the chloride and bicarbonate present in the plasma, leaving a lot of questions regarding the physiologic function of this isoform. On the other hand, the high activity isoform hCA II (also known as the "rapid" blood enzyme, to distinguish

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it from the “slow” one, hCA I) is involved in the secretion of electrolytes in a multitude of tissues, such as the bicarbonate-rich aqueous humor in the anterior chamber of the eyes, and the cerebrospinal fluid, but also in pH and CO₂ homeostasis all over the body, as mentioned above. Other functions include urine formation and bicarbonate reabsorption in the kidney tubules; biosynthetic reactions, such as gluconeogenesis, lipogenesis, and ureagenesis; bone resorption and calcification; and probably many other less well understood physiological/pathological processes.

Indeed, a dysregulation of the activity of these isoforms in one or more tissues has important pathologic consequences, such as glaucoma, when excessive aqueous humor is secreted within the eye, with the subsequent increase in the intraocular pressure (IOP) and edema, when not enough fluids are secreted/eliminated in the urine, leading to fluid accumulation in the body, processes in which CA II together with several other isoforms such as CA IV, XII, and XIV, are involved in the kidneys, epilepsy (the involvement of CA II and other brain CA isoforms in this disease is poorly understood and certainly not irrelevant), and some forms of cancer, in which CA II was observed to be overexpressed, alone or together with other isoforms such as CA IX and XII. CA II is also involved in other pathologies, such as acute mountain sickness and apparently, atherosclerosis and osteoporosis.

Primary sulfonamides constitute the main class of CA inhibitors (CAIs), with a number of such derivatives in clinical use for decades, mainly as antiglaucoma agents, diuretics, antiepileptics, or antibesity drugs. Recently, some sulfonamides with CA inhibitory properties entered phase I clinical trials as antitumor/antimetastatic agents targeting hypoxic tumors in which two CA isoforms, CA IX and XII, are overexpressed. The search for sulfonamide CAIs with various potentials in therapeutics is a dynamic research field, with many new classes being reported constantly and investigated in detail for inhibitory effects against mammalian and nonmammalian CAs.

Here, we report a class of 4-sulfamoylphenyl-ω-aminoalkyl ethers, a poorly investigated chemotype in the CAIs landscape, with interesting properties as antiglaucoma agents as well as for the design of fluorescent enzyme inhibitors with potential use for imaging CAIs in various tissues.

■ RESULTS AND DISCUSSION

Chemistry and Drug Design. Benzenesulfonamides constitute a highly investigated class of CAIs, with most such compounds reported so far being derivatives of sulfanilamide, homosulfanilamide, or 4-aminobenzylbenzenesulfonamide. Derivatization of the primary aliphatic/aromatic amino group from these compounds by its transformation into carboxamides, secondary sulfonamides, ureas, thioureas, or by reaction with pyrrolyl salts has led to a considerable number of new derivatives that showed excellent inhibitory properties against CA isoforms of medicinal interest, such as CA II, IV, VA/VB, IX, or XII. This is generally known as the tail approach for designing CAIs. Surprisingly, very few benzenesulfonamides incorporating ether or thioether moieties have been reported so far. In fact, only one paper, by Vernier et al., considered these chemotypes for the design of sulfonamide-based CAIs. In a very interesting study, these authors reported compounds of the type Ar-X-Ar’-SO₂NH₂, where X was O or S, and Ar, Ar’ aromatic/heterocyclic six-membered rings, which showed highly effective inhibitory properties against CA isoforms involved in important physiologic processes, such as CA II and IV. These derivatives showed improved water solubility compared with structurally similar sulfanilamide derivatives, possessed low nanomolar inhibitory action against CA II, and were shown to penetrate eye tissues, arriving at the ciliary processes where the enzyme is present within the eye, and participating in aqueous humor secretion. Unfortunately no in vivo antiglaucoma studies have been performed with those compounds that possessed physicochemical properties appropriate for an antiglaucoma drug candidate.

Considering these facts, we decided to explore the synthesis and properties of ethers incorporating the benzenesulfonamide “head” and aliphatic ether moieties of the type H₂N—(CH₂)ₙ—O—C₆H₄—SO₂NH₂. As mentioned above, such ethers were not investigated as CAIs until now, and considering the aromatic derivatives reported by the Pfizer group, the presence of aliphatic, amino moieties should also promote the water solubility of the compounds. In fact, a considerable pharmacologic problem of the first generation CAIs, such as acetazolamide, AZA; methazolamide, MAZ; or dichlorophenamide, DCP, was their poor water solubility. Only the second generation drugs, such as dorzolamide, DRZ; and brinzolamide, BRZ, have an improved water solubility because these two topically acting antiglaucoma drugs are administered as hydrochloride salts (both weak amines).

Thus, we designed the following strategy for obtaining the 4-sulfamoylphenyl ω-aminoalkyl ethers reported in this paper (Scheme 1). Reaction of ω-amino-alcohols 1a–e (n = 2–6) with tert-butylxycarbonyl anhydride afforded the Boc-protected amines 2a–e, which by Mitsunobu reaction with 4-hydroxybenzenesulfonamide 3 led to the Boc-protected derivatives 4a–e. Removal of the protecting group in the presence of trifluoroacetic acid (TFA) afforded the trifluoroacetate salts of the 4-sulfamoylphenyl ω-aminoalkyl ethers 5a–e (Scheme 1). The alkyl chain present in the new derivatives ranged from 2 to 6 carbon atoms to investigate the influence of the spacer length for the enzyme inhibitory properties of the new derivatives.

Another aspect in the design of CAIs is related to the use of such compounds as diagnostic tools, for example, for the imaging of tumors in which some CA isoforms are overexpressed. We have reported, for example, fluorescein-based sulfonamides (obtained again from sulfanilamide, homosulfanilamide or 4-aminobenzylbenzenesulfonamide, which were reacted with fluorescein isothiocyanate) that were essential for demonstrating the role of CA IX/XII in the acidification of the extracellular tumor milieu and also in the proof-of-concept
studies regarding the druggability of these novel antitumor targets. However, like most aromatic thioureas, the fluorescent sulfonamides reported earlier showed a rather low water solubility, which may be a limiting factor for some of their applications. Thus, we report here novel derivatives that were prepared by reaction of the 4-sulfamoylphenyl ω-aminoalkyl ethers 5a–e with fluorescein isothiocyanate 6 or [9-(2-carboxy-4-isothiocyanato-phenyl)-6-dimethylaminoxanthen-3-ylidene]-dimethylammonium chloride 8, leading to the novel fluorescent compounds of types 7a–c and 9a,b, respectively (Scheme 1). The last compounds (9a,b) incorporate a fluorophore that was not investigated earlier for its interaction with CAs.

**Carbonic Anhydrase Inhibition.** Inhibition data against four physiologically significant CA isoforms, that is, h (human) hCA I, II, IX, and XII, are shown in Table 1. The following structure–activity relationship (SAR) can be drawn from the data of Table 1:

(i) The slow human isoform hCA I effectively inhibited by some of the sulfonamides investigated here, such as 4a–4e and 7a, 7b, which showed $K_i$ values in the low nanomolar range (5.3–41.2 nM), whereas other derivatives (e.g., 5d, 5e, and 7c) were medium potency inhibitors, with $K_i$'s of 52.5–90.9 nM. Like acetazolamide AAZ, some of the new compounds, among which 5a–5c and 9a, 9b were less effective hCA I inhibitors, with $K_i$'s of 151–826 nM. Thus, the best hCA I inhibitors were the Boc-protected derivatives 4, which showed a rather compact behavior of very effective inhibitor, except for the compound with the 4-carbon-atoms linker (4c) which was less effective compared with its congeners 4a, b, d, and e. The deprotected amines 5 were less effective as hCA I inhibitors compared with the corresponding Boc derivatives (Table 1). Among the fluorescent CAIs reported here, the fluorescein-containing compounds 7a and 7b were effective hCA I inhibitors, whereas the tetramethylrhodamine derivatives 9a and 9b were much less effective as hCA I inhibitors.
Table 1. hCA I, II, IX, and XII Inhibition Data of the Newly Synthesized Sulfonamides 4a–9b and Acetazolamide AAZ as Standard, by the Stopped Flow CO2 Hydrase Assay

| compd | hCA I (nM) | hCA II (nM) | hCA IX (nM) | hCA XII (nM) |
|------|-----------|-------------|-------------|-------------|
| 4a   | 5.3       | 5.0         | 8.3         | 7.2         |
| 4b   | 6.6       | 5.1         | 5.8         | 6.5         |
| 4c   | 41.2      | 5.7         | 7.7         | 6.6         |
| 4d   | 7.9       | 5.5         | 7.1         | 5.7         |
| 4e   | 6.1       | 5.2         | 6.9         | 6.4         |
| 5a   | 649       | 66.5        | 8.7         | 88.5        |
| 5b   | 452       | 36.6        | 17.9        | 9.6         |
| 5c   | 286       | 8.9         | 32.6        | 7.5         |
| 5d   | 52.5      | 8.8         | 6.6         | 7.3         |
| 5e   | 63.0      | 3.9         | 6.5         | 6.5         |
| 7a   | 18.0      | 5.0         | 8.5         | 8.6         |
| 7b   | 17.1      | 4.6         | 7.2         | 5.7         |
| 7c   | 90.9      | 3.9         | 8.8         | 7.1         |
| 9a   | 826       | 215         | 9.6         | 609         |
| 9b   | 151       | 43.3        | 7.9         | 36.7        |
| AAZ  | 250       | 12.1        | 25.0        | 5.7         |

*Mean from different assays; errors are in the range of ±10% of the reported value.

Inhibitors compared with the fluorescein derivatives mentioned above.

(ii) The physiologically dominant hCA II very effectively inhibited by most sulfonamides reported here. Indeed, just four compounds (5a and 5b) as well as 9a,b showed medium potency activity, with \( K_i \)’s of 36.6–215 nM. The remaining sulfonamides showed very effective hCA II inhibitory properties, with \( K_i \)’s ranging between 3.9 and 8.9 nM (Table 1) and are thus more effective than the clinically used drug acetazolamide AAZ. The SAR is rather clear-cut: the five BOC-protected derivatives 4a–4e showed a very compact behavior with basically no variation of the inhibitory power, with the length of the linker from 2 to 6 CH₂ moieties. However, the situation is changed for the amines 5a–5e, which on one hand were weaker hCA II inhibitors compared with the corresponding Boc-protected derivatives and on the other hand showed an increase in the inhibitory power with an increase in the linker chain from 2 to 6 CH₂ moieties. Indeed, between compounds 5a and 5e, there is a 17-fold difference in the inhibitory activity against this isomor. As for hCA I inhibition, again, the fluorescein-tailed sulfonamides 7a–7c were much more inhibitory compared with the tetramethylrhodamine derivatives 9a and 9b.

(iii) The tumor-associated, transmembrane isoform hCA IX was very well inhibited by all derivatives reported here, with \( K_i \)’s of 5.8–32.6 nM. The SAR is almost impossible to delineate because these compounds show excellent inhibitory activity. For example, the Boc-protected derivatives 4a–e have a minimal variation of the inhibition constants, ranging between 5.8 and 8.3 nM. This variation is slightly higher for the amines 5 (between 6.5 and 32.6 nM) and is again almost absent for the fluorescent sulfonamides 7 and 9. It is interesting to note that for this isoform, both the fluorescein and the tetramethylrhodamine derivatives were equally effective as CAIs.

(iv) The other transmembrane isoform investigated here, hCA XII, was also effectively inhibited by most of the new sulfonamides reported in this paper. Two compounds, 5a and 9b, were medium potency inhibitors (\( K_i \)’s of 36.7–88.5 nM), and one (9a) was an ineffective inhibitor (\( K_i \) of 609 nM). The remaining sulfonamides investigated here showed excellent hCA XII inhibitory activity, with inhibition constants ranging between 5.4 and 9.6 nM, again with no obvious SAR to be discussed (Table 1).

(v) Although most of these sulfonamides were effective CAIs against all four isoforms investigated here, several interesting selectivity cases were observed: for example, 9a is a hCA IX-selective sulfonamide inhibitor, with a \( K_i \) of 9.6 nM against the tumor-associated isomor and >215 nM against hCA I, II, and XII (Table 1). Compound 5b effectively inhibits the two transmembrane isomers (\( K_i \)’s of 9.6–17.9 nM); it is a much less effective inhibitor of the two cytosolic isomers hCA I and II (\( K_i \)’s of 36.6–452 nM).

X-ray Crystallography. To rationalize some of the inhibition data presented above, two of the novel sulfonamides reported here, 4c (incorporating the Boc-aminobutyl moiety) and 5c (incorporating the 4-aminobutyl fragment) were crystallized with hCA II, and their crystal structures were resolved at a high resolution (Table 2). Both inhibitors were observed bound within the enzyme active site, coordinating to the Zn(II) ion by means of the deprotonated nitrogen of the sulfonamide moiety (Figures 1 and 2), like all other sulfonamide or sulfamates investigated so far by means of this technique. The phenyl ring and the rather long, hydrophobic alkyl tails of both inhibitors were observed to be involved within the enzyme active site (as shown in Figures 1–3), such as Val121, Phe131, Leu198, Pro201, and Pro202. The tail of compound 5c extends farther out into the enzyme’s hydrophobic cleft, allowing it to form more stabilizing interactions with amino acids, such as Pro201 and Leu204; however, the shorter tail of compound 4c is unable to perform such interactions (Figure 3).

It should be mentioned that for the Boc-protected derivative 4c, the electron density of the tail region was not completely

Table 2. Crystallographic Statistics for the hCA II Adducts of 4c and 5c

| PDB ID | hCA II-4c | hCA II-5c |
|--------|-----------|-----------|
| space group | P2₁ | P2₁ |
| unit-cell parameters (Å, deg) | a = 42.4, b = 41.3, c = 71.7, \( \beta = 104.1 \) | a = 42.5, b = 41.3, c = 72.1, \( \beta = 104.3 \) |
| resolution (Å) | 1.80 (1.86–1.80) | 1.63 (1.69–1.63) |
| total no. reflections | 71,626 | 105,407 |
| individual reflections | 22,386 | 30,415 |
| redundancy | 3.2 | 3.5 |
| completeness | 98.8 (99.5) | 99.7 (97.9) |
| R_in | 0.163 | 0.086 |
| R_index | 0.224/0.260 | 0.178/0.206 |
| r.m.s. for bond lengths/angles (Å, deg) | 0.006/1.10 | 0.010/1.29 |
| av B-factors (Å²) | 12.3/16.7 | 5.3/9.1 |
| no. protein atoms | 2086 | 2114 |
| no. water molecules | 54 | 127 |
| Ramachandran statistics most favored and additional/generously allowed | 89.4/10.5/0.5 | 87.6/11.5/0.9 |

*Values in parentheses represent highest resolution bin. \( R_{	ext{free}} = (\sum_{i=1}^{n} F_i - |\sum_{i=1}^{n} F_i| Away) / \sum_{i=1}^{n} |F_i| \). \( R_{	ext{free}} \) is calculated in the same way as \( R_{	ext{cryst}} \) except it is for data omitted from refinement (5% of reflections for all data sets).
defined (Figure 1), probably because of its high flexibility and disorder when complexed to the enzyme. In contrast, for the amine 5c, all atoms from the tail region had the electron density well-defined, proving that this region is less disordered compared with the Boc-protected compound 4c (Figure 2). To account for this disorder and to ensure the ligand was built into the density correctly, the map for compound 4c was contoured at a lower sigma level (0.6) than that of 5c (1.0).

The fact that the tails of these compounds lie only in the hydrophobic cleft of the hCA II active site is a noteworthy finding, because we showed in earlier papers that compounds having their tails lying only in the hydrophobic half generally do not possess isoform-selective inhibitory properties, and this is also confirmed by the present findings (although neither 4c nor 5c is highly effective as hCA I inhibitors, Table 1). In fact, these two compounds inhibit hCA II, IX, and XII in rather similar ranges and are effective inhibitors against these three isoforms. Because our interest was to obtain compound with antiglaucoma activity, for which both hCA II and XII effective inhibitory properties are desired, we consider the present observations of real interest.

**Antiglaucoma Activity.** Both the Boc-protected and the amino derivative sulfonamides reported here showed excellent water solubility and could be formulated as 2% eye drops at the neutral pH value (dorzolamide, DRZ, the clinically used drug is a hydrochloride salt with a pH of the eye drops of 5.5 which produces eye irritation and stinging as side effects). We have investigated the intraocular pressure (IOP) lowering properties of some of these compounds, more precisely, 4c and 5c (for which the X-ray in adduct with hCA II was reported; see the Discussion section, above), in an animal model of glaucoma. Indeed, both compounds were low nanomolar inhibitors of isoforms hCA II (responsible for aqueous humor secretion) and hCA XII (isoform that is overexpressed in the eyes of glaucomatous patients). As seen from the data of Figure 4, the Boc-protected derivative 4c showed a small decrease of IOP (of 1−2.5 mmHg) when given topically to the eye of the animals, whereas the free amine 5c (as a trifluoroacetate salt) was more effective, with an IOP decrease of 4.4 mmHg at 2 h postadministration, more effective than DRZ, the standard drug, which caused an IOP drop of 4 mmHg at 60 min postadministration. Another notable difference between 5c and DRZ was the fact that the new compound investigated here had a prolonged efficacy compared to DRZ, for which after 4 h no IOP decrease was seen. In contrast, 5c showed efficacy even after 4 h postadministration, with an IOP drop of 3 mmHg at that time point. It should be mentioned that the animal model employed here is of normotensive rabbits, and this is why the absolute IOP drops are not very high, but the advantage of...
this model is that the measurements can be done rapidly and are highly reproducible.44a

CONCLUSIONS

We report a series of new 4-sulfamoylphenyl-ω-aminooalkyl ethers that have been prepared by Mitsunobu reaction. Interesting SAR has been observed for the inhibition of four physiologically relevant CA isoforms: hCA I, II, IX, and XII. Many of the new compounds were highly effective inhibitors of all these isoforms, in the low nanomolar range, with few isoform-selective compounds also identified. These findings have been rationalized by resolving the X-ray crystal structures of two of the new sulfonamides. The tails of these derivatives were observed bound only in the hydrophobic half of the enzyme active site, making van der Waals contacts with amino moieties. Several fluorescent sulfonamides have also been reported that incorporate either fluorescein—thiourea or tetramethylrhodamine—thiourea moieties, which also effectively inhibited some CA isoforms investigated here.

EXPERIMENTAL PROTOCOLS

Chemistry. Anhydrous solvents and all reagents were purchased from Sigma-Aldrich, Alfa Aesar, and TCI. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringe techniques to transfer moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringe techniques to transfer moisture-sensitive compounds. Nuclear magnetic resonance (1H NMR, 13C NMR, DEPT-90, HSQC, HMBC) spectra were recorded using a Bruker solutions. Enzyme active site, making van der Waals contacts with amino moieties. Several fluorescent sulfonamides have also been reported that incorporate either fluorescein—thiourea or tetramethylrhodamine—thiourea moieties, which also effectively inhibited some CA isoforms investigated here.

General Procedure for the Synthesis of O-Alkylbenzenesulfonamides 5a−e via Mitsunobu coupling. a. General Procedure for Boc Protection. Aminooalcohol 1a−e (1.0 equiv) was dissolved in dichloromethane (DCM) and treated with a 1 M NaOH aqueous solution or diisopropyl ethyamine (DIEA) (1.0 equiv), then di-tert-butyl dicarbonate (1.0 equiv) was added, and the mixture was vigorously stirred O.N. until consumption of starting materials (TLC monitoring). The reaction was quenched with a 1 M hydrochloric acid aqueous solution, neutralized with NaHCO3 aqueous solution, and extracted with ethyl acetate (3 × 15 mL). The combined organic layers were washed with H2O (3 × 20 mL), dried over Na2SO4, filtered, and concentrated under vacuo to give the titled product.

b. General Procedure for Mitsunobu Coupling. Boc-aminooalcohol 2a−e (1.0 equiv) was dissolved in dry THF and transferred to a two neck-flask via cannula, followed by addition of Ph3P (1.0 equiv) and 4-hydroxysulfsalzefenamide (1.0 equiv). Then the solution was cooled to 0 °C, and diisopropyl azodicarboxylate (DIAD) (1.1 equiv) was added dropwise. The reaction was warmed to r.t. and stirred at the same temperature until the starting materials were consumed (TLC monitoring), quenched with slush, and extracted with ethyl acetate (3 × 15 mL). The combined organic layers were washed with H2O (3 × 20 mL), dried over Na2SO4, filtered, and concentrated under vacuo to give a residue that was purified by silica gel column chromatography followed by crystallization when necessary.

c. General Procedure for Boc Deprotection. Compounds 4a−e (1.0 equiv) was dissolved in DCM or 1,4-dioxane and treated with TFA. The reaction was stirred at r.t. until the starting material was consumed (TLC monitoring). The solvent was removed under vacuo, and the obtained residue was crystallized from IPA or triturated from diethyl ether to obtain the titled compound as a white solid.

Synthesis of tert-Butyl 2-Hydroxyethylcarbamate 2a. Ethanolamine 1a (1.0 g, 1.0 equiv) was dissolved in DCM (16.5 mL) and treated with a 1 M aqueous solution of NaOH (1.0 equiv), and then di-tert-butyl dicarbonate (1.0 equiv) was added. The reaction mixture was treated according to the general procedure a, previously reported, to give the titled compound 2a as a colorless liquid, which was used as it is.

tert-Butyl 2-Hydroxyethylcarbamate 2a: 66% yield; silica gel TLC Rf 0.18 (ethyl acetate/n-hexane 50% v/v); δ1H (400 MHz, DMSO-d6) 1.41 (9H, s), 3.0 (2H, q, J = 6.0), 3.38 (2H, t, J = 6.0), 4.6 (1H, t, J = 6.0), exchange with D2O, OH), 6.71 (1H, brt, exchange with D2O, NH); δ13C (100 MHz, DMSO-d6) 29.2, 43.6, 61.0, 78.4, 156.6. Experimental data are in agreement with reported data.

Synthesis of tert-Butyl 3-Hydroxypropylcarbamate 2b. 3-Amino-1-propanol 1b (0.98 g, 1.0 equiv) was dissolved in DCM (13 mL) and treated with a 1 M aqueous solution of NaOH (1.0 equiv), and then di-tert-butyl dicarbonate (1.0 equiv) was added. The reaction mixture was treated according to the general procedure a, previously reported, to give the titled compound 2b as a colorless liquid, which was used as it is.

Figure 4. Drop of intraocular pressure (ΔIOP, mmHg) versus time (min) in hypertonic saline-induced ocular hypertension in rabbits, treated with 50 μL of 2% solution of compounds 4c, 5c, and DRZ as the standard drug and vehicle. Errors were within 10−15% of the reported IOP values (from three different measurements for each of the four animals in the study group) and were statistically significant (p = 0.045 by the Student’s t test).
treated with a 1 M aqueous solution of NaOH (1.0 equiv), and then di-tert-butyl dicarbonate (1.0 equiv) was added. The reaction mixture was treated according to the general procedure a, previously reported, to give the titled compound 2b as a colorless liquid, which was used as it is.

tert-Butyl 3-Hydroxypropylcarbamate 2b: 80% yield; silica gel TLC Rf 0.16 (ethyl acetate/n-hexane 50% v/v); δH (400 MHz, DMSO-d6) 1.41 (9H, s), 1.55 (2H, pent, J 6.4), 3.0 (2H, q, J 6.4), 3.42 (2H, q, J 6.0), 4.4 (1H, t, J 6.0, exchange with D2O, OH), 6.76 (1H, brt, exchange with D2O, NH); δC (100 MHz, DMSO-d6) 27.2, 32.9, 38.1, 60.7, 78.3, 156.5. Experimental data are in agreement with reported data.

Synthesis of tert-Butyl 4-Hydroxybutylcarbamate 2c: 4-Amino-1-butanol 1c (1.45 g, 1.0 equiv) was dissolved in DCM (16 mL) and treated with DIPEA (1.0 equiv) and then di-tert-butyl dicarbonate (1.0 equiv). The reaction mixture was treated according to the general procedure a, previously reported, to give the titled compound 2c as a yellow liquid, which was used as it is.

tert-Butyl 4-Hydroxybutylcarbamate 2c: 70% yield; silica gel TLC Rf 0.16 (ethyl acetate/n-hexane 50% v/v); δH (400 MHz, DMSO-d6) 1.41 (13H, s), 2.93 (2H, m), 3.40 (2H, m), 4.39 (1H, t, J 6.0, exchange with D2O, OH), 6.68 (1H, t, J 6.0, exchange with D2O, NH); δC (100 MHz, DMSO-d6) 27.2, 29.2, 30.5, 33.4, 40.7, 61.6, 78.2, 156.5. Experimental data are in agreement with reported data.

Synthesis of tert-Butyl 5-Hydroxypentylcarbamate 2d: 5-Amino-1-pentanol 1d (1.5 g, 1.0 equiv) was dissolved in DCM (14.5 mL) and treated with DIPEA (1.0 equiv) and then di-tert-butyl dicarbonate (1.0 equiv). The reaction mixture was treated according to the general procedure a, previously reported, to give the titled compound 2d as a yellow liquid, which was used as it is.

tert-Butyl 5-Hydroxypentylcarbamate 2d: 77% yield; silica gel TLC Rf 0.3 (ethyl acetate/n-hexane 60% v/v); δH (400 MHz, DMSO-d6) 1.22—1.32 (2H, m), 1.34—1.48 (13H, m) 2.92 (2H, q, J 6.4), 3.39 (2H, m), 4.36 (1H, t, J 5.2, exchange with D2O, OH), 6.79 (1H, brt, exchange with D2O, NH); δC (100 MHz, DMSO-d6) 23.8, 29.2, 30.3, 33.1, 40.8, 61.6, 78.2, 156.5. Experimental data are in agreement with reported data.

tert-Butyl 6-Hydroxyhexylcarbamate 2e: 6-Amino-1-hexanol 1e (0.1 g, 1 equiv) was dissolved in DCM (8.5 mL) and treated with DIPEA (1.0 equiv) and di-tert-butyl dicarbonate (1.0 equiv). The reaction mixture was treated according to the general procedure a, previously reported, to give the titled compound 2e as a colorless oil, which was used as it is.

tert-Butyl 6-Hydroxyhexylcarbamate 2e: 81% yield; silica gel TLC Rf 0.2 (ethyl acetate/n-hexane 50% v/v); δH (400 MHz, DMSO-d6) 1.23—1.34 (4H, m), 1.34—1.48 (13H, m) 2.89 (2H, q, J 6.7), 3.36 (2H, q, J 5.2), 4.3 (1H, t, J 5.2, exchange with D2O, OH), 6.76 (1H, brt, exchange with D2O, NH); δC (100 MHz, DMSO-d6) 26.2, 27.1, 29.2, 30.5, 33.4, 40.7, 61.6, 78.2, 156.5. Experimental data are in agreement with reported data.

Synthesis of tert-Butyl 2-(4-Sulfamoylphenoxy)-ethylcarbamate 4a: tert-Butyl 2-hydroxyethylcarbamate 2a (1.0 g, 1.0 equiv) was dissolved in dry THF (9.5 mL) and was treated with PhP2 (1.0 equiv), 4-hydroxybenzenesulfonamide 3 (1.0 equiv), and DIAD (1.1 equiv) according to the general procedure b, previously reported. The reaction was stirred at r.t. until starting materials were consumed (TLC monitoring). The reaction was quenched with slush and extracted with ethyl acetate (3 × 15 mL). The combined organic layers were washed with H2O (3 × 20 mL), dried over Na2SO4, filtered, and concentrated under vacuo, and the obtained residue was purified by silica gel column chromatography eluting with ethyl acetate/n-hexane 60% v/v, followed by crystallization in EtOH/H2O mixture to afford the titled compound 4a as a white solid.

tert-Butyl 2-(4-Sulfamoylphenoxy)-ethylcarbamate 4a: 40% yield, silica gel TLC Rf 0.4 (ethyl acetate/n-hexane 60% v/v); mp 148—149 °C; δH (400 MHz, DMSO-d6) 1.42 (9H, s), 3.33 (2H, t, J 5.6), 4.07 (2H, t, J 5.6), 7.07 (1H, bbr, exchange with D2O, NH), 7.1 (2H, d, J 8.8), 7.24 (2H, s, exchange with D2O, SO2NH), 7.77 (2H, d, J 8.8); δC (100 MHz, DMSO-d6) 29.4, 40.3, 68.0, 79.2, 115.7, 128.9, 137.2, 156.9, 162.0. Elemental analysis: calc C 49.35, H 6.37, N 8.8); found C 49.43, H 6.03, N 8.97; m/z (ESI negative) 315.6 [M − H]−.

Synthesis of tert-Butyl 3-(4-Sulfamoylphenoxy)-propylcarbamate 4b: tert-Butyl 3-hydroxypropylcarbamate 2b (0.88 g, 1.0 equiv) was dissolved in dry THF (4.7 mL) and was treated with PhP2 (1.0 equiv), 4-hydroxybenzenesulfonamide 3 (1.0 equiv), and DIAD (1.1 equiv) according to the general procedure b, previously reported. The reaction was stirred at r.t. until starting materials were consumed (TLC monitoring). The reaction was quenched with slush and extracted with ethyl acetate (3 × 15 mL). The combined organic layers were washed with H2O (3 × 20 mL), dried over Na2SO4, filtered, and concentrated under vacuo, and the obtained residue was purified by silica gel column chromatography eluting with ethyl acetate/n-hexane 55% v/v, followed by crystallization in IPA to afford the titled compound 4b as a white solid.

tert-Butyl 3-(4-Sulfamoylphenoxy)-propylcarbamate 4b: 21% yield, silica gel TLC Rf 0.35 (ethyl acetate/n-hexane 55% v/v); mp 134—135 °C; δH (400 MHz, DMSO-d6) 1.41 (9H, s), 1.88 (2H, pent, J 6.4), 3.10 (2H, q, J 6.4), 4.08 (2H, t, J 6.4), 6.95 (1H, t, J 6.4, exchange with D2O, NH), 7.09 (2H, d, J 8.8), 7.23 (2H, s, exchange with D2O, SO2NH), 7.77 (2H, d, J 8.8); δC (100 MHz, DMSO-d6) 29.2, 30.0, 37.7, 66.8, 78.5, 115.3, 128.6, 137.0, 156.6, 161.9. Elemental analysis: calc C 50.89, H 6.71, N 8.48; found C 51.09, H 7.01, N 8.64, S 9.54; m/z (ESI negative) 329.40 [M − SO2NH]−.
dissolved in dry THF (10.0 mL) and was treated with Ph3P (1.0 equiv), 4-hydroxybenzenesulfonamide 3 (1.0 equiv), and DIAD (1.1 equiv) according to the general procedure b, previously reported. The reaction was stirred at r.t. until starting materials were consumed (TLC monitoring). The reaction was quenched with slush and extracted with ethyl acetate (3 × 15 mL). The combined organic layers were washed with H2O (3 × 20 mL), dried over Na2SO4, filtered, and concentrated under vacuo, and the obtained residue was purified by silica gel column chromatography eluting with 60% v/v ethyl acetate/n-hexane to afford the titled compound 4e as a white solid.

tert-Butyl 4-(4-Sulfamoylphenoxy)-butylcarbamate 4c: 22% yield, silica gel TLC Rf 0.40 (ethyl acetate/n-hexane 60% v/v); mp 93–94 °C; δH (400 MHz, DMSO-d6) 1.41 (9H, s), 1.56 (2H, m), 1.74 (2H, m), 3.10 (2H, q, J 6.5), 4.08 (2H, t, J 6.5), 6.95 (1H, t, J 5.2, exchange with D2O, NH), 7.09 (2H, d, J 8.8), 7.23 (2H, s, exchange with D2O, SO2N), 7.77 (2H, d, J 8.8); δC (100 MHz, DMSO-d6) 26.9, 27.0, 29.2, 30.2, 68.6, 78.4, 115.4, 128.6, 137.0, 156.6, 162.0; Elemental analysis: calcd C 36.37, H 3.97, N 8.48, S 9.71; found C 36.76, H 3.97, N 8.48, S 9.71; [M – H]+. Synthesis of tert-Butyl 5-(4-Sulfamoylphenoxy)-pentylcarbamate 4d: tert-Butyl 5-hydroxypentylcarbamate 2d (1.2 g, 1.0 equiv) was dissolved in dry THF (9.0 mL) and was treated with Ph3P (1.0 equiv), 4-hydroxybenzenesulfonamide 3 (1.0 equiv), and DIAD (1.1 equiv) according to the general procedure b, previously reported. The reaction was stirred at r.t. until starting materials were consumed (TLC monitoring). The reaction was quenched with slush and extracted with ethyl acetate (3 × 20 mL), dried over Na2SO4, filtered, and concentrated under vacuo, and the obtained residue was purified by silica gel column chromatography, eluting with 60% v/v ethyl acetate/n-hexane to afford the titled compound 4d as a white solid.

tert-Butyl 5-(4-Sulfamoylphenoxy)-pentylcarbamate 4d: 27% yield, silica gel TLC Rf 0.42 (ethyl acetate/n-hexane 60% v/v); mp 95–96 °C; δH (400 MHz, DMSO-d6) 1.37–1.51 (13H, m), 1.73 (2H, pent, J 6.8), 2.94 (2H, q, J 6.4), 4.04 (2H, t, J 6.4), 6.80 (1H, t, J 5.7, exchange with D2O, NH), 7.08 (2H, d, J 8.8), 7.20 (2H, s, exchange with D2O, SO2N), 7.75 (2H, d, J 8.8); δC (100 MHz, DMSO-d6) 23.6, 29.1, 31.2, 30.1, 40.6, 66.7, 78.2, 115.3, 128.6, 136.9, 156.5, 162.0; Elemental analysis: calcd C 35.61, H 7.31, N 7.82, S 8.95; found C 35.50, H 7.01, N 7.87, S 8.69; m/z (ESI negative) 357.60 [M – H]−.

tert-Butyl 2-(4-Sulfamoylphenoxy)-ethylcarbamate 4a (0.5 g, 1.0 equiv) in 10 mL of DCM. The reaction was stirred at r.t. according to the general procedure c, previously reported until starting material was consumed (TLC monitoring). The solvents were evaporated under vacuo, and the obtained residue was triturated with diethyl ether and dried under vacuum to afford the titled compound 5a as a white solid.

2-(4-Sulfamoylphenoxy)-ethylammonium Trifluoroacetate Salt 5a. TFA (7.0 equiv) was added to a stirring mixture of tert-butyl 2-(4-sulfamoylphenoxy)-ethylcarbamate 4a (0.5 g, 1.0 equiv) and DCM (10 mL). The reaction was stirred at r.t. for 3 h until starting material was consumed (TLC monitoring). The solvents were evaporated under vacuo, and the obtained residue was triturated with diethyl ether and dried under vacuum to afford the titled compound 5a as a white solid.

tert-Butyl 4-(4-Sulfamoylphenoxy)-hexylcarbamate 4e: tert-Butyl 6-hydroxyhexylcarbamate 2e (1.37 g, 1.0 equiv) was dissolved in dry THF (9.0 mL) and was treated with Ph3P (1.0 equiv), 4-hydroxybenzenesulfonamide 3 (1.0 equiv), and DIAD (1.1 equiv) according to the general procedure b, previously reported. The reaction was stirred at r.t. until starting materials were consumed (TLC monitoring). The reaction was quenched with slush and extracted with ethyl acetate (3 × 15 mL). The combined organic layers were washed with H2O (3 × 20 mL), dried over Na2SO4, filtered, and concentrated under vacuo, and the obtained residue was purified by silica gel column chromatography, eluting with 50% v/v ethyl acetate/n-hexane to afford the titled compound 4e as a white solid.

tert-Butyl 6-(4-Sulfamoylphenoxy)-hexylcarbamate 4e: 42% yield; silica gel TLC Rf 0.35 (ethyl acetate/n-hexane 50% v/v); 101–102 °C; δH (400 MHz, DMSO-d6) 1.41 (17H, m), 1.75 (2H, q, J 8.0), 2.94 (2H, q, J 8.0), 4.08 (1H, t, J 5.2, exchange with D2O, OH), 6.80 (1H, brt, exchange with D2O, NH), 7.11 (2H, d, J 8.5), 7.23 (2H, s, exchange with D2O, SO2N), 7.77 (2H, d, J 8.5); δC (100 MHz, DMSO-d6) 23.6, 24.8, 26.4, 29.2, 30.1, 39.5, 68.3, 78.2, 115.4, 128.5, 136.9, 156.3, 162.1; Elemental analysis: calcd C 54.82, H 7.58, N 7.52, S 8.56; found C 54.66, H 7.58, N 7.52, S 8.56; m/z (ESI negative) 371.25 [M – H]−.

Synthesis of 2-(4-Sulfamoylphenoxy)-ethylammonium Trifluoroacetate Salt 5a. TFA (7.0 equiv) was added to a stirring mixture of tert-butyl 2-(4-sulfamoylphenoxy)-ethylcarbamate 4a (0.5 g, 1.0 equiv) in 10 mL of DCM. The reaction was stirred at r.t. according to the general procedure c, previously reported until starting material was consumed (TLC monitoring). The solvents were evaporated under vacuo, and the obtained residue was triturated with diethyl ether and dried under vacuum to afford the titled compound 5a as a white solid.

2-(4-Sulfamoylphenoxy)-ethylammonium Trifluoroacetate Salt 5a. 88% yield, mp 142–143 °C; δH (400 MHz, DMSO-d6) 3.30 (2H, t, J 5.2), 4.27 (2H, t, J 5.2), 7.17 (2H, d, J 8.8), 7.29 (2H, s, exchange with D2O, SO2N), 7.81 (2H, d, J 8.8), 8.02 (3H, brt, exchange with D2O, NH); δC (100 MHz, DMSO-d6) 39.2, 65.7, 115.7, 118.2 (d, J 13C–F 299), 128.7, 137.8, 159.4 (q, J 13C–F 31), 161.2; δC (376 MHz, DMSO-d6) −73.5 (3F, s); Elemental analysis: calcd C 36.37, H 3.97, N 8.48, S 9.71; found C 36.76, H 3.72, N 8.08, S 10.02; m/z (ESI positive) 217.08 [M – CF3COO]−.

Synthesis of 3-(4-Sulfamoylphenoxy)-propylcarbamate 5b. tert-Butyl 3-(4-sulfamoylphenoxy)-propylcarbamate 4b (0.8 g, 1.0 equiv) was dissolved in 1,4-dioxane (10.0 mL), followed by addition of TFA (5.00 equiv). The reaction was treated according to the general procedure c, previously reported (TLC monitoring).
The solvents were evaporated under vacuum, and the obtained residue was triturated with diethyl ether and dried under vacuum to afford the titled compound 5b as a white solid.

### 3-(4-Sulfamoylphenoxy)-propylammonium Trifluoroacetate Salt 5b:
91% yield, mp 129–130 °C; δH (400 MHz, DMSO-d6) 2.05 (2H, pent, J 6.4), 3.00 (2H, m), 4.17 (2H, t, J 6.0), 7.12 (2H, d, J 8.8), 7.26 (2H, s, exchange with D2O, SO3NH), 7.78 (3H, br, exchange with D2O, −NH3), 7.79 (2H, d, J 8.8); δC (100 MHz, DMSO-d6) 27.6, 37.1, 66.0, 115.43, 118.2 (d, J 3CF-F 297), 128.6, 137.3, 159.4 (q, J 3CF-F 31) 161.6; δF (376 MHz, DMSO-d6) −73.64 (3F, s). Elemental analysis: calcd C 38.37, H 4.39, N 8.14, S 9.31; found C 38.42, H 4.60, N 7.95, S 9.66; m/z (ESI positive) 231.30 [M − CF3COO]+.

### Synthesis of 4-(4-Sulfamoylphenoxy)-butylammonium Trifluoroacetate Salt 5c.
ter-Butyl 4-(4-sulfamoylphenoxy)-butylcarbonate 4c (0.38 g, 1.0 equiv) was dissolved in DCM (5.0 mL), followed by addition of TFA (12.0 equiv). The reaction was treated according to the general procedure c previously reported (TLC monitoring). The solvents were evaporated under vacuum, and the obtained residue was crystallized from IPA to afford the titled compound 5c as a white solid.

### 4-(4-Sulfamoyl-phenoxy)-butylammonium Trifluoroacetate Salt 5c:
50% yield, mp 110–111 °C; δH (400 MHz, DMSO-d6) 1.74 (2H, m), 1.83 (2H, m), 2.90 (2H, t, J 7.4), 4.11 (2H, t, J 6.0), 7.10 (2H, d, J 8.0), 7.25 (2H, s, exchange with D2O, SO3NH), 7.79 (3H, SH, m, Ar-H, NH3); δC (100 MHz, DMSO-d6) 24.8, 26.4, 39.5, 68.3, 115.4, 118.2 (d, J 3CF-F 298) 128.6, 137.1, 159.15 (q, J 3CF-F 31) 161.8; δF (376 MHz, DMSO-d6) −73.4 (3F, s). Elemental analysis: calcd C 40.22, H 4.78, N 7.82, S 8.95; found C 40.30, H 4.63, N 7.72, S 8.96; m/z (ESI positive) 245.17 [M − CF3COO]+.

### Synthesis of 5-(4-Sulfamoylphenoxy)-pentylammonium Trifluoroacetate Salt 5d.
ter-Butyl 5-(4-sulfamoylphenoxy)-pentylcarbonate 4d (0.46 g, 1.0 equiv) was dissolved in DCM (5.0 mL), followed by addition of TFA (7.0 equiv). The reaction was treated according to the general procedure c previously reported (TLC monitoring). The solvents were evaporated under vacuum, and the obtained residue was triturated from diethyl ether to afford the titled compound 5d as a white solid.

### 5-(4-Sulfamoylphenoxo)-pentylammonium Trifluoroacetate Salt 5d:
90% yield; mp 121–122 °C; δH (400 MHz, DMSO-d6) 1.50 (2H, m), 1.63 (2H, m), 1.79 (2H, m), 2.85 (2H, m), 4.09 (2H, t, J 7.6), 7.10 (2H, d, J 9.0), 7.24 (2H, s, exchange with D2O, SO3NH), 7.71 (3H, br, exchange with D2O, NH3), 7.77 (2H, d, J 9.0); δC (100 MHz, DMSO-d6) 23.4, 27.6, 28.9, 39.6, 68.6, 115.3, 118.1 (d, J 3CF-F 298), 128.6, 137.0, 159.1 (q, J 3CF-F 31), 161.9; δF (376 MHz, DMSO-d6) −73.5 (3F, s). Elemental analysis: calcd C 41.93, H 5.14, N 7.52, S 8.61; found C 41.73, H 5.23, N 7.25, S 8.84; m/z (ESI positive) 259.17 [M-CF3COO]+.

### Synthesis of 6-(4-Sulfamoylphenoxy)-hexylammonium Trifluoroacetate Salt 5e.
ter-Butyl 6-(4-sulfamoylphenoxy)-hexylcarbonate 4e (0.10 g, 1.0 equiv) was dissolved in DCM (1.8 mL), followed by addition of TFA (5.0 equiv). The reaction was treated according to the general procedure c previously reported (TLC monitoring). The solvents were evaporated under vacuum, and the obtained residue was crystallized from IPA to afford the titled compound 5e as a white solid.

### 6-(4-Sulfamoylphenoxo)-hexylammonium Trifluoroacetate Salt 5e.
30% yield; mp 122–123 °C; δH (400 MHz, DMSO-d6) 1.45 (4H, m), 1.59 (2H, pent, J 7.5), 1.76 (2H, pent, J 7.5), 2.82 (2H, t, J 7.5), 4.08 (2H, t, J 6.5), 7.11 (2H, d, J 9.0), 7.25 (2H, brs, exchange with D2O, SO3NH), 7.63 (3H, br, exchange with D2O, NH3), 7.77 (2H, d, J 9.0); δC (100 MHz, DMSO-d6) 25.9, 26.4, 27.9, 30.7, 39.7, 68.7, 115.4, 118.1 (d, J 3CF-F 298), 128.6, 137.0, 159.3 (q, J 3CF-F 31), 162.0; δF (376 MHz, DMSO-d6) −73.5 (1F, s). Elemental analysis: calcd C 43.52, H 5.48, N 7.25, S 8.30; found C 43.19, H 5.24, N 6.95, S 8.16; m/z (ESI positive) 273.40 [M − CF3COO]+.

### General Procedure for Synthesis of Fluorescent Tagged Sulfonamides 7a–c.
3. The O-Alkylbenzenesulfonamide salt 5 (1.0 equiv) and 2-(6-hydroxy-3-oxo-3H-xanthene-9-yl)-5-sulfothiocyanate acid 6 (1.0 equiv) were poured into a two-neck flask, and dry DMA (1.0 mL) was added, followed by addition of TEA (1.5 equiv). The reaction was stirred at r.t. until starting materials were consumed (TLC monitoring). It was then quenched with aq NaOH and filtered, and concentrated in vacuum to afford the titled compound 7 as an orange powder.

### Synthesis of 2-(6-Hydroxy-3-oxo-3H-xanthene-9-yl)-5-[3-[2-(4-sulfamoylphenoxy)-ethyl]-thioiureido]-benzoic Acid 7a.
2-(4-Sulfamopylphenoxo)-ethylanmonium trifluoroacetate salt 5a (50 mg, 1.0 equiv) and 2-(6-hydroxy-3-oxo-3H-xanthene-9-yl)-5-sulfothiocyanate acid 6 (1.0 equiv) were treated according to the general procedure previously reported to afford the titled compound 7a as an orange powder.

### 2-(6-Hydroxy-3-oxo-3H-xanthene-9-yl)-5-[3-[2-(4-sulfamoylphenoxy)-ethyl]-thioiureido]-benzoic Acid 7a.
7a; 2-(4-Sulfamopylphenoxo)-ethylanmonium trifluoroacetate salt 5a (50 mg, 1.0 equiv) and 2-(6-hydroxy-3-oxo-3H-xanthene-9-yl)-5-sulfothiocyanate acid 6 (1.0 equiv) were treated according to the general procedure previously reported to afford the titled compound 7a as an orange powder.
117.6, 125.1, 127.6, 128.7, 130.0, 130.6, 137.4, 142.1, 148.3, 152.9,
160.5, 161.7, 169.5, 181.8 (C=S). Elemental analysis: calcd C 57.51,
H 3.83, N 6.94, S 10.59; found C 57.56, H 4.03, N 6.68, S 10.84;

m/z (ESI positive) 606.50 [M + H]+.

Synthesis of 2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-[3-(4-sulfamoylphenoxy)-propyl-thioureido]-benzoic Acid 7b. 3-(4-Sulfamoylphenoxy)-propylammonium trifluoroacetate salt 5b (50 mg, 1.0 equiv) and 2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-isothiocyanatobenzoic acid 6 (1.0 equiv) were treated according to the general procedure e, previously reported, to afford the titled compound 7b as an orange powder.

2-(6-Hydroxy-3-oxo-3H-xanthen-9-yl)-5-{3-[4-(4-sulfamoylphenoxy)-butyl]-thioureido}-benzoic Acid 7c. 4-(4-Sulfamoylphenoxy)-butylammonium trifluoroacetate salt 5c (50 mg, 1.0 equiv) and 2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-isothiocyanatobenzoic acid 6 (1.0 equiv) were treated according to the general procedure e, previously reported, to afford the titled compound 7c as an orange powder.

General Procedure for Synthesis of Florescent Tagged O-Alkyl Benzenesulphonamides 9a,b. Compound 5 (1.0 equiv) and 9-(2-carboxy-4-isothiocyanatophenyl)-6-dimethylaminoxanthen-3-ylidene][dimethylammonium chloride 8 (1.0 equiv) were poured into a two-neck flask, and dry DMA (1.0 mL) was added, followed by addition of TEA (1.5 equiv). The reaction was stirred at r.t. until starting materials were consumed (TLC monitoring), and then it was quenched with slush and a 6 M aqueous hydrochloric acid solution. The precipitate formed was centrifuged, collected, washed with diethyl ether (3 x 10 mL), and then dried under vacuum to afford the titled compound 9 as a red powder.

Synthesis of 9-(2-Carboxy-4-[3-(4-sulfamoylphenoxy)ethyl]-thioureido)phenyl]-6-dimethylaminoxanthen-3-ylidene][dimethylammonium Chloride 9a. 2-(4-Sulfamoyl-phenoxy)ethylammonium
trifluoroacetate salt 5a (6.9 mg, 1.0 equiv) and [9-(2-carboxy-4-isothiocyanatophenyl)-6-dimethylamino-3-yldiene]-dimethylammonium chloride 8 (1.0 equiv) were treated according to the previously reported procedure to afford the titled compound 9a as a red powder.

9-(2-Carboxy-4-[3-(4-sulfamoylphenoxo)-ethyl]-thioureido)-phenyl)-6-dimethylamino-3-yldiene]-dimethylammonium Chloride 9a. 70% yield; silica gel TLC Rf 0.40 (MeOH/DCM 20% v/v); mp 177–178 °C (dec); δH (400 MHz, DMSO-d6) 3.30 (12H, s), 3.99 (2H, brt), 4.33 (2H, J 6.2), 6.98 (3H, m), 7.08–7.25 (8H, m), 7.04 (1H, d, J 8.8), 7.75 (1H, d, J 8.8), 7.79 (2H, d, J 8.8), 8.77 (1H, brs), 7.94 (1H, d, J 8.8), 8.58 (1H, brt, exchange with D2O, NH), 10.51 (1H, brs, exchange with D2O, NH), 10.65 (1H, s); δC (100 MHz, DMSO-d6) 28.8, 35.0, 41.4, 41.5, 66.8, 97.2, 114.2, 115.5, 122.6, 124.5, 125.9, 128.7, 131.7, 137.5, 142.6, 157.6, 160.2, 161.4, 161.6, 166.8, 167.1 (C=O), 181.5 (C=O); m/z (ESI positive) 660.50 [M – Cl]–

Synthesis of 9a. 9-(2-Carboxy-4-[3-(4-sulfamoyl-phenoxo)-propyl]-thioureido)-phenyl)-6-dimethylamino-3-yldiene]-dimethylammonium Chloride 9b. 3-(4-Sulfamoylphenoxo)-propylammonium trifluoroacetate salt 5b (7.2 mg, 1.0 equiv) and [9-(2-carboxy-4-isothiocyanatophenyl)-6-dimethylamino-3-yldiene]-dimethylammonium chloride 8 (1.0 equiv) were treated according to the previously reported procedure to afford the titled compound 9b as a red powder.

9-(2-Carboxy-4-[3-(4-sulfamoyl-phenoxo)-propyl]-thioureido)-phenyl)-6-dimethylaminoxanthen-3-yldiene]-dimethylammonium Chloride 9b. 74% yield; silica gel TLC Rf 0.30 (MeOH/DCM 20% v/v); mp 192–193 °C (dec); δH (400 MHz, DMSO-d6) 3.30 (12H, s), 4.13 (2H, t, J 6.2), 4.21 (2H, t, J 6.2), 6.98 (3H, m), 7.08–7.25 (8H, m), 7.40 (1H, d, J 8.8), 7.75 (1H, d, J 8.8), 7.79 (2H, d, J 8.8), 8.77 (1H, brs), 7.94 (1H, d, J 8.8), 8.58 (1H, brt, exchange with D2O, NH), 10.51 (1H, brs, exchange with D2O, NH), 10.65 (1H, s); δC (100 MHz, DMSO-d6) 28.8, 35.0, 41.4, 41.5, 66.8, 97.2, 114.2, 115.5, 122.6, 124.2, 127.0, 128.6, 131.7, 137.1, 142.6, 157.7, 160.8, 161.9, 161.6, 166.8, 167.1 (C=O), 181.5 (C=O); m/z (ESI positive) 674.42 [M – Cl]–

X-ray Crystallography. Co-Crystalization. Two microliters of a 600 mM concentration of 4c was added to a 500 μL 1.6 M sodium citrate, 50 mM Tris–HCl pH 7.8 reservoir solution. One microliter of this reservoir solution was added to 5 μL of CA II at a final concentration of 10 mg/mL so that the final drug concentration was at 0.24 mM. Hanging drops were set up, and crystals were seen within 5 days. This was repeated for 5c.

**Diffraction Data and Collection.** Diffraction data for CA II-4c and CA II-5c complexes were collected on an in-house R-Axis IV++ image plate detector using a RU-H3R rotating Cu anode (Kα = 1.5418 Å) operating at 50 kV and 22 mA. Images were collected every 1° with an exposure time of 5 min at a detector distance of 100 mm. The crystal data were integrated, merged and scaled using HKL2000.

**Structure Determination.** Phasing was carried out in the PHENIX suite of programs using the Auto Molecular Replacement procedure to obtain the initial phases using a previously solved HCA II structure with water molecules removed (PDB code 3KS3). The graphics program COOT was used to view the electron density map, and the structure was adjusted on the basis of the calculated electron density. Topology files of the inhibitors were generated using the PRODRG server, and these files were used to model the drug into the density generated. Refinement was continued using PHENIX/REFINE until the Rfree and Rwork were minimized. The geometric restraints of the final model were analyzed using PROCHECK. The data diffraction and final model refinement statistics are summarized in Table 2.

**Normotensive Rabbit IOP Lowering Studies.** Male New Zealand albino rabbits weighing 1500–2000 g were used in these studies. Animals were anaesthetized using Zoletil (tiletamine chloride + zoletapram chloride, 3 mg/kg body weight, i.m. injection) and injected with 0.05 mL hypertonic saline solution (5% in distilled water) into the vitreous of both eyes. IOP was measured by using a digital tonometer (Tomo-Pen Avia Tonometer, Reichert Inc. Depew, NY, USA) prior to hypertonic saline injection (basal) at 1, 2, and 4 h after administration of the drug. Animals were anaesthetized using Zoletil (tiletamine chloride + zoletapram chloride, 3 mg/kg body weight, i.m. injection) and injected with 0.05 mL hypertonic saline solution (5% in distilled water) into the vitreous of both eyes. IOP was measured by using a digital tonometer (Tomo-Pen Avia Tonometer, Reichert Inc. Depew, NY, USA) prior to hypertonic saline injection (basal) at 1, 2, and 4 h after administration of the drug. Vehicle (phosphate buffer, pH 7.0 plus DMSO 2%) or drugs were instilled immediately after the injection of hypertonic saline into the conjunctive pocket. Eyes were randomly assigned to different groups. Four different animals were used for the tested compounds. Experiments with animals were conducted in agreement with current ethical guidelines and norms approved by the ethical committee of our university.

**ASSOCIATED CONTENT**

**Accession Codes**

4RFC and 4RFD.
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CA, carbonic anhydrase; CAI, CA inhibitor; DCM, dichloromethane; DIAD, diisopropyl azodicarboxylate; DIPEA, diisopropylethylamine; DMA, dimethylecetamide; IOP, intraocular pressure; IPA, propan-2-ol; $K_p$ inhibition constant; SAR, structure–activity relationship; TFA, trifluoroacetic acid

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