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Design, Synthesis, and Evaluation of NO-Donor Containing Carbonic Anhydrase Inhibitors To Lower Intraocular Pressure

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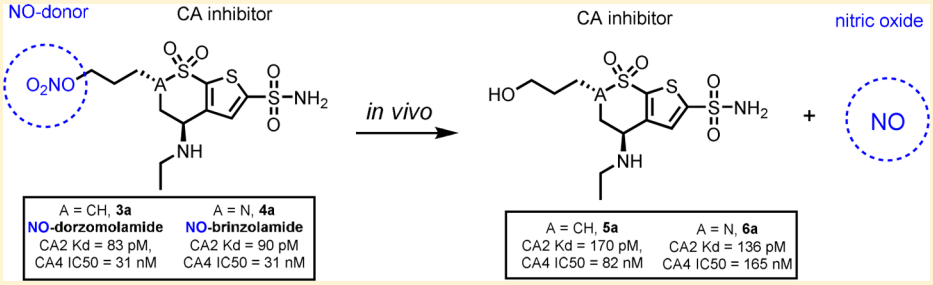


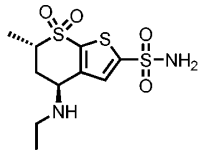
ABSTRACT: The antiglaucoma drugs dorzolamide (**1**) and brinzolamide (**2**) lower intraocular pressure (IOP) by inhibiting the carbonic anhydrase (CA) enzyme to reduce aqueous humor production. The introduction of a nitric oxide (NO) donor into the alkyl side chain of dorzolamide (**1**) and brinzolamide (**2**) has led to the discovery of NO-dorzolamide **3a** and NO-brinzolamide **4a**, which could lower IOP through two mechanisms: CA inhibition to decrease aqueous humor secretion (reduce inflow) and NO release to increase aqueous humor drainage (increase outflow). Compounds **3a** and **4a** have shown improved efficacy of lowering IOP in both rabbits and monkeys compared to brinzolamide (**2**).

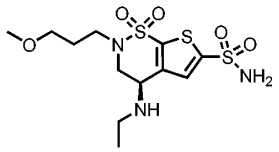
INTRODUCTION

Glaucoma is an eye disease characterized by a progressive loss of vision due to irreversible damage to the optic nerve. The chief pathophysiological feature of glaucoma is increased intraocular pressure (IOP), and it has been demonstrated that lowering IOP could control glaucoma and prevent vision loss.[1] IOP is determined primarily by the dynamic equilibrium between the production of aqueous humor (inflow) in the ciliary body and its efflux (outflow), mainly through the trabecular meshwork and Schlemm's canal.[2]

Aqueous humor is produced by the ciliary epithelial cells located in the ciliary body, and its production is regulated by the carbonic anhydrase (CA) enzymes.[3] Inhibition of CA enzymes decreases bicarbonate secretion and consequently reduces aqueous humor secretion by the ciliary epithelial cells into the posterior chamber, leading to a reduction of IOP.[4] Therefore, systemic carbonic anhydrase inhibitors (CAIs), such as acetazolamide, have been developed for the treatment of glaucoma.[5] However, systemic CAIs generally cause undesired side effects because CA isoforms II, IV, XII are present in many other tissues and organs.[6] To avoid these side effects, topically effective CAIs have been developed in the past 2 decades with two drugs available on the market: dorzolamide (**1**)[7] and brinzolamide (**2**).[8]

While inhibiting the secretion of aqueous humor has become an effective therapy for lowering IOP, another option is to increase the drainage of aqueous humor out of the eye. It is


dorzolamide (**1**)


brinzolamide (**2**)

reported that nitric oxide (NO) is able to increase the aqueous humor outflow rate, leading to IOP lowering.[9] For example, topical application of NO-donors, such as nitrovasodilators,[10] nipradilol,[11] sodium nitroprusside,[12] SIN-1, and SNAP[13] to rabbit eyes reduces IOP through the conventional outflow pathway. Furthermore, IOP lowering is correlated to the intraocular NO production from NO-donors in the rabbit eyes.[13]

It is common practice to use two or three different topical antiglaucoma drugs in combination when a favorable response of lowering IOP is not obtained with monotherapy, but the research on lowering IOP through multiple mechanisms achieved by a single small molecule remains an unexplored arena.[14] Herein, we report the design and synthesis of NO-donor containing CAIs (NO-CAIs) to lower IOP through two mechanisms: carbonic anhydrase inhibition to decrease

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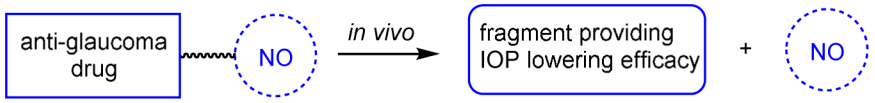
Scheme 1. Concept of Incorporating NO-Donor with Antiglaucoma Drugs



aqueous humor secretion (reduce inflow) and NO release to increase aqueous humor drainage (increase outflow).

RESULTS AND DISCUSSION

Design of NO-Donor Containing Carbonic Anhydrase Inhibitors. To design dual mechanism inhibitors to lower IOP, we decided to take advantage of the IOP lowering efficacy resulting from NO release.[10−13] As shown in Scheme 1, when an NO-donor is incorporated into an appropriate antiglaucoma drug, the new molecule may provide two mechanisms to lower IOP if NO can be intraocularly released. Obviously, a few attributes are required of the chosen antiglaucoma drug in order for the NO-donor containing antiglaucoma drug (NO-antiglaucoma drug) to be effective. First, since NO increases aqueous humor outflow, the NO-antiglaucoma drug may maximize the IOP lowering efficacy if the antiglaucoma drug reduces aqueous humor inflow. Second, the NO-antiglaucoma drug has to be topically administrated because of the in vivo instability; therefore, a topical antiglaucoma drug is preferred. Third, in order to achieve optimal levels of intraocular NO production from the NO-antiglaucoma drug, the drug should possess a relatively high formulated concentration. For example, Xalatan contains only 0.005% formulated latanoprost,[15] and the corresponding NO-latanoprost is unlikely to achieve a high level of in vivo NO release. Finally, both the parent NO-antiglaucoma drug and its corresponding fragment resulting from in vivo NO release should have sufficient potency to elicit efficacy.

Of the approximately 20 antiglaucoma drugs on the market, dorzolamide (**1**) and brinzolamide (**2**) distinguished themselves as ideal drugs to incorporate NO-donors.[16] Both dorzolamide (**1**) and brinzolamide (**2**) inhibit CA enzymes to reduce aqueous humor production, utilize topical administration, and possess relatively high concentrations (2% for dorzolamide and 1% for brinzolamide) in the formulation.[16] In addition, the cocrystal structure analyses of dorzolamide (**1**) and brinzolamide (**2**) with the CA enzyme reveal that introduction of an NO-donor on the alkyl side chains, i.e., the methyl group from dorzolamide (**1**) and the methoxypropyl group from brinzolamide (**2**), would have a minimum impact on binding affinity (Figure 1). Those alkyl side chains reside on the edge of the binding pocket and point toward the solvent, while the sulfonamides interact with the protein in the deep binding pocket.

NO-CAIs **3a−c** and **4a** bearing an NO-donor at the end of the alkyl side chain were designed based on evaluation of expected potency, stability, solubility, and synthetic accessibility (Scheme 2). Compounds **3a−c** were derived from dorzolamide (**1**) with a three-carbon, two-carbon, and one-carbon linker, while compound **4a** was modified from brinzolamide (**2**) with a three-carbon linker. A nitrate ester was chosen as a NO-donor mainly because the alkyl nitrate group could intraocularly decompose to release NO,[9−13] and its relatively small size would help NO-CAIs inherit topical druglike properties from their parent CAIs for topical administration. As NO-CAIs **3a−c** and **4a** penetrate into eye tissues after topical application, they could gradually decompose and generate des-NO fragments

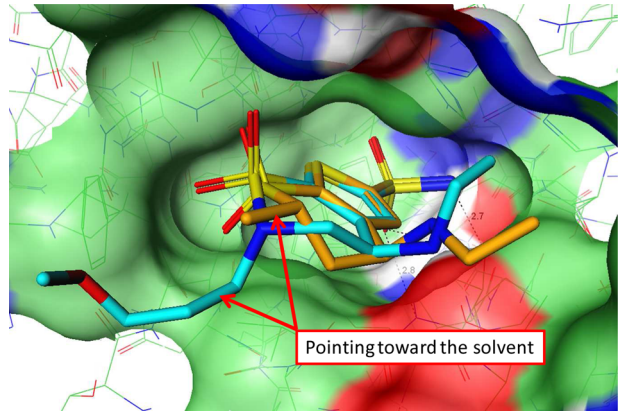


Figure 1. Cocrystal structures of dorzolamide (**1**) (orange, PDB code 1c1l) and brinzolamide (**2**) (cyan, PDB code 3znc) with the CA enzyme.

(**5a−c** and **6a**) while releasing NO. Ideally, both NO-CAIs (**3a−c** and **4a**) and their fragments (**5a−c** and **6a**) would be potent.

Synthesis of NO-CAIs 3a−c and 4a. The synthesis of NO-CAIs **3a−c** is outlined in Schemes 3−5.[7b] The thiopyranone **16** was efficiently constructed from commercially available starting materials (Scheme 3). For the synthesis of **16a**, PCC oxidation of 4-methoxybutan-1-ol followed by a Wittig reaction afforded the trans alkene **8** in 50% yield over two steps. The Michael addition of thiophene-2-thiol to alkene **8** proceeded very slowly to generate ester **9**. This Michael reaction was run neat with 10% Et_3N and still required 2 days to reach completion. When ester **9** was hydrolyzed in 6 M aqueous HCl at 100 °C, it resulted in low conversion and partially decomposed after being refluxed for an extended period of time. The low conversion was due to the poor solubility of ester **9** in 6 M aqueous HCl; the problem was solved by adding glacial acetic acid as a cosolvent. Other mixed solvents, such as aqueous THF or aqueous dioxane, failed to improve the conversion. The crude hydrolyzed product was then treated with 1.0 equiv of Tf_2O in toluene at 0 °C to form the desired thiopyranone **16a** in 53% yield. For the synthesis of **16b**, selective ketone reduction of β-ketoester **10** using $NaBH_4$ at −78 °C, followed by a tosyl protection, afforded ester **11** in ∼40% yield over two steps. S_N2 substitution by thiophene-2-thiol generated ester **12**, which was then treated with standard hydrolysis and cyclization conditions (steps i and j, Scheme 3) to afford thiopyranone **16b**. For the synthesis of **16c**, commercially available ester **13** was reacted with Ag_2O in MeOH, followed by Michael addition of 1.0 equiv of thiophene-2-thiol, generating ester **15** in 80% yield over two steps. Ester **15** underwent our standard hydrolysis/cyclization conditions (steps i and j, Scheme 3) to form thiopyranone **16c**.

With thiopyranone **16** at hand, the subsequent sulfonamidation using optimized reaction conditions afforded sulfonamide **17** in 49−68% yields (Scheme 4). In order to simplify purification and obtain reasonable isolated yields, the sulfonamidation steps required flash purified thiopyrane **16**

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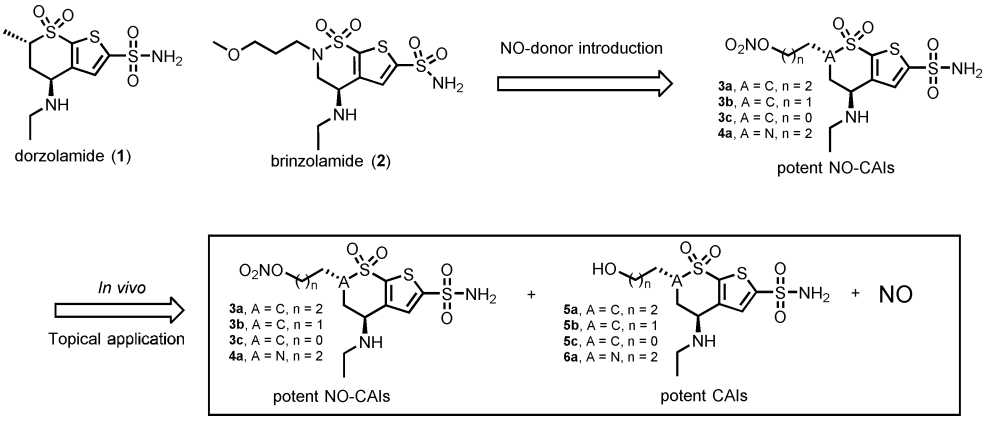
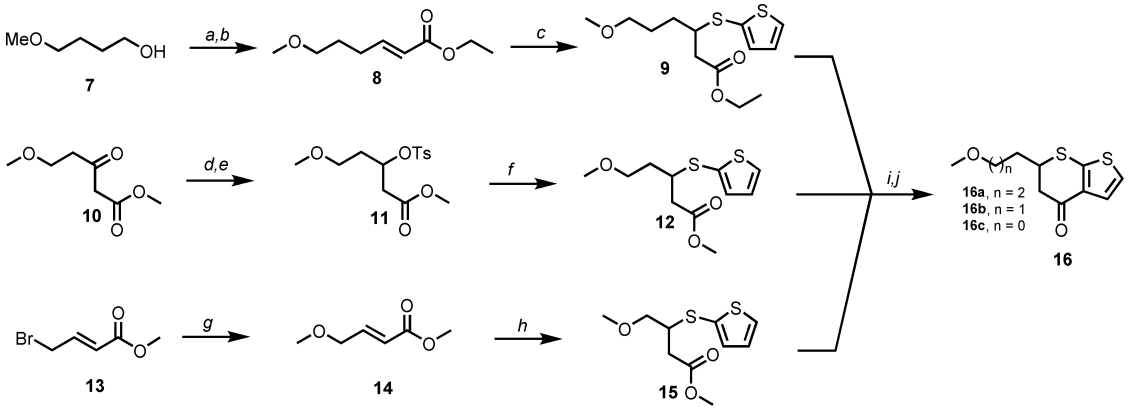
Scheme 2. Design of NO-CAIs 3a−c and 4a



Scheme 3. Synthesis of Thiopyranones 16a−c[a]



[a]Reagents and conditions: (a) 1.7 equiv of PCC, CH_2Cl_2, 25 °C, 3.5 h, 66%; (b) 1.8 equiv of $Ph_3P{=}CO_2Et$, 0 °C, 30 min, CH_2Cl_2, 77%; (c) 1.15 equiv of thiophene-2-thiol, 1−10% Et_3N, 25 °C, 2 d, 89%; (d) 1.1 equiv of $NaBH_4$, MeOH, −78 °C 2 h, 82%; (e) 1.2 equiv of TsCl, pyridine, 25 °C, 30 h, 50%; (f) 1.05 equiv of thiophene-2-thiol, 1.2 equiv of Et_3N, 25 °C, 2−3 h, 84%; (g) 0.75 equiv of Ag_2O, MeOH, 25 °C, sonication, 12 h, (h) 1.0 equiv of thiophene-2-thiol, 0.1 equiv of Et_3N, 25 °C, 12 h, 80% over two steps; (i) 6 M HCl/HOAc (v/v 7:1), 100 °C, 4 h; (j) 1.0 equiv of Tf_2O, toluene, 0 °C 30 min, 25 °C, 2 h, 28−67% over two steps.

because it was unstable even at 0 °C. Sulfonamide **17** was then treated with 2.2 equiv of Oxone in MeOH/H_2O to afford **18** in 71−100% yield. Interestingly, the reduction of **18** using $NaBH_4$ in EtOH generated exclusively alcohol **19** as a cis diastereoisomer in 77−95% yield. Alcohol **19** was exposed to Ritter reaction conditions (4:1 CH_3CN/conc H_2SO_4),[7a] followed by amide reduction using BH_3−THF to afford amine **20** in a 5:1 trans/cis ratio. Chiral separation of amine **20** using supercritical fluid chromatography (SFC) produced enantiomerically pure (−)-**20** in 99% ee.

Interestingly, sulfonamides **20a−c** showed different reactivity toward 48% aq HBr (Scheme 4). When sulfonamide **20a** (three-carbon linker) was treated with 48% aq HBr at 80 °C, the reaction reached completion within 2 days, generating a quantitative yield of the brominated product **21** with complete consumption of alcohol **5a**. Sulfonamide **20b** (two-carbon linker) was also completely converted into the brominated product **22** under 48% aq HBr at 85 °C without remaining alcohol **5b**; however, a much longer reaction time (7 days) was required. Bromides **21** and **22** were then treated with excess $AgNO_3$ in CH_3CN at 50 °C to form NO-CAIs **3a** and **3b** in 78−86% yield. On the other hand, treatment of sulfonamide **20c** with 48% aq HBr failed to deliver the expected brominated product **23**. Instead, the corresponding alcohol **5c** was obtained in 100% yield, even at higher reaction temperatures (85−110 °C) and prolonged reaction times. The failure of direct brominating sulfonamide **20c** was likely due to the steric hindrance of the reactive carbon. In addition the neighboring electron-rich sulfone motif could further prevent the approach of an electronegative nucleophile by electronic repulsion.

After numerous attempts, a successful route to access compound **3c** was outlined in Scheme 5, in which the nitro group was introduced prior to the sulfone formation. As shown in Scheme 5, reduction of sulfonamide **17c** afforded the alcohol **24** in 81% yield as the cis diastereoisomer. Alcohol **24** was exposed under strong Ritter amidation conditions (4:1 CH_3CN/conc H_2SO_4) that were previously used for sulfone **19** (Scheme 4), but it decomposed immediately and gave no desired amidation product **25**. After numerous attempts, mild Ritter reaction conditions were developed and successfully converted alcohol **24** to amide **25** in 80% yield using catalytic amounts of H_2SO_4 (0.7 equiv) at 25 °C. The reaction was also performed under dilute conditions (in 0.035 M CH_3CN) to avoid dimer formation. Interestingly, this mild Ritter amidation condition afforded a trans isomer exclusively. Reduction of amide **25** with BH_3−THF proceeded smoothly to afford amine **26** in 70% yield. When amine **26** was reacted with 48% aq HBr at 80 °C for 12 h, bromide **27** was formed successfully in a

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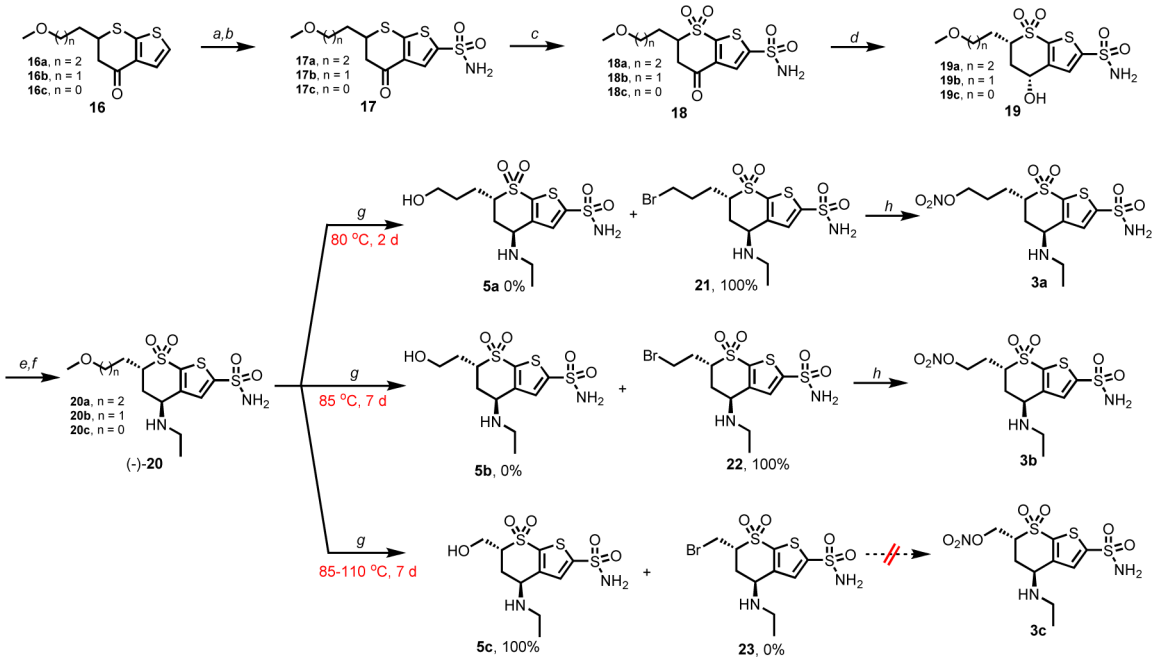
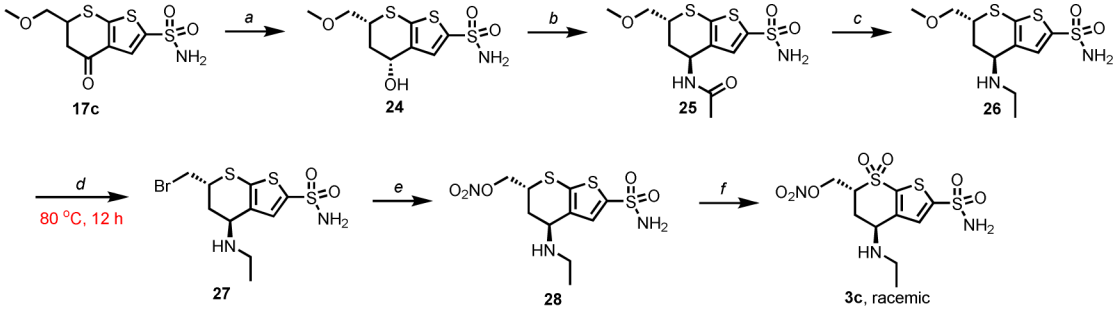
Scheme 4. Complete Synthesis of NO-CAIs 3a and 3b[a]



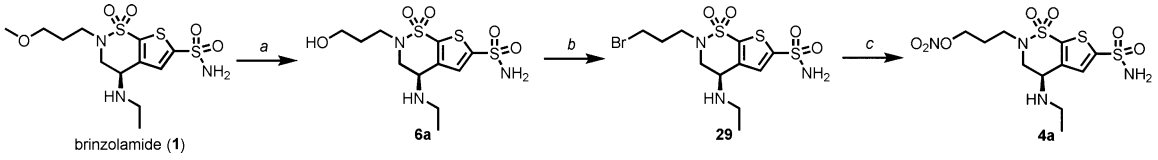
[a]Reagents and conditions: (a) 1.05 equiv of $ClSO_3H$, CH_2Cl_2, −5 °C for 30 min, 25 °C for 3 h; then 1.10 equiv of PCl_5, 0−25 °C, 30 min; (b) 0.5 M NH_3 in dioxane/7.0 M NH_3 in MeOH (v/v 5:2), 0 °C, 2 h, 49−68% overall yield; (c) 2.2 equiv of Oxone, MeOH/water (v/v 5:2), 25 °C, 12 h, 71−100%; (d) 1.3 equiv of $NaBH_4$, EtOH, 0 °C, 15 min; 2 N HCl, 25 °C, 77−95%; (e) CH_3CN/conc H_2SO_4 (v/v 4:1), 25 °C, 48 h, 44−65%; (f) 3.4 equiv of BH_3, THF, 25 °C, 48 h; 2.5 M H_2SO_4, 50 °C or 2 N HCl, 25 °C, 7 h; then chiral separation by SFC, 99% ee, 20−27% overall yield; (g) 48% aq HBr, reaction temperature and yield are indicated in the scheme; (h) 7.7 equiv of $AgNO_3$, CH_3CN, 50 °C, 24−38 h, 78−86%.

Scheme 5. Complete Synthesis of Racemic NO-CAI 3c[a]



[a]Reagents and conditions: (a) 1.3 equiv of $NaBH_4$, EtOH, 0 °C, 15 min; 2 N HCl, 25 °C, 81%; (b) 0.7 equiv of conc H_2SO_4, CH_3CN, 0.035 M, 25 °C, 2 d, 80%; (c) 3.4 equiv of BH_3, THF, 25 °C, 12 h; 2.5 M H_2SO_4, 50 °C, 2 h, 70%; (d) 48% aq HBr, 80 °C, 12 h, quant; (e) 8.0 equiv of $AgNO_3$, CH_3CN, 25 °C, 12 h; (f) 3.0 equiv of Oxone, MeOH/H_2O (v/v 1:1), 25 °C, 12 h, 50% over two steps.

Scheme 6. Synthesis of NO-CAI 2a[a]



[a]Reagents and conditions: (a) 4.0 equiv of BBr_3, CH_2Cl_2, −78 °C, 10 min, 66%; (b) 48% aq HBr, 80 °C, 18 h, 54%; (c) 5.0 equiv of $AgNO_3$, conc HNO_3, CH_3CN, 60 °C, 48 h, 28%.

quantitative yield. The nitration of bromide **27** proceeded at 25 °C to generate **28**, and subsequent oxidation with Oxone formed racemic NO-CAI **3c** in 50% yield over two steps.

The synthesis of NO-CAI **4a** is outlined in Scheme 6.[8b] Commercially available brinzolamide (**1**) was reacted with BBr_3 at −78 °C to afford alcohol **6a** in 66% yield. The subsequent bromination using 48% aq HBr at 80 °C generated bromide **29** in 54% yield. Bromide **29** was then reacted with an excess amount of $AgNO_3$ to form NO-CAI **4a** in 28% yield.

In Vitro and in Vivo Efficacy of NO-CAIs 3a−c and 4a. Compounds **3a−c** and **4a** were profiled together with dorzolamide (**1**) and brinzolamide (**2**) as indicated in Table

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1. Compounds **3a**–**c** and **4a** indeed possessed similar CA2 and CA4 enzymatic potency to their parent antiglaucoma drugs

Table 1. Enzymatic Potency, Lipophilicity, and Solubility of Dorzolamide (1), Brinzolamide (2), 3a–c, and 4a

compd[a]	CA II Kd (nM)	CA IV IC_{50} (nM)	clogD [b]	solubility (mg/mL)
dorzolamide (**1**)	0.043	43	−0.5	>20
3a	0.083	31	0.3	11.3
3b	0.034	47	−0.2	
3c	0.80	156	−0.5	
5a	0.170	82	−0.9	
brinzolamide (**2**)	0.089	45	−0.5	0.5
4a	0.090	31	0.1	2.2
6a	0.136	165	−0.8	

[a]Compound **3c** was racemic, and all other compounds were enantiomerically pure. [b]Calculated log *D*.

dorzolamide (**1**) and brinzolamide (**2**), respectively. The des-NO fragments **5a** and **6a** were also potent but with a modest loss in potency compared to their corresponding parent NO-CAIs **3a** and **4a**, likely due to the decreased lipophilicity.[17] The calculated log *D* of NO-CAIs, dorzolamide (**1**), and brinzolamide (**2**) demonstrated that the introduction of a nitro group had a modest effect on the lipophilicity. Compound **3c** had similar lipophilicity to dorzolamide (**1**). With the introduction of more lipophilic CH_2 groups, **3a** and **3b** became slightly more lipophilic than dorzolamide (**1**). Compound **4a** possessed a little higher clogD than brinzolamide (**2**). The solubility of **3a** and **4a** was also very comparable to **1** and **2**, respectively.

With the overall consideration of efficacy, formulation, and compound supply, NO-CAIs **3a** and **4a** emerged as candidates for rabbit pharmacokinetic (PK) studies using a formulated 2% suspension in Carbopol/Cremaphor,[18] derived from the commercial brinzolamide (**2**) formulation.[19] Figure 2 summarizes the rabbit PK for NO-CAIs **3a** and **4a** at the 4 h time point after topical treatment with 2 drops (2 × 25 μL) of formulated **3a** or **4a**. Both NO-CAIs **3a** and **4a** successfully penetrated into the iris ciliary body and decomposed into their corresponding des-NO fragments **5a** and **6a** as predicted. The formation of des-NO fragments **5a** or **6a** was used as an indicator for NO release, since direct detection of NO formation in vivo has proved to be very challenging.[20] A much higher concentration of **3a** relative to **4a** was measured in the iris ciliary body, which suggested that **3a** has superior in vivo stability to **4a**, resulting in less efficiency in NO release. In aqueous humor, while a relatively high concentration of des-NO fragments **5a** and **6a** was found, only trace amounts of parent NO-CAIs **3a** and **4a** were detected.

The percent inhibition of CA activity was evaluated via a pH STAT method.[21] For NO-CAI **4a**, its CA inhibition was determined and compared to brinzolamide (**2**) at 2, 4, 6, 8 h postdose after topical treatment with 2 drops (2 × 25 μL) of formulated **4a** (Figure 3). While nearly 90% CA inhibition was attained at 2 h postdose with compound **4a**, it gradually decreased but still maintained about 45% inhibition after 8 h. Apparently, the high CA affinity of des-NO fragment **6a** also contributed to the overall CA inhibition, since compound **4a** gradually decomposed to form **6a** in the iris ciliary body and only a trace amount of **4a** was left after 4 h, as indicated in Figure 2. Consistently, NO-CAI **4a** possessed slightly improved efficiency of CA inhibition compared to brinzolamide (**2**) across all four measured time points. Note that, in our hands, compound **4a** could be formulated up to a 2% w/v suspension in Carbopol/Cremaphor by which maximal CA inhibition was achieved. On the other hand, 1%, 2%, 3% formulated brinzolamide (**2**) resulted in similar CA inhibition and efficacy

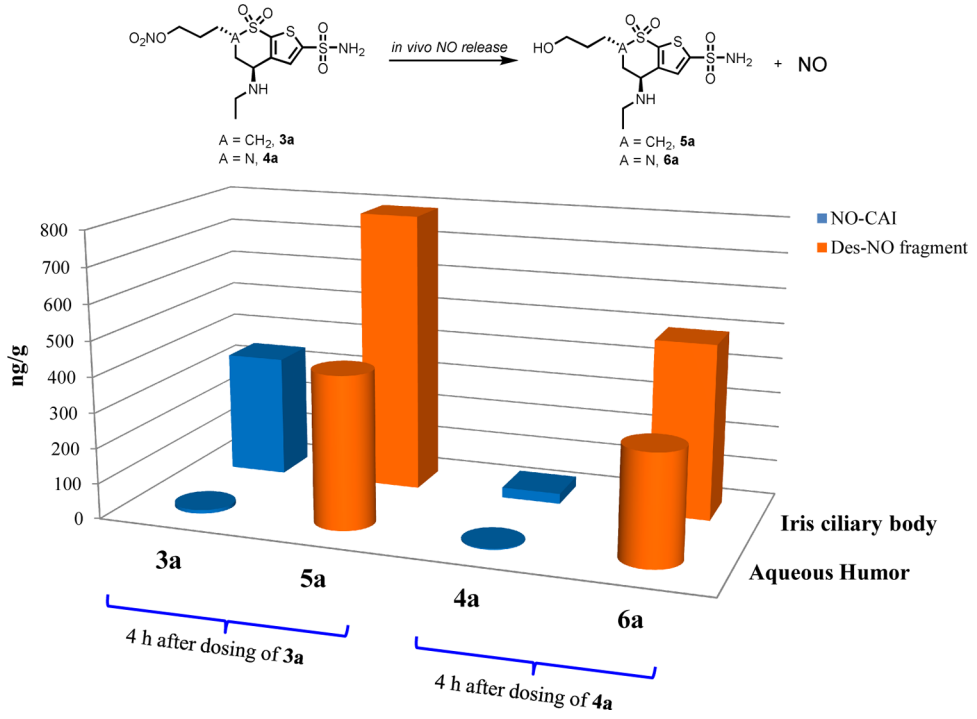

Figure 2. Concentration of NO-CAIs (**3a** and **4a**) and their corresponding des-NO fragments (**5a** and **6a**) in both iris ciliary body and aqueous humor (N = 3 eye) in 4 h after topical treatment of 2 drops (2 × 25 μL) of **3a** or **4a** with a formulated 2% w/v suspension in Carbopol/Cremaphor.

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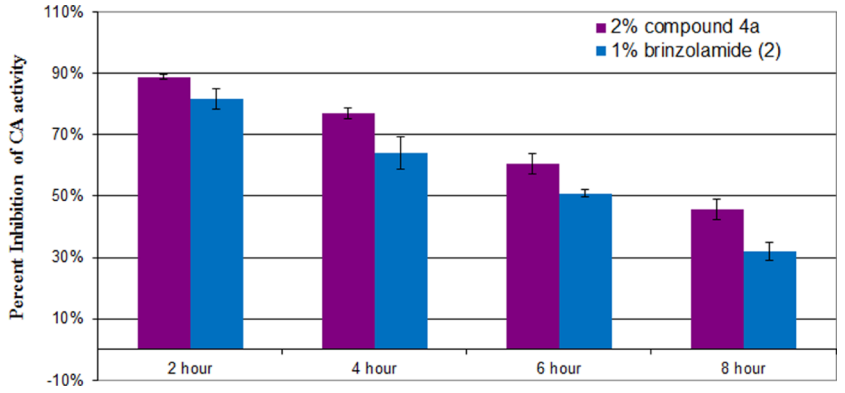

Figure 3. Percent inhibition of CA activities in the rabbit iris ciliary body (n = 3 eye) after topical treatment with 2 drops (2 × 25 μL) of formulated NO-CAI **4a** or brinzolamide (**2**) via pH Stat under the condition of 100 mL/min CO_2.

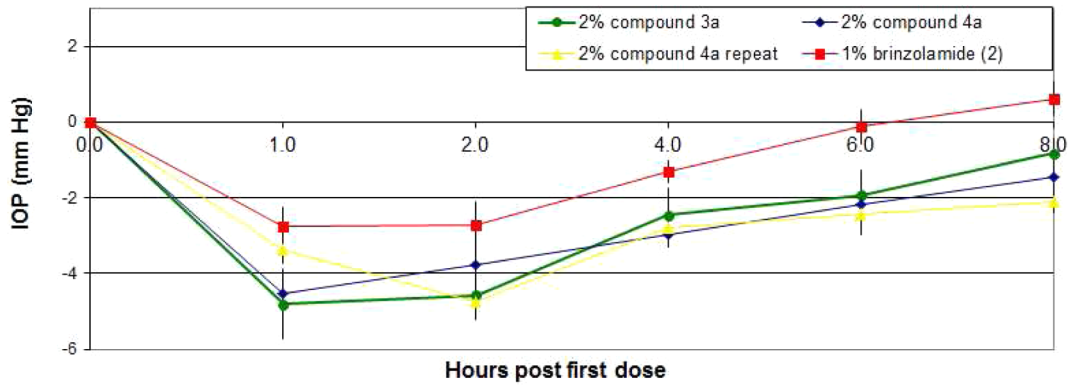

Figure 4. Efficacy of lowering IOP under topical treatment with 2 drops (2 × 25 μL) of formulated **3a**, **4a**, or brinzolamide (**2**) in the rabbit noncontact tonometry (N = 3 eye).

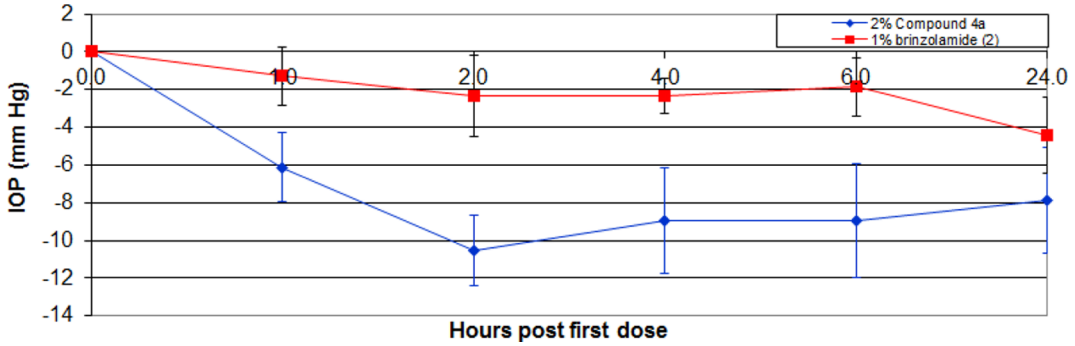

Figure 5. Efficacy of lowering IOP under topical treatment with 2 drops (2 × 25 μL) of formulated **3a** or brinzolamide (**2**) in the OHT primate tonometry (n = 3 eye).

of IOP lowering, indicating that 1% formulated brinzolamide (**2**) has achieved its maximal CA inhibition.[22] Therefore, in our in vivo experiments, 1% commercial brinzolamide ophthalmic suspension[19] was used to compare with 2% formulated NO-CAI **3a**/**4a**, with each reaching its maximal CA inhibition and efficacy of lowering IOP. For NO-CAI **3a**, the CA inhibition was only determined at the 4 h time point after topical treatment with 2 drops (2 × 25 μL) of formulated **3a**, resulting in very similar CA activity inhibition (data not shown) to compound **4a**.

The efficacy of lowering IOP for NO-CAIs **3a** and **4a** was then evaluated in rabbits and monkeys in comparison to brinzolamide (**2**). Using rabbit tonometry (Figure 4), IOP was measured and was shown to decline and hit trough levels in ∼1−2 h under topical treatment with 2 drops (2 × 25 μL) of formulated **3a** or **4a**. This drop was followed by a gradual IOP increase beyond 2 h postdose. While exhibiting similar efficacy in lowering IOP relative to each other, compounds **3a** and **4a** displayed improved efficacy in lowering IOP compared to brinzolamide (**2**) in rabbit tonometry. At trough, NO-CAIs **3a** and **4a** lowered IOP to a maximum of 5 mmHg, which is roughly twice the magnitude of effect shown by brinzolamide (**2**). Although 2% formulated **3a** or **4a** possessed slightly higher greater CA inhibition than 1% formulated brinzolamide (**2**), it is likely that NO release from **3a** and **4a** in the rabbit tonometry contributed to the overall efficacy of lowering IOP. Interestingly, the NO effect on lowering IOP became much more pronounced in the ocular hypertension (OHT) primate

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tonometry. As shown in Figure 5, when monkeys were treated with 2 drops (2 × 25 μL) of formulated **4a**, a significant IOP decrease of 10.5 mmHg was observed at 2 h postdose, and IOP lowering was preserved for 24 h following treatment. In contrast, treatment with 2 drops (2 × 25 μL) of brinzolamide (**2**) lowered IOP by only about 2 mmHg. Given that brinzolamide (**2**) shows close to maximal CA inhibition when measured in the pH stat assay, the significant efficacy increase from **4a** is likely to involve in a second mechanism in addition to just inhibiting CA. It appears that in vivo NO release from **4a** to increase aqueous humor drainage plays a key role in lowering IOP in the OHT primate tonometry.

CONCLUSION

NO-CAIs **3a−c** and **4a** were designed to maximize efficacy of IOP lowering by combining two mechanisms, reducing inflow by CA inhibition to decrease aqueous humor secretion, and increasing outflow by NO release to increase aqueous humor drainage. A nitro group was utilized as an NO-donor and incorporated in the side chains of two topically applied CAIs dorzolamide (**1**) and brinzolamide (**2**) with appropriate carbon linkers. While compound **4a** was made from commercially available brinzolamide (**1**) in three steps, the synthesis of compounds **3a−c** was completed in 12−13 linear steps which featured a few key reactions, such as sulfonamidation, a Ritter reaction, and bromination. The synthesized compounds **3a−c** and **4a** displayed good CA enzymatic potency. In addition, the rabbit PK illustrated that NO-CAIs **3a** and **4a** successfully penetrated and decomposed in the iris ciliary body with a slightly higher efficiency of CA inhibition. The efficacy of lowering IOP from NO-CAIs **3a** and **4a** was evaluated in both rabbits and monkeys in comparison to brinzolamide (**2**), and improved efficacy was observed. In particular, the significant improvement in efficacy from **4a** in the OHT primate tonometry revealed that the dual mechanism inhibitors have great potential to be an effective therapy for lowering IOP.

EXPERIMENTAL METHODS

Starting materials and other reagents were purchased from commercial suppliers and were used without further purification unless otherwise indicated. All reactions were performed under a positive pressure of nitrogen or argon or with a drying tube, at ambient temperature (unless otherwise stated), in anhydrous solvents, unless otherwise indicated. Analytical thin-layer chromatography was performed on glass-backed silica gel 60_F 254 plates (Analtech (0.25 mm)) and eluted with the appropriate solvent ratios (v/v). The reactions were assayed by high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) and terminated as judged by the consumption of starting material. The TLC plates were visualized by UV, phosphomolybdic acid stain, or iodine stain. Microwave assisted reactions were run in a Biotage Initiator. ^{1}H NMR spectra were recorded on a Bruker instrument operating at 400 MHz unless otherwise indicated. ^{1}H NMR spectra are obtained as DMSO-d_6 or $CDCl_3$ solutions as indicated (reported in ppm), using chloroform as the reference standard (7.25 ppm) or DMSO-d_6 (2.50 ppm). Other NMR solvents were used as needed. When peak multiplicities are reported, the following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broadened, dd = doublet of doublets, dt = doublet of triplets. Coupling constants, when given, are reported in hertz. The mass spectra were obtained using liquid chromatography mass spectrometry (LCMS) on an Agilent instrument using atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI). All test compounds showed >95% purity as determined by combustion analysis or by high-performance liquid chromatography (HPLC). HPLC conditions were as follows: XBridge C18 column at 80 °C, 4.6 mm × 150 mm, 5 μm, 5%−95% MeOH/H_2O buffered with 0.2% formic acid/0.4% ammonium formate, 3 min run, flow rate 1.2 mL/min, UV detection (λ = 254, 224 nm). Combustion analyses were performed by Atlantic Microlab, Inc. (Norcross, Georgia).

Synthesis of Compound 3a. *(E)-6-Methoxy-hex-2-enoic Acid Ethyl Ester (**8**).* To an orange suspension of PCC (60 g) in CH_2Cl_2 (700 mL) was added 4-methoxybutan-1-ol (compound **7**, 17 g) slowly at 25 °C. The resulting brown suspension was stirred at 25 °C for 3.5 h. The reaction mixture was diluted with 1.5 L of Et_2O and violently stirred at 25 °C for 10 min. The resulting gray suspension was filtered through Celite. The reaction container was washed with 2 × 200 mL of Et_2O. The organic solutions were combined and passed through Celite again. The filtrate was concentrated and the residue was columned on silica gel using 1:1 hexane/EtOAc to afford 11.0 g of 4-methoxybutyraldehyde in 66% yield as colorless oil.

To a clear solution of $Ph_3P{=}CO_2Et$ (67.5 g, 194 mmol, 1.80 equiv) in CH_2Cl_2 (400 mL) was added a solution of 4-methoxybutyraldehyde (11.0 g) in CH_2Cl_2 (25.0 mL) at 0 °C under N_2. The reaction was not complete after being stirred at 0 °C for 30 min. Another 23 g (66 mmol) of $Ph_3P{=}CO_2Et$ was added, and the mixture was stirred at 0 °C for 30 min to reach completion. The solvent was removed under reduced pressure, and the residue was diluted with 100 mL of hexane to form a white suspension. The suspension was filtered to remove phosphorus salts. The filtrate was concentrated under reduced pressure and columned on silica gel using 4:1 hexane/EtOAc to afford 13.1 g of the desired product in 71% yield as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 6.97 (dt, J = 15.6, 7.0 Hz, 1H), 5.84 (dt, J = 15.6, 1.5 Hz, 1H), 4.19 (q, J = 7.1 Hz, 2H), 3.40 (t, J = 6.3 Hz, 2H), 3.34 (s, 3 H), 2.26−2.32 (m, 2H), 1.71−1.77 (m, 2H), 1.30 (t, J = 7.1 Hz, 3H).

*6-Methoxy-3-(thiophen-2-ylsulfanyl)hexanoic Acid Ethyl Ester (**9**).* To a mixture of compound **8** (13.1 g, 76.1 mmol, 1.0 equiv), thiophene-2-thiol (7.06 mL, 76.1 mmol, 1.0 equiv), and THF (4 mL) was added Et_3N (0.2 mL, 1.47 mmol, 2 mol %) at 25 °C. The crude ^{1}H NMR indicated that about 20% of starting material left after the reaction was stirred at 25 °C for 16 h under N_2. Therefore, another 1.06 mL of thiophene-2-thiol (11.4 mmol, 0.15 equiv) was added and the resulting mixture was stirred at 25 °C for 12 h to reach completion. The mixture was columned on silica gel using 10:1 heptane/EtOAc (partially separated, three columns were run). In total, we obtained 19.5 g of the desired product in 89% yield as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.41 (dd, J = 1.3, 5.3 Hz, 1H), 7.17 (dd, J = 1.3, 3.8 Hz, 1H), 7.02 (dd, J = 3.5, 5.3 Hz, 1H), 4.10−4.22 (m, 2H), 3.39−3.42 (m, 2H), 3.34 (s, 3H), 3.19−3.26 (m, 1H), 2.62 (dd, J = 7.6, 15.6 Hz, 1H), 2.49 (dd, J = 7.1, 15.8 Hz, 1 H), 1.86−1.97 (m, 1H), 1.50−1.80 (m, 3H), 1.28 (t, J = 7.0 Hz, 3H).

*6-(3-Methoxypropyl)-5,6-dihydrothieno[2,3-b]thiopyran-4-one (**16a**).* A mixture of compound **9** (17.0 g), 6.0 M aq HCl (350 mL), and HOAc (50 mL) was warmed to 100 °C. After being refluxed at 100 °C for 4 h, the reaction was complete and was cooled to 25 °C. The reaction mixture was concentrated, and the residue was diluted with brine (300 mL) and toluene (200 mL). The organic layer was separated, dried over Na_2SO_4, filtered, and concentrated to afford a pale yellow oil. The resulting crude hydrolyzed product was dissolved in dry toluene (250 mL), and Tf_2O (10.9 mL, 64.8 mmol, 1.0 equiv) was slowly added at 0 °C. A colorless solution was obtained. The reaction mixture turned slowly from colorless to gray and eventually to brown. The reaction was stirred at 0 °C for 30 min and at 25 °C for 2.0 h. Excess amounts of Tf_2O were destroyed with water. The mixture was diluted by EtOAc (500 mL), washed with brine (400 mL), dried over Na_2SO_4, filtered, and concentrated. The resulting black residue was columned on silica gel using 2:1 heptane/EtOAc to afford 7.5 g of the desired product in 53% as a pale yellow oil. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.45 (d, J = 5.3 Hz, 1H), 7.02 (d, J = 5.5 Hz, 1H), 3.67−3.75 (m, 1H), 3.38−3.43 (m, 2H), 3.33 (s, 3H), 2.94 (dd, J = 16.6, 3.1, 1H), 2.74 (dd, J = 16.6, 11.1, 1H), 1.69−1.89 (m, 4H).

*6-(3-Methoxypropyl)-4-oxo-5,6-dihydro-4H-thieno[2,3-b]-thiopyran-2-sulfonic Acid Amide (**17a**).* Chlorosulfuric acid (0.796 mL, 11.9 mmol, 1.05 equiv) was added into precooled DCM (20 mL).

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The diluted colorless $ClSO_3H$−DCM solution was added slowly into a precooled solution of compound **16a** (2.75 g, 11.35 mmol, 1.0 equiv) in DCM (100 mL) at −10 °C (NaCl−ice bath). A green clear solution was obtained. The mixture was stirred at −5 °C for 30 min and 25 °C for 3.0 h to form a pale orange solution. PCl_5 (2.60 g, 12.50 mmol, 1.10 equiv) was added portionwise at 0 °C, and an orange solution was obtained. After being stirred at 25 °C for 30 min, the reaction mixture turned from orange color into deep blue color. The reaction was dumped into a funnel that contained ice. Water (200 mL) and DCM (100 mL) were added. The organic layer became a blue color. The organic solution was quickly separated, dried over Na_2SO_4, filtered, and concentrated to a deep blue solution in a volume of around 15 mL. To the solution were added slowly 50 mL of 0.5 M NH_3 in dioxane and 20 mL of 7.0 M NH_3 in MeOH at 0 °C. The reaction was stirred at 0 °C until it reached completion. The mixture was concentrated, and the residue was dissolved with EtOAc (200 mL) and brine (200 mL). The organic layer was separated, dried over Na_2SO_4, filtered, and concentrated to afford a yellow solid. The resulting yellow solid was triturated twice with 20 mL of 1:1 EtOAc/hexane to afford 2.20 g of the pure desired product in 60% yield as a pale yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.83 (s, 2H), 7.65 (s, 1H), 3.92−4.00 (m, 1H), 3.32 (t, J = 7.3 Hz, 2H), 3.21 (s, 3H), 2.94 (dd, J = 3.3, 16.9 Hz, 1H), 2.77 (dd, J = 10.0, 16.9 Hz, 1H), 1.70−1.80 (m, 2H), 1.60−1.65 (m, 2H).

*6-(3-Methoxypropyl)-4,7,7-trioxo-4,5,6,7-tetrahydro-7λ^6-thieno-[2,3-b]thiopyran-2-sulfonic Acid Amide (**18a**).* A solution of Oxone (8.42 g, 13.7 mmol, 2.20 equiv) in 40.0 mL of water was added slowly into a solution of compound **17a** (2.0 g, 6.22 mmol, 1.0 equiv) in 100 mL of MeOH at 0 °C. The reaction was stirred at 25 °C for 12 h to reach completion. Methanol was removed under reduced pressure. The resulting yellow suspension was diluted with 100 mL of water. The solid was filtered and dried under reduced pressure at 60 °C to afford 2.20 g of the desired product in 100% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.21 (s, 2H), 7.75 (s, 1H), 4.37−4.45 (m, 1H), 3.35 (t, J = 5.7 Hz, 2H), 3.23 (s, 3H), 3.19−3.23 (m, 2H), 2.03−2.10 (m, 1H), 1.63−1.80 (m, 3H).

*cis-4-Hydroxy-6-(3-methoxypropyl)-7,7-dioxo-4,5,6,7-tetrahydro-7λ^6-thieno[2,3-b]thiopyran-2-sulfonic Acid Amide (**19a**).* To a solution of compound **18a** (2.00 g, 5.66 mmol, 1.0 equiv) in EtOH (100 mL) was added $NaBH_4$ (0.278 g, 7.35 mmol, 1.30 equiv) slowly at 0 °C. The reaction was stirred at 0 °C for 15 min to reach completion. The solvent was removed under reduced pressure. The oily residue was diluted with water (50 mL) and acidified with 2 N HCl to pH = 2−3 to form lots of precipitate. The precipitate was filtered, washed with small amounts of water, and dried to afford 1.7 g of the desired compound in 77% yield as a pale yellow solid. The cis stereochemistry was confirmed by NOSEY experiment. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.05 (s, 2H), 7.56 (s, 1H), 6.08 (br s, 1H), 4.82−4.84 (m, 1H), 3.7−3.80 (m, 1 H), 3.37 (t, J = 5.9 Hz, 2H), 3.24 (s, 3H), 2.42−2.46 (m, 1H), 1.97−2.14 (m, 2H), 1.58−1.80 (m, 3H).

*(4S,6S)-4-Ethylamino-6-(3-methoxy-propyl)-7,7-dioxo-4,5,6,7-tetrahydro-7λ^6-thieno[2,3-b]thiopyran-2-sulfonic Acid Amide (**20a**).* To a suspension of compound **19a** (3.00 g, 8.44 mmol, 1.0 equiv) in anhydrous MeCN (100 mL) under ice−NaCl bath was added slowly 25 mL of conc H_2SO_4. A transparent pale orange solution was then obtained. The reaction was stirred at 25 °C for 48 h to reach completion. It was dumped into a mixture of water (200 mL) and EtOAc (200 mL) at 0 °C and the organic layer was separated. The aqueous solution was washed with 2 × 100 mL of EtOAc. Organic solutions were combined, dried over $MgSO_4$, and concentrated to afford the crude product as brown sticky oil. It was columned on silica gel using 15:1 and 10:1 DCM/MeOH to afford 2.20 g of an amide intermediate in 65% yield as a white solid. The product is a mixture of trans/cis isomers in a 5:1 ratio. ^{1}H NMR for trans isomer (400 MHz, DMSO-d_6) δ ppm 8.62 (d, J = 8.30 Hz, 1H), 8.06 (br s, 2H), 7.41 (s, 1H), 5.16−5.21 (m, 1H), 3.77−3.85 (m, 1H), 3.37 (t, J = 5.8 Hz, 2H), 3.23 (s, 3H), 2.41−2.50 (m, 1H), 2.32−2.38 (m, 1H), 1.96−2.05 (m, 1H), 1.87 (s, 3H), 1.57−1.77 (m, 3H).

The amide intermediate (a mixture of trans/cis isomers in a 5:1 ratio, 1.80 g, 4.53 mmol, 1.0 equiv) was added into a solution of BH_3 (1.45 mL, 15.3 mmol, 3.37 equiv) in 15 mL of THF at −10 °C. The reaction was warmed to 25 °C, and a clear pale yellow solution was obtained. It was stirred at 25 °C for 2 d to reach completion. The reaction mixture was added into 20 mL of aq H_2SO_4 (2.5 M) at 0 °C. After being stirred at 50 °C for 7 h, the mixture was cooled into 0 °C and neutralized by using 10 and 1.0 M NaOH to tune pH to ∼8. The neutralized mixture was extracted with 3 × 100 mL of 9:1 $CHCl_3$/MeOH. The organic solutions were combined, dried over $MgSO_4$, and concentrated to afford 1.74 g of the indicated compound as a mixture of trans/cis isomers in a roughly 5:1 ratio. SFC technology was employed for purification and chiral separation. The desired enatiomerically pure compound (−)-**20a** was isolated in 27% yield (470 mg) as a white solid with >99% ee. The obs is −0.011 and $[\alpha]_D$ is −29.86. LC/MS: (APCI) 383 (M^+ + 1). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.96 (s, 2H), 7.55 (s, 1H), 3.90−3.95 (m, 1H), 3.81−3.88 (m, 1H), 3.37 (t, J = 6.0 Hz, 2H), 3.25 (s, 3H), 2.63−2.67 (m, 1H), 2.42−2.56 (m, 3H), 2.21−2.28 (m, 1H), 1.96−2.04 (m, 1H), 1.54−1.80 (m, 3H), 1.03 (t, J = 7.0 Hz, 3H).

*(4S,6S)-6-(3-Bromopropyl)-4-ethylamino-7,7-dioxo-4,5,6,7-tetrahydro-7λ^6-thieno[2,3-b]thiopyran-2-sulfonic Acid Amide (**21**).* At 0 °C, compound (−)-**20a** (white solid, 200 mg, 0.523 mmol) was dissolved in 20 mL of HBr. A colorless solution was obtained. The mixture was stirred 80 °C for 48 h to reach completion. It was cooled to 25 °C and the solvent was removed under reduced pressure to afford the desired product as a white solid in the form of HBr salts in quantitative yield. LCMS (APCI) m/z 431 (M^+ + 1). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.88 (br s, 2H), 8.18 (s, 2H), 7.86 (s, 1H), 4.71−4.74 (m, 1H), 4.02−4.06 (m, 1H), 3.63 (t, J = 5.8 Hz, 2H), 3.20−3.25 (m, 1H), 3.04−3.09 (m, 1H), 2.58−2.75 (m, 2H), 2.04−2.13 (m, 3H), 1.74−1.81 (m, 1H), 1.24 (t, J = 7.3 Hz, 3H).

*(4S,6S)-4-Ethylamino-6-(3-nitrooxy-propyl)-7,7-dioxo-4,5,6,7-tetrahydro-7λ^6-thieno[2,3-b]thiopyran-2-sulfonic Acid Amide (**3a**).* To a pale yellow homogeneous solution of compound **21** (HBr salt, 268 mg, 0.523 mmol) in MeCN (15.0 mL) was added in one portion of $AgNO_3$ (444 mg, 2.61 mmol, 5.0 equiv) at 0 °C. The reaction was stirred at 50 °C for 20 h, and about $^1/_3$ of SM was left. Another 240 mg of $AgNO_3$ (2.70 mmol, 1.41 equiv) was added, and the resulting suspension was stirred at 58 °C for 18 h to reach completion. The reaction was cooled to 25 °C, and the solvent was removed under reduced pressure. The residue was dissolved in EtOAc (40 mL) and passed through Celite. The filtrate was concentrated and purified by HPLC (water/MeCN/HOAc) to afford the desired product in 78% yield as a white solid. LCMS (APCI) m/z 414 (M^+ + 1). ^{1}H NMR for the product in the form of HCl salt (400 MHz, DMSO-d_6) δ ppm 9.39 (s, 1H), 9.16 (s, 1H), 8.19 (s, 2H), 7.91 (s, 1H), 4.71 (br s 1H), 4.62 (t, J = 6.0 Hz, 2H), 4.16−4.24 (m, 1H), 3.17−3.25 (m, 1H), 3.01−3.09 (m, 1H), 2.75−2.79 (m, 1H), 2.56−2.67 (m, 1H), 1.91−2.10 (m, 3H), 1.72−1.81 (m, 1H), 1.26 (t, J = 7.1 Hz, 3H).

Synthesis of Compound 3b. *Methyl 3-Hydroxy-5-methoxypentanoate.* To a solution of methyl 5-methoxy-3-oxopentanoate (compound **10**, 36.0 g, 225 mmol, 1.0 equiv) in 500 mL of MeOH at −78 °C was added portionwise $NaBH_4$ (10.4 g, 247 mmol, 1.1 equiv). During the addition, lots of bubbles were generated. Aftering being stirred at −78 °C for 2 h, the reaction mixture was concentrated under reduced pressure, diluted with 1 N HCl and extracted with EtOAc twice. The combined organic layers were concentrated to afford 30 g of the desired compound as yellow oil in 82% yield.

*Methyl 5-Methoxy-3-{[(4-methylphenyl)sulfonyl]oxy}pentanoate (**11**).* To a solution of methyl 3-hydroxy-5-methoxypentanoate (30.0 g, 185 mmol, 1.0 equiv) in pyridine (200 mL) at 0 °C was added TsCl (43.2 g, 222 mmol, 1.2 equiv). The resulting orange solution was stirred at rt for 30 h, and the reaction turned into brown color. The reaction mixture was diluted with 500 mL of EtOAc and neutralized with 2 N HCl and 1 N HCl. The organic layer was separated, washed with saturated $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and concentrated. The resulting residue was columned on silica gel using 4:1 and 2:1 heptane/EtOAc to afford 23 g of the desired compound in 50% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.81 (d, J = 8.06 Hz, 2H), 7.35 (d, J = 8.56 Hz, 2H), 5.03 (quin, J = 6.23 Hz, 1H), 3.61 (s,

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3H), 3.33−3.40 (m, 1H), 3.23−3.32 (m, 1H), 3.17 (s, 3H), 2.65−2.81 (m, 2H), 2.45 (s, 3H).

*Methyl 5-Methoxy-3-(2-thienylthio)pentanoate (**12**).* To a solution of compound **11** (21.70 g, 68.59 mmol, 1.0 equiv) in 6.0 mL of THF (very small amounts) was added 2-thiophenethiol (7.05 mL, 72.0 mmol, 1.05 equiv). The resulting yellow solution was stirred at 25 °C for 10 min, and Et_3N (11.5 mL, 1.2 equiv) was added at 0 °C. Lots of solids were generated. The suspension was stirred at 25 °C for 2−3 h to reach completion. The mixture was diluted with EtOAc (400 mL) and washed with brine (500 mL). The organic layer was collected, dried over Na_2SO_4, filtered, and concentrated. The residue was columned on silica gel using 10:1 and 4:1 heptane/EtOAc) to afford 15.0 g of the desired compound in 84% yield as yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.41 (dd, J = 5.41, 1.13 Hz, 1H), 7.17 (dd, J = 3.53, 1.26 Hz, 1H), 7.00−7.04 (m, 1H), 3.71 (s, 3H), 3.48−3.67 (m, 2H), 3.36−3.43 (m, 1H), 3.35 (s, 3H), 2.53−2.68 (m, 2H), 1.75−1.87 (m, 2H).

*6-(2-Methoxyethyl)-5,6-dihydro-4H-thieno[2,3-b]thiopyran-4-one (**16b**).* A mixture of compound **12** (15.0 g, 57.6 mmol), 6.0 M aqueous HCl (300 mL), and HOAc (50 mL) was heated to 100 °C. After being refluxed at 100 °C for 4 h, the reaction was complete. The mixture was cooled to 25 °C, and the mixture was concentrated under reduced pressure. The orange sticky oily residue was diluted with toluene (200 mL), dried over Na_2SO_4, filtered, and concentrated to afford 14.0 g of the hydrolyzed intermediate as a pale yellow oil. The crude hydrolyzed intermediate (14 g, 56.8 mmol) was dissolved in dry toluene (300 mL), and Tf_2O (10.5 mL, 62.5 mmol, 1.10 equiv) was added slowly at 0 °C to give a colorless solution. The reaction mixture turned slowly from colorless to gray and eventually to brown. After being stirred at 0 °C for 30 min, the ice bath was removed and the reaction was stirred at 25 °C for 1.5 h to reach completion. The excess amounts of Tf_2O were destroyed by 10 mL of water. The reaction mixture was then diluted with 100 mL of EtOAc, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The black oily residue was purified on silica gel using 4:1 and 2:1 heptane/EtOAc to afford 8.60 g of the desired product as yellow oil in 67% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.45 (d, J = 5.54 Hz, 1H), 7.02 (d, J = 5.29 Hz, 1H), 3.84−3.92 (m, 1H), 3.45−3.58 (m, 2H), 3.35 (s, 3H), 2.98 (dd, J = 16.74, 3.40 Hz, 1H), 2.75 (dd, J = 16.87, 10.07 Hz, 1H), 1.98−2.07 (m, 2H).

*6-(2-Methoxyethyl)-4-oxo-5,6-dihydro-4H-thieno[2,3-b]-thiopyran-2-sulfonamide (**17b**).* $ClSO_3H$ (1.59 mL, 23.8 mmol, 1.2 equiv) was added into a precooled DCM (20 mL). The resulting diluted colorless $ClSO_3H$−DCM solution was added slowly into a precooled solution of compound **16b** (4.53 g, 19.9 mmol) in 100 mL of DCM at NaCl−ice bath. The resulting deep brown mixture was stirred at rt for 6 h to reach completion. PCl_5 (4.96 g, 23.8 mmol, 1.2 equiv) was then added into the reaction mixture portionwise at 0 °C. The resulting orange solution was stirred at 25 °C for 0.5 h, during which the reaction mixture turned from orange color to deep blue color. The reaction mixture was poured into a separatory funnel that contained ice. Another 200 mL of water and 100 mL of DCM were added. The organic layer turned into a blue color, which was collected quickly, dried over Na_2SO_4, filtered, and concentrated to be a 15 mL blue solution. At 0 °C, to the 15 mL of blue solution were added dioxane (100 mL), precooled 0.5 M NH_3 in dioxane (50 mL), and 7.0 M NH_3 in MeOH (50 mL). The resulting suspension was stirred at rt for 30 min to reach completion. The solid was filtered, and the filtrate was concentrated. The concentrated residue was diluted with EtOAc (200 mL) and washed with brine (200 mL). The organic layer was collected and concentrated under reduced pressure. The resulting residue was kept in the freezer for solidification. The solid was triturated with heptane/EtOH three times to afford 3.8 g of the desired product in 60% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.82 (br s, 1H), 7.65 (s, 1 H), 3.95−4.05 (m, 1H), 3.39−3.50 (m, 2H), 3.24 (s, 3H), 2.91−3.00 (m, 1H), 2.75−2.84 (m, 1H), 1.85−2.06 (m, 2H).

*6-(2-Methoxyethyl)-4-oxo-5,6-dihydro-4H-thieno[2,3-b]-thiopyran-2-sulfonamide 7,7-Dioxide (**18b**).* A solution of Oxone (13.20 g, 21.5 mmol, 2.2 equiv) in 60 mL of water was added slowly into a solution of compound **17b** (3.00 g, 9.80 mmol, 1.0 equiv) in 150 mL of MeOH at 0 °C. The resulting white suspension was then stirred at 25 °C overnight. The solvent (MeOH) was removed under reduced pressure. The residue was diluted with 100 mL of water and filtered. The solid was collected and dried under reduced pressure at 60 °C for 3 h to afford 2.45 g of the desired product in 74% yield as a yellow solid. LCMS (ACPI) m/z 338 ($M^+ - 1$). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.21 (s, 2H), 7.74 (s, 1H), 4.39−4.56 (m, 1H), 3.47−3.56 (m, 2H), 3.24 (s, 3H), 3.22−3.26 (m, 2H), 2.20−2.29 (m, 1H), 1.81−1.91 (m, 1H).

*cis-4-Hydroxy-6-(2-methoxyethyl)-5,6-dihydro-4H-thieno[2,3-b]-thiopyran-2-sulfonamide 7,7-Dioxide (**19b**).* To a suspension of compound **18b** (2.41 g, 7.10 mmol, 1.0 equiv) in 90 mL of EtOH at 0 °C was added slowly $NaBH_4$ (0.322 g, 8.52 mmol, 1.20 equiv). The resulting pale yellow solution was stirred at 0 °C for 15 min to reach completion. EtOH was removed under reduced pressure. To the oily residue was added 50 mL of water. The solution was acidified with 2 N HCl to pH = 2−3 (small amounts of 2 N HCl were needed) to crush out the product. The white solid was filtered, washed with 10 mL of water, and dried under reduced pressure to give 2.0 g of the desired product in 82% yield. The product is exclusively cis isomer. The cis stereochemistry was determined by NOSEY experiments. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.03 (s, 2H), 7.56 (s, 1H), 6.05 (d, J = 7.05 Hz, 1H), 4.85 (ddd, J = 10.45, 6.67, 5.54 Hz, 1H), 3.75−3.87 (m, 1H), 3.55 (t, J = 6.29 Hz, 2H), 3.27 (s, 3H), 2.48−2.54 (m, 1H), 2.05−2.26 (m, 2H), 1.81 (dd, J = 14.35, 7.81 Hz, 1H).

*(−)-(4S,6R)-4-(Ethylamino)-6-(2-methoxyethyl)-5,6-dihydro-4H-thieno[2,3-b]thiopyran-2-sulfonamide 7,7-Dioxide (**20b**).* To a suspension of compound **19b** (2.00 g, 5.86 mmol, 1.0 equiv) in anhydrous MeCN (36 mL) under ice−NaCl bath was added slowly 9 mL of conc H_2SO_4. The resulting transparent pale orange solution was stirred at 25 °C for 48 h (CAUTION: excess reaction time will make the reaction messier) to reach completion. The reaction mixture was diluted with a mixture of water (150 mL) and EtOAc (150 mL) at 0 °C. The organic solution was separated, and the aqueous solution was washed with 2 × 100 mL of EtOAc. Organic layers were combined, dried over Na_2SO_4, and concentrated to form a brown sticky oil. The oily compound was purified on silica gel using 10:1 DCM/MeOH to afford 1.60 g (44% yield) of the desired amide intermediate as a trans/cis mixture in 5:1 ratio. LCMS (APCI, negative) m/z 381 ($M^+ - 1$). ^{1}H NMR for trans isomer (400 MHz, DMSO-d_6) δ ppm 8.63 (d, J = 8.31 Hz, 1H), 8.06 (br s, 2H), 7.41 (s, 1H), 5.16−5.23 (m, 1H), 3.81−3.91 (m, 1H), 3.46−3.60 (m, 2H), 3.26 (s, 3H), 2.39−2.48 (m, 2H), 2.13−2.26 (m, 1H), 1.86 (s, 3H), 1.77−1.85 (m, 1H).

The obtained amide intermediate (trans/cis in 5:1 ratio, 1.60 g, 4.2 mmol) was added into a solution of BH_3 (1.40 mL, 14.2 mmol, 3.37 equiv) in 12 mL of THF at 0 °C. After being stirred at rt for 48 h, the reaction mixture was poured into 130 mL of aq H_2SO_4 (2.5 M) at 0 °C. Lots of white sticky solids were generated, which were slowly redissolved. The mixture was then heated at 50 °C for 2 h to completely decompose amine−borane complexes. The mixture was cooled to 0 °C and basified by 10 M NaOH and 2.0 M NaOH to pH ≈ 8. The resulting mixture was then stirred at rt for another 1 h and extracted with 3 × 100 mL of 9:1 $CHCl_3$/MeOH. The organic solutions were combined, dried over $MgSO_4$, and concentrated to afford 1.10 g of the desired product as a white solid. From ^{1}H NMR, the product consists of a mixture of trans/cis isomers in a 5:1 ratio. The racemic product was separated by SFC to give 310 mg (20% yield) of enatiomerically pure (−)-**20b**. LCMS (APCI) m/z 369.0 (M^+ + 1). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.01 (s, 2H), 7.57 (s, 1H), 3.88−3.97 (m, 2H), 3.51−3.58 (m, 2H), 3.27 (s, 3H), 2.46−2.70 (m, 3H), 2.31−2.45 (m, 1H), 2.13−2.30 (m, 2H), 1.72−1.83 (m, 1H), 1.03 (t, J = 7.05 Hz, 3H).

*(4S,6R)-6-(2-Bromoethyl)-4-(ethylamino)-5,6-dihydro-4H-thieno-[2,3-b]thiopyran-2-sulfonamide 7,7-Dioxide (**22**).* A solution of compound (−)-**20b** (220 mg, 0.597 mmol) in 25 mL of 48% aqueous HBr was stirred at 85 °C for 5 days. The reaction was cooled to 25 °C and all the solvent was removed under reduced pressure to afford 298 mg of the desired product in a HBr salt form as a yellow solid in 100% yield. LCMS (APCI) m/z 416.8 (M^+ + 1). ^{1}H NMR (400 MHz,

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DMSO-d_6) δ ppm 8.98 (br s, 2H), 8.20 (s, 2H), 7.87 (s, 1H), 4.73−4.84 (m, 1H), 4.12−4.22 (m, 1H), 3.76 (t, J = 7.05 Hz, 2H), 3.16−3.29 (m, 1H), 3.01−3.14 (m, 1H), 2.73−2.84 (m, 1H), 2.57−2.70 (m, 1H), 2.42−2.49 (m, 1H), 2.21−2.35 (m, 1H), 1.24 (t, J = 7.18 Hz, 3H).

*(−)-2-[(4S,6R)-2-(Aminosulfonyl)-4-(ethylamino)-7,7-dioxido-5,6-dihydro-4H-thieno[2,3-b]thiopyran-6-yl]ethyl Nitrate (**3b**).* To a pale yellow homogeneous solution of compound **22** (HBr salt, 290 mg, 0.582 mmol) in MeCN (20 mL) at 0 °C was added $AgNO_3$ (770 mg, 4.5 mmol, 7.8 equiv). The resulting suspension was heated at 50 °C for 24 h to reach completion. The reaction was diluted with CH_3CN and filtered through Celite. The filtrate was concentrated to give a yellow solid, which was purified by HPLC to afford 200 mg of the desired product in 86% yield. LCMS (APCI) m/z 400.0 (M^+ + 1). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.88 (br s, 2H), 8.20 (s, 2H), 7.85 (s, 1H), 4.67−4.85 (m, 3H), 4.09−4.20 (m, 1H), 3.19−3.32 (m, 1H), 2.98−3.14 (m, 1H), 2.58−2.81 (m, 2H), 2.31−2.44 (m, 1H), 2.10−2.21 (m, 1H), 1.23 (t, J = 7.05 Hz, 3H).

Synthesis of Compound 3c. *Methyl 4-Methoxy-3-(2-thienylthio)butanoate (**15**).* To a solution of methyl *trans*-4-bromo-2-butenoate (compound **13**, 38.5 g, 215 mmol) in anhydrous MeOH (100 mL) was added Ag_2O (37.4 g, 161 mmol, 0.75 equiv). The reaction mixture was sonicated overnight to reach completion. After filtration to remove solid and rinsing solid with 50 mL of MeOH, the filtrate was mixed with 2-thiophenethiol (25.0 g, 215 mmol, 1.0 equiv) and Et_3N (2.18 g, 21.5 mmol, 0.1 equiv). The reaction mixture was stirred at room temperature overnight. After removal of solvent, the residue was purified on silica gel column using hexane/EtOAc to afford 42.46 g of the desired product as oil in 80% yield over two steps. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.68−7.77 (dd, J = 1.2, 5.3 Hz, 1H), 7.18−7.27 (dd, J = 1.3, 3.2 Hz, 1H), 7.05−7.16 (dd, J = 5.3, 3.5 Hz, 1H), 3.62 (s, 3H), 3.41−3.51 (m, 1H), 3.30−3.41 (m, 2 H), 3.25 (s, 3H), 2.62−2.75 (m, 1H), 2.39−2.49 (m, 1H).

*6-(Methoxymethyl)-5,6-dihydro-4H-thieno[2,3-b]thiopyran-4-one (**16c**).* To a mixture of compound **15** (41.3 g, 168 mmol) in 1,4-dioxane (660 mL) was added 6 N aqueous HCl. After being stirred at 80 °C for 16 h, the reaction mixture was allowed to cool to room temperature, diluted with water (600 mL), and extracted with EtOAc (3 × 400 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated. The residue was passed through a large silica gel plug, eluted with 2 L of 40% EtAOc/hexanes, and concentrated to dryness. The resulting 42.4 g of acid product was diluted with toluene (600 mL) and treated with trifluoroacetic anhydride (151 mL, 1.1 mol, 6.0 equiv). After being stirred at 25 °C for 3 days, the mixture was passed through a silica gel plug, eluted with 500 mL of 25% EtOAc/hexane, and concentrated to dryness. Crude material was purified via normal phase chromatography, eluting with 5−25% EtOAc/hexane to afford 13.79 g of the desired compound as a brown oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.84 (d, J = 5.4 Hz, 1H), 7.05 (d, J = 5.3 Hz, 1H), 3.89−4.03 (m, 1H), 3.60−3.69 (m, 2H), 3.43 (s, 3H), 2.83−2.94 (m, 2H).

*6-(Methoxymethyl)-4-oxo-5,6-dihydro-4H-thieno[2,3-b]-thiopyran-2-sulfonamide (**17c**).* $ClSO_3H$ (2.40 mL, 35.3 mmol, 1.05 equiv) was added into a precooled DCM (20 mL). The diluted colorless $ClSO_3H$−DCM solution was added slowly into a precooled solution of compound **16c** (7.20 g, 34.9 mmol) in DCM (200 mL) at NaCl−ice bath. A deep brown solution was obtained. After being stirred at −5 to 0 °C for 10 min and at 25 °C for 3 h, a brown solution was obtained. The LCMS indicated that the reaction was complete. PCl_5 (7.35 g, 35.3 mmol, 1.05 equiv) was added portionwise at 0 °C, and an orange solution was obtained. The ice bath was removed after the completion of addition. After being stirred at 25 °C for 0.5 h, the reaction mixture turned form orange color to deep blue color. The reaction was dumped into a separatory funnel that contained ice. Another 200 mL of water and 100 mL of DCM were added. The organic layer became a blue color, which was collected quickly and dried over Na_2SO_4, filtered, and concentrated to be about 15 mL of deep blue solution. The solution was placed in 0 °C, and 100 mL of precooled (0 °C) 0.5 M NH_3 in dioxane was added, followed by added 150 mL of 7.0 M NH_3 in MeOH. After being stirred at 0 °C for 2 h, the solid was filtered and the filtrate was concentrated. The residue was diluted with 200 mL of EtOAc and washed with 200 mL of brine. The organic layer was collected and the solvent was the removed to afford a yellow residue, which was triturated twice with EtOAc/heptane to afford 5.0 g of the pure desired product in 49% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.83 (br s, 2H), 7.64 (s, 1H), 4.17−4.30 (m, 1H), 3.53−3.69 (m, 2H), 3.28 (s, 3H), 2.84−2.91 (m, 1H), 2.75−2.84 (m, 1H).

*4-Hydroxy-6-(methoxymethyl)-5,6-dihydro-4H-thieno[2,3-b]-thiopyran-2-sulfonamide (**24**).* Compound **17c** (1.50 g, 5.22 mmol, 1.0 equiv) was suspended in 20 mL of EtOH and cooled to 0 °C. $NaBH_4$ (243 mg, 6.78 mmol, 1.3 equiv) was added portionwise. The pale yellow solution was stirred at 0 °C for 15 min and 25 °C for another 1 h to reach completion. EtOH was removed under reduced pressure. An amount of 20 mL of water was added into the oily residue, and the mixture was acidified with 2 N HCl to pH 5−7. At this point, white solids were precipitated out. The solid was filtered and washed with 5 mL of water. The solid was collected and dried under reduced pressure to give 1.25 g of the desired product in 81% yield as a pale yellow solid. The cis isomer is the major product, which contains <5% trans isomer. The cis stereochemistry was confirmed by 2D NMR. LCMS (APCI) m/z 294 (M^+ − 1). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.59 (s, 2H), 7.44 (s, 1H), 5.65 (d, J = 6.04 Hz, 1H), 4.67 (ddd, J = 9.63, 5.29, 4.97 Hz, 1H), 3.89−3.99 (m, 1H), 3.67 (dd, J = 10.20, 4.66 Hz, 1H), 3.47 (t, J = 9.57 Hz, 1H), 3.28 (s, 3 H), 2.25 (ddd, J = 13.22, 5.16, 2.27 Hz, 1H), 1.70 (ddd, J = 13.22, 10.45, 10.32 Hz, 1H).

*N-[2-(Aminosulfonyl)-6-(methoxymethyl)-5,6-dihydro-4H-thieno-[2,3-b]thiopyran-4-yl]acetamide (**25**).* H_2SO_4 (0.168 mL, 0.70 equiv) was dissolved in anhydrous CH_3CN (7.0 mL) at 0 °C. A solution of H_2SO_4−CH_3CN was added to a clear solution of compound **24** (1.00 g, 3.39 mmol, 1.0 equiv) in 90 mL of anhydrous CH_3CN at 0 °C. After being stirred at 0 °C for 2−3 h, the reaction was continued running at 25 °C for 2 days. The reaction mixture was then concentrated to give a light green suspension. The suspension was placed still at least 3 h for precipitation. The suspension was then filtered and washed with EtOAc to afford 900 mg of the amide product in 80% yield. The trans stereochemistry was confirmed by 2D NMR. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.37 (d, J = 8.31 Hz, 1H), 7.63 (br s, 2H), 7.50−7.76 (m, 2H), 7.27 (s, 1H), 5.04 (ddd, J = 8.50, 4.53, 4.34 Hz, 1H), 3.78−3.88 (m, 1H), 3.67 (dd, J = 10.07, 5.29 Hz, 1H), 3.44−3.54 (m, 1H), 3.30 (s, 3H), 2.02−2.11 (m, 1H), 1.85−1.93 (m, 1H), 1.84 (s, 3H).

*4-(Ethylamino)-6-(methoxymethyl)-5,6-dihydro-4H-thieno[2,3-b]thiopyran-2-sulfonamide (**26**).* Neat solid compound **25** (900 mg, 2.68 mmol, 1.0 equiv) was added into a solution of BH_3−DMS (0.863 mL, 9.10 mmol, 3.40 equiv) in THF (12.0 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 5 min, and a yellow transparent solution was obtained. The reaction was then stirred at 50 °C for 12 h to reach completion. The reaction was cooled to 0 °C and then poured into 20 mL of aq H_2SO_4 (2.5 M). After being stirred at 25 °C for 20 min and at 50 °C for 2 h, all amine−borane complexes were converted into the desired product as indicated by LCMS. The mixture was cooled to 0 °C and was neutralized by using 10 M NaOH and 2.0 M NaOH to tune pH to ∼8. The neutralized mixture was then stirred at 25 °C for another 1 h. The reaction mixture was then extracted with 3 × 30 mL of 7:1 $CHCl_3$/MeOH. The organic solutions were combined, dried over Na_2SO_4, concentrated, and purified by HPLC to afford 600 mg of the desired product in 70% yield as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.20 (s, 1H), 7.58 (br s, 2H), 7.40 (s, 1H), 3.88−3.97 (m, 1H), 3.84 (t, J = 3.78 Hz, 1H), 3.68 (dd, J = 10.07, 5.04 Hz, 1H), 3.48 (dd, J = 9.82, 8.81 Hz, 1H), 3.30 (s, 3H), 2.64−2.73 (m, 1H), 2.53−2.61 (m, 1H), 2.17−2.24 (m, 1H), 1.68 (ddd, J = 14.23, 10.95, 3.78 Hz, 1H), 1.04 (t, J = 7.05 Hz, 3H).

*6-(Bromomethyl)-4-(ethylamino)-5,6-dihydro-4H-thieno[2,3-b]-thiopyran-2-sulfonamide (**27**).* A solution of compound **26** (100 mg, 0.31 mmol) in 5.0 mL of 48% aqueous HBr was stirred at 80 °C for 24 h to reach completion. The resulting pale green solution was concentrated to afford 141 mg of the desired product as a HBr salt in 100% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.72 (br s, 2H),

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7.76 (s, 2H), 7.65 (s, 1H), 4.62 (br s, 1H), 3.96−4.10 (m, 2H), 3.81−3.89 (m, 1H), 3.19 (br s, 1H), 2.99 (br s, 1H), 2.63 (d, J = 2.27 Hz, 1H), 2.10 (ddd, J = 15.11, 11.33, 4.28 Hz, 1H), 1.23 (t, J = 7.18 Hz, 3 H).

*[trans-2-(Aminosulfonyl)-4-(ethylamino)-7,7-dioxido-5,6-dihydro-4H-thieno[2,3-b]thiopyran-6-yl]methyl Nitrate (**3c**).* A mixture of compound **27** (HBr salt, 141 mg, 0.31 mmol), silver nitrate (421 mg, 2.47 mmol, 8.0 equiv), and CH_3CN (5.0 mL) was stirred at 25 °C for 12 h to reach completion. The resulting suspension was filtered to afford a crude compound **28**, which was subjected for the next Oxone oxidation without further purification. To a solution of crude compound **28** in 3 mL of MeOH was added slowly a solution of Oxone (0.78 mmol, 2.5 equiv) in water (3.0 mL) at 0 °C. A white suspension was obtained. The resulting suspension was stirred at 25 °C for 12 h to reach completion. The reaction mixture was carefully diluted with 20 mL of water and extracted with 2 × 30 mL of DCM. The organic layers were combined, dried over Na_2SO_4, concentrated, and purified by HPLC to give 60 mg of the desired product as a yellow solid in 50% yield. LCMS (APCI) 385.9 (M^+ + 1). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.47 (br s, 1H), 9.34 (br s, 1H), 8.22 (s, 2H), 7.97 (s, 1H), 4.98−5.14 (m, 2H), 4.70−4.82 (m, 2H), 3.16−3.33 (m, 1H), 3.00−3.12 (m, 1H), 2.80−2.90 (m, 1H), 2.65−2.76 (m, 1H), 1.27 (t, J = 6.7 Hz, 3H).

Synthesis of Compound 4a. *(R)-4-(Ethylamino)-2-(3-hydroxypropyl)-3,4-dihydro-2H-thieno[3,2-e][1,2]thiazine-6-sulfonamide 1,1-Dioxide (**6a**).* To a slurry of compound **1** (397 mg, 1.04 mmol, 1.0 equiv) in 20 mL of DCM under N_2 at −78 °C was added a solution of BBr_3 (1.0 M, 4.1 mL, 4.10 mmol, 3.94 equiv) dropwise. The slurry was allowed to stir for 10 min at −78 °C and gradually warmed to 25 °C. After being stirred at 25 °C for 24 h, it was carefully quenched by 20 mL of MeOH at 0 °C, and the resulting colorless homogeneous mixture was then stirred at 25 °C for 30 min. The solution was then concentrated to provide an oil that was azeotroped with 3 × 10 mL of MeOH. The crude oil was purified by reverse HPLC (1−30%, CH_3CN/H_2O, 0.1% AcOH) to afford 255 mg of the desired product in 66% yield as a white solid. LCMS (ESI) m/z 370.0 (M^+ + 1). ^{1}H NMR (300 MHz, D_2O) δ ppm 7.82 (s, 1H), 4.76−4.80 (m, 1H), 4.23 (dd, J = 15.6, 4.3 Hz, 1H), 4.09 (dd, J = 15.6, 5.8 Hz, 1H), 3.68 (t, J = 6.0 Hz, 2H), 3.53 (dt, J = 14.3, 7.3 Hz, 1H), 3.38 (dt, J = 13.8, 6.8 Hz, 1H), 3.14 (q J = 7.3 Hz, 2H), 1.82−2.01 (m, 2H), 1.28 (t, J = 7.2 Hz, 3H).

*(R)-2-(3-Bromopropyl)-4-(ethylamino)-3,4-dihydro-2H-thieno-[3,2-e][1,2]thiazine-6-sulfonamide 1,1-Dioxide (**29**).* A solution of compound **6a** (1.05 g, 2.74 mmol) in 10 mL of HBr (48%) was stirred at 80 °C for 18 h to reach completion. The resulting brown homogeneous solution was concentrated under vacuum and the residue was purified by reverse HPLC (1−30%, CH_3CN/H_2O, 0.1% AcOH) to afford 738 mg the desired product in 54% yield as a white solid. LCMS (ESI) m/z 432.0 (M^+ + 1). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.21 (s, 2H), 7.99 (s, 1H), 4.97 (S, 1H), 4.05−4.23 (m, 2H), 3.40−3.71 (m, 4H), 3.23−3.39 (m, 1H), 2.98−3.21 (m, 2H), 2.17 (dt, J = 13.2, 6.6 Hz, 2H), 1.25 (t, J = 7.1 Hz, 3H).

*(R)-3-(4-(Ethylamino)-1,1-dioxido-6-sulfamoyl-3,4-dihydro-2H-thieno[3,2-e][1,2]thiazin-2-yl)propyl Nitrate (**4a**).* To a solution of compound **29** (738 mg, 1.49 mmol) in 25 mL of CH_3CN were added nitric acid (1.0 mL) and $AgNO_3$ (726 mg, 3.0 equiv). The mixture was stirred at 60 °C for 24 h, at which point additional $AgNO_3$ (508 mg, 2.0 equiv) was added in one portion. The resulting mixture was then stirred at 60 °C for additional 24 h to reach completion. The mixture was cooled to 25 °C and diluted with EtOAc (50 mL) and brine (20 mL). The organic layer was collected, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by reverse HPLC (1−30%, CH_3CN/H_2O, 0.1% AcOH) to afford 174 mg of the desired product in 28% yield. LMCS (ESI) m/z 415.0 (M^+ + 1). ^{1}H NMR(300 MHz, DMSO-d_6) δ ppm 8.05 (br s, 2H), 7.69 (s, 1H), 4.59 (t, J = 6.1 Hz, 2H), 4.14 (br s, 1H), 3.84 (s, 2H), 3.45 (dt, J = 14.1, 7.3 Hz, 1H), 3.21−3.32 (m, 1H), 2.56−2.67 (m, 2H), 2.02 (dt, J = 12.8, 6.4 Hz, 2H), 1.04 (t, J = 6.4 Hz, 3H).

Determination of CA II Kd Using Fluorescence Polarization Tight Binding Assay. Compounds were diluted in DMSO at a concentration of 1 mM, 50 μM, and then 0.5 μM and transferred to a 96-well polypropylene plate for further serial dilutions (1:3 dilutions, 11 points) in duplicate. Final compound concentration in CA II Kd FP tight binding assay is 0.01 μM. Assays were conducted in a final volume of 100 μL in 50 mM Tris/HCl (pH 7.6), 100 mM Na_2SO_4, and 0.005% Tween-20 in a 96-well Microfluor 1, black assay plate. Labeled BODIPY558/568-acetazolamide was used as the tracer. Binding inhibition was determined by pipetting 8 μL of human CA II (1.5 nM) into assay plate containing 2 μL of compound and 2 μL of tracer (0.5 nM) in 88 μL of assay buffer. The assay plate was incubated at room temperature for 1 h and read in the fluorescence polarization reader (Molecular Devices, Analyst) at 524/45 nm (excitation) and 595/60 nm (emission). The Kd binding was calculated using GraphPad Prism and Morrison tight binding ligand equation.

Determination of CA IV IC_{50} Using Fluorescence Polarization Assay. Compounds were dissolved in DMSO at a concentration of 1 mM, then to 250 μM in DMSO and transferred to a 96-well plate for further dilutions (1:3 dilutions, 11 points) in duplicate. Final compound highest concentration in CA IV FP assay is 5 μM. Assays were conducted in a final volume of 100 μL in 50 mM Tris/HCl (pH 7.6), 100 mM Na_2SO_4, and 0.005% Tween-20 in a 96-well black assay plate. BODIPY558/568-acetazolamide (catalog no. B-12270 from Invitrogen-Molecular Probes, discontinued item) was used as the tracer. Binding inhibition was determined by pipetting 8 μL of human CA IV (25 nM) into assay plate contained 2 μL of compound and 2 μL of tracer (2 nM) in 88 μL of assay buffer. The assay plate was incubated at ambient temperature for 30 min and read in the fluorescence polarization reader (Molecular Devices, Analyst) at 524/45 nm (excitation), 595/60 nm (emission), and 561 nm (beam splitter). The IC_{50}, the inhibitor concentration resulting in 50% inhibition of the enzyme activity, was calculated using GraphPad Prism or similar in-house software with the IC_{50} curve fitting using the four-parameter logistic equation.

Formulation Methods. Compounds **3a** and **4a** have physical properties similar to those of brinzolamide (**2**) and are poorly soluble in water, making them suitable for a topical ophthalmic suspension formulation. The compounds were formulated at up to 2% w/v final concentration as sterile, aqueous suspensions to be readily suspended and slow settled, following shaking. The final pH was set between 5.5 and 7.5 and the osmolality at 300 mOsm/kg. Each milliliter of formulation contained up to 20 mg of the respective active ingredient and was preserved with benzalkonium chloride (0.2 mg). The vehicle was composed of aqueous 4.5% w/v mannitol, 0.35% w/v carbomer 974P, 1−5% w/v polyethoxylated castor oil, 0.01% w/v edetate disodium, with hydrochloric acid and/or sodium hydroxide to adjust pH if needed. The active ingredients were homogenized and suspended in the aforementioned vehicle to achieve dosing concentrations (0.1−20 mg/mL) required for preclinical evaluation using a magnetic stir bar and magnetic stir plate with 700 rpm mixing speed for 24 h. All the excipients (except for Cremophor EL) used to formulate compounds **3a** and **4a** were USP/NF grade, obtained from Sigma-Aldrich (St. Louis, MO).

Ocular Pharmacokinetic Assessments in Rabbits. Pigmented male Dutch-belted (DB) rabbits ($n \geq 3$ rabbits/time point) weighing 1.5−2.0 kg were used. All animals received a 2 × 25 μL topical dose of formulated **3a** or **4a** in each eye. At 4 h time point, the rabbits were euthanized and enucleated and ocular tissues were dissected. Compounds **3a** and **5a**, or **4a** and **6b**, were isolated from the homogenized rabbit ocular tissue (cornea, iris ciliary body, and aqueous humor) by protein precipitation and quantitated using high performance liquid chromatography/mass spectrometry (LC/MS/MS). The LC/MS/MS system used consisted of two Shimadzu LC-10AD HPLC pumps (Columbia, MD), a CTC HTS PAL autosampler (LEAP, Carbrboro, NC), and a Sciex API 4000 triple quadrupole mass spectrometer (Life Technologies, Foster City, CA). Peak area determination, calculation of the ratio between the analyte to internal standard peak area, and determination of sample concentrations were done using Analyst software (Analyst 1.4.1, Life Technologies, Foster City, CA).

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Inhibition of CA Activity by Using a PH-STAT Method. Pigmented male Dutch-belted (DB) rabbits ($n \geq 3$ rabbits/time point) weighing 1.5−2.0 kg were used. All animals received a 2 × 25 μL topical dose of formulated **3a**, **4a**, or brinzolamide (**2**) in each eye. At the certain time points, the rabbits were euthanized and enucleated and ocular tissues were dissected. The ciliary bodies were rinsed with water and mechanically homogenized with small glass beads in buffer containing 4-(hydroxylethyl)-1-piperazineethanesulfonic acid (HEPES) 0.01 M and tris[hydroxylmethyl]aminomethane hydrochloride (TRIZMA hydrochloride), 0.01 M, with Na_2SO_4, 0.1 M, adjusted to pH 7.5. The sample was agitated for 2 h and then centrifuged at 12 000 rpm for 15 min. The supernatant was used for CA activity determination, which was assessed using the pH stat assay.[21] This assay measures the rate of hydration of CO_2 by determining the rate at which a standard solution of NaOH has to be added to Tris-HCl buffer, pH 8.6, to maintain a constant pH as CO_2 is bubbled into the buffer. Enzymatic activity is proportional to the volume of 0.025 N NaOH that is required to maintain the pH at a value of 8.3.

Intraocular Pressure Measurements. IOP was measured in conscious normotensive Dutch-belted (DB) rabbits or unilaterally lasered ocular hypertensive cynomolgus monkeys following topical ocular dosing of test articles (vehicle and active compound containing formulations). Each animal was dosed topically in the right eye (OD) with 2 × 25 μL drops of freshly formulated NO-CAI (**3a** or **4a**) or brinzolamide (**2**). As controls, contralateral eyes were treated with placebo vehicle only. All test and control articles were administered in the morning (9:00 a.m.) in a masked fashion. IOP was measured using a model 30 classic pneumatonometer (Medtronic, Minneapolis, MN) before administration of test articles (0 h) and then at predetermined time points after test article administration. Animals received 30 μL of topical 0.25% tetracaine HCl (anesthetic, Henry Schein, USA) prior to each IOP measurement. An average of three IOP measurements was taken at each time point for both eyes of an animal. All animal-related procedures were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic research and were approved by the appropriate animal care and use committees of each institute.

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Notes

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ABBREVIATIONS USED

IOP, intraocular pressure; CA, carbonic anhydrase; CAI, carbonic anhydrase inhibitor; NO, nitric oxide; NO-CAI, NO-donor containing carbonic anhydrase inhibitor; NO-antiglaucoma drug, NO-donor containing antiglaucoma drug

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(18) The 2% suspension in carbopol/cremaphor was utilized because this formulation afforded the most efficient CA activity inhibition for both compounds **3a** and **4a**. See the section Experimental Methods for the formulation procedure.
(19) Each milliliter of 1% AZOPT (brinzolamide ophthalmic suspension) contains the following. Active ingredient: brinzolamide, 10 mg. Preservative: benzalkonium chloride, 0.01 mg. Inactives: mannitol, carbomer 974P, tyloxapol, edetate disodium, sodium chloride, purified water, with hydrochloric acid and/or sodium hydroxide to adjust pH.
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