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Analogs of the ATP-Sensitive Potassium (K_{ATP}) Channel Opener Cromakalim with in Vivo Ocular Hypotensive Activity

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Abstract

K_{ATP} channel openers have emerged as potential therapeutics for the treatment of glaucoma, lowering intraocular pressure in animal models and cultured human anterior segments. We have prepared water soluble phosphate and dipeptide derivatives of the K_{ATP} channel opener cromakalim and evaluated their IOP lowering capabilities in vivo. In general, the phosphate derivatives proved to be more chemically robust and efficacious at lowering IOP with once daily dosing in a normotensive mouse model. Two of these phosphate derivatives were further evaluated in a normotensive rabbit model, with a significant difference in activity observed. No toxic effects on cell structure or alterations in morphology of the aqueous humor outflow pathway were observed after treatment with the most efficacious compound (3*S*,4*R*)−**2**, suggesting that it is a strong candidate for development as an ocular hypotensive agent.

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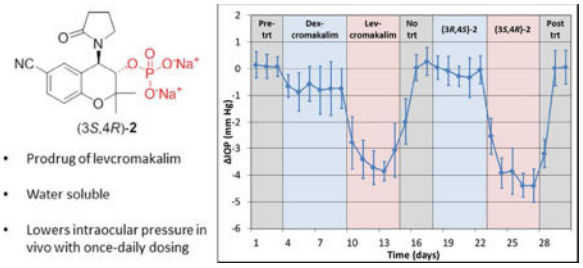

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Supporting Information

Synthesis of compounds (±)-**2** and (−)-**2**. Table S1, effect of compounds (3*S*,4*R*)-**2**, (±)-**2**, and (±)-**4** on IOP in mice. X-ray crystal structure of compound **14**.

Crystallographic Information

A CIF file with the X-ray crystal structure of **14** has been deposited with the Cambridge Crystallographic Data Centre (CCDC 1476916).

Author Contributions

The laboratories of Michael P. Fautsch and Peter I. Dosa contributed equally to work described in this manuscript.

Introduction

Glaucoma is the leading cause of irreversible blindness, affecting an estimated 64 million people worldwide.[1] This number is expected to rise to 112 million by 2040 as the global population of the elderly increases. The most common form of the disease is primary open-angle glaucoma (POAG) which is responsible for approximately 74% of glaucoma cases.[2] Elevated intraocular pressure (IOP) is the most prevalent and only treatable risk factor for the disease.[3–5] All therapies for glaucoma include administration of ocular hypotensive agents to the eye or surgically modifying the outflow pathway to lower IOP. Several long-term clinical trials have demonstrated that lowering IOP slows the progression of glaucoma clinically, even in patients who have normal IOP.[6–8]

Aqueous humor is produced in the eye by the non-pigmented ciliary body epithelium and supplies nutrients to the avascular tissues of the anterior chamber of the eye. In essence, it serves the same function to the front of the eye that blood does to the rest of the body. Aqueous humor exits the eye by one of two outflow routes: the conventional (trabecular) pathway or the uveoscleral pathway. IOP is generated by the balance between the amount of aqueous humor produced and the amount of aqueous humor that is removed from the front part of the eye. [9–11] In adult humans, the conventional outflow pathway, comprising the trabecular meshwork, Schlemm's canal and the associated collector channels is responsible for the drainage of 80% or more of the secreted aqueous humor from the anterior chamber.[5, 12–13] Most cases of POAG are believed to be caused by an increase in resistance to the drainage of aqueous humor through the conventional pathway, leading to a build-up of fluid in the eye and a corresponding rise in IOP, which in turn damages the optic nerve.[10]

Existing classes of therapeutics lower IOP by either reducing the production of aqueous humor (β-adrenergic receptor blockers and carbonic anhydrase inhibitors), by increasing the outflow of the fluid from the eye (prostaglandin agonists and cholinergic drugs) or by a combination of the two mechanisms (α-adrenergic agonists).[14–15] These therapeutics often need to be used in combination to achieve an IOP-lowering effect of the desired magnitude. While there are now several existing classes of therapeutics to lower IOP, each class comes with its own set of side effects such as ocular adverse events for prostaglandins, cardiac and respiratory side effects for β-blockers, and local allergic reactions with α-adrenergic agonists.[15–16] Additionally, none of the current classes of ocular hypotensive agents target the trabecular meshwork, which is the site of increased outflow resistance in glaucoma. As a result, the search for new drugs to treat glaucoma is still a very active area of research.[14,17–19]

Recently, we identified functional ATP-sensitive potassium (K_{ATP}) channels in the human and murine outflow pathways and demonstrated that K_{ATP} channel openers including diazoxide and cromakalim (Figure 1) lower IOP in cultured human anterior segments and in vivo in mice.[20–22] K_{ATP} channels are hetero-octameric complexes comprised of four regulatory sulfonylurea (SUR) receptor subunits and four pore forming inwardly rectifying potassium (K_{ir}) channel subunits. There are three SUR subtypes (SUR1, SUR2A, and SUR2B) as well as two K_{ir} subunits (K_{ir}6.1 and K_{ir}6.2). The subunits that form K_{ATP} channels vary by tissue; for instance K_{ATP} channels are comprised primarily of SUR2A and

$K_{ir}6.2$ in ventricular myocytes,[23] SUR2B and $K_{ir}6.1$ in adipose tissue,[24] and SUR1 and $K_{ir}6.2$ in pancreatic beta-cells.[25] Utilizing pharmacologic K_{ATP} channel openers with selective subunit specificity, and a combination of immunohistochemistry and treatment of $K_{ir}6.2^{(-/-)}$ mice, we determined that the IOP lowering effect of K_{ATP} channel openers was due to their action on K_{ATP} channels comprised of SUR2B and $K_{ir}6.2$ subunits.[21] This combination of SUR and K_{ir} subunits has also been identified in other cell types such as cardiomyocytes.[26]

One of the most promising aspects of our results with diazoxide and cromakalim was the strong ocular hypotensive effect in vivo of these drugs with only once daily dosing. This is important because patient non-compliance is one of the major causes of treatment failure for glaucoma and once a day dosing can help increase compliance.[16, 27] In addition, cromakalim showed a significant additive effect when used in combination with latanoprost,[22] a prostaglandin analog that is one of the first-line drugs used to lower IOP in glaucoma. However, cromakalim and diazoxide have limited aqueous solubility and in our in vivo studies we used DMSO and Cremophor EL to solubilize the K_{ATP} channel openers and permeabilize the cornea so that they could be administered topically.[20–22] Because the toxicity of DMSO makes its clinical use in eye drops undesirable, [28–29] we synthesized water soluble prodrugs of K_{ATP} channel openers and evaluated whether they could be administered topically to lower IOP. Having aqueous solubility is advantageous for topical ophthalmic drug delivery because it allows the minimum amount of fluid to be administered to the eye, which in turn reduces the amount of drug lost to lacrimal drainage.[30] The synthesis of novel prodrugs also allows for the generation of potentially patentable new chemical entities, easing the path to clinical development.[31] We chose to focus our derivatization efforts on cromakalim as it was significantly more potent than diazoxide in reducing IOP at lower concentrations in our human anterior segments,[20, 22] consistent with the relative potencies of the two drugs in activating recombinant SUR2B/$K_{ir}6.2$ subunit containing K_{ATP} channels (3 μM vs. 200 μM).[32] In addition, the secondary alcohol of cromakalim lends itself to chemical conjugations that can be used to improve solubility. Herein we report the synthesis of a series of phosphate and dipeptide derivatives of cromakalim and examine their ability to lower IOP in vivo.

Results and Discussion

Synthesis of Phosphate Analogs of Cromakalim

Our initial efforts were focused on synthesizing phosphate prodrugs of cromakalim because typically these types of prodrugs are relatively easy to prepare, add significantly to the aqueous solubility of the parent drug, and display good chemical stability.[33–34] In addition, a phosphate prodrug (prednisolone sodium phosphate) is already marketed for ophthalmic use. [35–36]

There are two main classes of phosphate ester prodrugs that are commonly used: one in which the phosphate ester is directly attached to the parent compound through an alcohol and one in which the phosphate ester is attached through an oxymethyl spacer group (known as oxymethylphosphate or OMP prodrugs). Both types of prodrugs are activated by alkaline phosphatase, but OMP prodrugs are typically activated more rapidly for steric reasons and

release an equivalent of formaldehyde in addition to the parent drug upon bio-activation.[33–34] Alkaline phosphatase is present in the cornea[37] and previous investigations into phosphate-based prodrugs for ophthalmic use have found complete release of the active parent compounds prior to or during corneal absorption.[30, 36] Juntunen and coworkers determined that a phosphate ester of a lipophilic cannabinoid analog had significantly higher flux across rabbit corneas than the parent compound administered as a suspension.[30]

Cromakalim is a racemic compound, with its activity derived almost entirely from its (3*S*, 4*R*)-enantiomer levcromakalim.[38–39] We initially prepared both the directly-linked and OMP prodrugs of cromakalim in racemic form (compounds **2** and **4**, respectively), and based on the results of our initial animal studies, we proceeded to synthesize the individual enantiomers of **2**.

To obtain the directly-linked phosphate ester prodrug (3*S*,4*R*)-**2**, levcromakalim was phosphitylated with dibenzyl *N,N*-dimethylphosphoramidite[40] in the presence of tetrazole. The resulting phosphite triester was oxidized in situ with H_2O_2 to furnish phosphate triester (3*S*,4*R*)-**1** (Scheme 1). Because attempts to debenzylate **1** using catalytic Pd/C and hydrogen led to extensive decomposition, **1** was instead deprotected using TMSBr.[41] The resulting material was purified by reverse-phase chromatography and converted to a sodium salt by ion-exchange column using Dowex resin.[42] The (3*R*,4*S*)-enantiomer of **2** was prepared by the same synthetic route using dexcromakalim[43] instead of levcromakalim as the starting material. Compound **2** was readily soluble in water at 10 mg/mL and was stable in D_2O solution for extended periods of time (> 6 months). Addition of alkaline phosphatase (in a buffer containing $ZnCl_2$, $MgCl_2$ and sodium glycine) to an aqueous solution of (3*S*,4*R*)-**2** led to the release of levcromakalim, confirming that (3*S*,4*R*)-**2** can act as a prodrug of levcromakalim.

The synthesis of OMP prodrug (±)-**4** began with conversion of cromakalim into the methylthiomethyl derivative (±)-**3** via a Pummerer rearrangement using DMSO, acetic acid and acetic anhydride[42] Next, (±)-**3** was stirred with *N*-iodosuccinimide (NIS) and orthophosphoric acid[44] to furnish (±)-**4**, which was obtained as a sodium salt after ion-exchange filtration through Dowex resin. As some OMP prodrugs can be chemically unstable in aqueous solution,[42] the stability of compound **4** was monitored by NMR spectroscopy. No signs of decomposition were observed after one week in D_2O solution by either ^1H or ^{31}P NMR.

We also prepared the more lipophilic CF_3-substituted analog (3*S*,4*R*)-**9**, as previous studies have suggested that moderately lipophilic compounds may penetrate the cornea better than highly hydrophilic compounds [45–46] Compound **9** is a prodrug of compound **7** (Scheme 3), which as a racemate was reported to have more potent activity than cromakalim at relaxing airway smooth muscle in guinea pigs.[47] The enantioselective synthesis of (3*S*,4*R*)-**9** began with 4-(trifluoromethyl)phenol,[48] which was condensed with 1,1-diethoxy-3-methyl-2-butene in the presence of catalytic base[49] to furnish 6-trifluoromethyl-2,2-dimethylchromene (compound **5**). Jacobsen epoxidation yielded (*S,S*)-**6**, which was purified by silica gel chromatography and then recrystallized to increase the enantiomeric purity of the compound.[43] The epoxide was reacted with 2-pyrrolidone and NaH to furnish (3*S*,4*R*)-**7**.

HPLC analysis determined the enantiomeric excess of (3*S*,4*R*)-**7** to be 99%. The synthesis of (3*S*,4*R*)-**9** was completed using the same sequence of steps used to obtain **2** from levcromakalim.

Synthesis of Dipeptide-Conjugated Analogs

In addition to the phosphate-based prodrugs described above, we also prepared a set of analogs in which levcromakalim was conjugated to a dipeptide. These dipeptide analogs offer several advantages with regards to ocular bioavailability that make their preparation attractive. First, they have the potential to be actively transported into the eye by oligopeptide transporters located within the cornea[45, 50] Second, they are less polar than the highly hydrophilic phosphate prodrugs, and thus would be expected to better penetrate the eye by passive transport mechanisms.

In our initial efforts, we attempted to synthesize Gly-Sar and Gly-Leu conjugates of levcromakalim, as these dipeptides are known to be good substrates for the oligopeptide transporter PepT1.[51] However, the synthesis of these compounds proved to be challenging, likely due to the crowded steric environment near the alcohol of levcromakalim, and unfortunately we were unable to obtain significant amounts of the Gly-Sar conjugate. The best conditions identified for preparing the Gly-Leu conjugate started by coupling Boc-protected Gly-Leu to levcromakalim using several equivalents of HATU and DMAP (Scheme 4). This procedure led to a mixture of epimers (**10** and **12**). After separation by silica gel chromatography, epimers **10** and **12** were individually Boc-deprotected and purified by reverse-phase chromatography, yielding compounds **11** and **13**, respectively, as formic acid salts. Our initial effort to assign the stereochemistry of the two epimers consisted of coupling Boc-Gly-D-Leu-OH with levcromakalim. However, this led to a mixture of **10** and **12** in the same ratio that was obtained with Boc-Gly-Leu-OH, meaning that it was not possible to assume the major product of each reaction contained an unepimerized leucine residue. Instead, X-ray crystallography was used to definitively assign stereochemistry. Since we were unable to obtain suitable X-ray quality single crystals from compounds **10–13**, a more crystalline product was synthesized by coupling compound **11** to 4-bromobenzoic acid (Scheme 4). Analysis of an X-ray crystal structure of the resulting amide **14** determined that **11** was the conjugate of levcromakalim and Gly-D-Leu-OH (see Supporting Information).

Because the in vivo release of levcromakalim from prodrugs **11** and **13** by esterases could potentially be hindered by the sterically crowded environment near the ester in these prodrugs, we also prepared compound (3*S*,4*R*)-**15**, in which the Gly-Leu dipeptide was conjugated to levcromakalim via an acetalester.[52–53] Analogous to OMP prodrug **4**, this compound would release the parent drug and an equivalent of formaldehyde upon bioactivation by esterases. To synthesize this compound, (3*S*,4*R*)-**3** was prepared as a single enantiomer from levcromakalim by the same method that was used to obtain racemic **3** from cromakalim (Scheme 2). Next, this methylthiomethyl derivative was treated with sulfuryl chloride followed by addition of Boc-Gly-Leu-OH to obtain the Boc-protected conjugate (Scheme 5). Deprotection with HCl yielded the desired (3*S*,4*R*)-**15** as an HCl salt.

Intraocular Pressure Lowering Effects of Cromakalim Prodrugs in Normotensive Mice

We have previously reported that cromakalim reduces IOP in normotensive C57BL/6 wild type mice by 3.19 ± 0.41 mm Hg compared to vehicle treated contralateral eyes over a 5 day treatment period with once daily topical administration of 5 mM cromakalim (5 μL) dissolved in a mixture of DMSO, Cremophor EL and phosphate-buffered saline (PBS).[22] To evaluate the cromakalim phosphate-based prodrugs, we treated >7 month old C57BL/6 retired breeders daily for 7 consecutive days with (±)-**2** or (±)-**4** dissolved in PBS. Compounds were administered topically once daily as a 5 μl bolus to one eye while vehicle (PBS) was administered to the contralateral eye. IOP was measured and recorded at times corresponding to 1, 4 and 23 hours after treatment in conscious, non-anesthetized mice using a hand held rebound tonometer (Icare Tonolab; Colonial Medical Supply, Franconia, NH). The average of the 1, 4 and 23 hour readings was used as the daily IOP and expressed as change in IOP (ΔIOP=IOP of treated – IOP of control eye in mm Hg) with respect to the vehicle treated contralateral eye. Treatment with (±)-**2** (2.5 mM) resulted in a significant reduction in IOP (2.50 ± 0.16 mm Hg reduction, n=5, $p<0.001$) following a once daily topical administration for 7 consecutive days (Figure 2A, purple line). In contrast, OMP prodrug (±)-**4** used at a concentration of 2.5 mM (1.81 ± 0.85 mm Hg reduction, n=5, $p<0.001$) (Figure 2A, green line) was both less potent than (±)-**2** at lowering IOP and took a greater number of administrations to reach its maximum effect. In both experiments contralateral eyes treated with vehicle (PBS) showed a slight, but not statistically significant, increase in pressure compared to pre-treatment baseline measurements (see Table S1 in Supporting Information).

Based on the ocular hypotensive effects of (±)-**2** in mice, we synthesized and tested its individual enantiomers. Treatment of C57BL/6 mice with (3*S*,4*R*)-**2**, the enantiomer of **2** derived from levcromakalim, resulted in a significant reduction in IOP (3.14 ± 0.42 mm Hg, n=10, $p<0.001$) following a topical once daily administration of a 5 μl bolus of a 2.5 mM solution in PBS for 7 consecutive days (Figure 2A, red line). Comparison of IOP at 1, 4, and 23 hours after daily administration of (3*S*,4*R*)-**2** showed no significant difference, suggesting that the compound may be suitable for once daily dosing (Figure 3). Additionally, cessation of treatment resulted in IOP returning to baseline within 48 hours. Notably, the robust IOP-lowering ability of an aqueous solution of (3*S*,4*R*)-**2** in the absence of DMSO and Cremophor EL shows that these permeabilizing agents are not necessary for a strong in vivo effect. Furthermore, treatment with (3*S*,4*R*)-**2** did not alter morphology of the aqueous humor outflow pathway and did not show any toxic effects on cell structure (Figure 2B). Taken together, these results suggest that (3*S*,4*R*)-**2** is a strong candidate as an ocular hypotensive agent due to its aqueous solubility, once daily dosing, IOP reduction comparable to the parent compound cromakalim[22], and no observable toxicity following treatment. Additionally, (3*S*,4*R*)-**2** shows similar IOP reduction to other clinically approved drugs that are currently used in first-line treatment of glaucoma (e.g. latanoprost).[54]

We next examined and compared the IOP lowering ability of levcromakalim and (3*S*,4*R*)-**2** to their respective enantiomers dexcromakalim and (3*R*,4*S*)-**2** (Figure 4A). Following establishment of baseline IOP, mice were treated once daily with a topical application of dexcromakalim (initially dissolved in DMSO at a 100 mM concentration and diluted to a

5mM solution with 10% cremophor EL in PBS) for 6 days, followed by 5 days of treatment with levcromakalim (5 mM solution prepared the same as dexcromakalim). Subsequently, IOP was allowed to return to baseline and 5 mM (3*R*,4*S*)-**2** (dissolved in PBS) was added once daily for 5 days followed by 5 mM (3*S*,4*R*)-**2** (dissolved in PBS) for another 5 days. Results show a significant but weak reduction of IOP with dexcromakalim (0.73 ± 0.42 mm Hg reduction, n=10, p<0.001) compared to a robust IOP reduction with levcromakalim (3.37 ± 0.44 mm Hg, n=10, p<0.001). Similarly, treatment with (3*R*,4*S*)-**2** showed minimal IOP reduction (0.14 ± 0.26 mm Hg reduction, n=10, p=0.12) whereas (3*S*,4*R*)-**2** lowered IOP by 3.82 ± 0.35 (n=10, p<0.001) which was similar in magnitude to that observed with its parent compound levcromakalim. Analysis of the aqueous outflow pathways in mouse eyes following treatment did not show any observable changes to cell structure or number when compared to vehicle treated control (Figure 4B).

The more lipophilic CF_3-substituted analog (3*S*,4*R*)-**9** also showed very good IOP reduction when administered topically at 5 mM to mouse eyes, lowering IOP by 3.91 ± 0.29 mmHg compared to vehicle treated contralateral eyes over a 6 day treatment period (Figure 5, purple line). Like (3*S*,4*R*)-**2**, the CF_3-substituted analog showed very little difference between IOP reduction measured at 1, 4, and 23 hours after each dose which is an ideal profile for once daily dosing. However, after treatment was stopped it took more than 72 h for IOP to return to baseline with (3*S*,4*R*)-**9**, which was longer than that observed with (3*S*,4*R*)-**2**.

In contrast to the phosphate-based cromakalim analogs, the dipeptide derivatives **11** (Figure 6, blue line), **13** (red line), and **15** (green line) administered once daily at 5 mM in PBS all performed poorly at lowering IOP in vivo in mice. Although IOP changes were statistically significant when compared to the contralateral eye, all dipeptide compounds exhibited less efficacy and took longer to produce a maximum IOP reduction in comparison to the phosphate-based analogs (**11**, 1.57 ± 0.55 mm Hg, n=5, p=0.003; **13**, 2.45 ± 0.27 mm Hg, n=9, p<0.001; **15**, 1.93 ± 0.39 mm Hg, n=5, p<0.001). In addition, compound **13** showed inconsistent IOP reduction throughout the course of the treatment regiment, suggesting it may be unstable in solution. This is not unexpected, as previously synthesized dipeptide analogs have shown pH-dependent chemical stability profiles, with good stability observed only under moderately acidic conditions (pH 4–6).[50] Because of these less than optimal results, the development of the dipeptide derivatives was discontinued and the phosphate-based analogs advanced into further testing.

Intraocular Pressure Lowering Effects of Compounds in Normotensive Rabbits

Mice have significantly thinner corneas than humans (approximately 130 μM vs. 535 μM),[55] making it easier for topically applied therapeutics to enter the eye. In contrast, rabbits have a thicker cornea than mice (approximately 380 μM)[56] and thus may serve as a better model for evaluating the efficacy of the phosphate-based analogs. Accordingly, both (3*S*,4*R*)-**2** and (3*S*,4*R*)-**9** dissolved in PBS were applied topically to normotensive Dutch-belted pigmented rabbits at a 2.5 mM concentration in a 50 μL bolus once daily for four days, followed by daily dosing at 5 mM for an additional four days (Figure 7). While both compounds showed similar efficacy in the normotensive mouse model, (3*S*,4*R*)-**2** showed better IOP lowering

potency than (3*S*,4*R*)-**9** in the rabbit model at 2.5 mM (2.02 ± 0.63 mm Hg versus 1.06 ± 0.39 mm Hg) and at 5 mM (2.26 ± 0.57 mm Hg versus 1.60 ± 0.52 mm Hg for (3*S*,4*R*)-**9**).

Summary

K_{ATP} channels have emerged as a new therapeutic target for the treatment of glaucoma.[20–22] We have synthesized a set of phosphate and dipeptide derivatives of the K_{ATP} channel opener cromakalim and evaluated their IOP lowering capabilities in vivo. In general, the phosphate derivatives proved to be more chemically robust and efficacious at lowering IOP in a normotensive mouse model. These compounds also had high aqueous solubility, which should facilitate their clinical development. Two of these derivatives, (3*S*,4*R*)-**2** and (3*S*,4*R*)-**9** were further evaluated in a normotensive rabbit model, with a significant difference in activity observed. Because of the efficacy of compound (3*S*,4*R*)-**2** at lowering IOP in both mouse and rabbit models, we are continuing its development as a potential clinical candidate for the treatment of glaucoma.

Experimental Protocols

Chemistry

NMR spectra were recorded using a Bruker 400 spectrometer. ^{1}H NMR data are reported as follows: chemical shift in parts per million downfield of tetramethylsilane (TMS), multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, quint = quintet and m = multiplet), coupling constant (Hz), and integrated value. Coupling constants listed as J_{31P} disappeared when ^{1}H NMR spectra were obtained with ^{31}P decoupling. ^{31}P NMR spectra were taken with complete proton decoupling and were referenced to 85% phosphoric acid, which was added to the NMR tube in a sealed capillary tube. Cromakalim was synthesized by the method of Ashwood[38] and levcromakalim by the method of Jacobsen.[43] All compounds tested in vivo were found to have > 95% purity by HPLC or LC/MS analysis.

Dibenzyl ((3*S*,4*R*)-6-cyano-2,2-dimethyl-4-(2-oxopyrrolidin-1-yl)chroman-3-yl) phosphate (1)

To a stirred suspension of levcromakalim (150 mg, 0.175 mmol) in CH_2Cl_2 (15 mL) was added 0.45 M tetrazole in acetonitrile (11.7 mL, 5.28 mmol) followed by dibenzyl *N,N*-dimethylphosphoramidite (0.600 mL, 2.3 mmol). The reaction mixture was stirred at rt for 2.5 h, then cooled to 0 °C. THF (15 mL) was added, followed by dropwise addition of 30% H_2O_2 (3 mL). After stirring for an additional 15 min., saturated aqueous $Na_2S_2O_3$ (60 mL) was added slowly. The mixture was diluted with water (100 mL) and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The crude material was purified by flash column chromatography on silica gel (5–30% acetone in hexanes as eluent). Impure fractions were repurified as above and combined with the pure material to obtain the title compound as a white foam (0.272 g, 86% yield). ^{1}H NMR (400 MHz, $CDCl_3$): 7.46 (d, J = 8.5 Hz, 1H), 7.40–7.30 (m, 10H), 7.26 (s, 1H), 6.91 (d, J = 8.5 Hz, 1H), 5.55 (d, J = 9.9 Hz, 1H), 5.10–4.96 (m, 4H), 4.60 (dd, J = 9.9 Hz, J_{31P} = 10 Hz, 1H), 3.56–3.46 (m, 1H), 2.94–2.83 (m, 1H), 2.51–2.39 (m, 1H), 2.35–2.23 (m, 1H), 2.02–1.88 (m, 2H), 1.51 (s, 3H), 1.28 (s, 3H). ^{13}C NMR (100

MHz, $CDCl_3$): 18.0, 18.8, 26.7, 31.0, 42.3, 50.2, 69.7 (d, J = 5.7 Hz), 69.8 (d, J = 5.9 Hz), 75.1 (d, J = 6.2 Hz), 79.1 (d, J = 5.0 Hz), 104.9, 118.7, 118.8, 120.4, 127.9, 128.2, 128.65, 128.67, 128.74, 128.75, 131.8, 133.3, 135.3 (d, J = 4.3 Hz), 135.4 (d, J = 5.3 Hz), 156.8, 177.2. ^{31}P NMR (162 MHz, $CDCl_3$): −0.86. HRMS *m/z* calcd for $C_{30}H_{31}N_2NaO_6P$ $[M + Na]^+$ 569.1812, found 569.1831. $[\alpha]_D^{22}$ −8.1 (*c* 1.0, $CHCl_3$).

Sodium (3*S*,4*R*)-6-cyano-2,2-dimethyl-4-(2-oxopyrrolidin-1-yl)chroman-3-yl phosphate [(+)-2]

To a solution of (−)-**1** (122.3 mg, 0.224 mmol) in dry CH_2Cl_2 (6 mL) was added TMSBr (100 μL, 0.758 mmol) by syringe. After stirring for overnight, the reaction mixture was concentrated under reduced pressure. The resulting residue was purified by chromatography (0% acetonitrile/40 mM triethylammonium acetate buffer to 100% acetonitrile, C_{18} column) to yield a white solid after lyophilization. To prepare the sodium salt of (+)-**2**, a 1 cm wide column was filled with 12 cm of DOWEX 50W2 (50–100 mesh, strongly acidic) ion exchange resin. The column was prepared by sequentially washing with 1:1 acetonitrile/water, 1M aqueous $NaHCO_3$ (lots of gas evolution), water, and then finally 1:1 acetonitrile/water. The reaction product was dissolved in 1:1 acetonitrile/water and loaded onto the column, which was eluted with 1:1 acetonitrile/water. The product containing fractions were lyophilized to furnish the title compound as a white solid (63.5 mg, 69% yield). ^{1}H NMR (400 MHz, D_2O): 7.63 (dd, J = 8.6, 1.8 Hz, 1H), 7.46 (bs, 1H), 7.00 (d, J = 8.6 Hz, 1H), 5.26 (d, J = 9.8 Hz, 1H), 4.46 (dd, J = 10.0, J_{31P} = 10 Hz, 1H), 3.66–3.56 (m, 1H), 3.21–3.07 (m, 1H), 2.67–2.51 (m, 2H), 2.20–1.99 (m, 2H), 1.58 (s, 3H), 1.32 (s, 3H). ^{13}C NMR (100 MHz, D_2O): 17.9, 18.6, 26.7, 31.9, 52.1, 72.8 (d, J = 6.4 Hz), 81.1 (d, J = 4.3 Hz), 104.2, 119.1, 120.3, 121.1, 132.8, 134.4, 157.5, 181.3. ^{31}P NMR (162 MHz, D_2O): 0.30. HRMS *m/z* calcd for $C_{16}H_{18}N_2Na_2O_6P$ $[M + H]^+$ 411.0692, found 411.0686. $[\alpha]_D^{22}$ +22.9 (*c* 1.0, H_2O). Racemic **2** was prepared starting from cromakalim by the same set of procedures used to synthesize (+)-**2** from levcromakalim (see Supporting Information).

(±)-(3*S*,4*R*)-2,2-Dimethyl-3-((methylthio)methoxy)-4-(2-oxopyrrolidin-1-yl)chromane-6-carbonitrile (3)

To a solution of racemic cromakalim (188.2 mg, 0.657 mmol) in DMSO (10 mL) was added acetic anhydride (10 mL) and acetic acid (6 mL). After stirring at rt for 24 hours, the reaction mixture was diluted with water (400 mL) and carefully neutralized with solid $NaHCO_3$. The mixture was extracted with ethyl acetate (3 × 400 mL). The organic layers were then each further extracted with water (400 mL), combined, dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Purification by flash chromatography (30% to 100% ethyl acetate/hexanes) on silica gel furnished 202.5 mg of (±)-**3** (89% yield) as a white solid. Use of levcromakalim as the starting material in this procedure yielded (+)-**3** as product [optical rotation $[\alpha]_D^{22}$ +21.0 (*c* 1.0, $CHCl_3$); mp 152–154 °C]. ^{1}H NMR (400 MHz, $CDCl_3$): 7.45 (d, J = 8.5 Hz, 1H), 7.25 (bs, 1H), 6.89 (d, J = 8.5 Hz, 1H), 5.40 (d, J = 9.5 Hz, 1H), 4.83 (d, J = 12.0 Hz, 1H), 4.69 (d, J = 12.0 Hz, 1H), 3.80 (d, J = 9.5 Hz, 1H), 3.53–3.44 (m, 1H), 3.00–2.90 (m, 1H), 2.65–2.44 (m, 2H), 2.18 (s, 3H), 2.16–1.98 (m, 2H), 1.54 (s, 3H), 1.28 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 14.3, 17.9, 19.1, 27.1, 31.2, 43.0, 50.7, 74.7, 76.4, 80.0, 104.6, 118.8, 118.9, 121.5, 132.0, 133.0, 157.2, 176.5. HRMS *m/z* calcd for $C_{18}H_{22}N_2NaO_3S$ $[M + Na]^+$ 369.1243, found 369.1233.

(±)-Sodium (((3S,4R)-6-cyano-2,2-dimethyl-4-(2-oxopyrrolidin-1-yl)chroman-3-yl)oxy)methyl phosphate (4)

To a stirred suspension of (±)-**3** (30.8 mg, 0.089 mmol), phosphoric acid (74.1 mg, 0.756 mmol) and 4 Å molecular sieves (239 mg) in THF (3 mL) at 0 °C, was added a solution of *N*-iodosuccinimide (32.9 mg, 0.146 mmol) in THF (1 mL). After warming to rt over 2 h, a TLC showed starting material remaining, so additional NIS was added (37 mg, 0.164 mmol). An hour later, the mixture was decanted to remove the sieves. Aqueous sodium thiosulfate was added until the color disappeared and then 0.5 mL of 1 M triethylammonium acetate buffer was added. The THF was removed under reduced pressure and the resulting residue purified by chromatography (0% acetonitrile/20 mM triethylammonium acetate buffer to 100% acetonitrile, C_{18} column) to yield 13.4 mg of brown solid after lyophilization. The sodium salt of **4** was prepared by the same procedure described above for **2**. White solid (7.7 mg, 20% yield from **3**). ^{1}H NMR (400 MHz, D_2O): 7.64 (d, J = 8.6 Hz, 1H), 7.49 (s, 1H), 7.01 (d, J = 8.6 Hz, 1H), 5.26 (d, J = 9 Hz, 1H), 5.07 (dd, J = 5.3, J_{31P} = 6 Hz, 1H), 5.02 (dd, J = 5.3, J_{31P} = 6 Hz, 1H), 4.14 (d, J = 10.0 Hz, 1H), 3.72–3.60 (m, 1H), 3.26–3.08 (m, 1H), 2.82–2.68 (m, 1H), 2.66–2.54 (m, 1H), 2.32–2.17 (m, 1H), 2.15–1.99 (m, 1H), 1.61 (s, 3H), 1.30 (s, 3H). LC/MS calculated for $(C_{17}H_{21}N_2O_7P\text{-}H)^-$, 395.1; observed, 395.5.

6-Trifluoromethyl-2,2-dimethylchromene (5)

To a 100 mL 3-necked round bottom flask equipped with a distillation head was added 5,5-diethoxy-2-methylpent-2-ene (5.9 mL, 29.0 mmol) in *p*-xylene (60 mL), followed by 4-(trifluoromethyl)phenol (3.0 g, 18.5 mmol) and 3-picoline (0.60 mL, 6.2 mmol). The reaction flask was heated to an internal temperature of 95 °C for 12 h., then heated at an internal temperature of 115 °C for 7 h., and cooled to room temperature. The reaction mixture was diluted with EtOAc (30 mL) and washed with 1M HCl (3 × 20 mL). The organic layer was washed with saturated aqueous $NaHCO_3$ (2 × 20 mL) and brine (20 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude material was purified by flash column chromatography on silica gel (0–10% EtOAc in hexanes as eluent) to obtain the title compound (1.03 g, 24% yield) as a clear, colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (dd, J = 8.4, 2.2 Hz, 1H), 7.22 (d, J = 2.2 Hz, 1H), 6.83 (d, J = 8.4 Hz, 1H), 6.33 (d, J = 9.8 Hz, 1H), 5.69 (d, J = 9.9 Hz, 1H), 1.46 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.4, 77.4, 116.6, 121.3, 121.6, 123.0 (q, J = 32.4 Hz), 123.6 (q, J = 3.8 Hz), 124.6 (q, J = 269.5 Hz), 126.4 (q, J = 3.7 Hz), 132.0, 155.8.

(1a*S*,7b*S*)-2,2-dimethyl-6-(trifluoromethyl)-1a,7b–dihydro-2H–oxireno[2,3-c]chromene (6)

A solution of bleach (NaOCl) buffered to pH ≈ 11.3 was prepared by addition of 0.05 M Na_2HPO_4 and 1 N NaOH. To 30 mL of this solution cooled to 0 °C was added a solution of **5** (1.26 g, 5.52 mmol) and (*S,S*)-Jacobsen's catalyst (500 mg, 0.79 mmol) in CH_2Cl_2 (20 mL). The mixture was stirred vigorously at 0 °C for 6 hours, after which it was diluted with EtOAc (250 mL) and extracted with water (200 mL) followed by brine (200 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Purification by flash chromatography (0–30% ethyl acetate/hexanes) on silica gel furnished 1.008 g product as a yellow solid. The solid was dissolved in hot ethanol (11 mL) and then

water (8 mL) was added slowly to the solution. After cooling to rt, 747.9 mg of **6** (55%) was obtained as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): 7.61 (d, J = 2.0 Hz, 1H), 7.49 (dd, J = 8.5, 1.8 Hz, 1H), 6.88 (d, J = 8.5 Hz, 1H), 3.93 (d, J = 4.3 Hz, 1H), 3.52 (d, J = 4.4 Hz, 1H), 1.60 (s, 3H), 1.28 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 22.8, 25.6, 50.4, 62.5, 74.0, 118.3, 120.3, 123.3 (q, J = 32.7 Hz), 124.2 (q, J = 268.3 Hz), 127.0 (q, J = 3.9 Hz), 127.5 (q, J = 3.7 Hz), 155.4.

1-((3*S*,4*R*)-3-Hydroxy-2,2-dimethyl-6-(trifluoromethyl)chroman-4-yl)pyrrolidin-2-one (7)

To a stirred solution of (*S,S*)-**6** (735 mg, 3.01 mmol) and 2-pyrrolidinone (0.350 mL, 4.61 mmol) in DMSO (5 mL) was added NaH (60% dispersion in mineral oil, 190 mg, 4.75 mmol). After 2 hours, the mixture was diluted with water (125 mL) and extracted with EtOAc (3 × 150 mL). Each organic extract was then further extracted with water (2 × 125 mL). The organic extracts were combined, dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Purification by flash chromatography (30–100% ethyl acetate/hexanes) on silica gel furnished **7** as a white solid (483.8 mg, 48.8%). The enantiomeric excess of **7** was determined to be 98.9% by HPLC analysis using a Chiralcel OD column (90% hexanes/10% IPA, 1 ml/min., 220 nM). ^{1}H NMR (400 MHz, $CDCl_3$): 7.42 (bd, J = 8.7 Hz, 1H), 7.17 (bs, 1H), 6.90 (d, J = 8.6 Hz, 1H), 5.31 (d, J = 10.3 Hz, 1H), 3.83 (d, J = 6.6 Hz, 1H), 3.76 (dd, J = 10.1, 6.6 Hz, 1H), 3.39–3.29 (m, 1H), 3.11–2.99 (m, 1H), 2.65–2.48 (m, 2H), 2.21–1.97 (m, 2H), 1.54 (s, 3H), 1.27 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 18.2, 18.4, 26.7, 31.4, 42.7, 52.2, 70.7, 79.9, 118.1, 119.6, 123.1 (q, J = 32.9 Hz), 124.2 (q, J = 271.5 Hz), 124.5 (q, J = 3.8 Hz), 126.4 (q, J = 3.6 Hz), 156.4, 178.2. $[\alpha]_D^{22}$ −17.1 (*c* 1.0, $CHCl_3$). Mp 219–222 °C. HRMS m/z calcd for $C_{16}H_{18}F_3N_1NaO_3$ $[M+Na]^+$ 352.1136, found 352.1144.

Dibenzyl ((3*S*,4*R*)-2,2-dimethyl-4-(2-oxopyrrolidin-1-yl)-6-(trifluoromethyl)chroman-3-yl) phosphate (8)

Compound (3*S*,4*R*)-**8** was prepared from (3*S*,4*R*)-**7** by the same procedure described above for the synthesis of **1** from levcromakalim. Clear colorless oil, 63% yield. ^{1}H NMR (400 MHz, $CDCl_3$): 7.43 (d, J = 8.6 Hz, 1H), 7.40–7.30 (m, 10H), 7.19 (s, 1H), 6.91 (d, J = 8.6 Hz, 1H), 5.58 (d, J = 9.9 Hz, 1H), 5.10–4.96 (m, 4H), 4.60 (dd, J = 9.9 Hz, J_{31P} = 10 Hz, 1H), 3.54–3.44 (m, 1H), 2.93–2.84 (m, 1H), 2.51–2.39 (m, 1H), 2.36–2.24 (m, 1H), 2.03–1.83 (m, 2H), 1.51 (s, 3H), 1.28 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 18.2, 18.7, 26.8, 31.2, 42.3, 50.5, 69.6 (d, J = 5.5 Hz), 69.7 (d, J = 5.9 Hz), 75.6 (d, J = 6.4 Hz), 78.5 (d, J = 4.9 Hz), 118.2, 119.4, 123.8 (q, J = 33.0 Hz), 124.1 (q, J = 271.1 Hz), 124.6 (q, J = 3.6 Hz), 126.6 (q, J = 3.6 Hz), 127.9, 128.3, 128.63, 128.66, 128.69, 128.70, 135.46 (d, J = 2.7 Hz), 135.54 (d, J = 1.4 Hz), 155.8, 177.2. ^{31}P NMR (162 MHz, $CDCl_3$): −0.75. HRMS *m/z* calcd for $C_{30}H_{31}F_3N_1NaO_6P$ $[M + Na]^+$ 612.1733, found 612.1745.

Sodium (3*S*,4*R*)-2,2-dimethyl-4-(2-oxopyrrolidin-1-yl)-6-(trifluoromethyl)chroman-3-yl phosphate (9)

Compound (3*S*,4*R*)-**9** was prepared from (3*S*,4*R*)-**8** by the same procedure described above for the synthesis of **2** from **1**. White solid, 80% yield. ^{1}H NMR (400 MHz, D_2O): 7.58 (dd, J = 8.6, 1.4 Hz, 1H), 7.30 (bs, 1H), 7.00 (d, J = 8.6 Hz, 1H), 5.22 (d, J = 9.9 Hz, 1H), 4.41 (dd, J = 10.0, J_{31P} = 10 Hz, 1H), 3.77–3.65 (m, 1H), 3.17–3.03 (m, 1H), 2.74–2.61 (m, 1H),

2.60–2.48 (m, 1H), 2.21–2.08 (m, 1H), 2.08–1.94 (m, 1H), 1.60 (s, 3H), 1.30 (s, 3H). ^{13}C NMR (126 MHz, D_2O): 17.4, 17.9, 23.2, 26.4, 31.5, 44.0, 52.1, 71.5 (d, J = 6.1 Hz), 80.6 (d, J = 3.5 Hz), 117.7, 120.2, 123.0 (q, J = 33.1 Hz), 124.2 (q, J = 270 Hz), 124.5 (q, J = 3.8 Hz), 126.6 (q, J = 3.6 Hz), 155.6, 180.7. ^{31}P NMR (162 MHz, D_2O): 2.09. HRMS *m/z* calcd for $C_{16}H_{18}F_3NNa_2O_6P$ $[M + H]^+$ 454.0614, found 454.0604.

(3*S*,4*R*)-6-Cyano-2,2-dimethyl-4-(2-oxopyrrolidin-1-yl)chroman-3-yl (tert-butoxycarbonyl)glycyl-D-leucinate (10) and (3*S*,4*R*)-6-Cyano-2,2-dimethyl-4-(2-oxopyrrolidin-1-yl)chroman-3-yl (tert-butoxycarbonyl)glycyl-L-leucinate (12)

A suspension of levcromakalim (80.6 mg, 0.281 mmol), DMAP (308.9 mg, 2.53 mmol), and Boc-Gly-Leu-OH (334.7 mg, 1.16 mmol) was stirred for 10 min. in DCM (5 mL). HATU (473.7 mg, 1.25 mmol) was added and the mixture stirred at rt. After 24 h., the solvent was removed under reduced pressure and the resulting residue was purified by chromatography (25% to 75% ethyl acetate/hexanes) on silica gel to furnish 103 mg of **10**, followed by 31.9 mg of **12**, both as waxy white solids. Compound **10**: ^{1}H NMR (400 MHz, $CDCl_3$): 7.48 (dd, J = 8.6, 1.6 Hz, 1H), 7.31 (bs, 1H), 6.93 (d, J = 8.6 Hz, 1H), 6.57 (d, J = 7.4 Hz, 1H), 5.76 (bs, 1H), 5.44 (d, J = 10.5 Hz, 1H), 5.22 (d, J = 10.5 Hz, 1H), 4.56–4.43 (m, 1H), 3.93 (dd, J = 16.7, 6.4 Hz, 1H), 3.75 (dd, J = 16.6, 5.9 Hz, 1H), 3.35–3.25 (m, 1H), 3.00–2.89 (m, 1H), 2.62–2.43 (m, 2H), 2.12–1.95 (m, 2H), 1.74–1.48 (m, 3H), 1.46 (s, 9H), 1.42 (s, 3H), 1.34 (s, 3H), 0.99 (d, J = 6.4 Hz, 3H), 0.95 (d, J = 6.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 18.2, 19.6, 21.7, 22.7, 24.9, 26.2, 28.3, 31.0, 40.5, 42.4, 44.4, 48.9, 51.6, 69.7, 78.5, 80.1, 104.9, 118.8, 119.0, 120.0, 131.8, 133.3, 156.6, 156.9, 170.3, 171.1, 177.4. LC/MS calculated for $C_{29}H_{40}N_4O_7 + H^+$, 557.3; observed, 557.5. Compound **12**: ^{1}H NMR (400 MHz, $CDCl_3$): 7.48 (dd, J = 8.3, 1.5 Hz, 1H), 7.25 (s, 1H), 6.94 (d, J = 8.6 Hz, 1H), 6.65–6.53 (m, 1H), 5.46 (d, J = 10.0 Hz, 1H), 5.33–5.21 (m, 1H), 5.14 (d, J = 10.1 Hz, 1H), 4.67–4.57 (m, 1H), 3.91–3.81 (m, 2H), 3.36–3.26 (m, 1H), 3.00–2.90 (m, 1H), 2.60–2.38 (m, 2H), 2.09–1.97 (m, 2H), 1.74–1.50 (m, 3H), 1.45 (s, 9H), 1.41 (s, 3H), 1.33 (s, 3H), 0.96 (d, J = 6.0 Hz, 3H), 0.94 (d, J = 6.0 Hz, 3H). LC/MS calculated for $C_{29}H_{40}N_4O_7 + H^+$, 557.3; observed, 557.5.

(3*S*,4*R*)-6-Cyano-2,2-dimethyl-4-(2-oxopyrrolidin-1-yl)chroman-3-yl glycyl-D-leucinate (11)

Compound **10** (26 mg, 0.047 mmol) was dissolved in 4 M HCl in dioxane (5 mL) and stirred for 30 min. The reaction mixture was concentrated under reduced pressure. The resulting residue was purified by chromatography (10% acetonitrile/water with 0.1% formic acid to 100% acetonitrile, C_{18} column) to yield 5.0 mg after lyophilization (21% yield) as a formic acid salt. ^{1}H NMR (400 MHz, DMSO-d_6): 8.41 (d, J = 6.5 Hz, 1H), 8.18 (s, 1H), 7.69 (dd, J = 8.6, 1.8 Hz, 1H), 7.49 (bs, 1H), 7.04 (d, J = 8.6 Hz, 1H), 5.30 (d, J = 10.8 Hz, 1H), 5.18 (d, J = 10.5 Hz, 1H), 4.31–4.19 (m, 1H), 3.34–3.29 (m, 2H), 3.20–3.10 (m, 1H), 2.99–2.89 (m, 1H), 2.51–2.46 (m, 1H), 2.28–2.16 (m, 1H), 2.05–1.92 (m, 1H), 1.88–1.74 (m, 1H), 1.71–1.59 (m, 1H), 1.59–1.42 (m, 2H), 1.37 (s, 3H), 1.32 (s, 3H), 0.91 (d, J = 6.6 Hz, 3H), 0.86 (d, J = 6.5 Hz, 3H). LC/MS calculated for $C_{24}H_{32}N_4O_5 + H^+$, 457.2; observed, 457.4.

(3*S*,4*R*)-6-cyano-2,2-dimethyl-4-(2-oxopyrrolidin-1-yl)chroman-3-yl glycyl-L-leucinate (13)

Compound **12** (18.5 mg, 0.033 mmol) was dissolved in 4 M HCl in dioxane (5 mL) and stirred for 30 min. The reaction mixture was concentrated under reduced pressure. The resulting residue was purified by chromatography (10% acetonitrile/water with 0.1% formic acid to 100% acetonitrile, C_{18} column) to yield 4.1 mg after lyophilization (25% yield) as a formic acid salt. ^{1}H NMR (400 MHz, DMSO-d_6): 8.32 (d, *J* = 6.6 Hz, 1H), 8.26 (s, 1H), 7.68 (dd, *J* = 8.5, 1.9 Hz, 1H), 7.49 (bs, 1H), 7.03 (d, *J* = 8.6 Hz, 1H), 5.27 (d, *J* = 10.8 Hz, 1H), 5.17 (d, *J* = 10.3 Hz, 1H), 4.26–4.16 (m, 1H), 3.24–3.12 (m, 1H), 3.00–2.89 (m, 1H), 2.51–2.41 (m, 1H), 2.31–2.19 (m, 1H), 2.02–1.90 (m, 1H), 1.89–1.78 (m, 1H), 1.70–1.56 (m, 2H), 1.53–1.40 (m, 1H), 1.33 (s, 3H), 1.27 (s, 3H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.87 (d, *J* = 6.3 Hz, 3H). LC/MS calculated for $C_{24}H_{32}N_4O_5 + H^+$, 457.2; observed, 457.4.

(3*S*,4*R*)-6-cyano-2,2-dimethyl-4-(2-oxopyrrolidin-1-yl)chroman-3-yl (4-bromobenzoyl)glycyl-D-leucinate (14)

Compound **10** (29.9 mg, 0.054 mmol) was dissolved in 4 M HCl in dioxane (2.5 mL) and stirred for 30 min. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in DCM (5 mL) and 4-bromobenzoic acid (11.4 mg, 0.057 mmol) was added followed by DMAP (134 mg, 1.10 mmol) and HATU (92.1 mg, 0.242 mmol). After 24 h., the solvent was removed under reduced pressure and the resulting residue was purified by chromatography (10% to 100% ethyl acetate/hexanes) on silica gel to furnish 23.1 mg of **14** (67% yield) as a white solid. Crystals suitable for X-Ray crystallography were obtained from DCM/pentane. ^{1}H NMR (400 MHz, $CDCl_3$): 8.30 (t, *J* = 6.5 Hz, 1H), 7.92 (d, *J* = 8.5 Hz, 2H), 7.55 (d, *J* = 8.5 Hz, 2H), 7.49 (dd, *J* = 8.5, 1.7 Hz, 1H), 7.21 (d, *J* = 1.3 Hz, 1H), 6.89 (d, *J* = 8.5 Hz, 1H), 6.72 (d, *J* = 6.8 Hz, 1H), 5.19 (ABq, *J* = 11 Hz, 2H), 4.51–4.39 (m, 2H), 3.77 (dd, *J* = 15.2, 5.1 Hz, 1H), 3.37–3.27 (m, 1H), 2.99–2.91 (m, 1H), 2.82–2.71 (m, 1H), 2.54–2.44 (m, 1H), 2.22–1.94 (m, 2H), 1.79–1.67 (m, 1H), 1.62–1.42 (m, 2H), 1.30 (s, 3H), 1.00 (d, *J* = 6.6 Hz, 3H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.89 (s, 3H). Mp 240–241 °C.

(((3*S*,4*R*)-6-cyano-2,2-dimethyl-4-(2-oxopyrrolidin-1-yl)chroman-3-yl)oxy)methyl glycyl-L-leucinate (15)

To a solution of (3*S*,4*R*)-**3** (57.0 mg, 0.165 mmol) in DCE (1.5 mL) was added sulfuryl chloride (0.95 mL of 1 M in DCM solution, 0.95 mmol). After stirring for 90 min., the solvent was removed under reduced pressure and the resulting residue was left under vacuum for 10 min. The residue was then dissolved in acetonitrile (1 mL) and a solution of Boc-Gly-Leu-OH (110.8 mg, 0.384 mmol) in acetonitrile (3 mL) and DIEA (300 μL, 1.72 mmol) added by syringe. After 3 h., the solvent was removed under reduced pressure and the resulting residue was purified by chromatography (30% to 100% ethyl acetate/hexanes) on silica gel to furnish 96.1 mg of (((3*S*,4*R*)-6-cyano-2,2-dimethyl-4-(2-oxopyrrolidin-1-yl)chroman-3-yl)oxy)methyl (tert-butoxycarbonyl)glycyl-L-leucinate as a white solid (99% yield). ^{1}H NMR (400 MHz, $CDCl_3$): 7.46 (d, *J* = 8.3, 1H), 7.23 (bs, 1H), 6.89 (d, *J* = 8.5 Hz, 1H), 6.47 (d, *J* = 6.4 Hz, 1H), 5.46–5.32 (m, 3H), 5.17–5.04 (m, 1H), 4.65–4.54 (m, 1H), 3.89 (d, *J* = 9.6 Hz, 1H), 3.87–3.73 (m, 2H), 3.45–3.34 (m, 1H), 3.08–2.96 (m, 1H), 2.67–2.49 (m, 2H), 2.21–2.06 (m, 2H), 1.73–1.48 (m, 3H), 1.48 (s, 3H), 1.45 (s, 9H), 1.27 (s, 3H), 0.95 (d, *J* = 4.5 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): 18.2, 19.0, 21.5, 23.0, 24.8, 26.7,

28.3, 31.1, 41.0, 43.0, 44.4, 50.7, 51.0, 76.4, 79.2, 80.5, 89.4, 104.8, 118.8, 118.9, 120.9, 131.7, 133.2, 156.1, 157.0, 169.6, 172.5, 176.5. LC/MS calculated for $C_{30}H_{42}N_4O_8 + H^+$, 587.3; observed, 587.3.

The Boc-protected conjugate (42.4 mg, 0.072 mmol) was dissolved in 4 M HCl in dioxane (2 mL) and stirred for 30 min. The reaction mixture was concentrated under reduced pressure. The resulting residue was washed with diethyl ether, which was then discarded. The remaining material was purified by chromatography (10% acetonitrile/water to 100% acetonitrile, C_{18} column). The product containing fractions were lyophilized after a small amount of dilute HCl was added to them. 8.8 mg white solid (23% yield) as an HCl salt. ^{1}H NMR (400 MHz, DMSO-d_6): 8.81 (d, J = 7.3, 1H), 8.04 (bs, 3H), 7.65 (d, J = 8.7, 1H), 7.45 (s, 1H), 6.99 (d, J = 8.4 Hz, 1H), 5.44 (d, J = 5.8 Hz, 1H), 5.21 (d, J = 6.2 Hz, 1H), 5.13 (d, J = 9.2 Hz, 1H), 4.44–4.33 (m, 1H), 4.07 (d, J = 9.6 Hz, 1H), 3.64 (d, J = 16.3 Hz, 1H), 3.57 (d, J = 16.4 Hz, 1H), 3.46–3.33 (m, 1H), 3.05–2.92 (m, 1H), 2.60–2.44 (m, 1H), 2.42–2.30 (m, 1H), 2.11–1.92 (m, 2H), 1.75–1.62 (m, 1H), 1.62–1.51 (m, 2H), 1.47 (s, 3H), 1.18 (s, 3H), 0.92 (d, J = 6.5 Hz, 3H), 0.87 (d, J = 5.8 Hz, 3H). LC/MS calculated for $C_{25}H_{34}N_4O_6 + H^+$, 487.3; observed, 487.4.

In Vivo Experiments

All animal studies adhered to the tenets of the *Association for Research in Vision and Ophthalmology* (ARVO) Statement for the use of animals in ophthalmic and vision research. All protocols utilizing animals were reviewed and approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC A43414, A65613, A67713, A42713).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Used

DCE	1,2-dichloroethane
DCM	dichloromethane
DIEA	*N,N*-diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMSO	dimethyl sulfoxide

HATU	1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate
IOP	intraocular pressure
K_{ATP} channel	ATP-sensitive potassium channel
K_{ir} channel	inwardly rectifying potassium channel
NIS	*N*-iodosuccinimide
OMP	oxymethylphosphate
PBS	phosphate-buffered saline
POAG	primary open angle glaucoma
SUR	sulfonylurea
THF	tetrahydrofuran
TMSBr	trimethylsilyl bromide
Sar	sarcosine

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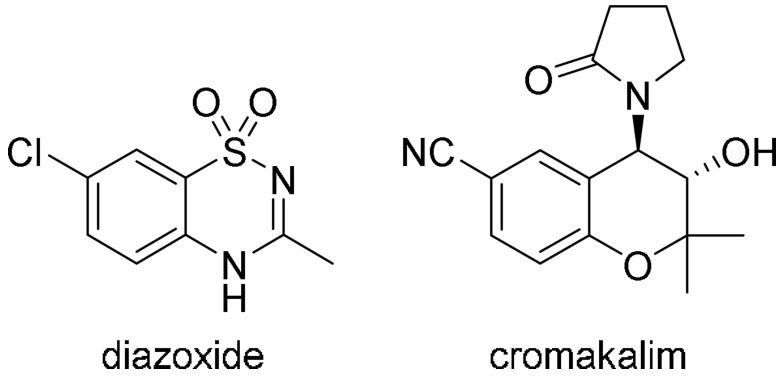


Figure 1.
Structures of K_{ATP} channel openers diazoxide and cromakalim.

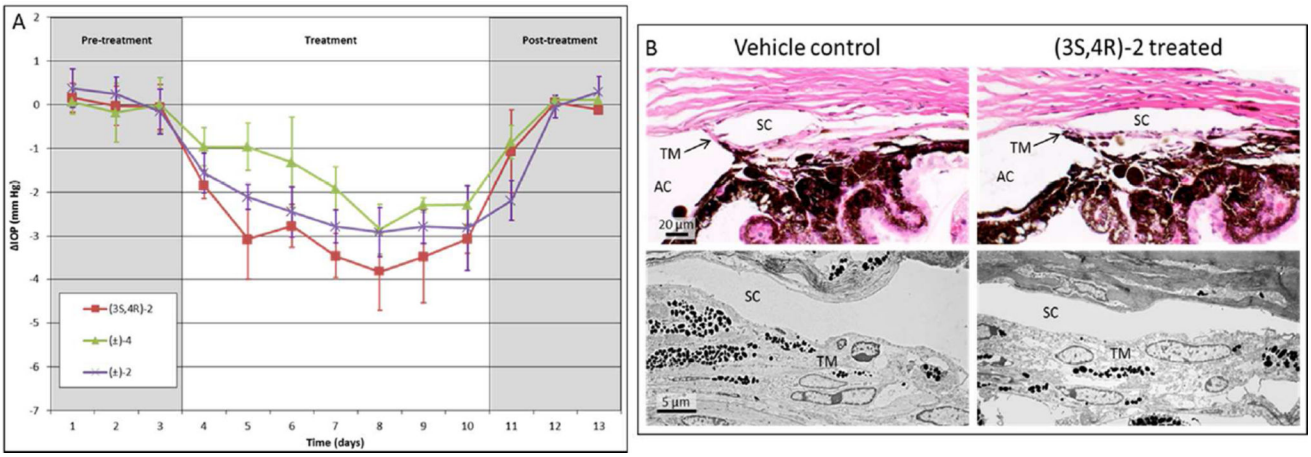


Figure 2.

(A) Comparison of IOP lowering effects of cromakalim prodrugs following once daily treatment for 7 consecutive days. (±)-**2**, 2.5 mM in PBS, n=5 (days 1–8) and n=3 (days 9–13), purple line; (±)-**4**, 2.5 mM in PBS, n=5 (all days), green line; (3*S*,4*R*)-**2**, 2.5 mM in PBS, n=10 (days 1–10) and n=6 (days 11–13), red line. Note that several animals were sacrificed prior to post-treatment to examine ocular histology and morphology. (B) Representative images of the aqueous outflow pathway in mice following treatment with (3*S*,4*R*)-**2** or vehicle control. Hematoxylin and eosin stained sections show that tissue morphology is maintained in (3*S*,4*R*)-**2** treated eyes. Transmission electron micrographs show that no observable changes occurred to cell structure or number in the aqueous outflow pathway following treatment with (3*S*,4*R*)-**2**. TM, trabecular meshwork; SC, Schlemm's canal; AC, anterior chamber.

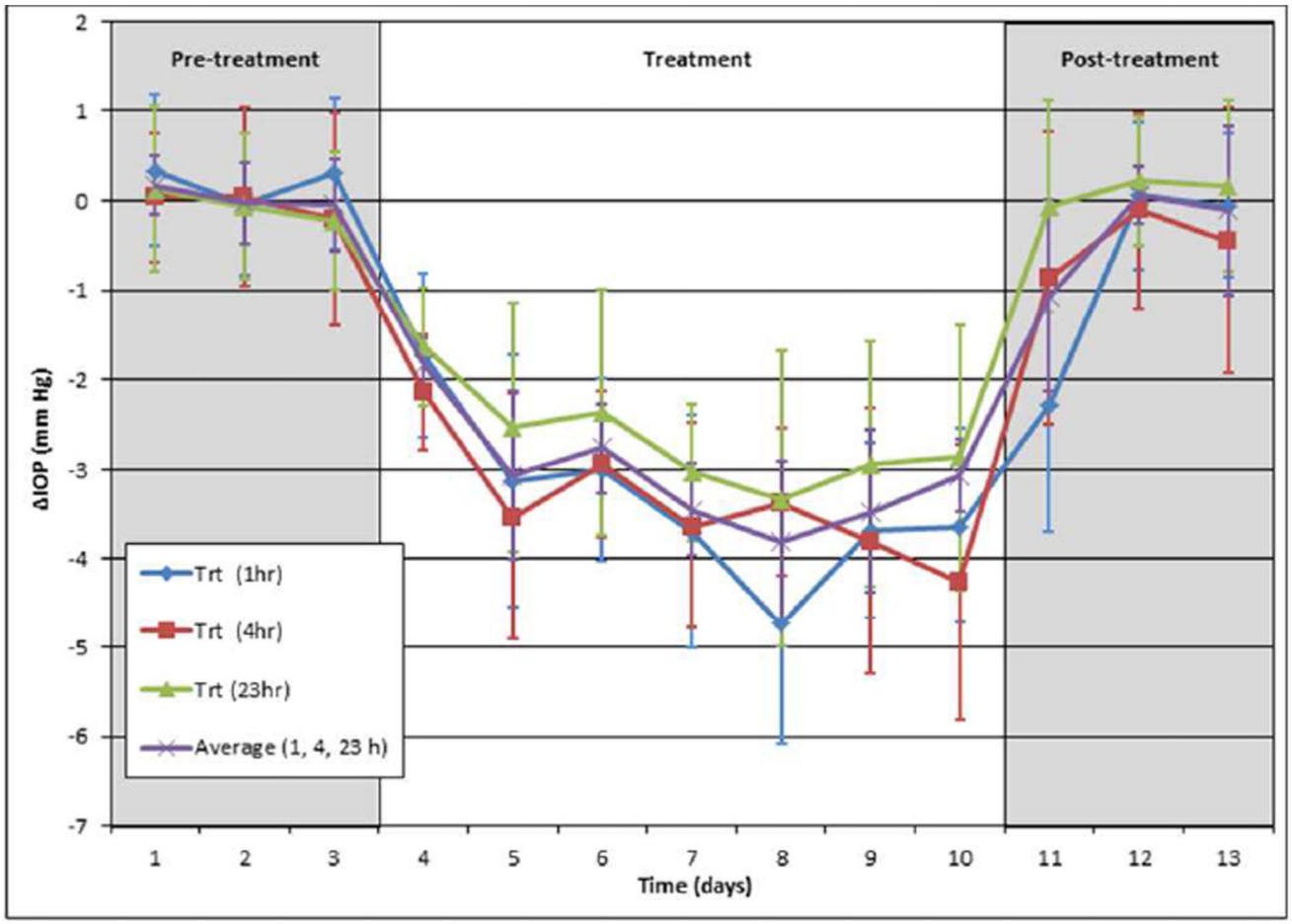


Figure 3.

C57BL/6 mice treated with (3*S*,4*R*)-**2** (2.5 mM in PBS) at 1, 4, and 23 h post-treatment, n=10 (for days 1–10) and n=6 (for days 11–13)

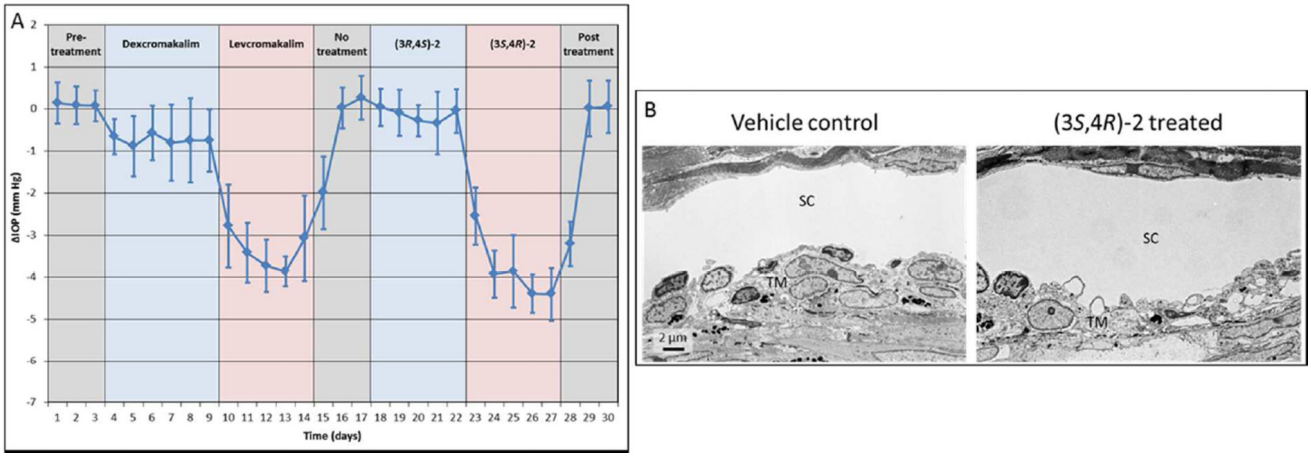


Figure 4.

(A) Comparison of IOP-lowering ability of individual enantiomers of cromakalim and (±)-**2**: n=10 (days 1–27) and n=6 (days 28–30). Dexcromakalim and levcromakalim were administered in a mixture of DMSO, cremophor EL, and PBS. (3*R*,4*S*)-**2** and (3*S*,4*R*)-2 were dissolved in PBS. All drugs were used at 5 mM and added as a 5 μl bolus. Note: four mice were sacrificed on day 27 to examine ocular histology and morphology. (B) Histologic analysis showed no difference in cellular morphology of the aqueous outflow pathway between vehicle control and treated eyes.

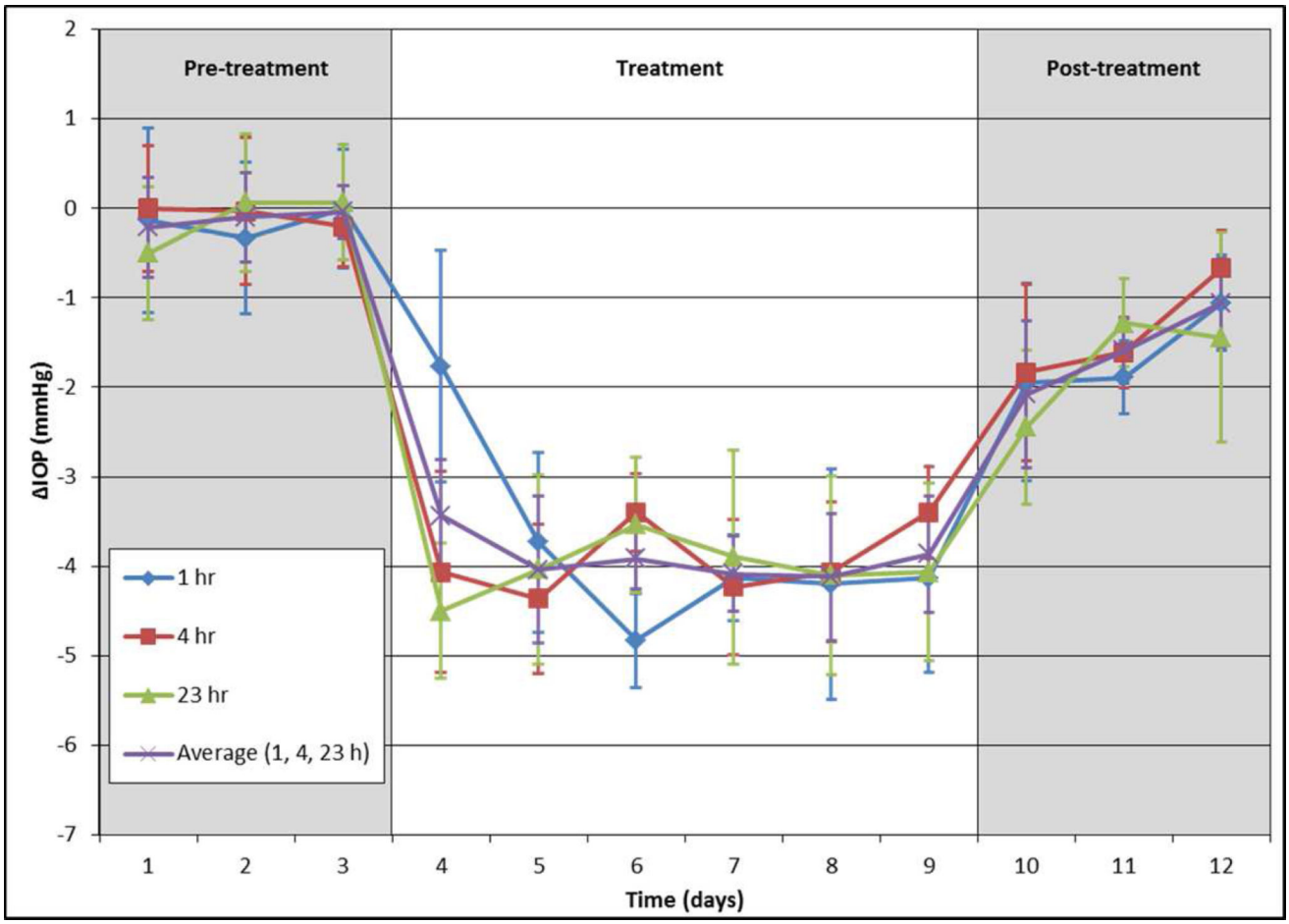


Figure 5.

C57BL/6 mice treated with once daily 5 mM solution of (3*S*,4*R*)-**9** in PBS at 1, 4, and 23 h post-treatment, n=10 (for days 1–9) and n=6 (for days 10–12).

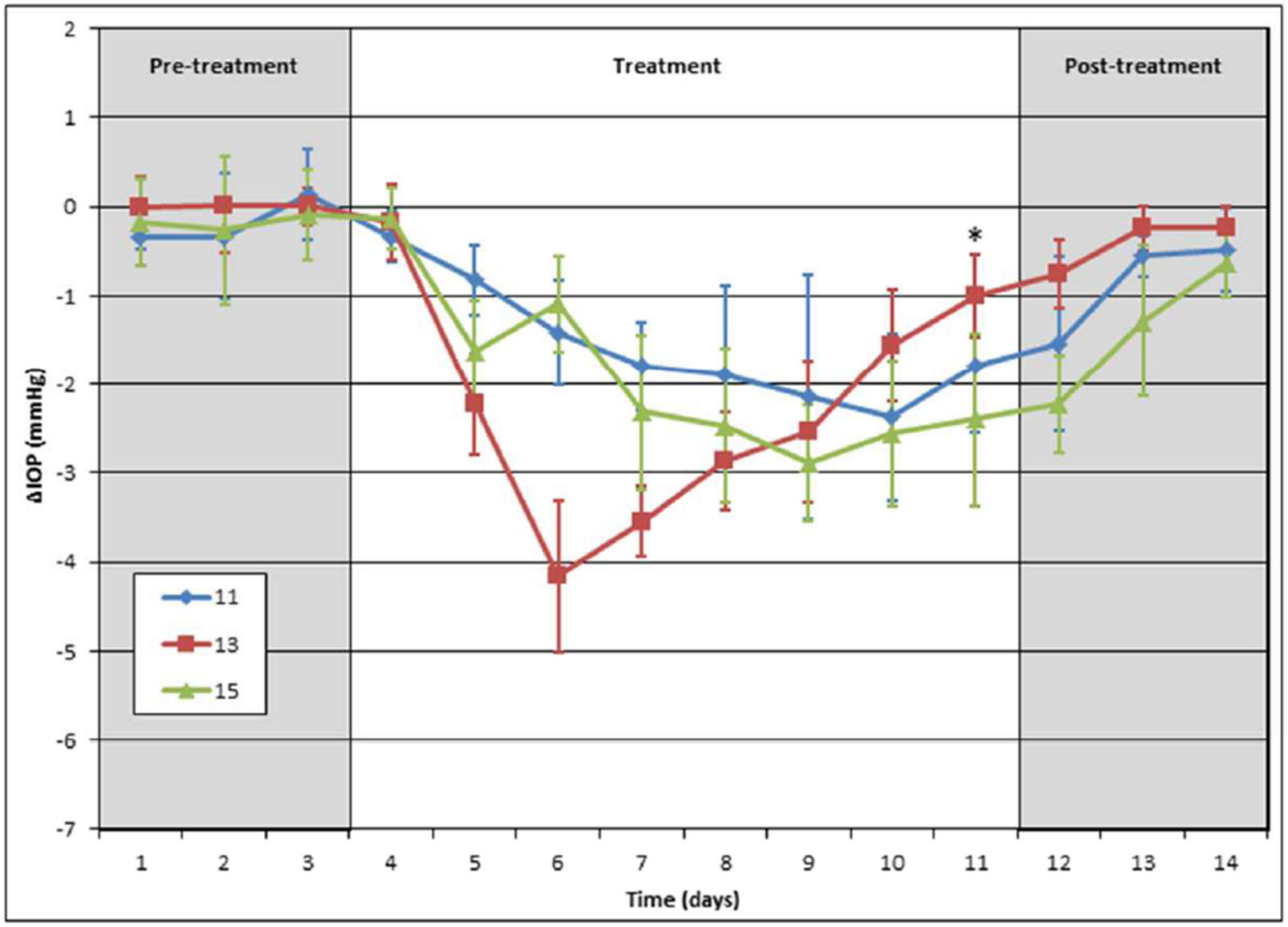


Figure 6.

C57BL/6 mice treated with dipeptides **11** (n=5); **13** (n=9, for days 1–11, n=5 for days 12–14), **15** (n=5). All were dissolved in PBS and treated at 5 mM dose. *No drug added to mice treated with **13** on day 11.

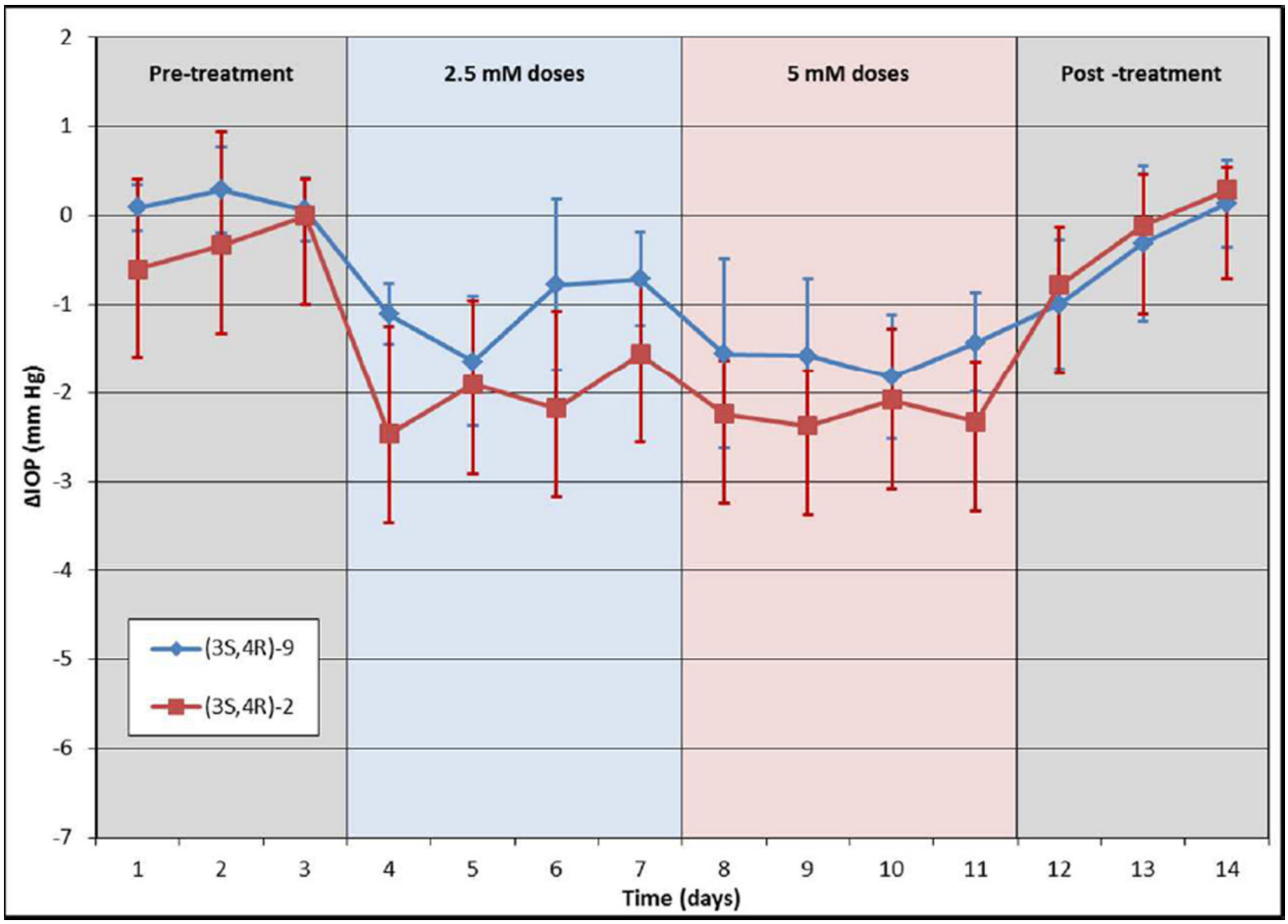
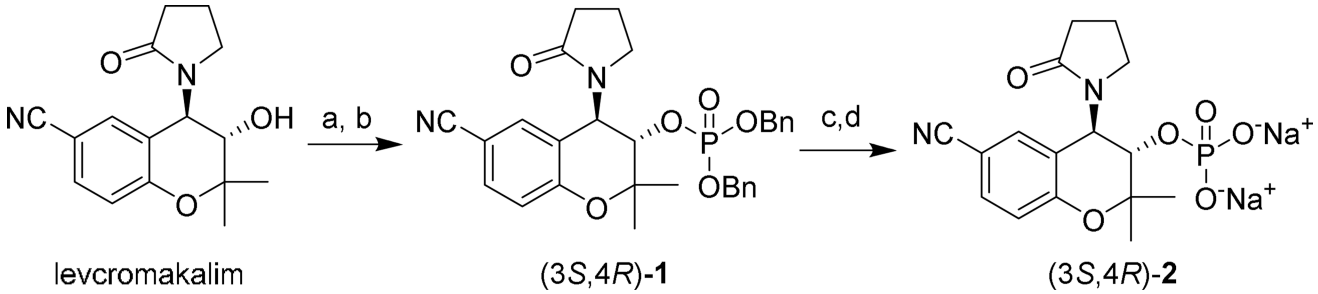


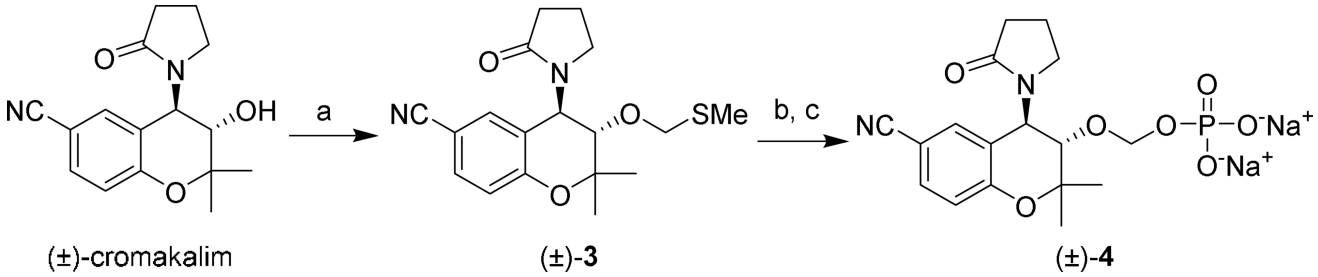
Figure 7.

Both (3*S*,4*R*)-**2** (red line; n=5) and (3*S*,4*R*)-**9** (blue line; n=5) dosed at 2.5 and 5 mM lowered IOP in normotensive Dutch-belted pigmented rabbits, but (3*S*,4*R*)-**2** produced a greater and more consistent reduction over the treatment period. Compounds were administered topically once daily at 2.5 mM for 4 days followed by 5 mM for 4 days. Data points are an average of IOP measurements taken at 1, 4, and 23 hours after each dose and are expressed as the average absolute difference in IOP (in mmHg) between the treated and control eyes.



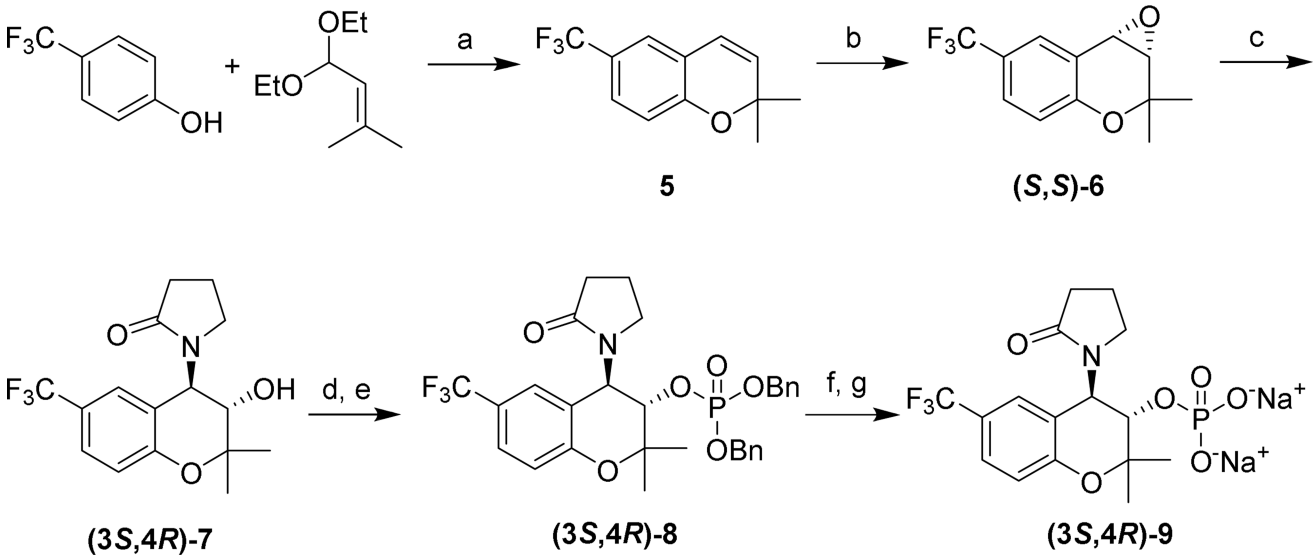
Scheme 1. Synthesis of Directly-Linked Phosphate Cromakalim Analog (3*S*,4*R*)-2[a]

[a]Reagents and conditions: (a) dibenzyl *N,N*-dimethylphosphoramidite, tetrazole, CH_2Cl_2, CH_3CN; (b) 30% H_2O_2, THF, 0 °C; (c) TMSBr, CH_2Cl_2; (d) ion exchange using Na^+-Dowex resin.



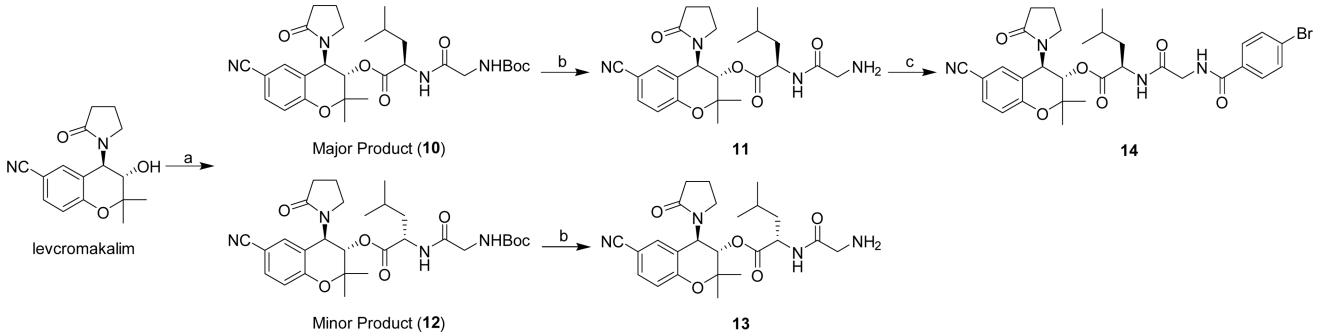
Scheme 2. Synthesis of Cromakalim Oxymethylphosphate Analog (±)-4[a]

[a]Reagents and conditions: (a) DMSO, Ac_2O, AcOH; (b) H_3PO_4 *N*-iodosuccinimide, molecular sieves, THF; (c) ion exchange using Na^+-Dowex resin.



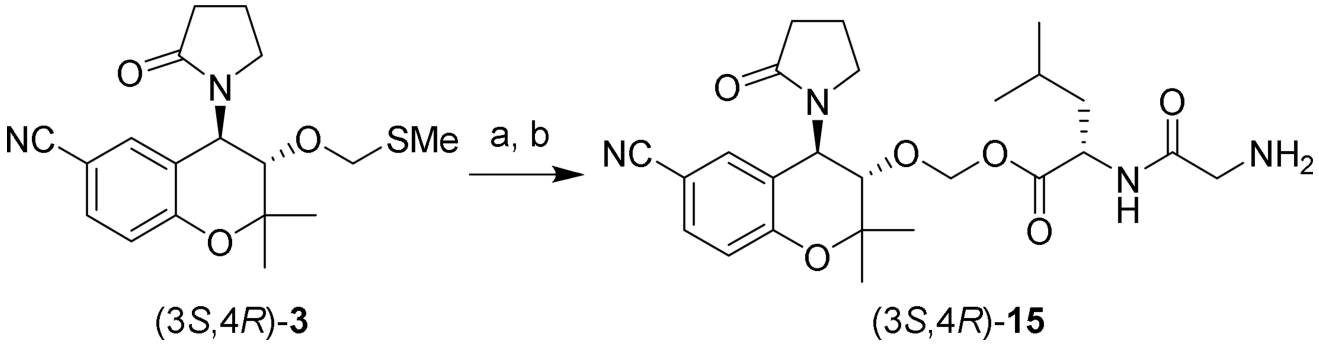
Scheme 3. Enantioselective Synthesis of CF_3-Analog (3*S*,4*R*)-9[a]

[a]Reagents and conditions: (a) 3-picoline, *p*-xylene, Δ; (b) (*S,S*)-Jacobsen's catalyst, NaOCl, CH_2Cl_2, H_2O, pH ≈ 11.3, 0 °C; (c) 2-pyrrolidone, NaH, DMSO; (d) dibenzyl *N,N*-dimethylphosphoramidite, tetrazole, CH_2Cl_2, CH_3CN; (e) 30% H_2O_2, THF, 0 °C; (f) TMSBr, CH_2Cl_2; (g) ion exchange using Na^+-Dowex resin.



Scheme 4. Synthesis of Levcromakalim Analogs Directly Conjugated to Dipeptides[a]

[a]Reagents and conditions: (a) Boc-Gly-Leu-OH, HATU, DMAP, CH_2Cl_2; (b) 4M HCl in dioxane; (c) 4-bromobenzoic acid, HATU, DMAP, CH_2Cl_2.



Scheme 5. Synthesis of a Levcromakalim Analog Conjugated to a Dipeptide through an Acetalester Linker[a]

[a]Reagents and conditions: (a) sulfuryl chloride, DCE, Boc-Gly-Leu-OH, DIEA; (b) 4M HCl in dioxane.