# Carbonic Anhydrase-II Inhibition. What are the True Enzyme–Inhibitory Properties of the Sulfamide Cognate of Topiramate?

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*Received December 13, 2007*

The marketed drug topiramate (**1**) is a moderate inhibitor of carbonic anhydrase-II (CA-II) ($K_i$ or $K_d$ = 0.3–0.6 $\mu$M), whereas sulfamide cognate **2** is a comparatively weak inhibitor ($K_i$ or $K_d$ = 25–650 $\mu$M). From an X-ray cocrystal structure of **2**·CA-II, Winum et al. (*J. Med. Chem.* **2006**, *49*, 7024) proposed that an adverse steric interaction between the C8 methyl group in **2** and Ala-65 of CA-II is responsible for the diminished CA-II inhibitory potency of **2**. We performed a straightforward test of this Ala-65 effect by synthesizing and examining ligand **3**, which lacks the offending (pro-*S* or C8) methyl substituent in **2**. We also prepared and evaluated related sulfamides **5**, **7**, and **9**. In a CA-II inhibition assay (4-nitrophenyl acetate), the $K_i$ for **3** was ~300 $\mu$M, indicating very weak inhibition, close to that for **2** (4NPA, $K_i$ = 340 $\mu$M). In a CA-II binding assay (ThermoFluor), the $K_d$ for **3** was >57 $\mu$M, indicating very weak binding, lower than the affinity of **2** ($K_d$ = 25 $\mu$M). Our results draw into question the proposed steric interaction between the C8 methyl of **2** and Ala-65 of CA-II.

Certain carbonic anhydrase (CA) enzymes (EC 4.2.1.1)[1,2] are inhibited to varying degrees by some useful therapeutic agents.[2,3] Because of our interest in topiramate (**1**), which is a marketed drug for the treatment of epilepsy and migraine,[4] we have been examining the relative effectiveness of isosteric sulfamate ($-OSO_2NH_2$) and sulfamide ($-NHSO_2NH_2$) derivatives for inhibition of human CA-II, by using both enzyme kinetic and thermodynamic methods (Chart 1).[5–8] Our collective results to date indicate, unambiguously, that a sulfamate group is much more effective than the sulfamide group for inhibiting human CA-II. For example, topiramate (**1**) had $K_i$ and $K_d$ values in the range of 0.3–0.6 $\mu$M, whereas its sulfamide cognate **2** had $K_i$ and $K_d$ values in the range of 25–650 $\mu$M.[5–8] Other direct comparisons of sulfamates with their cognate sulfamides have echoed this general pattern.[5–8] To accommodate these observations, we have suggested that the much weaker CA-II inhibitory potency of the sulfamides might be attributed to their higher p$K_a$ relative to the sulfamates.[5,6] By way of illustration, the p$K_a$ values for **1** and **2** are 8.7 and 10.7, respectively, which represents a sizable 100-fold difference in acidity.[5] Given the lower acidity of **2**, its anionic form ($SO_2NH^-$), which is essential for binding to Zn(II) in the CA-II active site, will have a lower thermodynamic stability and a correspondingly lower population.[9–11]

The recently published X-ray cocrystal structure of **2** complexed with human CA-II shows a binding mode analogous to that of the X-ray structure of **1** complexed with human CA-II.[12] The sulfamide group is bound through its terminal nitrogen atom to Zn(II) in the active site, and there are other close contacts involving hydrogen bonds to Asn-62, His-94, Gln-92, Thr-199, and Thr-200. In this X-ray report,[12] the weak CA-II binding of **2** was ascribed to a "clash between one methyl group of the inhibitor and Ala-65".[13] Furthermore, it was proposed that this specific Ala-65 interaction could be used as a "means for designing inhibitors with low affinity [for CA-II]". While such a structural finding in the solid state may be interesting, we wondered if it would be applicable also to enzyme kinetics and ligand binding in the solution phase. Basically, the proposed Ala-65 effect begs the question: What would happen if the ligand's offending C8 methyl[13] group were eliminated? Given an adverse steric interaction between the C8-methyl of **2** and the Ala-65 side chain (methyl group) of the enzyme, a ligand analogue devoid of this problem should exhibit *markedly enhanced CA-II inhibition*. As a consequence, there is a clear-cut test of whether the proposed Ala-65 effect is real or not.

We decided to conduct some key experiments to address this issue. First, we examined mono(des-methyl) sulfamide **3**, *with the problematic pro-S methyl group removed*, for its potency in inhibiting CA-II ($K_i$) *and* in binding to CA-II ($K_d$). For comparison, we also measured the mono(des-methyl) sulfamate **4**. As a further test, we applied this protocol to bis(des-methyl) sulfamide **5** and bis(des-methyl) sulfamate **6**, as well as to the related sulfamide/sulfamate pairs **7/8** and **9/10**, in which the 4,5-ketal region of **2/1** is altered further. This investigation led to the following conclusions. (1) There is no basis for a serious, adverse steric interaction between the C8 methyl in **2** and the Ala-65 residue of CA-II in the real-world situation in solution. (2) The Ala-65 interaction is likely to be of minimal value in drug design, such as for obtaining selective CA inhibitors. (3) Sulfamides are decidedly much weaker inhibitors of CA-II than their cognate sulfamates, as we have pointed out before.[5–7]

## Results and Discussion

**Alteration of the 4,5-Ring of 1 and 2.** A very direct way to test the claim[12] about there being an adverse steric interaction between the C8 methyl group[13] of **2** and the Ala-65 of CA-II would be to eliminate the offending methyl from the ligand by replacing it with a hydrogen atom. Thus, we synthesized the mono(des-methyl) analogue, **3**, from diol **11** (Scheme 1) and tested the ability of **3** to inhibit CA-II (Table 1). We used the 4-nitrophenyl acetate (4NPA) hydrolysis assay to determine CA-II inhibition because this method provides a greater dynamic range than the $CO_2$ hydration assay *for compounds with very weak CA inhibitory potency*.[8,15] Sulfamide **3** had a very weak

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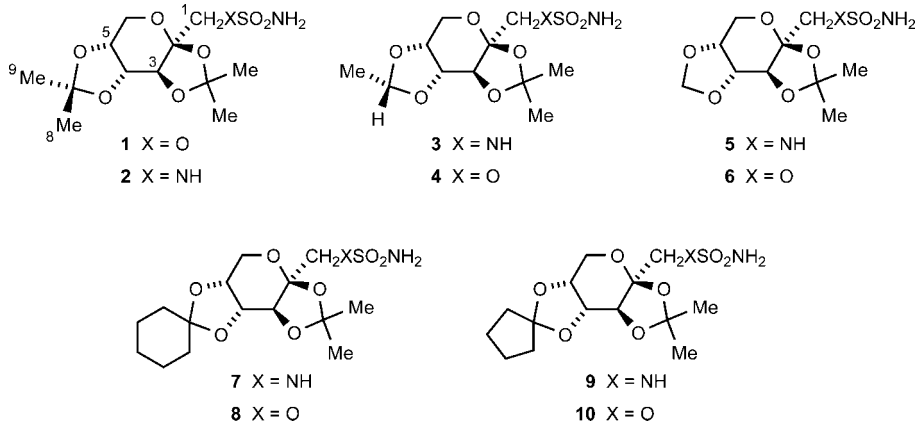
10.1021/jm7015649 CCC: $40.75 
Published on Web 03/26/2008

**Chart 1.** Structures **1**–**10**




**Scheme 1.** Synthesis of Sulfamide **3**

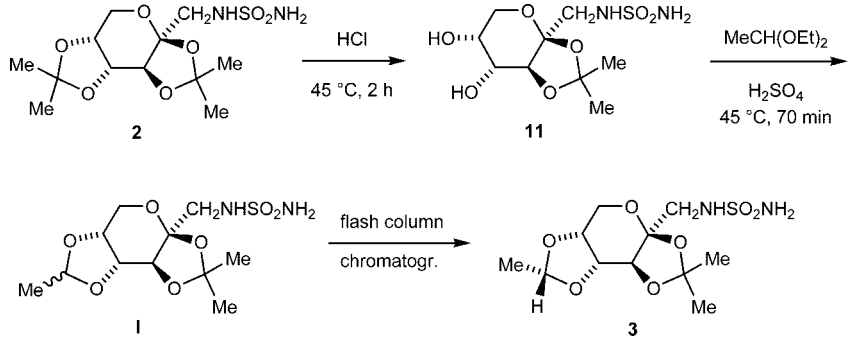



**Table 1.** CA-II Inhibition Data and Binding Affinities

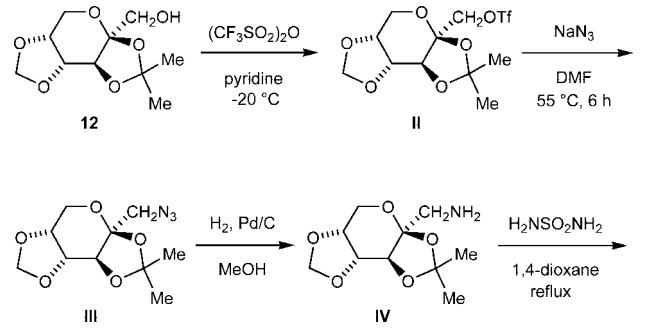
| compd | 4NPA $K_i$[a] ($\mu$M) | $K_d$[b] ($\mu$M) |
|---|---|---|
| **1** | 0.48[c]/0.45[d] | 0.29[e] |
| **2** | 340[c]/490[d] | 25[e] |
| **3** | ~300[f] | >57 |
| **4** | 0.20 | 0.30 |
| **5** | ~300[f] | >57 |
| **6** | 0.35 | 0.30 |
| **7** | >300[g] | 50 |
| **8** | 1.6 | 0.90 |
| **9** | >300[g] | >57 |
| **10** | 1.6 | 0.50 |
| zonis[h] | 6.8 | ND |

[a] Inhibition of human CA-II as determined by measuring the inhibition of hydrolysis of 4-nitrophenylacetate (4NPA). The data for **1** and **2** were previously published (refs 7 and 8); the data for **3**–**12** are newly generated. [b] The binding affinities for human CA-II were determined by ThermoFluor; ND, not determined. [c] This value was taken from ref 7. [d] This value was taken from ref 8. [e] This ThermoFluor value was taken from ref 6. [f] 40–60% inhibition at 300 $\mu$M. [g] 25–35% inhibition at 300 $\mu$M. [h] Reference compound zonisamide.

$K_i$ value for CA-II inhibition of ~300 $\mu$M, close to that for parent compound **2** (4NPA, $K_i = 340$ $\mu$M[7]). On the other hand, sulfamate **4**, which corresponds to **3**,[14] had a much more potent $K_i$ value of 0.20 $\mu$M, which is 2-fold better than that for parent compound **1** (4NPA, $K_i = 0.48$ $\mu$M[7]).

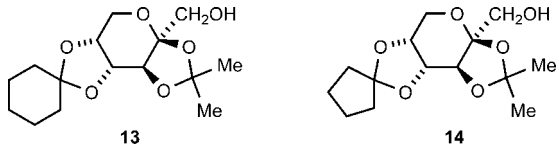
To probe this key point further, we sought to obtain thermodynamically based $K_d$ values as a direct measure of ligand affinity. In this vein, we employed the ThermoFluor method, which affords a thermal melting curve for CA-II and ligand from the change in fluorescence intensity of 1-anilino-8-naphthalenesulfonic acid as a function of temperature (Table 1).[16,17] Importantly, thermodynamic measurements by ThermoFluor, or by other methods such as isothermal titration calorimetry (ITC), are derived *under equilibrium conditions in the absence of substrate*, thereby avoiding any concerns about (1) slow, tight binding kinetics and (2) variation in enzymatic rates under different conditions (including different substrates). This alternative method is crucial in the particular situation at hand because of the wide disparity in CA-II inhibition ($K_i$ or $IC_{50}$) values for diverse sulfamates and sulfamides reported by us[5–8] and by another research group.[18] The binding affinity of CA-II for **3** was virtually undetectable ($K_d > 57$ $\mu$M), making it *even weaker* than that previously observed for parent compound **2** ($K_d = 25$ $\mu$M[6]). The corresponding sulfamate, **4**, had a $K_d$ value of 0.30 $\mu$M, which is essentially the same as that for topiramate, **1** ($K_d = 0.29$ $\mu$M[6]).

**Scheme 2.** Synthesis of Sulfamide **5**




In a follow-up study, we prepared bis(des-methyl) sulfamide **5**, in which the pro-*S* (C8) and pro-*R* (C9) methyl groups are missing, from alcohol **12**[14] (Scheme 2) and measured its potency for CA-II inhibition and binding (Table 1). For comparison, we also examined bis(des-methyl) sulfamate **6**.[14] The very weak $K_i$ value for **5** of ~300 $\mu$M is similar to that for sulfamide **3** (and in the range of that for parent compound **2**). The corresponding sulfamate, **6**, was substantially more potent than

**Chart 2.** Structures **13** and **14**




the corresponding sulfamide, with a $K_i$ value of 0.35 $\mu$M. The binding affinity of **5** for CA-II was virtually undetectable ($K_d$ > 57 $\mu$M), making it a poor ligand, again even weaker than parent compound **2**. The corresponding sulfamate, **6**, had a $K_d$ value of 0.30 $\mu$M, which is essentially the same as that for topiramate, **1** ($K_d$ = 0.29 $\mu$M[6]).

We extended our investigation to related sulfamide/sulfamate pairs **7/8** and **9/10**, in which the 4,5-ketal region of **2/1** is in a spirocyclic arrangement. The sulfamates were reported by us earlier,[14,19] and the sulfamides were synthesized according to the route depicted in Scheme 2, employing the method used for the preparation of **2**.[5] Thus, sulfamides **7** and **9** were obtained from starting alcohols **13**[19] and **14**,[14] respectively (Chart 2). Compounds **7** and **9** had very weak $K_i$ and $K_d$ values (Table 1), in the realm of parent sulfamide **2**. The corresponding sulfamates, **8** and **10**, had moderately potent $K_i$ and $K_d$ values (Table 1).

Overall, our CA-II enzyme inhibition results with compounds **1**−**10** indicate that sulfamides are decidedly much weaker CA-II inhibitors than their cognate sulfamates, as we have noted previously.[5–7] Since the enzyme kinetics results are consistent with the CA-II binding affinities from ThermoFluor, our enzyme kinetics determinations are soundly corroborated by a totally independent method. The weaker CA-II inhibitory potency of the sulfamides is probably associated with their weaker acidity relative to the sulfamates (by ~2 p$K_a$ units). Our results with sulfamides **2**, **3**, and **5** clearly indicate that there is no experimental basis in solution for a severe steric interaction between the C8 methyl group of **2** and the side chain of Ala-65 in CA-II.

Perhaps the proposal by Winum et al.[12] of a serious, adverse steric interaction between the C8 methyl in **2** and the Ala-65 residue of CA-II may only be pertinent to a *condensed state*, as afforded by the crystal lattice of **2**·CA-II. Although X-ray cocrystals can yield useful information for enzyme complexes with inhibitors as weak as 10 $\mu$M, and computer-assisted docking parameters for marginally potent compounds can be appealing, the static X-ray picture may capture the ligand−protein complex in an unnatural state.[20] Thus, in structure-based drug design one may find that $K_i$ values do not necessarily reflect the docked structures that are determined. For sulfamide **2**, the diminished $K_i$ value relative to that for topiramate (**1**) is not likely to be caused by the perceived "clash between one methyl group of the inhibitor and Ala-65", but can be explained by differences in p$K^a$ (2 log units). Anyway, regardless of the reason, the interaction analysis for the X-ray cocrystal structure of **2**·CA-II[12] is not consistent with our enzyme inhibition and binding results in solution phase.

## Conclusion

We performed a straightforward test of the proposed[12] Ala-65 effect of **2** in its complex with CA-II. Enzyme inhibition and equilibrium binding results with sulfamides **3** and **5**, both of which lack the pro-*S* (C8) methyl group that is present in **2** and viewed to interact adversely with the Ala-65 side chain of CA-II, supply unambiguous evidence that the Ala-65 effect[12] does not exist in solution phase. Specifically, **3** and **5** did not exhibit substantially more potent $K_i$ or $K_d$ values than **2** for CA-II inhibition or binding, as would be anticipated by removal of the offending C8 methyl substituent. The enzyme inhibition results for corresponding sulfamates **4** and **6** reinforce our previous observations[5–7] that sulfamides are distinctly much weaker inhibitors of CA-II than their cognate sulfamates. Data for the related sulfamide/sulfamate pairs **7/8** and **9/10** also support this viewpoint. At this juncture, we further suggest that the proposed Ala-65 interaction[12] would have minimal value in drug design for obtaining selective CA inhibitors.

## Experimental Section

**General Chemical Procedures.** Details for general methods are provided in our previous papers[5,6] and the Supporting Information.[21] The methodology employed to prepare sulfamide products from their corresponding alcohol precursors is provided in our previous papers.[5,6]

**Materials.** 4-Nitrophenyl acetate (4-NPA) and human carbonic anhydrase-II (purified from erythrocytes) were purchased from Sigma-Aldrich (St. Louis, MO). 1-Anilino-8-naphthalenesulfonic acid (ANS) was obtained from Molecular Probes at Invitrogen Corp. (Carlsbad, CA).

**1-[(Aminosulfonyl)amino]-1-deoxy-2,3-*O*-(isopropylidene)-4,5-*O*-ethylidene-β-D-fructopyranose (3).** Sulfamide **2**[5] (8.0 g, 0.0237 mol) in THF (80 mL) was treated with 3 N HCl (80 mL, 0.24 mol), and the reaction was stirred at 45 °C for 2 h. With ice bath cooling, solid sodium carbonate was added until pH 9.0; then solid sodium chloride was added and the THF solution was separated. The solids and aqueous solution were extracted three times with THF, and the combined THF solution was dried ($Na_2SO_4$) and concentrated in vacuo to an oil (4.43 g). This crude 4,5-diol (**11**) was purified by flash column chromatography (ethyl acetate/MeOH, 20:1) and concentrated in vacuo to yield white solid diol **11** (2.40 g): $^1$H NMR (DMSO) $\delta$ 1.30/1.41 (2 s, 6H), 3.20 (m, 2H), 3.33 (s, $H_2O$), 3.55 (m, 2H), 3.77 (m, 1H), 4.0 (m, 2H), 4.80 (s, 1H), 5.23 (s, 1H), 6.05 (s, 1H), 6.47 (s, 2H); MS (neg) *m*/*z* 297 (M−1). The diol (1.5 g, 5.0 mmol) was dissolved in acetaldehyde diethyl acetal (15 mL, 105 mmol), and sulfuric acid was added (95−98%, 75 $\mu$L, 1.4 mmol) and the reaction was stirred at 45 °C for 70 min. The reaction was cooled to room temperature, diluted with acetaldehyde diethyl acetal (15 mL), cooled ice bath, and neutralized by addition of solid sodium carbonate until pH 7. The solids were filtered, and the filtrate was concentrated in vacuo to oil intermediate **I**, a mixture of two diastereomers highly enriched in **3**. Crude **I** was purified twice by flash column chromatography (1,2-dichloroethane/ethanol, 20:1; then ethyl acetate/heptane, 2:1). The resultant oil was dissolved in methylene chloride and evaporated in vacuo to give a white flaky solid of isomerically pure **3** (203 mg). MS (pos): *m*/*z* 325 ($MH^+$). [α]$_D$: −28.5 (*c* 1.10, MeOH). $^1$H NMR ($CDCl_3$) $\delta$: 1.40 (s, 3H), 1.47 (d, $J$ = 4.9 Hz, 3H), 1.54 (s, 3H), 3.36 (dd, $J$ = 4.8, 13.8 Hz, 1H), 3.55 (dd, $J$ = 6.8, 13.8 Hz, 1H), 3.85 (m, 2H), 4.15 (d, $J$ = 8.0 Hz, 1H), 4.30 (d, $J$ = 2.4 Hz, 1H), 4.45 (dd, $J$ = 2.3, 8.1 Hz, 1H), 4.69 (s, 2H), 5.02 (m, 2H). Anal. Calcd for $C_{11}H_{20}N_2O_7S \cdot 0.10CH_2Cl_2$: C, 40.05; H, 6.12; N, 8.42. Found: C, 39.75; H, 6.10; N, 8.08.

**Synthesis of 5.** Alcohol **12**[14] was converted into **5**, via intermediates **II**−**IV**, by employing the same method that was used to synthesize **2**[5] (Scheme 2). MS (pos): *m*/*z* 311 ($MH^+$). $^1$H NMR ($CDCl_3$) $\delta$: 1.41 (s, 3H), 1.55 (s, 3H), 3.34 (dd, $J$ = 4.7, 14.0 Hz, 1H), 3.56 (dd, $J$ = 7.4, 14.0 Hz, 1H), 3.90 (m, 2H), 4.18 (d, $J$ = 8.2 Hz, 1H), 4.33 (d, $J$ = 2.4 Hz, 1H), 4.43 (dd, $J$ = 2.4, 8.1 Hz, 1H), 4.75 (s, 2H), 4.79 (s, 1H), 4.98 (m, 1H), 5.24 (s, 1H). Anal. Calcd for $C_{10}H_{18}N_2O_7S$: C, 38.70; H, 5.85; N, 9.03. Found: C, 38.71; H, 5.83; N, 8.88.

**Synthesis of 7.** Alcohol **13**[19] was converted into **7** by employing the same method that was used to synthesize **2**[5] and **5** (Scheme 2). MS (pos): *m*/*z* 379 ($MH^+$). $^1$H NMR ($CDCl_3$) $\delta$: 1.33 (s, 3H), 1.47 (s, 3H), 1.3–1.7 (m, 10H), 3.32 (d, $J$ = 13.5 Hz, 1H), 3.46 (br d, $J$ = 13.6 Hz, 1H), 3.75 (m, 2H), 4.17 (dd, $J$ = 1.1, 7.8 Hz, 1H), 4.23 (d, $J$ = 2.5 Hz, 1H), 4.52 (dd, $J$ = 2.5, 7.8 Hz, 1H), 4.65 (br s, 2H), 5.0 (br s, 1H). Anal. Calcd for $C_{15}H_{26}N_2O_7S$: C, 47.61; H, 6.92; N, 7.40. Found: C, 47.15; H, 6.84; N, 7.06.

**Synthesis of 9.** Alcohol **14**[14] was converted into **9** by employing the same method that was used to synthesize **2**[5] and **5** (Scheme 2).

MS (pos): *m/z* 365 ($MH^+$). $^1$H NMR ($CDCl_3$) $\delta$: 1.39 (s, 3H), 1.54 (s, 3H), 1.70 (m, 6H), 2.0 (m, 2H), 3.33 (dd, $J$ = 4.5, 13.6 Hz, 1H), 3.56 (dd, $J$ = 6.8, 13.7 Hz, 1H), 3.80 (dd, $J$ = 0.5, 13.1 Hz, 1H), 3.89 (dd, $J$ = 1.8, 13.1 Hz, 1H), 4.16 (dd, $J$ = 1.0, 8.2, 1H), 4.29 (d, $J$ = 2.3 Hz, 1H), 4.49 (dd, $J$ = 2.3, 8.0 Hz, 1), 4.66 (s, 2H), 5.03 (m, 1H). Anal. Calcd for $C_{14}H_{24}N_2O_7S$: C, 46.14; H, 6.64; N, 7.69. Found: C, 46.08; H, 6.79; N, 7.41.

**Carbonic Anhydrase Inhibition. 4-Nitrophenylacetate Hydrolysis Assays.** Purified human CA-II was used. Inhibition of CA-II was determined for both assays according to the procedures that were described previously in detail.[5,7] The temperature for the 4-NPA hydrolysis assay was 23 °C.

**ThermoFluor Studies.** ThermoFluor measurements were carried out by using available instruments (developed in house), according to the reported methodology.[6,16,17] In brief, solutions (4 $\mu$L) of human CA-II (0.1 mg/mL) in the indicated buffer plus 100 $\mu$M ANS and test compound (0–100 $\mu$M) were dispensed into black 384-well polypropylene PCR microplates and overlaid with 1 $\mu$L of silicon oil to prevent evaporation. Plates were heated at a rate of 1 °C/min on a thermal block and illuminated with light in the range of 380–400 nm; fluorescence (450−500 nm) was measured by using an overhead CCD (charge-coupled device). $K_d$ values were calculated as previously described.[17] Similar ThermoFluor results were obtained with various buffer conditions (Table S1, Supporting Information).[21]

**Acknowledgment.** We thank Diane Gauthier for expert technical assistance.

**Supporting Information Available:** General procedures and additional ThermoFluor studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) This C8 methyl is the pro-*S* methyl on the 4,5-cyclic ketal.

(14) In the crystallographic study of **2**·CA-II (with 1.9 Å resolution), no electron density was observed for the C8 methyl group in question.[12] Maryanoff, B. E.; Costanzo, M. J.; Nortey, S. O.; Greco, M. N.; Shank, R. P.; Schupsky, J. J.; Ortegon, M. E.; Vaught, J. L. Structure−activity studies on anticonvulsant sugar sulfamates related to topiramate. Enhanced potency with cyclic sulfate derivatives. *J. Med. Chem.* **1998**, *41*, 1315–1343.

(15) This point relates to having a Hill slope in the analysis of the kinetics data that is not greatly less than unity.

(16) (a) Pantoliano, M. W.; Petrella, E. C.; Kwasnoski, J. D.; Lobanov, V. S.; Myslik, J.; Graf, E.; Carver, T.; Asel, E.; Springer, B. A.; Lane, P.; Salemme, F. R. High-density miniaturized thermal shift assays as a general strategy for drug discovery. *J. Biomol. Screen.* **2001**, *6*, 429–440. (b) The midpoint transition temperature, $T_m$, is a measure of protein stability that is determined from the melting curve. Binding affinity ($K_a = 1/K_d$) is directly related to changes in the increase in protein stability and $T_m$, as induced by the test compound (L).[16a] The magnitude of the change in $T_m$ is proportional to both ligand concentration, log[L], and binding affinity. This plate-based technology allows one to establish $T_m$ values rapidly under a wide range of experimental conditions, with as many as 32 different ligands in the same experiment. The binding affinity at the $T_m$, $K_{a,Tm}$, is calculated with the aid of calorimetrically measured parameters for protein stability, and is then extrapolated to 37 °C.[17] The ThermoFluor method has been validated for CA-II with six sulfonamide-inhibitor ligands.[17] The ThermoFluor results correlated well with ITC results. (c) The ThermoFluor assay was developed by 3-Dimensional Pharmaceuticals, Inc., which has merged into Johnson & Johnson Pharmaceutical Research & Development, LLC. "ThermoFluor" is a registered trademark in the United States and certain other countries.

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(20) (a) Henzler-Wildman, K.; Kern, D. Dynamic personalities of proteins. *Nature* **2007**, *450*, 964–972. (b) The dynamic structures of proteins can govern their function, such that a multidimensional energy landscape of conformational states (thermodynamics) and energy barriers between them (kinetics) is needed to understand proteins in action.[20a]

(21) See the Supporting Information information at the end of this paper.

JM7015649