

Supporting Information

INHIBITION OF CARBONIC ANHYDRASE II BY THIOXOLONE: A MECHANISTIC AND STRUCTURAL STUDY

Albert A. Barrese III, Caroli Genis, S. Zoe Fisher, Jared N. Orwenyo, Mudalige Thilak Kumara, Subodh K. Dutta, Eric Phillips, James J. Kiddle, Chingkuang Tu, David N. Silverman, Lakshmanan Govindasamy, Mavis Agbandje-McKenna, Robert McKenna, and Brian C. Tripp

Preparation of 4-Mercaptobenzene-1,3-diol (Compound 3) by Acid Hydrolysis of Thioxolone (Compound 1). The thioxolone hydrolysis product observed in the preliminary crystal structure, 4-mercaptobenzene-1,3-diol, was not commercially available. Therefore, it was synthetically prepared from thioxolone by acid hydrolysis and purified by silica column chromatography. Four grams of 99% pure thioxolone (Compound 1; 6-hydroxybenzo [*d*][1,3]oxathiol-2-one) were suspended in 50 ml of deionized (DI) water and dissolved by addition of 1 M sodium hydroxide in drop-wise manner. A solution of 0.5 M sulfuric acid was added in a drop-wise manner until a cloudy solution was generated. The resulting solution was stored at 4 °C overnight to allow crystallization to occur. The resulting white, needle-like crystals were isolated by vacuum filtering using a Buchner funnel with Whatman No 1 qualitative 70 mm filter paper. The recrystallized thioxolone crystals were transferred to a 500 ml three-necked round bottom flask and resuspended in 70 ml of DI water. The flask was sealed with a rubber septum and connected to a condensor. The flask and sample were purged of oxygen by flowing nitrogen gas through the system for 30 min. A volume of 30 ml of 0.5

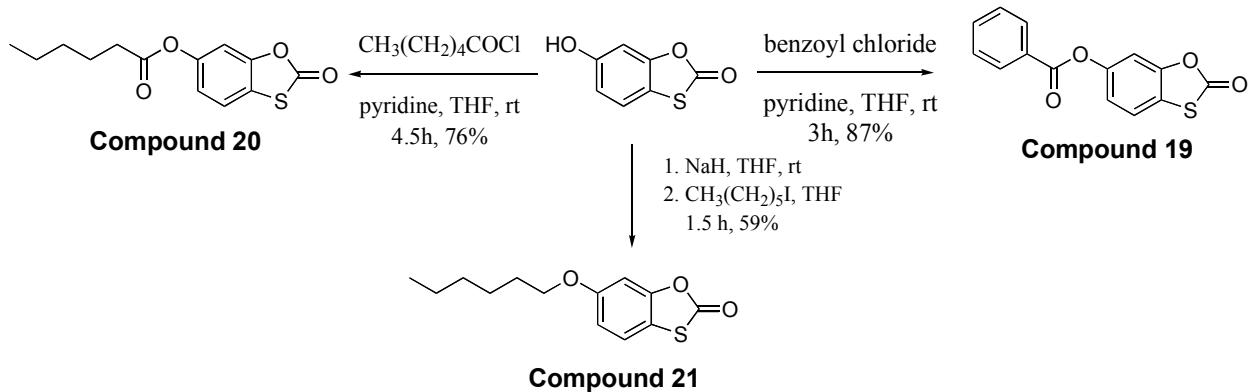
M sulfuric acid was injected into the system through the rubber septum cap. The system was again purged with nitrogen for a further fifteen min to ensure that all of the oxygen was removed. Nitrogen purging was then stopped and a nitrogen-filled rubber balloon was connected to the top of the condenser through the rubber septum cap. The rubber balloon was observed to expand during the hydrolysis reaction, indicating the generation of a gas, most likely CO₂ resulting from release and equilibration of carbonic acid with the solvent. The system was refluxed for four hours at 100 °C and then allowed to cool down to room temperature. Needle-like crystals formed as the solution cooled; these crystals were separated and collected by vacuum filtration with a Buchner funnel with Whatman No 1 qualitative 70 mm filter paper. The solvent fraction of the mixture was also collected and stored in a smaller 100 ml round bottom flask and stored at room temperature for further analysis.

The resulting crystals of 4-mercaptobenzene-1,3-diol hydrolysis product were dissolved in chloroform and analyzed by thin layer chromatography (TLC) (Uniplate, Analtech Inc., Newark, DE) using 10% acetone, 90% chloroform (vol./vol.) mixture and either directly visualized with a UV light or by staining via incubating the plate with iodine gas in a sealed container (data not shown) and liquid chromatography-mass spectrometry (LC-MS). The solvent fraction was found to contain more 4-mercaptobenzene-1,3-diol and was therefore solvent extracted in two steps using 50 ml of chloroform in each step. The solid product was concentrated and dried to solid by rotoevaporation of the chloroform. The dried compound was dissolved in 5 ml of a mixture of 5% methanol and 95% (vol./vol.) chloroform mixture.

A glass chromatography column with a porous glass frit bottom (2 cm diameter x 50

cm length) was packed with 400 Å mesh column chromatography silica gel suspended in chloroform to give a packed gel bed height of 20 cm. The hydrolysis product from the final recrystallization step was loaded onto the column and eluted isocratically with a mixture of 10% methanol and 90% chloroform. The eluting fractions were collected in 10 ml aliquots and analyzed for the presence and purity of products with TLC. The purity of products was further confirmed by LC-MS. The purified samples were then pooled and placed in a 50 ml round bottom flask for rotovaporation. The resulting semi-solid product was then resuspended in 1 ml of methanol and transferred into a pre-weighed 5 ml glass test tube. The test tube was then placed back inside the 50 ml round bottom flask and the sample was again rotovaporated. After all of the methanol had been evaporated, the test tube was weighed and the final mass of the sample product was then calculated. The purified 4-mercaptopbenzene-1,3-diol samples were analyzed by LC-MS to insure that none of the thioxolone starting compound was present, before they were combined and concentrated by rotovaporation. The purified samples yielded *m/z* values of 139-140, corresponding to the ionized states of the final monomer product, 4-mercaptopbenzene-1,3-diol, and the disulfide-linked dimer, bis-4-mercaptopbenzene-1,3-diol.

Synthesis of Thioxolone Structural Analogs, Compounds 19-21. Three thioxolone analogs, compounds **19-21** (Table 1) were synthesized using thioxolone as a starting compound (Scheme 1).



Scheme 1. Synthesis of thioxolone analogs, compounds **19-21**.

Synthesis of 2-Oxobenzo[*d*] [1,3]oxathiol-6-yl benzoate (Compound 19). A solution of thioxolone (Compound **1**; 6-hydroxybenzo [*d*][1,3]oxathiol-2-one; 505 mg, 3.0 mmol) in tetrahydrofuran (THF) (2 ml) was stirred with pyridine (261 mg, 3.3 mmol) at room temperature. To the stirring solution was added benzoyl chloride (422 mg, 3.0 mmol) via a syringe. The solution was then stirred for 3 h. Subsequently, water (25 ml) was added and the mixture extracted twice with ether. The combined organic layers were washed once with 10% NaHCO₃, once with saturated NaCl, dried over MgSO₄, and the solvent removed under reduced pressure. Column chromatography (4:1 hexane/ethylacetate) yielded 711 mg (87%). ¹H NMR (CDCl₃, 400 MHz) δ 8.83 (1H, d, *J* = 5.1 Hz), 8.20 (1H, d, *J* = 8.4 Hz), 7.80 (1H, d, *J* = 8.8 Hz), 7.75 (1H, m), 7.62 (2H, t, 7.7 Hz), 7.50 (1H, d, *J* = 2.2 Hz), 7.32 (1H, dd, *J* = 8.4, 2.2 Hz). ¹³C NMR (CDCl₃, 400 MHz) δ 168.1, 164.3, 150.7, 148.5, 145.8, 141.1, 134.1, 130.1, 129.5, 128.9, 127.4, 123.6, 119.4, 106.9. Analytical calculation for C₁₄H₈O₄S: C, 61.76; H, 2.96. Found: C, 61.69; H, 3.11.

Synthesis of 2-Oxobenzo[*d*] [1,3]oxathiol-6-yl hexanoate (Compound 20). A solution of thioxolone (Compound 1; 6-hydroxybenzo [*d*][1,3]oxathiol-2-one; 505 mg, 3.0 mmol) in THF (2 ml) was stirred with pyridine (261 mg, 3.3 mmol) at room temperature. To the stirring solution was added hexanoyl chloride (404 mg, 3.0 mmol) via a syringe. The solution was then stirred for 4.5 h. Subsequently, water (25 ml) was added and the mixture extracted twice with ether. The combined organic layers were washed once with 10% NaHCO₃, once with saturated NaCl, dried over MgSO₄, and the solvent removed under reduced pressure. Column chromatography (4:1 hexane/ethylacetate) yielded 607 mg (76%). ¹H NMR (CDCl₃, 400 MHz) δ 7.38 (1H, d, *J* = 8.4 Hz), 7.11 (1H, d, *J* = 2.2 Hz), 7.00 (1H, dd, *J* = 8.4, 2.2 Hz), 2.57 (2H, t, *J* = 6.9 Hz), 1.76 (4H, m), 1.37 (4H, m), 0.93 (3H, t, *J* = 7.1 Hz). ¹³C NMR (CDCl₃, 400 MHz) δ 176.7, 164.3, 151.4, 148.5, 131.0, 123.9, 117.2, 108.8, 33.8, 31.2, 25.1, 23.9, 14.6. Analytical calculation for C₁₃H₁₆O₃S: C, 61.88; H, 6.39. Found: C, 62.04; H, 6.57.

Synthesis of 6-(Hexyloxy)benzo[*d*][1,3]oxathiol-2-one (Compound 21). To a stirring suspension of 60% NaH in mineral oil (120 mg, 3.0 mmol) in 2 ml of THF was added thioxolone (Compound 1; 6-hydroxybenzo [*d*][1,3]oxathiol-2-one; 505 mg, 3.0 mmol) dissolved in 2 ml of THF. The mixture was stirred for 15 minutes followed by the addition of 1-iodohexane (636 mg, 3.0 mmol) via a syringe. The solution was then stirred for an additional 1.5 h. The reaction mixture was then diluted with ether (15 ml) and extracted twice with water. The organic layer was dried over MgSO₄ and the solvent removed under reduced pressure to yield 447 mg (59%) of the product after column chromatography (4:1 hexane/ethylacetate). ¹H NMR (CDCl₃, 400 MHz) δ 7.29 (1H, d, *J* = 8.1 Hz), 7.04 (1H, d, *J* = 2.4 Hz), 6.95 (1H, dd, *J* = 8.1, 2.4 Hz), 3.18 (2H, t, *J* = 7.3

Hz), 1.79 (2H, m), 1.36 (2H, m), 1.28 (2H, m), 0.88 (3H, t, J = 6.6 Hz). ^{13}C NMR (CDCl₃, 400 MHz) δ 163.1, 150.2, 149.0, 128.5, 122.6, 116.8, 107.3, 66.9, 31.4, 29.5, 24.7, 21.4, 15.1. Analytical calculation for C₁₃H₁₄O₄S: C, 58.63; H, 5.30. Found: C, 58.69; H, 5.42.

Measurement of Apparent Inhibition Constants for Thioxolone Structural Analogs. An additional 13 structural analogs of thioxolone (compounds **6-18**) (Table 1) were obtained from Specs (Delft, The Netherlands). These compounds were commercially available and were chosen for their structural similarity to the original thioxolone structure, but had additional functional groups at several possible locations on the thioxolone scaffold, excluding the ester bonds in the five-member 1,3-oxathiol-2-one ring moiety. Three additional structural analogs (compounds **19-21**) were prepared synthetically using thioxolone as the starting compound via nucleophilic substitution reactions on appropriate halogenated substrates to yield the three target compounds (Supporting Information). All 16 analogs contained the original thioxolone ring structure (Table 1), but also had additional aliphatic and aromatic groups substituted at the C6 carbon hydroxyl group, using the nomenclature of Byres and Cox (53). Some analogs were also modified by halogenation of the C1 and/or C5 carbons.

All 16 thioxolone analogs were tested for inhibition of CA II esterase activity with 4-NPA to estimate their apparent inhibition constants (IC₅₀ values) relative to thioxolone after an incubation time of 15 minutes. The resulting ester hydrolysis products of these compounds should have similar structures to the thioxolone product, 4-mercaptobenzene-1,3-diol (compound **3**). However, inhibition first requires that the pro-

drug compound can correctly bind in the CA II active site to allow activation by ester/thioester cleavage to occur. Excluding the exceptionally weak inhibitor, compound **21**, and the non-inhibitory compounds **6** and **17**, the average apparent IC_{50} value for the remaining 13 analogs was $1.27 \pm 0.65 \mu\text{M}$. Thus, the apparent IC_{50} values for many of the analogs were slightly lower than, but similar in magnitude to, the IC_{50} value of $1.77 \mu\text{M}$ measured for thioxolone (compound **1**) after the same incubation time. These results clearly demonstrated that modification of the thioxolone structure by addition of other moieties could result in a moderately higher potency of inhibition.

Five thioxolone analogs, compounds **7**, **12**, **13**, **15**, and **16**, had the lowest IC_{50} values in the 4-NPA esterase assay, ranging from $0.6\text{--}0.9 \mu\text{M}$, with the lower values approaching the $0.44 \mu\text{M}$ IC_{50} value observed for the strong CA II inhibitor, acetazolamide (compound **2**), in the same assay. Compound **7** had the lowest IC_{50} value of $0.61 \mu\text{M}$. This compound differed from thioxolone in that it had a benzyl group linked to the C6 oxygen by an ester linkage; this additional moiety may improve its non-polar interactions with the CA II hydrophobic pocket. Compounds **8**, **10**, **11**, **14**, and **19** exhibited lower potencies of CA II inhibition, with IC_{50} values ranging from $1\text{--}2 \mu\text{M}$.

The observed differences in the inhibition properties of these compounds may result from the different groups remaining attached to the 4-mercaptopbenzene-1,3-diol scaffold. The observed variations in the IC_{50} values may also be a result of differences in the initial binding affinity of the pro-drug compound of the enzyme, varying rates of CA II ester hydrolysis of each compound to generate the final product, and secondary effects of other hydrolysis compounds generated from the rest of the scaffold. All 16 of the larger thioxolone analogs tested possessed a nucleophilic oxygen attached to the C6 carbon

(Table 1), analogous to the hydroxide group present in the original thioxolone structure (compound **1**). The majority of these analogs had a substituent attached at the C6 carbon via an ester linkage, although three compounds had an ether linkage at this position (compounds **10**, **11**, and **21**). No obvious correlation was apparent between potency of inhibition and the presence of an ester vs. ether oxygen bond at the C6 position; these three ether bonded analogs yielded IC_{50} values ranging from 1.0-12.7 μ M. However, analogs with an aromatic substituent (compounds **7**, **10**, **11**, **12**, **13**, **14**, **15**, **16**, and **19**) or cyclohexane (compound **8**) at the C6 position exhibited a higher potency of inhibition, with IC_{50} values of 0.61-1.45 μ M. Conversely, analogs with aliphatic C6 hydroxyl substituents (compounds **9**, **18**, **20**, and **21**) generally exhibited weaker inhibition, with larger IC_{50} values of 2.0-12.7 μ M, or no inhibition (compounds **6** and **17**); however, another factor may be responsible for the lack of inhibition, as discussed below. Six of the thioxolone analogs were also further modified by halogenation at the C5 position on the thioxolone ring structure, either by chlorine (compounds **7** and **8**) or bromine (compounds **6**, **16**, **17**, and **18**). Two of these analogs (compounds **6** and **17**) were also modified by bromination at the C1 position. The four compounds with chlorine or bromine present only at the C5 position functioned as inhibitors, with a wide range of IC_{50} values (0.6-2.0 μ M); including the most potent inhibitor, compound **7**). In contrast, Compounds **6** and **17**, with bromine attached at the C1 position, failed to exhibit any detectable inhibition of the enzyme esterase activity. This complete lack of inhibition was interpreted as a failure of these compounds to bind at the enzyme active site, an event required for subsequent ester bond cleavage and generation of the more strongly inhibitory thiol derivatives. The C1 bromine substituent is the most unique feature of

these two compounds; otherwise, a wide variety of functional groups were tolerated at the C5 and C6 positions. This analysis suggests that the C1 bromine group is responsible for this behavior, possibly due to a steric effect that prevents binding of the thioxolone analog to one or more CA II active site residues.

Table 1 - Supporting Information. Structures and apparent IC₅₀ values for inhibition of human carbonic anhydrase II by thioxolone analogs

Compound Number and Name	Molecular Structure	Molecular Formula	Mol. Mass (Da)	IC ₅₀ / K _i value (μM) ^{a,b}
1. thioxolone (6-hydroxybenzo[d][1,3]oxathiol-2-one)		C ₇ H ₄ O ₃ S	168.2	314 ± 69 ^c 1.77 ^d (1.06-2.95)
7. 5-chloro-2-oxobenzo[d][1,3]oxathiol-6-yl phenyl carbonate		C ₁₄ H ₇ ClO ₅ S	322.7	0.61 (0.51-0.74)
8. 5-chloro-2-oxobenzo[d][1,3]oxathiol-6-yl cyclohexyl-carbamate		C ₁₄ H ₁₄ ClNO ₄ S	327.8	0.98 (0.84-0.11)
9. 2-oxobenzo[d][1,3]oxathiol-6-yl butylcarbamate		C ₁₂ H ₁₃ NO ₄ S	267.3	2.13 (1.44-3.17)
10. 6-(2,6-dichloro-4-nitrophenoxy)benzo[d][1,3]oxathiol-2-one		C ₁₃ H ₅ Cl ₂ NO ₅ S	358.2	1.45 (0.87-2.44)

11. 6-(4-(methylsulfonyl)-2-nitrophenoxy)benzo[<i>d</i>][1,3]oxathiol-2-one		C ₁₄ H ₉ NO ₇ S ₂	367.4	1.03 (0.85-1.25)
12. 2-oxobenzo[<i>d</i>][1,3]oxathiol-6-yl 2,6-dichlorobenzoate		C ₁₄ H ₆ C ₁₂ O ₄ S	341.2	0.74 (0.35-1.56)
13. 2-oxobenzo[<i>d</i>][1,3]oxathiol-6-yl 4-nitrobenzoate		C ₁₄ H ₇ NO ₆ S	317.3	0.93 (0.50-1.74)
14. 2-oxobenzo[<i>d</i>][1,3]oxathiol-6-yl 3-(trifluoromethyl)benzoate		C ₁₅ H ₇ F ₃ O ₄ S	340.3	1.07 (0.91-1.24)
15. 2-oxobenzo[<i>d</i>][1,3]oxathiol-6-yl 4-chloro-3-nitrobenzoate		C ₁₄ H ₆ ClNO ₆ S	351.7	0.68 (0.50-0.93)
16. 5-bromo-2-oxobenzo[<i>d</i>][1,3]oxathiol-6-yl phenyl carbonate		C ₁₄ H ₇ BrO ₅ S	367.2	0.89 (0.57-1.37)
17. 5,7-dibromo-2-oxobenzo[<i>d</i>][1,3]oxathiol-6-yl methylcarbamate		C ₉ H ₅ Br ₂ NO ₄ S	383.0	no inhibition ^f
18. 5-bromo-2-oxobenzo[<i>d</i>][1,3]oxathiol-6-yl methyl carbonate		C ₉ H ₅ BrO ₅ S	305.1	2.01 (1.55-2.61)
19. 2-oxobenzo[<i>d</i>][1,3]oxathiol-6-yl benzoate		C ₁₅ H ₇ ClO ₅ S	334.7	0.85 (0.69-1.04)

20. 2-oxobenzo[<i>d</i>] [1,3]oxathiol-6-yl hexanoate		C ₁₃ H ₁₄ O ₄ S	266.3	2.76 (2.18-3.50)
21. 6-(hexyloxy) benzo[<i>d</i>][1,3]oxa- thiol-2-one		C ₁₃ H ₁₆ O ₃ S	252.3	12.7 (8.6-18.8)

^a Apparent IC₅₀ values for inhibition of 4-NPA esterase kinetic rate, determined by absorbance measurements at 348 nm.

^b Error range for fitted IC₅₀ value was defined as a 95% confidence interval.

^c Inhibitor constants (K_i values) for these compounds were determined using ¹⁸O-exchange activity studies.

^d IC₅₀ data from Iyer, et al. (1). Error range for fit IC₅₀ value was defined as a 95% confidence interval.

^e No data. No IC₅₀ data was determined for this compound using the 4-NPA esterase assay.

^f No detectable inhibition was observed for this compound in the CA II 4-NPA esterase IC₅₀ assay.