Synthesis of Novel Thiol-Containing Citric Acid Analogues. Kinetic Evaluation of These and Other Potential Active-Site-Directed and Mechanism-Based Inhibitors of ATP Citrate Lyase

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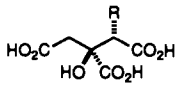
ATP citrate lyase is an enzyme involved in mammalian lipogenesis and cholesterogenesis. Inhibitors of the enzyme represent a potentially novel class of hypolipidemic agents. Citric acid analogues **5**–**16** bearing electrophilic and latent electrophilic substituents were synthesized and evaluated as irreversible inhibitors of the enzyme. The design of these agents was based on the *classical* enzymatic mechanism where an active-site nucleophile (thiol) was believed to be critically involved in catalysis. Reversible inhibition (K_i's ranging from *ca.* 20 to 500 μM) was observed for compounds **5**, **10**, and **12**–**16**. Compounds **6**–**9** and **11** had no appreciable affinity for enzyme (K_i > 1 mM). Time-dependent inactivation of the enzyme by **5**–**16** was not detected following long incubation times (>1 h, 37 °C) at 2 mM inhibitor concentrations.

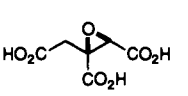
The formation of cytosolic acetyl CoA is an essential biosynthetic step required for *de novo* lipogenesis and cholesterogenesis in mammalian cells.[1] ATP citrate lyase (EC 4.1.3.8) is the enzyme which mediates the formation of this two-carbon donor unit employing citric acid (**1**), coenzyme A (CoA-SH), and ATP as substrates (Scheme 1).[2] It has been shown that *in vivo* dosing of rodents with (−)-hydroxycitrate **2** attenuates lipogenesis and cholesterogenesis in these animals.[3] We have demonstrated that the activity profile of **2** in the human cell line HepG2 parallels that observed for mevinolin.[4] (−)-Hydroxycitrate **2** is a naturally ocurring, potent (K_i = 150 nM), and selective inhibitor of human ATP citrate lyase.[5] These experimental results suggest this enzyme is an attractive target for hypolipidemic intervention, and we have recently described our attempts to inhibit the enzyme through the design of tight binding inhibitors.[6] Herein we describe the design, synthesis, and evaluation of electrophilic and latent electrophilic citric acid analogues as potential inhibitors of ATP citrate lyase.

At the outset of our discovery project, the accepted detailed mechanism for catalysis was reported to proceed through five discrete chemical transformations (Scheme 1).[7] The formation of a phospho–enzyme complex is the first step in catalysis.[2b,8] This occurs through transfer of phosphate from ATP to a nucleophilic histidine residue.[9] Following phosphorylation, enzyme-bound citric acid (**1**) (as its magnesium chelate)[10] is then activated as a carboxylate–phosphate anhydride, **i**.[11] The transfer of phosphate from histidine to the *pro-S* arm of citrate is a critical stereochemical feature of the activation reaction.[11] Anhydride **i** is subsequently attacked by an active-site nucleophile yielding a *covalently bound* citryl–enzyme adduct, **ii**, coincident with the release of inorganic phosphate (P_i).[8,11c] The identification of an active-site thiol as an absolute requirement of catalytic activity[8] makes this a likely candidate for the enzymatic nucleophile. Direct attack by CoA-SH on citryl–enzyme **ii** occurs to generate a noncovalent but tightly bound citryl–CoA bisubstrate, **iii**.[11a,b] The active-site nucleophile (thiol) is hence regenerated. Finally, retro-Claisen cleavage of the citryl–CoA intermediate causes release of the remaining products, oxaloacetate (**3**) and acetyl-CoA (**4**).[2,11]

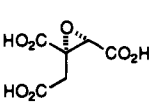
Given the perceived role of an active-site nucleophile in catalysis, we designed a variety of potential active-site-directed[12] and mechanism-based[12] inhibitors, **5**–**16**, of the enzyme. These citrate-derived agents were designed to covalently modify the active-site nucleophile and irreversibly inactivate the enzyme. Examples of potential active-site-directed ligands include epoxides **5** and **6**, Michael acceptors **7**–**9**, and chlorocitrates **10** and **11**. Disulfide-containing citrates **13** and **16** may



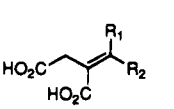
1: R = H
(-)-**2**: R = OH



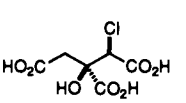
(-)-**5**



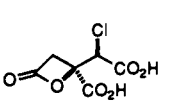
(+)-**6**



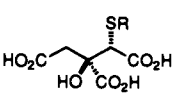
7: R_1 = H; R_2 = CO_2H
8: R_1 = CO_2H; R_2 = H
9: R_1 = R_2 = H



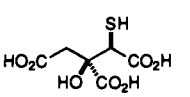
(±)-**10**



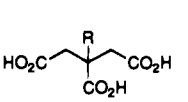
(±)-**11**



(-)-**12**: R = H
(-)-**13**: R = SMe



(-)-**14**



15: R = SH
16: R = SSMe

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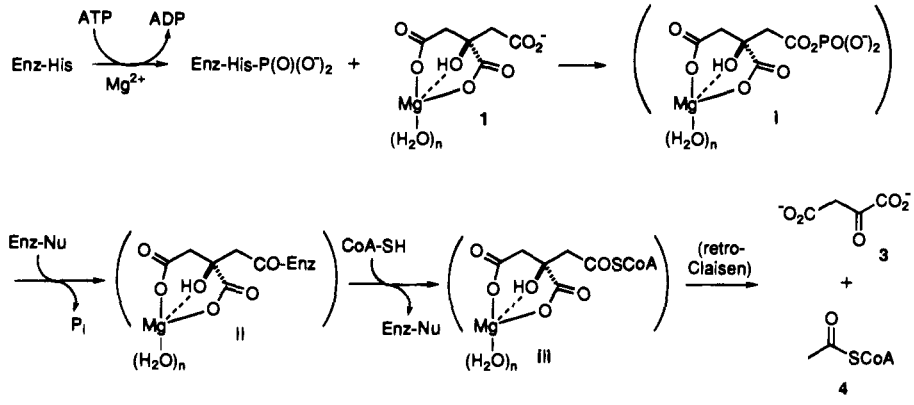
Scheme 1. Classical Mechanism for ATP Citrate Lyase Catalysis[7,11c]



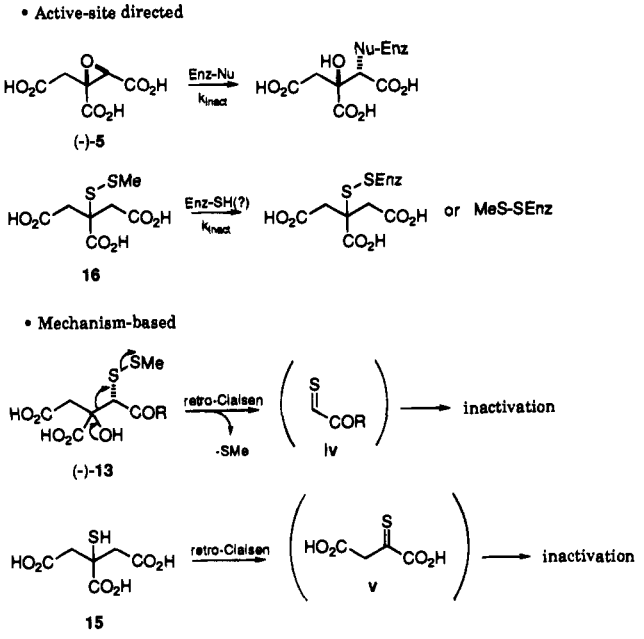
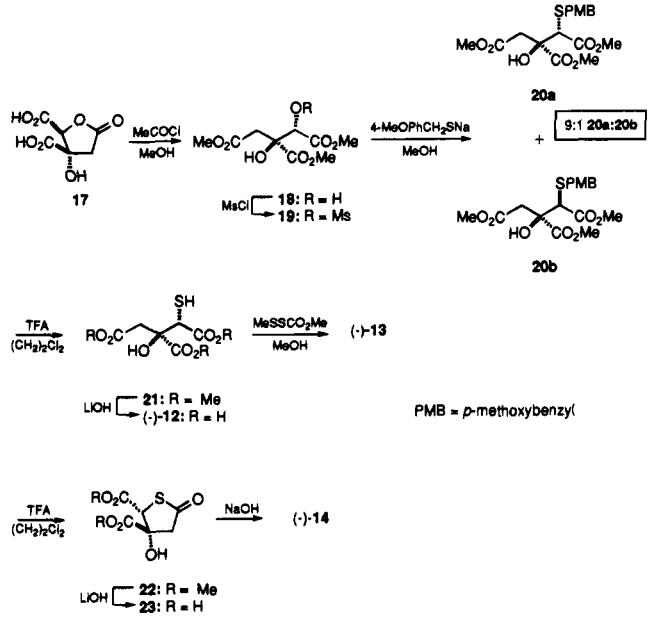


Figure 1. Potential modes of irreversible inactivation by citrate analogues.

also serve as active-site-directed irreversible inhibitors as these citrate analogues could potentially undergo direct disulfide exchange[13] if the active-site nucleophile were a thiol (Figure 1). Alternatively, if **13** were a substrate, then it was thought that the retro-Claisen cleavage might yield the electrophilic species **iv** and inactivate enzyme (Figure 1). If this latter mechanism was operative, disulfide **13** could be formally considered as a mechanism-based inhibitor of ATP citrate lyase. Citrate analogue **15** could also act as a mechanism-based inhibitor where potential turnover of **15** by the enzyme would give rise to reactive thiooxalate (Figure 1).

Upon considering the detailed catalytic mechanism presented in Scheme 1, one of our stereochemical criteria for inhibitor design was to prepare citrate analogues where the *pro-S* arm of the prochiral citrate substrate was exclusively modified. It was thought that positioning the electrophilic (or latent electrophilic) substituent in the *pro-S* arm would offer the greatest opportunity to covalently modify the active-site nucleophile (thiol). Such modifications may also provide selectivity over other citrate-utilizing enzymes (*e.g.*, aconitase). Inhibitors which were prepared in optically active form include epoxyaconitic acids **5** and **6** and the novel thiocitrates **12**–**16**.

Scheme 2. Synthesis of Citrate Analogues (−)-**12**, (−)-**13**, and (−)-**14**



Chemistry

The *cis*- and *trans*-aconitic and itaconic acids (**7**–**9**) were purchased from Aldrich Chemical Co. Epoxyaconitic acids (−)-**5** and (+)-**6**, chlorocitrate (±)-**10**, and the β-lactone (±)-**11** were prepared from **7** and **8** according to published procedures.[14] Hydroxycitrate (−)-**2** was isolated from a natural source.[15]

Preparation of Citrate Analogues (−)-12, (−)-13, and (−)-14. (−)-Garcinia lactone **17** (isolated from the fruit rind of *Garcinia cambogia*)[15a] was esterified in MeOH in the presence of HCl (Scheme 2).[15b] The triester **18** was treated with methanesulfonyl chloride (1 equiv) in pyridine to give the monosulfonate ester **19**. Reaction of **19** with sodium 4-methoxybenzylthiolate (1.1 equiv each of NaOMe and 4-MeOPhCH$_2$SH) in MeOH (70 °C, 2 h) furnished a 9:1 mixture of diastereomeric sulfides **20a**,**b**. The minor isomer, **20b**, crystallized exclusively from the reaction mixture. Further purification by recrystallization (mp 95–95.5 °C; Et$_2$O) and subsequent X-ray crystallographic analysis permitted the assignment of relative and absolute[16] stereochemistry in **20b** (ORTEP, Figure 2), and hence the major isomer, **20a** (oil), as indicated in Scheme 2. Interestingly, the minor isomer, **20b**, is derived from direct S_N2 displacement of the mesylate by the thiolate

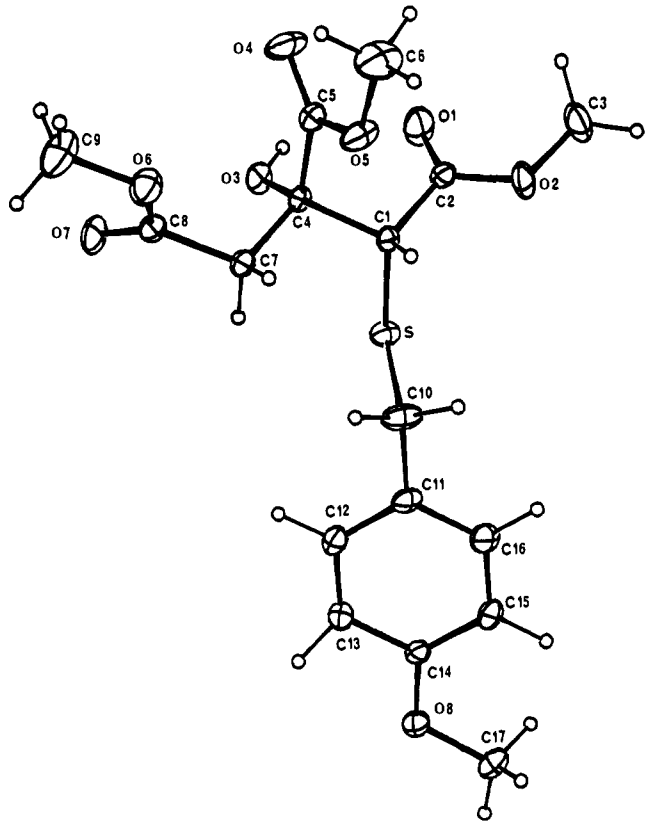


Figure 2. Molecular structure of triester **20b** as determined by X-ray crystallography (ORTEP diagram).

anion, while the major isomer, **20a**, retains the relative and absolute stereochemistry found in mesylate **19**. Presumable under the reaction conditions, a double inversion of stereochemistry occurs via the intermediacy of an epoxide giving rise to **20a** as the major isomer.[17] The mixture of sulfides **20a,b** was routinely subjected to trifluoroacetic acid (TFA)-mediated deprotection (1:1 TFA–$(CH_2)_2Cl_2$, 0.1 equiv of anisole, 80 °C, 3 h) to remove the 4-methoxybenzyl (PMB) protecting group. This treatment with TFA yielded thiol **21** (derived from **20a**) and thiolactone **22** (derived from **20b** via spontaneous thiolactonization). Thiol **21** was conveniently isolated and purified by dissolution in aqueous $NaHCO_3$, enabling neutral byproducts to be removed by extraction. Thiolactone **22** was separately obtained by analogous TFA deprotection of pure **20b**. Saponification of triester **21** was carried out using 10 equiv of LiOH in aqueous THF (1:2 THF–H_2O) to furnish the desired 2-mercaptocitrate (−)-**12**.[18] Methyl disulfide (−)-**13** was prepared in quantitative yield following exposure of (−)-**12** to *S*-methylsulfenyl *O*-methyl thiocarbonate in MeOH.[19] Thiol (−)-**14** was obtained from thiolactone **22** using a two-step saponification protocol which required initial diester hydrolysis followed by *in situ* thiolactone ring opening (**22** → **23** → (−)-**14**).

Preparation of Citrate Analogues 15 and 16. Methyl *tert*-butyl malonate (**24**) was alkylated with methyl bromoacetate (2 equiv each of NaH and $BrCH_2$-COOMe) in THF to give the mixed tetraester **25** (Scheme 3). Monoacid **26** was obtained upon selective removal of the *tert*-butyl ester in **25** (1:1 TFA–CH_2Cl_2, 25 °C, 1.5 h) which, following treatment with thionyl chloride, furnished the acid chloride **27**. Acylthioxanthate **28**, obtained upon exposure of acid chloride **27** to sodium ethylthioxanthate, underwent photochemical

Scheme 3. Synthesis of Citrate Analogues **15** and **16**

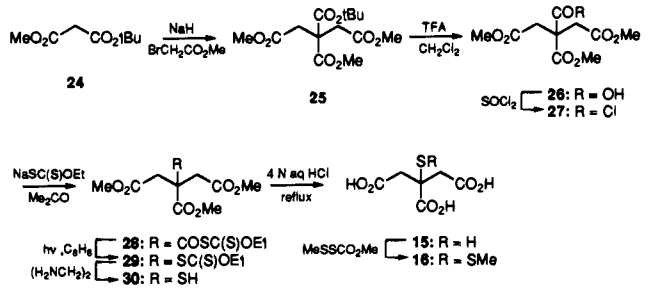


Table 1. Kinetic Results of Potential Inhibitors Evaluated against Rat ATP Citrate Lyase

compd	reversible binding[a] K_i (μM)	irreversible inactivation[a] ($k_i \times 10^{-3}$ h^{-1})
2	0.15	nd
5	18	nd
6	400	nd
7	>10000	nd
8	>10000	nd
9	>10000	nd
10	29	nd
11	340	nd
12	58	nd
13	34	nd
14	150	nd
15	295	nd
16	480	nd

[a] Reversible binding was measured by the inhibition of the carbon–carbon bond cleavage activity in citrate substrate. Time-dependent inactivation was not detectable (nd) above the thermal decay rate of 5.0×10^{-3} h^{-1} at 2 mM inhibitor at 2 °C. See the Experimental Section for details.

rearrangement in benzene[20] to give the *S*-citryl xanthate **29** in 22% isolated yield. The desired 2-mercaptocitrate **15** was generated from triester **29** following base ($H_2NCH_2CH_2NH_2$) and then acid-catalyzed hydrolysis (3 M aqueous HCl, 100 °C, 3 h; **29** → **30** → **15**). Conversion of **15** to methyl disulfide **16** was carried out as describe for the preparation of (−)-**13** from (−)-**12**.

Results and Discussion

A selection of potential active-site-directed and mechanism-based irreversible inhibitors derived from citric acid were prepared and evaluated as inhibitors of ATP citrate lyase. The results of the kinetic inhibition studies of these compounds against enzyme is presented in Table 1. Reversible inhibition against enzyme was seen for many of the analogues; time-dependent inactivation of enzyme, the hallmark of irreversible inhibition, was not observed for any of the citrate analogues.

Reversible inhibition constants (K_i) were measured for each of the citrate analogues. The *cis*- and *trans*-aconitic acids, **7** and **8**, show a complete absence of enzyme affinity. Modest reversible inhibition was observed for the *cis*- and *trans*-epoxyaconitic acids with the *cis*-epoxide (−)-**5** (K_i = 18 μM) showing a 22-fold enhancement in potency relative to the *trans*-epoxide (+)-**6** (K_i = 400 μM). This result is suggestive of a preferred *syn* orientation of the carboxylates in the active site of the enzyme. The contrasting affinities of **5** and **6** versus **7** and **8** provide evidence for the relative importance of a hydrogen-bond acceptor/donor in this region of the citrate skeleton. Neither a chlorine atom nor a thiol group positioned in the *pro-S* arm of citric acid affords any advantage over the single epoxide

oxygen in this regard. Chlorocitrate **10** and thiocitrates (−)-**12** and (−)-**13** possess modest reversible inhibition against enzyme. The K_i's for **10** (29 μM),[21] (−)-**12** (58 μM), and (−)-**13** (34 μM) are all within *ca.* 3-fold of the K_i measured for (−)-**5** (18 μM). Enhancement in inhibitory activity (>100-fold) is only realized upon introducing a hydroxyl group in the *pro-S* arm as in (−)-**2**. The potency of (−)-hydroxycitrate **2** (K_i = 150 nM) is highly dependent on absolute stereochemistry as only one of the four known hydroxycitrate stereoisomers exhibits appreciable affinity for ATP citrate lyase.[22] The secondary hydroxyl group in (−)-**2** probably serves as both a hydrogen-bond acceptor and donor in the active site of the enzyme.

The lack of time-dependent inactivation of ATP citrate lyase by compounds **5-16** was somewhat surprising. Considering the critical role of the active-site nucleophile in catalysis,[8,11c] citrate analogues **5-16** and our previously disclosed vinyl- and 2,2-difluorocitrates[23,24] represent a classical design strategy to inhibiting enzymes where nucleophilic catalysis is operative.[12] During the course of our endeavor, both rat and human ATP citrate lyase were cloned and expressed and their amino acid sequences determined.[9] The enzyme mechanism was also reinvestigated.[25] Information gleaned from these studies has led to the formulation of a revised mechanism for ATP citrate lyase catalysis.[6,25] In this revised mechanism, the citrate phosphate anhydride is attacked *directly* by CoA-SH to give citryl−SCoA bisubstrate and P_i, eliminating the requirement for the active-site nucleophile and the subsequent covalently bound citryl−enzyme complex. Hence, one possible explanation for the lack of time-dependent inactivation displayed by our inhibitors is due to the fact that there is no nucleophilic group which engages the *pro-S* carboxylate. Furthermore, on the basis of the inhibitor data presented here and elsewhere,[23,24] it may be unlikely that an active-site thiol (or any nucleophile) is in close proximity (bonding distance) to the citrate backbone.[26]

The work cited in this and previous communications[6,23,24] concludes our attempts to design potent reversible and irreversible citric acid-based inhibitors of ATP citrate lyase. Our research efforts are currently focused on the design of bisubstrate (citrate-SCoA) analogues as a means to inhibiting this enzyme. The details of this new design strategy and the biological profile of the novel inhibitors will be reported in due course.

Experimental Section

General Procedures. Melting points were determined on a Buchi capillary melting point apparatus and are uncorrected. Analytical samples were homogeneous by TLC performed on silica gel plates. Elemental analyses (C, H, N) of new compounds are within 0.4% of the theoretical values, ^{1}H NMR spectra were determined on Bruker AM 250 or AM 360 spectrometers using tetramethylsilane (TMS) as the internal standard. IR spectra were recorded on a Perkin-Elmer Model 298 instrument. Low- and high-resolution (EI) mass spectra were recorded on a VG-70-250-SEQ instrument. All structural assignments were consistant with IR, NMR, and mass spectra.

[*S*-[*R,*R**)]-1,2-Dihydroxy-1,2,3-propanetricarboxylic Acid Trimethyl Ester (18).** Garcinia lactone 17[15] (250 g, 1.2 mol) was dissolved in methanol (1 L), and the solution was cooled to 0 °C. Acetyl chloride (250 mL) was added dropwise, and the mixture was heated to 70 °C for 5 h. The reaction mixture was cooled to 10 °C, pyridine (250 mL) was added, and the solution was concentrated in vacuo. The residue was diluted with brine (500 mL), and then the mixture was extracted with CH_2Cl_2 (4 × 500 mL). The organic extracts were combined, washed with 1 M aqueous HCl and saturated aqueous $NaHCO_3$, and dried ($MgSO_4$). Removal of the solvent in vacuo gave triester **18** (166 g, 51%) as a viscous oil which was used without further purification: ^{1}H NMR ($CDCl_3$) δ 4.32 (d, 1H, J = 8.5 Hz, CH), 4.02 (s, 1H, OH), 3.87 (s, 6H, CO_2CH_3), 3.39 (d, 1H, J = 8.5 Hz, OH), 3.04 (s, 2H, CH_2).

[*S*-(*R,*R**)]-2-Hydroxy-1-[(methylsulfonyl)oxy]-1,2,3-propanetricarboxylic Acid Trimethyl Ester (19).** To a solution of triester **18** (140 g, 0.56 mol) in pyridine (500 mL) was added dropwise, at 0 °C, methanesulfonyl chloride (44 mL, 0.56 mol), and the resulting red reaction mixture was stirred for 3 h at 0 °C. The mixture was poured into ice/water containing concentrated hydrochloric acid (600 mL) and then washed with diethyl ether (2 × 300 mL). The diethyl ether extracts were discarded, and the aqueous solution was further extracted with CH_2Cl_2 (4 × 300 mL). The organic extracts were combined, washed with saturated aqueous $NaHCO_3$, and dried ($MgSO_4$). The solution was concentrated to *ca.* 700 mL, treated with activated carbon, filtered, and then concentrated in vacuo to give an oil, which solidified on trituration with diethyl ether. The residue was collected and thoroughly washed with diethyl ether until the liquors were colorless to provide sulfonate **19** (120.6 g, 66%) as a pale yellow solid. This material was used in the subsequent reaction without further purification. Recrystallization of a small sample gave analytically pure **19**: white solid; mp 88−89 °C (lit.[14a] mp 89−91 °C); $[\alpha]^{25}_D$ − 17.8° (*c* 0.4, MeOH); ^{1}H NMR ($CDCl_3$) δ 5.19 (s, 1H, CH), 4.15 (s, 1H, OH), 3.88 and 3.89 (singlets, 3H each, CO_2CH_3), 3.70 (s, 3H, CO_2CH_3), 3.17 (s, 3H, OSO_2CH_3), 3.17 and 3.06 (d, 2H, J = 16.4 Hz, CH_2).

[*S*-(*R,*S**)]-2-Hydroxy-1-[(4-methoxybenzyl)thio]-1,2,3-propanetricarboxylic Acid Trimethyl Ester (20a) and the [*R*-(*R**,*R**)]-Diastereomer (20b).** A solution of sodium methoxide in methanol (125 mL of a 2.2 M solution, 0.275 mol) was added to a solution of 4-methoxybenzenethiol (45.24 g, 0.275 mol) in methanol (125 mL). The solution of sodium thiolate so obtained was then added to a solution of mesylate **19** (82.1 g, 0.25 mol) in methanol (750 mL). The reaction mixture was heated to 70 °C for 2 h. The reaction mixture was concentrated in vacuo, and water (500 mL) was added. The solution was extracted with $CHCl_3$ (3 × 300 mL) and dried ($MgSO_4$). Concentration in vacuo gave a 9:1 mixture of sulfides **20a**:**20b** as an oil (98.8 g). Silica gel chromatography (0−90% diethyl ether−petroleum ether gradient) furnished analytically pure **20a,b**. **20a**: oil; ^{1}H NMR ($CDCl_3$) δ 4.15 (s, 1H, OH), 3.80 (m, 2H, SCH_2), 3.80, 3.78 and 3.75 (singlets, 3H each, CO_2CH_3), 3.64 (s, 3H, OCH_3), 3.48 (s, 1H, CH), 3.10 and 2.65 (doublets, 1H each, J = 16 Hz, CH_2).

20b: solid; mp 95−95.5 °C (diethyl ether); ^{1}H NMR ($CDCl_3$) δ 4.43 (s, 1H, OH), 3.85 (m, 2H, CH_2S), 3.80, 3.75 and 3.73 (singlets, 3H each, CO_2CH_3), 3.62 (s, 3H, OCH_3), 3.55 (s, 1H, CH), 3.13 and 2.68 (doublets, 1H each, J = 15.9 Hz, CH_2).

[*S*-(*R,*S**)]-2-Hydroxy-1-mercapto-1,2,3-propanetricarboxylic Acid Trimethyl Ester (21).** The mixture of **20a,b** obtained above (30 g, 80.2 mmol) was dissolved in 1,2-dichloroethane (300 mL) containing anisole (9 g, 0.083 mol. Trifluoroacetic acid (300 mL) was added; the mixture was heated to 80 °C for 3 h and then concentrated in vacuo. Saturated aqueous $NaHCO_3$ (200 mL) was added to the residue, and the solution was extracted with diethyl ether (3 × 100 mL). The aqueous solution was acidified with concentrated hydrochloric acid and extracted with CH_2Cl_2 (4 × 100 mL). The organic extracts were combined, dried ($MgSO_4$), and concentrated in vacuo to give a yellow residue. The residue was triturated with diethyl ether, collected, and washed thoroughly with diethyl ether to furnish analytically pure mercaptan **21** (2.9 g, 14%): mp 105−107 °C (diethyl ether); ^{1}H NMR ($CDCl_3$) δ 4.20 (s, 1H, OH), 3.74 (d, 1H, CO_2CH_3), 3.69 (s, 3H, CO_2CH_3), 3.15 and 2.91 (doublets, 1H each, J = 16 Hz, CH_2), 2.54 (d, 1H, J = 11.4 Hz, SH).

(2*R*)-*cis*-Tetrahydro-3-hydroxy-5-oxo-2,3-thiophenedicarboxylic Acid Dimethyl Ester (22). Sulfide **20b** (7.72 g, 20 mmol) was dissolved in 1,2-dichloroethane (100 mL)

containing anisole (2.48 g, 23 mmol). Trifluoroacetic acid (100 mL) was added; the mixture was heated to 80 °C for 3 h and then concentrated in vacuo. Saturated aqueous $NaHCO_3$ solution was added to the residue, and the solution was extracted with diethyl ether (3 × 10 mL). The diethyl ether extracts were combined and dried ($MgSO_4$), and the solvents were removed in vacuo. The residue so obtained was purified by silica gel chromatography (2.5% MeOH–CH_2Cl_2) to yield thiolactone **22** (2.36 g, 54%): oil; ^1H NMR ($CDCl_3$) δ 5.15 (s, 1H, CH), 4.10 (s 1H, OH), 3.93 and 3.81 (singlets, 3H each, CO_2CH_3) 3.11 and 2.91 (doublets, 1H each, J = 16.5 Hz, CH_2).

[*S*-(*R,*S**)]-2-Hydroxy-1-mercapto-1,2,3-propanetricarboxylic Acid Disodium Salt ((−)-12).** Trimethyl ester **21** (0.8 g, 3 mmol) was dissolved in freshly distilled THF (15 mL) under an atmosphere of nitrogen. To the solution was added freshly prepared, degassed, aqueous lithium hydroxide (30 mL of a 1 M solution), and the mixture was stirred at 30 °C for 48 h. The solution was washed with distilled diethyl ether and directly subjected to ion-exchange chromatography (BioRad 50W-X4 (H^+) resin; 50 g). The acidic eluant was collected, combined, and lyophilized. The residue so obtained was dissolved in distilled diethyl ether, filtered, and concentrated to furnish a colorless oil. The oil was further purified by ion-exchange chromatography (BioRad 50W-X2 (Na^+); 30 g). Fractions eluting at pH 3–4 were collected, combined, and lyophilized to give the disodium salt of the triacid **12** (0.69 g, 86%) amorphous solid; $[\alpha]^{25}_D$ −6.4° (*c* 1.0, H_2O); ^1H NMR (D_2O) δ 3.67 (s, 1H), CH), 3.06 and 2.79 (doublets, 1H each, J = 15.5 Hz, CH_2). Anal. ($C_6H_6O_7SNa_2$) C, H, S.

[*S*-(*R,*S**)]-2-Hydroxy-1-[(methylthio)thio]-1,2,3-propanetricarboxylic Acid Disodium Salt (13).** Tricarboxylic acid (−)-**12** (250 mg, 0.93 mmol) was dissolved in methanol (15 mL) containing water (3 mL). The solution was added to a solution of *S*-methysulfenyl *O*-methyl thiocarbonate[19] (200 mg, 1.45 mmol) in methanol (10 mL), and the reaction mixture was stirred for 2 h at 25 °C. The reaction mixture was concentrated to dryness, and the residue was triturated with acetonitrile. The solid material so obtained was collected, washed with acetonitrile, and dried in vacuo to give the disulfide (−)-**13** (217 mg, 74%): amorphous solid; $[\alpha]^{25}_D$ −58.4° (*c* 1.0, H_2O); ^1H NMR (D_2O) δ 3.79 (s, 1H, CH), 3.07 and 2.75 (doublets, 2H each, J = 15.7 Hz, CH_2), 2.43 (s, 3H, SCH_3). Anal. ($C_7H_{10}O_7S_2$) C, H, S.

(2*R*)-*cis*-Tetrahydro-3-hydroxy-5-oxo-2,3-thiophenedicarboxylic Acid (23). Thiolactone **22** (0.74 g, 3.39 mmol) was dissolved in freshly distilled THF (17 mL) under an atmosphere of nitrogen, and a solution of freshly prepared, degrassed aqueous lithium hydroxide (31 mL of a 1.0 M solution) was added. The reaction mixture was stirred at 30 °C for 24 h and then washed with distilled ether. The aqueous solution was directly subjected to ion-exchange chromatography on (BioRad 50W-X4 (H^+); 42 g), and the acidic eluant was collected and lyophilized. The residue was triturated with diethyl ether and filtered and the organic solvent concentrated in vacuo to give a mixture of free thiol triacid **14** and thiolactone diacid **23**. This mixture was dissolved in diethyl ether (15 mL) and added to a stirred suspension of silica gel (230–400 mesh, 6 g) in diethyl ether (25 mL) containing concentrated sulfuric acid (1 mL). The heterogeneous mixture was stirred for 6 h at 25 °C, filtered, and concentrated to give an oil which was further purified by silica gel chromatography (4:6:1 MeOH–CH_2Cl_2–HCO_2H) to give diacid thiolactone **23** (150 mg, 21%): solid; mp 84–86 °C (diethyl ether/petroleum ether); $[\alpha]^{25}_D$ −168° (*c* 0.5, H_2O); ^1H NMR (D_2O) δ 5.52 (s, 1H, CH), 3.46 and 2.96 (doublets, 1H each, J = 17.3 Hz, CH_2). Anal. ($C_6H_6O_6S$) C, H, S.

[*R*-(*R,*R**)]-2-Hydroxy-1-mercapto-1,2,3-propanetricarboxylic Acid Disodium Salt ((−)-14).** Triacid (−)-**14** was prepared *in situ* for enzyme evaluation upon treating an aqueous solution of thiolactone **23** with 3.3 equiv of aqueous NaOH (0.5 N solution) and, after 5 min, readjusting to neutral pH with 0.5 N aqueous HCl.[27] (−)-**14**: $[\alpha]^{25}_D$ −6.8° (*c* 0.25, 0.1 M NaOH).

1,2,2,3-Propanetetracarboxylic Acid 2-*tert*-Butyl Ester 1,2,3-Trimethyl Ester (25). Under a nitrogen atmosphere, a solution of *tert*-butyl methyl malonate (**24**) (25 g, 0.144 mol) in dry THF (100 mL) was added dropwise to a stirred suspension of NaH (11.48 g, 0.287 mol; 60% oil dispersion) in anhydrous THF (50 mL), while the temperature was maintained between 18 and 25 °C. The reaction mixture was stirred at 25 °C for 1.5 h followed by the dropwise addition of a solution of methyl bromoacetate (43.9 g, 0.287 mol) in anhydrous THF (50 mL). The temperature of the reaction mixture was maintained at 20 °C during the addition of methyl bromoacetate. The reaction mixture was stirred at 25 °C for 18 h and then cautiously poured onto ice/water. The solution was extracted with diethyl ether and then with CH_2Cl_2, and the combined organic extracts were dried ($MgSO_4$). Removal of the solvents in vacuo gave tetraester **25** (42.1 g, 92%). A portion of the material was purified further by distillation: oil; bp 150 °C; (0.1 mmHg); IR (liquid film) 1743 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.76 (s, 3H, tertiary CO_2CH_3), 3.68 (s, 6H, CO_2CH_3), 3.13 (s, 4H, CH_2), 1.44 (s, 9H, *t*-Bu); MS *m/z* (rel intensity) 263 (4), 245 (45), 218 (10), 57 (100).

1,2,2,3-Propanetetracarboxylic Acid 1,2,3-Trimethyl Ester (26). To a solution of tetraester **25** (41.39 g, 0.13 mol) in CH_2Cl_2 (115 mL) was added dropwise trifluoroacetic acid (115 mL). The reaction mixture was stirred at 25 °C for 1.5 h and then carefully neutralized with saturated aqueous $NaHCO_3$ (400 mL). The organic solution was discarded, and the aqueous layer was acidified to pH 2 with 12 M HCl and re-extracted with CH_2Cl_2. The organic extracts were combined, dried ($MgSO_4$), and concentrated in vacuo to give acid **26** (26.66 g, 78%): oil; IR (liquid film) 1739 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.80 (s 3H, tertiary CO_2CH_3), 3.69 (s, 6H, CO_2CH_3), 3.18 and 3.17 (singlets, 2H each, CH_2); MS *m/z* (rel intensity) 245 (5), 231 (10), 187 (×5), 127 (100), 59 (98). Anal. ($C_{10}H_{14}O_8$) C, H.

2-(Chlorocarbonyl)-1,2,3-propanetricarboxylic Acid Trimethyl Ester (27). To a solution of acid **26** (26.2 g, 0.1 mol) in CH_2Cl_2 (75 mL) was added a solution of thionyl chloride (47.6 g, 0.4 mol) in CH_2Cl_2 (75 mL) followed by the addition of a catalytic amount of DMF. The reaction mixture was heated to 40 °C for 2 h, and then the solvent and excess thionyl chloride were removed in vacuo to give the acid chloride **27** (26.63 g, 95%) which was used without further purification: oil; IR (liquid film) 1740, 1170-1210 cm^{-1}.

2-[[(Ethoxythioxomethyl)thio]carbonyl]-1,2,3-propanetricarboxylic Acid Trimethyl Ester (28). A solution of acid chloride **27** (26.63 g, 0.095 mol) in anhydrous acetone (190 mL) was cooled to −35 °C in a CH_3CN/dry ice bath. A solution of potassium ethylxanthogenate (16.73 g, 0.104 mol) in anhydrous acetone (170 mL) was added dropwise over a 30 min period, and the reaction mixture was then stirred at −35 °C for 1 h, before being allowed to warm to room temperature. The solvent was removed in vacuo and the residue partioned between water and CH_2Cl_2. The organic layer was separated, washed with 0.1 M aqueous Na_2CO_3 and water, and dried ($MgSO_4$). The solvent was evaporated in vacuo, and the residue was purified by silica gel chromatography (10–50% diethyl ether–petroleum ether gradient) to give the acylthioxanthate **28** (15.67 g, 45%): oil; IR (liquid film) 1740 cm^{-1}; ^1H NMR ($CDCl_3$) δ 4.69 (m, 2H, CH_2CH_3), 3.80 (s, 3H, tertiary CO_2CH_3), 3.69 (s, 6H, CO_2CH_3), 3.28 (s, 4H, CH_2), 1.48 (t, 3H, CH_2CH_3). Anal. ($C_{13}H_{18}O_8S_2$) C, H, S.

2-[(Ethoxythioxomethyl)thio]-1,2,3-propanetricarboxylic acid Trimethyl Ester (29). A solution of triester **28** (15.53 g, 0.042 mol) in anhydrous benzene (800 mL) was irradiated under an argon atmosphere with a 125 W mercury arc lamp for 20 min. The solvent was removed in vacuo, and the residue was purified by silica gel chromatography (5–40% diethyl ether–petroleum ether gradient) to furnish dithiocarbonate **29** (3.19 g, 22%): solid, mp 75–76 °C (ether/petroleum ether); IR (Nujol mull) 1752, 1738 cm^{-1}; ^1H NMR ($CDCl_3$) δ 4.64 (q, 2H, CH_2CH_3), 3.78 (s, 3H, tertiary CO_2CH_3), 3.69 (s, 6H, CO_2CH_3), 3.36 and 3.27 (doublets, 2H, each, J = 16.0 Hz), 1.44 (t, 3H, CH_2CH_3); MS *m/z* (rel intensity) 338 (M^+, 3), 309 (5), 217 (60), 185 (100), 153 (75). Anal. ($C_{12}H_{18}O_7S_2$) C, H, S.

2-Mercapto-1,2,3-propanetricarboxylate Trimethyl Ester (30). A solution of dithiocarbonate **29** (1.06 g, 3.1 mmol) in ethylenediamine (42 mL) was stirred under an argon atmosphere at 30 °C for 3 h and then poured into an ice/water solution of 1.0 M aqueous sulfuric acid. The pH of the soluion

was adjusted to pH 2, and the solution was extracted with CH_2Cl_2 and diethyl ether. The combined organic extracts were dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by silica gel chromatography (10–45% diethyl ether–petroleum ether gradient) to give mercaptan **30** (250 mg, 32%): oil; ^1H NMR ($CDCl_3$) δ 3.79 (s, 3H, tertiary CO_2CH_3), 3.70 (s, 6H, CO_2CH_3), 3.20 and 3.15 (doublets, 2H each, J = 16.5 Hz).

2-Mercapto-1,2,3-propanetricarboxylic Acid (15). A solution of triester **30** (240 mg, 0.96 mmol) in 4 M aqueous HCl (10 mL) was heated to 100 °C for 4 h. The solution was cooled to 25 °C and lyophilized. The residue was recrystallized (diethyl ether–petroleum ether) to give mercaptocitrate **15** (115 mg, 58%): solid (diethyl ether–petroleum ether); mp 162–164 °C; IR (Nujol mull) 1718, 1699 cm^{-1}; ^1H NMR (DMSO) δ 12.60 (br s, 3H, CO_2H), 3.70 (br s, 1H, SH), 2.99 (s, 4H, CH_2); MS (FAB positive ion) m/z 209 (M^+). Anal. ($C_6H_8O_6S$) C, H, S.

2-[(Methylthio)thio]-1,2,3-propanetricarboxylic Acid (16). A solution of *S*-methylsulfenyl *O*-methyl thiocarbonate[19] (246 mg, 1.78 mmol) in methanol (6 mL) was added to a solution of mercaptan **15** (190 mg, 0.91 mmol) in methanol (6 mL). The reaction mixture was stirred at 25 °C for 2 h and then concentrated in vacuo. The residue was dissolved in water (5 mL) and washed with $CHCl_3$. The aqueous solution was filtered and then lyophilized to give analytically pure disulfide **34** (110 mg, 47%): solid, mp 154–156 °C; IR (Nujol mull) 1704 cm^{-1}; ^1H NMR (D_2O) δ 3.21 and 3.17 (doublets, 2H each, J = 18 Hz, CH_2) 2.43 (s, 3H, $SSCH_3$); MS (FAB positive ion) m/z 255 (M^+). Anal. ($C_7H_{10}O_6S_2$) C, H, S.

Rat ATP Citrate Lysase Assay. ATP citrate lyase was purified from rat liver as previously described.[25] Reversible binding K_i and the determination of time-dependent inactivation were measured by inhibition of the carbon–carbon cleavage activity,[28] using 1.0 mM citrate ($S = K_m$) as previously described.[24] Under these conditions, the equation for competitive inhibition, $V = V_{max}[S]/\{K_m(1 + [I]/K_i) + S\}$, reduces to $V = V_{max}/(2 + [I]/K_i)$. Reversible inhibition data were fitted to this equation using the program Grafit.[29] For the time-dependent inhibition, enzyme was incubated with 2 mM inhibitor at 25 °C in Tris·HCl, pH 8.0, buffer. Aliquots were taken over a period of 48 h and immediately placed in assay cocktail containing saturating substrate. The resulting enzyme activities were fitted to the equation for first-order inactivation, $A_t = A_0e^{(-k_it)}$.

Supplementary Material Available: Experimental details of the X-ray structure determination of **20b**, including tables of fractional atomic coordinates, thermal parameters, interatomic distances, and angles (10 pages). Ordering information is given on any current masthead page.

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