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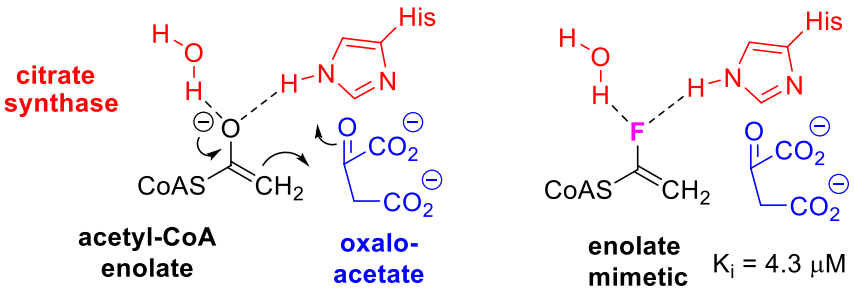

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vinylfluorine as a steric and spacial mimetic of an oxygen enolate

Acetyl coenzyme A analogues as rationally designed inhibitors of citrate synthase.

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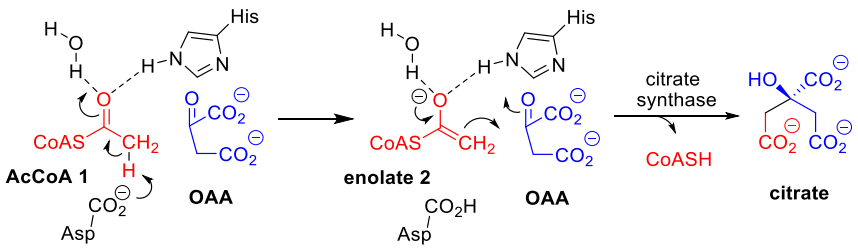
Abstract: In this study we probe inhibition of pig heart citrate synthase (E. C. 4.1.3.7) by synthesis of seven analogues designed to mimic either the proposed enolate intermediate in this enzyme reaction or develop from historical inhibitors. The most potent inhibitor was fluorovinyl thioether **9** (K_i = 4.3 μM), where a fluorine replaces the oxygen atom of the enolate. A comparison of the potency of **9** versus its non-fluorinated vinyl thioether analogue **10** (K_i = 68.3 μM), revealed a clear 'fluorine effect' favouring **9** by an order of magnitude. Inhibitors **11** and **12** were dethia analogues of **9** and **10**; these proved to be poor inhibitors. Methyl sulfoxide **13** was a moderate inhibitor (K_i = 11.1 μM) suggesting hydrogen bonding interactions in the enolate site. Finally two propenoate thioether isomers **14**(*E*) and **15**(*Z*) were explored as conformationally constrained carboxylates, but these were not inhibitors. All compounds were prepared by the synthesis of the appropriate pantetheinyl diol and then assembly of the coenzyme A structure using a three enzyme biotransformation protocol. A quantum mechanical study, modelling both inhibitors **9** and **10** into the active site indicated short CF···H contacts ~2.0Å, consistent with fluorine making two stabilising hydrogen bonds, and mimicking an enolate rather than an enol intermediate. Computation also indicated that when **9** binds to citrate synthase this increases the basicity of a key Asp carboxylate which becomes protonated.

Introduction: Citrate synthase is an enzyme of the citric acid cycle that catalyses the condensation of acetyl coenzyme A (AcCoA) **1** and oxaloacetate (OAA) to generate citrate, with concomitant release of coenzyme A (CoASH).[7] The enzyme mechanism has been studied in detail by experiment, enzyme X-ray structure analysis, [8] and computation[9] and the consensus, particularly from computation, suggests that the reaction progresses through an AcCoA enolate **2**, which is stabilised by hydrogen bonding contacts as illustrated in Scheme 1.



Scheme 1. The citrate synthase reaction progresses through an enolate intermediate **3**.[8,9]

Inhibitors **3** to **8** of citrate synthase have been evaluated[7b,10,11]over the years by modification of AcCoA **1** to anticipate mimicking intermediates of the reaction course, or to make electrostatic contacts within the active site. Figure 1 illustrates these on a hierarchy from nanomolar to micromolar inhibition constants (K_i). The most potent is a dethia-carboxylate **3** which carries a formal negative charge spatially located similar to that in intermediate enolate **2**. The amide **4** is both a good hydrogen bond donor and acceptor. Carboxylate **5**, which is negatively charged retains good activity, although

spacially it does not easily map onto enolate **2**, and this may account for the reduced activity relative to carboxylate **3**. Inhibitors **6** and **7** are less potent (K_i = micromolar). Ketone **6** will be less enolic than amide **4**, and the oxygens of alkylnitrates such as **7** are known to be relatively poor hydrogen bonding acceptors. Carboxylate **8** has modest potency, and despite its charge it does not obviously map onto enolate **2**.

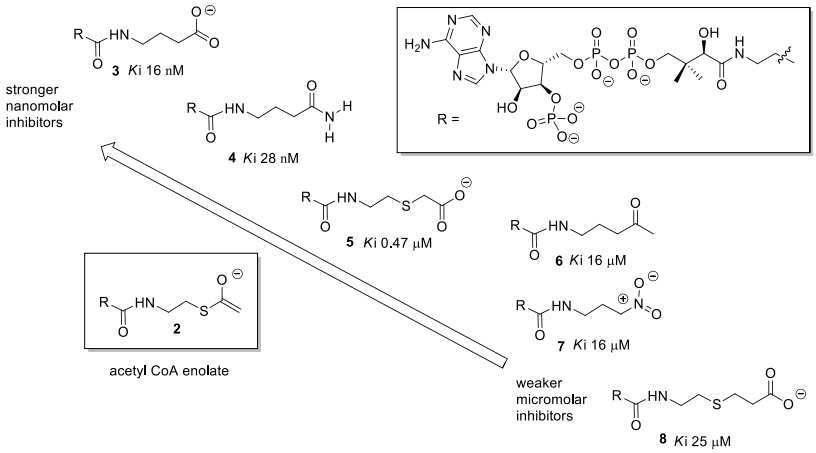


Figure 1. Historical inhibitors **3**-**8** [10,11] of citrate synthase drawn relative to a representation of the AcCoA enolate **2** intermediate.

In this study we have used citrate synthase as an arena in which to explore a range of modifications to the acyl moiety of AcCoA **1**, by comparing their capacity as inhibitors. The compounds range from fluorovinyl thioether **9** to sulfoxide **13** and conjugated carboxylates **14**(*E*) and **15**(*Z*) as illustrated in Figure 2. Other candidate inhibitors (**9** vs **11**) explore the effect of removing sulfur or of removing fluorine (**9** vs **10** and **11** vs **12**).

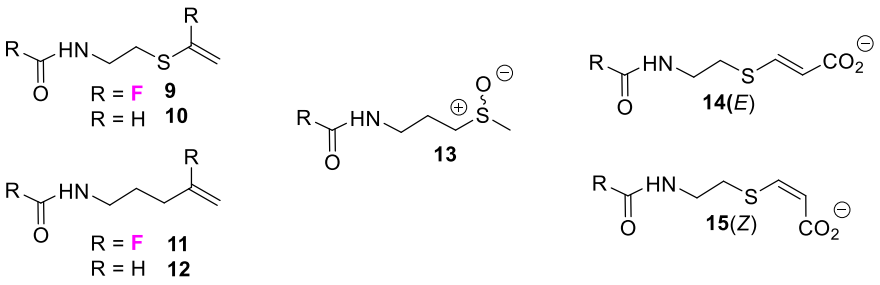


Figure 2. Candidate inhibitors **9** -**15**(*Z*) of citrate synthase.

We viewed fluorovinyl thioether **9** as a potential enolate mimetic where fluorine occupies the location of the oxygen in enolate **2**. Organic bound fluorine has been used in bioorganic chemistry to mimic oxygen functionality in a number of circumstances by direct atom replacement.[1] Fluorine and oxygen sit adjacent to each other in the Periodic Table. Both are highly electronegative (Pauling scale; F = 3.98 versus O = 3.44)[2] and these atoms have a close steric (van der Waals radii F = 1.47Å versus O = 1.52Å)[3] as well as electronic profile. Although fluorine is the more electronegative and more sterically compact of the two, they are the most closely analogous elements in this regard. A well-known example of this is the relatively widely described use of vinyl fluorides **16** as amide mimetics.[4] In such a case the steric

profile of the amide bond is maintained very closely, however it is not obvious that the electronic profile, and particularly the hydrogen bond donor and acceptor ability of the amide are well matched.

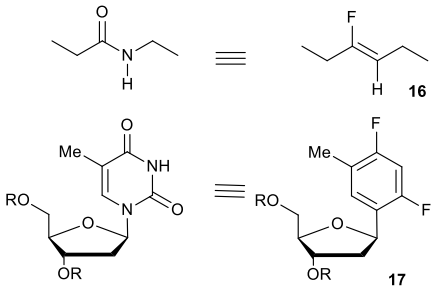
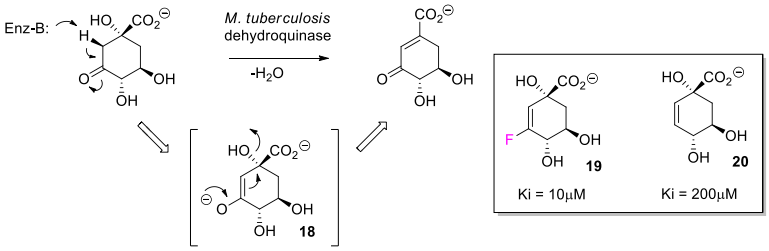


Figure 3. Steric and electronic organo- fluorine amide[4] and nucleoside mimetics.[5,6]

Also nucleoside analogues, such as **17** carrying a difluorotoluene moiety in place of thymidine, have been shown to be competent partners in Watson-Crick DNA base pairing interactions.[5] In these latter cases there has been a significant discussion[6] and bias towards an interpretation where such a replacement mimics a close steric profile and less so a well matched electronic profile, because fluorine is a poor hydrogen bonding acceptor and the aryl hydrogen, a poor donor.

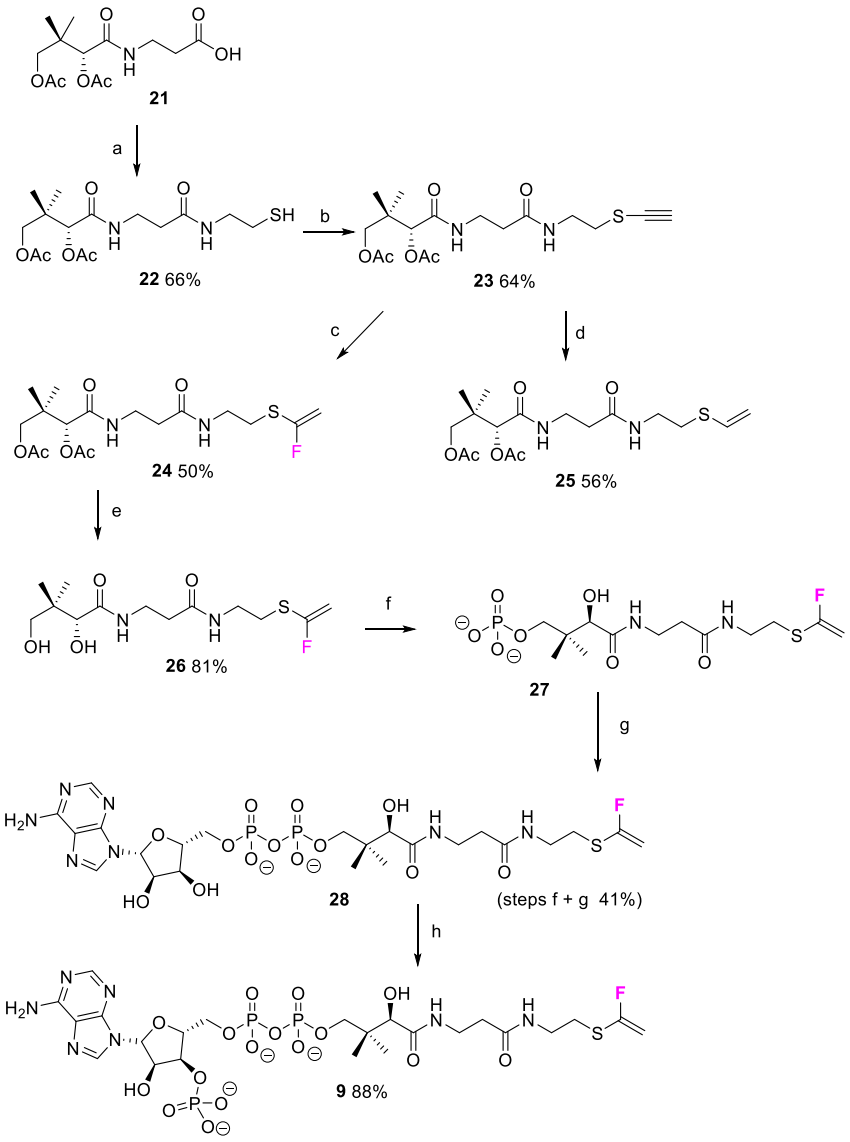


Scheme 2. Fluorovinyl cyclohexene **19** is a better inhibitor of the *M. tuberculosis* dehydroquinase than cyclohexene **20**, suggesting a fluorine effect in mimicking enolate **18**.[12]

The only related study we could find in enzymology using a vinyl fluoride as an enolate mimetic was a report describing vinyl fluoride **19** as an inhibitor of a shikimate dehydratase enzyme from *M. tuberculosis.*[12] Vinyl fluoride **19** was a significantly more potent inhibitor than cyclohexene **20** (K_i = 10 μM versus K_i = 200 μM), displaying a clear 'fluorine effect'. This enzyme reaction is proposed to proceed *via* intermediate enolate **18** suggesting some stabilisation of **19** through fluorine hydrogen bonding with active site residues. The same comparison was extended to the enzymes from *Streptomyces coelicolor* and *Salmonella typhi* in this study, however in those cases the differential was only a factor of two between **19** and **20** and perhaps this indicates less coordinated enolate stabilisation at those active sites. It is with this background that we were interested in exploring fluorovinyl thioether **9** as a mimetic of enolate **2**.

For sulfoxide **13**, the polarised oxygen should locate at the corresponding enolate site in the enzyme. Sulfoxide **13** has previously been reported [11] in the literature but it was never evaluated as an inhibitor of citrate synthase. Geometric isomers **14** and **15** represent confomationally constrained analogues of the historical inhibitor **8** and were designed as probes to explore the relevant binding conformation (Figure 1).

Results and Discussion: The fluorovinyl thioether motif has had very little currency in synthetic organic chemistry. We have recently explored routes to its synthesis as a necessary platform to progress this investigation.[13] One challenge was to find a method to prepare the motif attached to the pantetheine arm of coenzyme A to generate **9**. Wright *et. al.,*[14] have demonstrated that coenzyme A analogues can be assembled from appropriately modified pantetheinyl chains in a protocol using three ATP requiring enzymes, pantothenate kinase (panK), pantothenyl phosphate adenylate kinase (PPAT) and dephospho coenzyme A kinase (DPCK). If this enzyme protocol worked here, then the synthesis approach to prepare **9** is reduced to a preparation of fluorovinyl thioether **26**. Accordingly thioether **26** became the target and its synthesis was accomplished as illustrated in Scheme 3 starting from the protected pantothenic acid **21**.[14] Amide formation with cysteamine, generated the protected

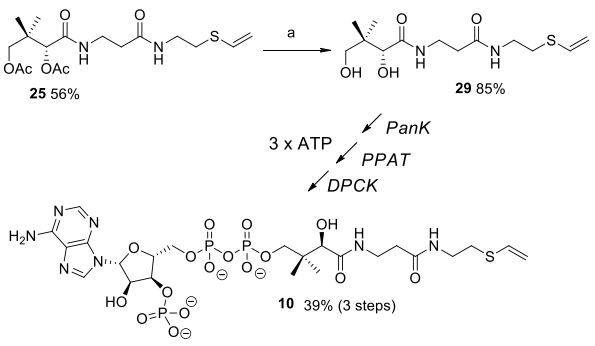


Scheme 3. Chemo-enzymatic synthesis of vinyl thioether **9**. a) 1,1'-dicarbonyldiimidazole, cysteamine.HCl, THF, reflux; b) EBX-TMS, THF, then TBAF.3H_2O; c) i. AgF, ii. I_2, iii. triethylamine, MeCN; d) Lindlar catalyst, 10 bar H_2, quinoline, pyridine; e) MeONa, MeOH; f) PanK, ATP; g) PPAT, ATP; h) DPCK, ATP.

pantetheine **22** in a straightforward manner, and then formation of acetylene thioether **23** was carried out using EBX-TMS by a modification of the method of Waser.[15] A key reaction involved the

hydrofluorination of **23**. This was achieved either by using $Et_3N.3HF$ and titanium tetrafluoride (TiF_4),[10a] or by treatment with AgF-I_2/TEA as illustrated in Scheme 3, the latter of which gave the more reliable conversion to **24** and generally a better yield.[10c] We have found that the fluorovinyl thioether moiety is relatively stable in neutral to basic media, however it is vulnerable to decomposition under acidic conditions. Thus acetate protecting groups were used when introducing the pantothenyl moiety rather than the more classical acetal protection of the diol, and the esters could be removed by treating **24** under basic methanolic conditions to afford **26**. This diol was purified by preparative HPLC.

The biotransformation of **26** to **9** was successfully achieved after over-expression of the PanK, PPAT and DPCK enzymes from *E. coli*. For the purpose of analysis, biotransformations were explored individually, such that the intermediate products **27** and **28** could be assigned retention times by HPLC, and could be fully characterised by LC-MS (See Supplementary Information). For practical purposes the most efficient protocol for preparative amounts of **9** involved an initial two enzyme biotransformation (PanK + PPAT) with ATP, and then purification of intermediate **28** by preparative HPLC (with LC-MS and NMR analysis). The final phosphorylation with DPCK and ATP then gave coenzyme A analogue **9**, which was also purified by preparative HPLC. It was important for the study to prepare the control coenzyme A analogue, the vinyl thioether **10** to explore any fluorine effect. The synthesis of **10** was accomplished from vinyl thioether **25**, a compound which was prepared by Lindlar partial hydrogenation of thioacetylene **23** (Scheme 3). Progression of vinyl thioether **25** by basic hydrolysis to **29** and then the three enzyme protocol, was carried out as illustrated in Scheme 4. Coenzyme A analogue **10** was also purified by preparative HPLC. The resultant ^{1}H-NMR spectra of **9** and **10** are illustrated in Figure 3. The vinyl protons are highlighted to illustrate the difference between these compounds.



Scheme 4. Chemo-enzymatic synthesis of vinyl thioether **10**. a) MeONa, MeOH.

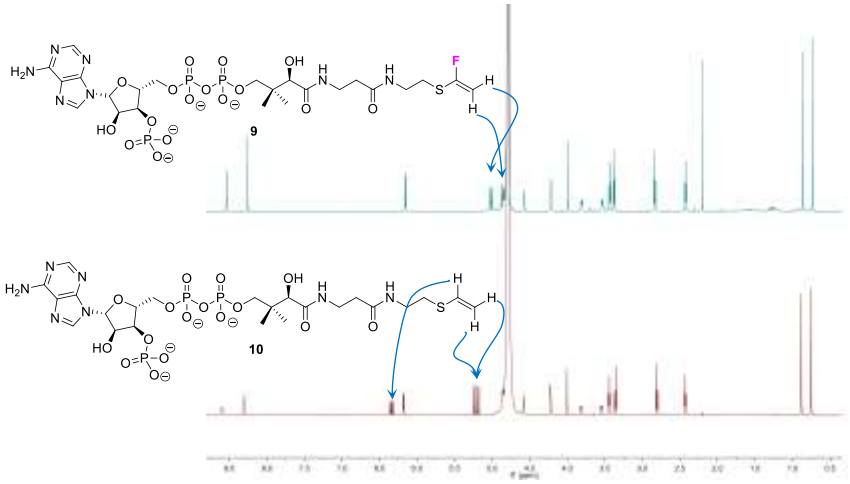
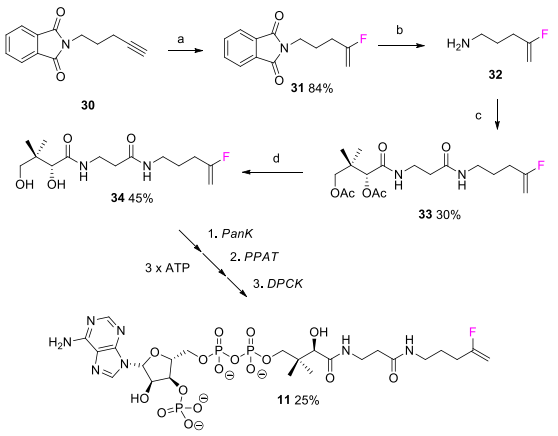


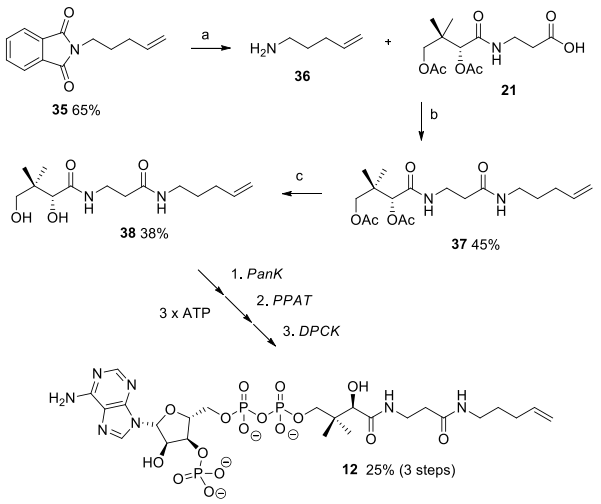
Figure 3 ^{1}H-NMR spectra of **9** (upper) and **10** (lower), showing clearly the difference in the vinyl moiety resonances of these acetyl co-enzyme A enolate analogues.

We also prepared the dethia analogues **11** and **12**, both with and without fluorine, to compare progressively the effect of removing the sulfur, and then to assess the relative potency of inhibition with and without fluorine. The synthesis is shown in Scheme 5.



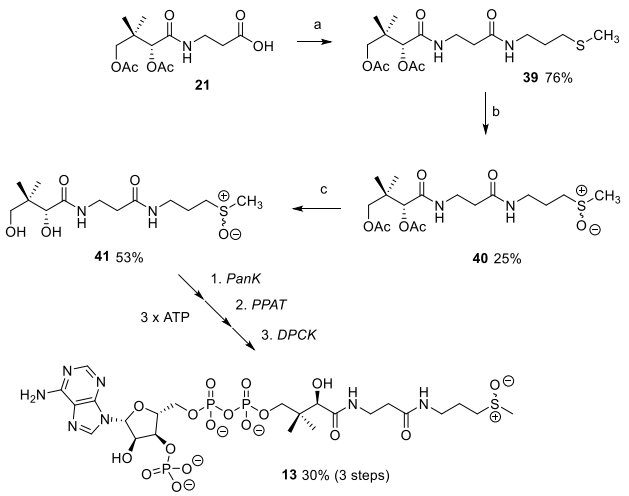
Scheme 5. Chemo-enzymatic synthesis of fluorovinyl dethia motif **11**. a) DMPU.HF, JohnPhos-Au-Pht , dry $CHCl_3$ b) hydrazine monohydrate, ethanol; c) **21**, HOBt, TEA, EDCI, DCM; d) MeONa, MeOH.

A key reaction in the synthesis of **11** involved hydrofluorination of the terminal acetylene of **30** under gold catalysis.[16] Release of the resultant fluorovinyl amine **32** was followed by coupling to carboxylate of **21**, and then subsequent deprotection under basic hydrolysis gave diol **34**. Diol **34** was then successfully transformed by the three enzyme protocol to the desired fluorovinyl dethia -CoA analogue **11.**



Scheme 6. Chemo-enzymatic synthesis of vinyl dethia motif **12**. a) Hydrazine monohydrate, ethanol; b) HOBt, TEA, EDCI, DCM; c) MeONa, MeOH.

The corresponding defluoro-hydro analogue **12** was prepared as illustrated in Scheme 6. Amine **36** was released after hydrazine treatment of pent-4-enyl-N-phthalimide **35**,[17] which was itself prepared as previously described.[18] Coupling of **36** with pantothenic acid **21** generated amide **37** and then basic hydrolysis provided access to diol **38** for biotransformation. In the event diol **38** proved a satisfactory substrate for the three enzyme biotransformation and **12** was purified by preparative HPLC in an overall 25% yield from **38**.

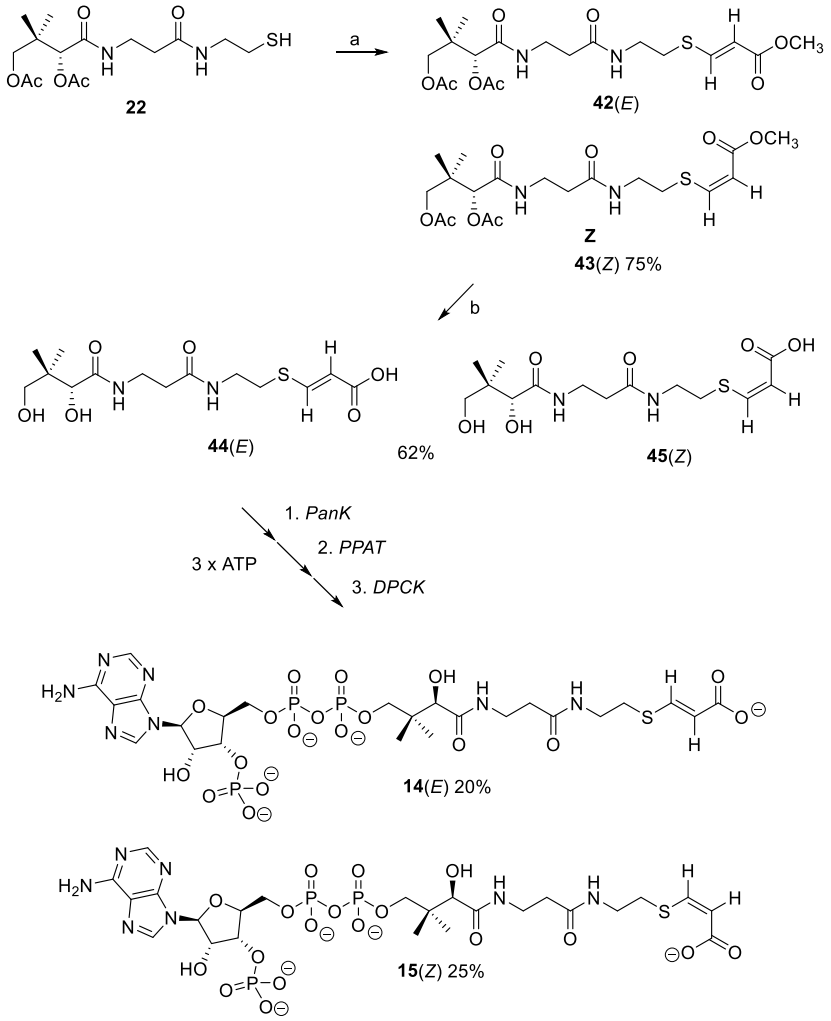


Scheme 7. Chemo-enzymatic route to sulfoxide **13**. a) 3-(methylthio) propylamine ,HOBt, TEA, EDCI, DCM; b) mCPBA, DCM; EtONa, EtOH; c) MeONa, MeOH.

Our synthesis approach to sulfoxide **13** is illustrated in Scheme 7. Sulfoxide **41** presented as the synthetic target for subsequent biotransformation. It was prepared by coupling 3-(methylthio) propylamine with carboxylic acid **21** to generate amide **39** and then by an oxidation at sulfur to give sulfoxide **40** as a mixture of isomers. It was not possible to distinguish the diastereoisomers by ^{1}H-NMR but we have to assume a 1:1 mixture. The hydrolysis of **40** under basic ethanolic conditions gave **41**. This diol was purified (as a mixture of stereoisomers) by preparative HPLC and was then progressed by the three enzyme biotransformation to the desired analogue **13**. This sulfoxide was finally purified by preparative HPLC and characterized by NMR and LC-MS.

Finally we have prepared the geometric isomers of propenoate thioether isomers **14**(*E*) and **15**(*Z*). These compounds relate spatially to historical inhibitor **8**, however unlike **8**, they are configurationally

fixed and have the potential to access two different conformations on binding to the active site of citrate synthase.



Scheme 8. Chemo-enzymatic synthesis propenoate thioether isomers **14**(*E*) and **15**(*Z*). a) Methyl propiolate, DBU, THF; b) MeONa, MeOH.

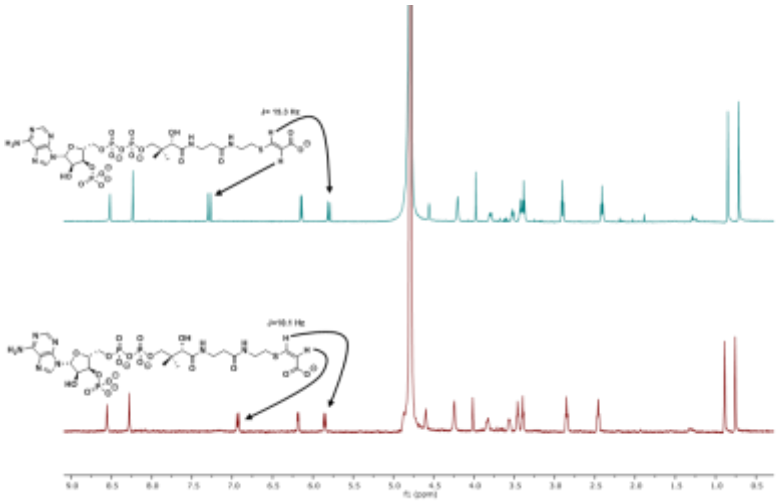


Figure 4 ^{1}H-NMR spectra of **14**(*E*) (upper) and **15**(*Z*) (lower), with the olefinic resonances of the two geometric isomers highlighted.

Candidate inhibitors **14**(*E*) and **15**(*Z*) were prepared by conjugate addition of the thiolate of **22** to commercially available propargylic ester. [19] The resultant isomers could be separated by HPLC after hydrolysis, however it proved most expedient to take them as a mixture through the biotransformation protocol and separate at the end. Thus **42**(*E*) and **43**(*Z*) underwent a global ester hydrolysis to generate carboxylic acids **44**(*E*) and **45**(*Z*) respectively as a mixture. This mixture was then subject to biotransformation. Both isomers progressed with no obvious isomer discrimination during the biotransformation and preparative HPLC allowed collection of individual isomers **14**(*E*) and **15**(*Z*). This completed the synthase of the seven candidate inhibitors.

	$K_{i\ app}$	IC_{50}
9	4.3 μM ± 0.6	42 μM ± 5.5
10	68.3 ± 4.5μM	657 ± 31 μM
11	44.8 ± 9.2μM	239 ± 47.9 μM
12	53.1 ± 6.5μM	282 ± 32.4 μM
13[ǂ]	11.1 ± 0.9μM	59 ± 4.4 μM
14(*E*)	≥ 500 μM	≥ 3 mM
15(*Z*)	≥ 500 μM	≥ 3 mM

[ǂ] *We assume a 1:1 mix of sulfoxide diastereoisomers **13** epimeric at sulfur*.

Table 1. Inhibition data for **9 -15** with pig heart citrate synthase (E. C. 4.1.3.7). Acetyl-CoA (50mM); oxaloacetate (250 mM), TRIS buffer (55mM). The apparent K_i's were determined using the Cheng-Prusoff equation as described in the SI. For full assay details see Supplementary Information. K_M value of acetyl CoA **1** was 5.5 μM)

With compounds **9** – **15** in hand, they were all assayed as inhibitors of pig heart citrate synthase (E. C. 4.1.3.7) following coenzyme A release from AcCoA **1**.[20] The resultant inhibition data is presented in Table 1, expressed as inhibition constants (K_i) as well as IC_{50}'s. Fluorovinyl thioether **9** (K_i = 4.3 μM) was the most potent inhibitor of the study and bound with an affinity similar to acetyl CoA **1** (K_M = 5.8μM). It was designed as a close biomimetic of AcCoA, enolate **2**, with fluorine replacing the enolate oxygen. A comparison with the defluoro-hydro analogue **10** (K_i = 68.3 μM) demonstrated that the fluorine in **9** made it an order of magnitude more potent than **10**, showing a clear 'fluorine effect'. This is similar to the differential previously observed for inhibition of the *M. tuberculosis* dehydratase [12] with shikimate analogues **19** and **20**. The dethia inhibitors **11** and **12** compare structurally with **9** and **10** but without the sulfur of the thioether. In both cases they showed modest inhibition, with some indication of a residual 'fluorine effect'. Sulfoxide **13** (K_i = 11.6 μM) was a relatively good, low micromolar inhibitor, with a slightly better efficacy than the historical inhibitors ketone **6** and nitroalkyl **7**, and perhaps indicating better ability as a hydrogen bonding acceptor by comparison. It should be noted that sulfoxide **13** was assayed as a mixture of diastereoisomers, and the inhibition values are an average of the two, where one isomer may be more potent than the other. Finally the propenoate thioether isomers **14**(*E*) and **15**(*Z*) were evaluated individually as citrate synthase inhibitors however they did not show any meaningful inhibition. These can be compared to the historical inhibitor **8** which is conformationally flexible, but the constrained geometries of **14**(*E*) and **15**(*Z*) do not appear to access any meaningful conformation on binding to citrate synthase.

QM Computational study: In order to gain some insight into the nature of the binding of the vinyl thioethers, with and without fluorine (**9** versus **10**) we performed QM calculations for a model taken from the X-ray structure of citrate synthase,[21] with AcCoA **1** and malate bound. This model contains

a truncated CoA moiety (Scheme 1) and all of the important residues, side chains or co-crystallates within 6 Å of the acetyl group. The malate was converted to oxalacetate (OAA) and the structures of the complex (Enz·OAA·AcCoA) was optimised with a two-layer QM/MM ONIOM scheme (BP86:PM6)[22] embedded in a polarisable continuum, where only part of AcCoA, the OAA, and the nearest water molecule and Asp375 side chain were allowed to relax (see computational details and Figure S1). The complexes with inhibitors **9** and **10** were obtained by changing the substituent attached to sulfur accordingly and re-optimising with the same atoms frozen as before. The free ligands **9** and **10** were (partially) optimised with a similar two-layer setup using a continuum solvent model with the parameters of water. Finally, energies were refined at a two-layered B3LYP level with two different basis sets (B3LYP/6-311+G**:B3LYP/SDD, where SDD denotes pseudoptentials with valence double-zeta basis sets).[23] With this set up, and where the binding pocket and binding modes are largely maintained, the relative binding strength of **9** and **10** was determined as illustrated in equation (1).

$$\text{Enz·OAA·}\mathbf{9} + \mathbf{10} \rightarrow \text{Enz·OAA·}\mathbf{10} + \mathbf{9} \qquad \Delta E = +3.5 \text{ kcal/mol} \qquad (1)$$

The outcome indicates that the vinylfluoride inhibitor **9** binds more strongly than **10** to citrate synthase. The computed structure for **9** indicates that the two CF…H hydrogen bonding distances are very short (2.02 Å F…H-His & 2.00 Å F…H-water) and at an optimal length for carbon bound fluorine hydrogen bonds.[24] Although fluorine is not considered to be a very good hydrogen bonding acceptor[24,25] it will certainly participate in hydrogen bonding interactions under optimal conditions, and this is consistent with the improved performance of **9**.

A similar calculation but this time comparing AcCoA **1** and fluorovinyl thioether **9** bound to citrate synthase suggests that the citrate synthase complex with **9** is higher in energy (+ 6.7 kcal/mol) than that with AcCoA **1** (equation 2).

$$\text{Enz·OAA·}\mathbf{1} + \mathbf{9} \rightarrow \text{Enz·OAA·}\mathbf{9} + \mathbf{1} \qquad \Delta E = +6.7 \text{ kcal/mol} \qquad (2)$$

This significant energy difference in favour of AcCoA **1** could arise in the model due to electrostatic repulsion between the negatively charged Asp375 carboxylate and the electron-rich fluorovinyl moiety of **9**, however the outcome is inconsistent with similar experimental binding affinities of **9** (*K*i ~ 4.3 μM) and AcCoA **1** (*K*m ~5.8 μM). There is the possibility that protonation of the Asp-375 carboxylate could result in a stronger binding of vinylfluoride **9**, as binding of the latter could increase the basicity of the carboxylate to such an extent that it becomes protonated. The reaction to model this scenario is formulated as shown in equation (3);

$$\text{Enz·OAA·}\mathbf{1} + \mathbf{9} + H^+ \rightarrow \text{Enz}^{H+}\text{·OAA·}\mathbf{9} + \mathbf{1}, \qquad \Delta G \ +1.2 \pm 0.8 \text{ kcal/mol} \qquad (3)$$

where Enz^{H+} denotes the enzyme model with Asp375 protonated (see Figure S3). Using a judiciously designed thermodynamic cycle and selected high-level *ab initio* and experimental data from the literature, we arrive at an estimate of $\Delta G = +1.2 \pm 0.8$ kcal/mol for this reaction (see computational details). In view of the many approximations made en route to this estimate, its quantitative accuracy is difficult to assess. We note, however, that this estimate is in reasonable qualitative agreement with experiment, and that this qualitative accord is only achieved when we assume a change in protonation state of Asp375 upon binding of vinylfluoride **9**.

This argument may also apply to the competition between the vinylfluoride (**9**) and its parent vinyl substrate (**10**), eq 2. However, the stabilisation/destabilisation from the Asp375 residue will be rather

similar for both ligands, so that their overall relative affinity should be much less dependent on the protonation state of that residue.

It should be noted that these results may be somewhat biased toward the AcCoA **1** model, because this model is closest to the structure in the solid that was used as a starting point. Also, differences in binding constants by one order of magnitude or less would correspond to a change in free energy of 1.3 kcal/mol or less at room temperature, a rather ambitious target for theory. For more quantitative modelling, more refined QM/MM calculations with proper equilibration and/or free-energy MD simulations should be performed.

In conclusion we have explored a range of rationally designed coenzyme A analogues as candidate inhibitors of citrate synthase. The fluorovinyl thioether **9** analogue was the most potent inhibitor studied and was designed as a mimetic for the enolate **2** of thioester AcCoA **1**. Computation indicated a good fit in the active site, however the low micromolar (*K*i 4.3 μM) rather than nanomolar level of inhibition of **9** overall, suggests that the fluorine interaction does not fully compensate that of the enolate oxygen. This is not unexpected given the fluorovinyl thioether moiety is neutral species whereas enolate **2** is charged, and electrostatic interactions will be of a different magnitude. However the study does reveal a significant 'fluorine effect' when comparing **9** with vinyl ether **10** as an inhibitor of citrate synthase. The computational analysis suggests that the fluorine can achieve optimal hydrogen bonding interactions in the enolate binding site. Computation also raised the idea that binding of **9** will lead to concomitant protonation of the Asp357 carboxylate of citrate synthase, as this model better equates the similar experimental affinity of AcCoA **1** and fluorovinyl thiother **9**. Sulfoxide analogue **13** was also a moderate inhibitor (*K*i 11.1 μM) of the enzyme, similar to historical inhibitors **6** and **7** and suggesting an ability also to act as a hydrogen bonding acceptor. These observations are consistent with the view that citrate synthase stabilises an enolate intermediate, rather than an enol.

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References

[1] (a) E. P. Gillis, K. J. Eastman, M. D. Hill, D. J. Donnelly, N. J. Meanwell, J. Med. Chem., **2015**, *58*, 8315-8359; (b) J. P. Bégué, D. Bonnet-Delpon (Eds), '*Bioorganic and Medicinal Chemistry of Fluorine'* Wiley: Hoboken, NJ, **2007**; (c) I. Ojima (Ed), '*Fluorine in Medicinal Chemistry and Chemical Biology'* I., Wiley-Blackwell, Chichester, U.K., **2009**.

[2] (a) L. Pauling, *J. Am. Chem. Soc*. **1932**, *54*, 3570; (b) L. Pauling, *'The Nature of the Chemical Bond*,' 3rd ed.; Cornell University Press: Ithaca, NY, **1960**.

[3] A. Bondi, *J. Phys. Chem*., **1964**, *68*, 441-451.

[4] (a) A. Choudhary, R. T. Raines, *ChemBioChem,* **2011**, *12*, 1801-1807; (b) R. J. Braham, S. L. R. Ellison, F. Schonholzer , W. A. Thomas, *Tetrahedron*, **1986**, *42*, 2101–2110.

[5] (a) C. F. Guerra, F. M. Bickelhaupt, *J. Chem. Phys*., **2003**, *119*, 4262-4273 ; (b) S. Moran, R. X.-F. Ren, S. Rumney IV, E. T. Kool, *J. Am. Chem. Soc.,* **1997**, *119*, 2056-2057; (b) D. Liu, S. Moran, E. T. Kool, *Chem. Biol.,* **1997**, *4*, 919 -926.

[6] E. T. Kool and H. O. Sintim, *Chem. Commun*., **2006**, 3665–3675.

[7] (a) H. Eggerer, *Biochem. Z*, 1965, 343, 111-138; (b) E. Bayer, B. Bauer, H. Eggerer, *Eur. J. Biochemistry*, **1981**, *120*, 155-160.

[8] (a) L. C. Kurz, G. Drysdale, M. Riley, M. A. Tomar, J. Chen, R. J. M. Russell, M. J. Danson, *Biochemistry*, **2000**, *39*, 2283 – 2296; (b) C. T. Evans, L. C. Kurz, S. J. Remington, P. A. Srere, *Biochemistry,* **1996**, *35*, 10661−10672; (c) S. J. Remington, *Curr. Top. Cell. Regul.,* **1992**, *33*, 209−229.

[9] (a) X. Zhang, S. J. Bennie, M. W. van der Kamp, D. R. Glowacki, F. R. Manby, A. J. Mulholland, *R. Soc. Open sci.,* **2018**, *5*, 171390; (b) S. J. Bennie, M. W. van der Kamp, R. C. R. Pennifold, M. Stella, F. R. Manby, A. J. Mulholland; *J. Chem. Theory Comput*., **2016**, *12*, 2689-2697; (c) A. Aleksandrov, E. Zvereva, M. Field, J. Phys. Chem. B., 2014, 118, 4505 – 4513; (d) M. W. van der Kamp, J. D. McGeagh, A. J. Mulholland, *Angew. Chem. Int. Ed*., **2011**, *50*, 10349 – 10351; (e) M. W. van der Kamp, F. Perruccio, A. J. Mulholland, *Chem., Commun.* **2008**, 1874−1876.

[10] (a) J. Xun, H. Huang, K. W. Vogel, D. G. Drueckhammer, *Bioorg. Chem*., **2005**, *33*, 90 – 107; (b) B. Schwartz, D. G. Drueckhammer, K. C. Usher, S. J. Remmington, *Biochemistry*, **1995**, *34*, 15459 – 15466.

[11] D. P. Martin, R. T. Bibart, D. G. Drueckhammer, *J. Am. Chem. Soc.,* **1994**, *116*, 4660 – 4668.

[12] M. Frederickson, A. W. Roszak, J. R. Coggins, A. J. Lapthorn, C. Abell, *Org. Biomol. Chem*., **2004**, *2*, 1592 – 1596.

[13] (a) D. Bello, R. A. Cormanich, D. O'Hagan, *Aus. J. Chem,* **2015**, *68*, 72 - 79; (b) F. Nahra, S. R Patrick, D. Bello, M. Brill, A. Obled, D. B. Cordes, A. M. Z. Slawin, D. O'Hagan, S. P. Nolan, *ChemCatChem*, **2015**, *7*, 240 – 244; (c) D. Bello, D.O'Hagan, *Beilstein J. Org. Chem*., **2015**, *11*, 1902 - 1909.

[14] (a) I. Nazi, K. P. Kotera, G. D. Wright, *Anal. Biochem*., **2004**, *324*, 100 - 105; (b) M. Tosin, D. Spiteller, J.B.Spencer, *ChemBioChem*., **2009**, *10*, 1714 - 1723.

[15] (a) R. Tomita, N. Al-Maharik, A. Rodil, M. Bühl, D, O'Hagan, *Org. Bio. Chem*., **2018**, *16*, 1113–1117.Y. Li, D. P. Hari, M. V. Vita and J. Waser, *Angew. Chem., Int. Ed.,* **2016**, *55*, 4436–4454; (b) R. Frei and J. Waser, *J. Am. Chem. Soc*., **2013**, *135*, 9620–9623.

[16] O.E. Okoromoba, J. Han, G.B. Hammond, B. Xu,*J. Am. Chem. Soc*., **2014**, *136*, 14381−14384.

[17] X. Qi, F. Yu, P. Chen, G. Liu, *Angew.Chem.Int.Ed.*, **2017**, *56, 12692-12696.*

[18] W. Du, Q. Gu, Y. Li, Z. Lin, D. Yang,*Org. Lett.*, **2017**, *19*, 316−319

[19] R.L. Carney, T.L. Brown, *U.S.Patent No. 4,877,899*, **1989**.

[20] V.M. Anoop; U. Basu, M.T. McCammon, L. McAlister-Henn and G. J. Taylor, Plant Physiol., **2003**, *132*, 2205–2217; (b) P. A. Srere, L. Gonen, and H. Brazil, Acta Chem. Scand., **1963**, *17*, 129–134.

[21] M. Karpusas, D. Holland, S. J. Remington, *Biochemistry*, **1991**, *30*, 6024 – 6031.

[22] S. Dapprich, I. Komáromi, K. S. Byun, K. Morokuma, M. J. Frisch, *J. Mol. Struct. (Theochem),* **1999**, *462*, 1-21.

[23] (a) A. D. Becke, *J. Chem. Phys*., **1993**, *98*, 5648 – 5652; (b) C. Lee, W. Yang, R. G. Parr, *Phys. Rev. B*., **1988**, *37*, 785 – 789.

[24] J. A. K. Howard, V. J. Hoy, D. O'Hagan, G. T. Smith, *Tetrahedron*, **1996**, *52*, 12613-12622.

[25] (a) S. K. Mishra, N. Suryaprakash, *Molecules,* **2017**, *22*, 423; (b) J. D. Dunitz, *ChemBioChem,* **2004**, *5*, 614–621; (c) J. D. Dunitz, R. Taylor, *Chem. Eur. J*. **1997**, *3*, 89–98.