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Michael Acceptor-Containing Coenzyme A Analogues As Inhibitors of the Atypical Coenzyme A Disulfide Reductase from *Staphylococcus aureus*

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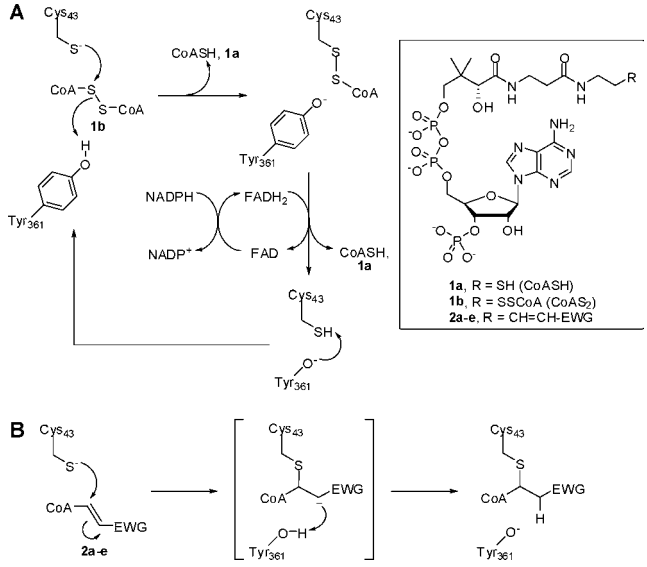
Abstract: Coenzyme A (CoA) analogues containing α,β-unsaturated ester, ketone, and sulfone moieties were prepared by chemo-enzymatic synthesis as inhibitors of coenzyme A disulfide reductase (CoADR), a proven and as yet unexploited drug target in *Staphylococcus aureus*. Among these Michael acceptor-containing CoA analogues, which were designed to target CoADR's single essential active site cysteine for conjugate addition, a phenyl vinyl sulfone-containing analogue showed the most potent inhibition with a competitive K_i of ∼40 nM, and time-dependent inactivation with a second-order rate of inactivation constant of ∼40 000 $s^{-1}\cdot M^{-1}$. Our results suggest that electrophilic substrate analogues should be considered as potential inhibitors of other medicinally relevant disulfide reductase enzymes.

Staphylococcus aureus, the causative agent of most nosocomial and many other opportunistic infections in humans, is rapidly becoming resistant to the current arsenal of available antibacterial agents, and the development of new antistaphylococcal treatments is therefore urgently needed. Interestingly, *S. aureus* offers a target for drug development that remains unexploited as yet, based on the unique thiol/disulfide redox system it uses to counteract environmental stress and to protect itself against the reactive oxygen species produced by the host's immune system. While most organisms (including humans) use glutathione and a flavin-dependent glutathione reductase (GR) enzyme to uphold their intracellular redox balance, *S. aureus* and some other Gram-positive bacteria such as *Bacillus anthracis* achieve the same result by maintaining high concentrations of the essential thiol-containing metabolite coenzyme A (CoA, **1a**) in its reduced form by means of a CoA disulfide reductase (CoADR) enzyme.[1] This distinction and the fact that various studies have highlighted the importance of CoADR in the growth, survival, and virulence of *S. aureus*[2] make this enzyme an attractive drug target.

Most pyridine nucleotide-disulfide oxidoreductases (PNDORs) such as GR and trypanothione reductase (TR), an enzyme unique to *Trypanosoma* spp. parasites that has also been extensively targeted by various drug development efforts, share a reaction mechanism that is based on two active site cysteine residues joined in a redox active disulfide linkage when the enzyme is in its resting state. Catalysis is initiated by reduction of this disulfide by the reduced FAD cofactor, followed by a thiol/disulfide interchange reaction between the active site cysteines and the disulfide substrate which results in formation of the reduced thiol products and the active site disulfide. However, CoADR is a mechanistically unique PNDOR as it utilizes only a single active site cysteine (Cys_{43} in *S. aureus* CoADR), which reacts directly with its substrate ($CoAS_2$, **1b**) to form a mixed enzyme−substrate disulfide. In contrast to other PNDORs, the enzyme maintains this mixed disulfide in its resting state, and catalysis is also initiated by its reaction with $FADH_2$ (Scheme 1A).[3,4] Based on these mechanistic differences we envisaged the development of new CoADR-selective inhibitors by targeting its essential cysteine with Michael acceptor-containing substrate analogues, a strategy that has been employed with great success in the discovery of cysteine protease inhibitors (Scheme 1B).[5]

Scheme 1. (A) CoADR Reaction Mechanism;[a] (B) Proposed Mechanism of Inhibition of CoADR by Michael Acceptor-Containing CoA Analogues **2a**−**e**



[a] CoA disulfide **1b** reacts with Cys_{43} to release CoASH **1a** while forming a mixed enzyme−substrate disulfide, which is subsequently reduced by $FADH_2$. Tyr_{361} has been proposed to act as a catalytic acid/base as shown.[3]

Accordingly we set out to synthesize CoA analogues **2a**−**e** that contain α,β-unsaturated ester, ketone, or vinyl sulfone moieties, as these routinely occur in potent cysteine protease inhibitors (Scheme 2). We used CoA as the recognition motif since the crystal structure of CoADR highlighted the importance of its constituent groups for substrate recognition, and also because the resting state of the enzyme has a CoA molecule bound to Cys_{43}.[3] Moreover, the Michael acceptor was positioned in such a manner that its electrophilic center correlated with the disulfide bond in the substrate **1b**. The warheads were obtained by preparing the protected amines **4a**−**c** and **6a**−**b** by utilizing either Wittig or Horner−Wadsworth−Emmons olefination reactions that gave the *E*-alkenes in all cases. These amines were subsequently deprotected and coupled to pantothenic acid **7** using standard procedures to give the pantothenamides **8a**−**e**. Finally, the CoA biosynthetic enzymes PanK, PPAT, and DPCK were used to transform **8a**−**e** to the corresponding CoA analogues **2a**−**e** using established protocols.[6]

In this manner four of the five CoA analogues were obtained in purified form; unfortunately the α,β-unsaturated ketone-containing

Scheme 2. Synthesis of the Michael Acceptor-Containing Pantothenamides **8a**−**e** and CoA Analogues **2a**−**e**

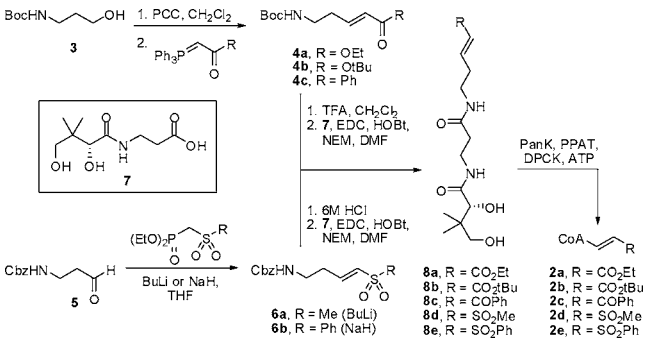


Table 1. Kinetic Parameters for the Inhibition of CoADR by CoA Analogues

CoA analogue	K_i[a] (μM)	k_{inact}/K_i[b] ($s^{-1} \cdot M^{-1}$)
2a	0.66 ± 0.12	219.1 ± 45.5
2b	5.16 ± 0.96	nd[c]
2d	0.30 ± 0.05	500.2 ± 89.8
2e	0.04 ± 0.01	39 690 ± 10 980

[a] For competitive inhibition. [b] Second-order rate of inactivation constants. [c] nd, Not determined.

analogue **2c**, although it was successfully prepared by biotransformation of **8c**, decomposed upon purification. The pantothenamides **8a**−**e** and the analogues **2a**−**e** were subsequently assayed for inhibition of CoADR using a concentration of 200 μM (**2c** was tested in crude form). While all five CoA analogues showed inhibition of CoADR activity, none of the pantothenamides had any effect, highlighting the essential requirement of CoA's adenosine and phosphate moieties for recognition and binding.

Although the CoA analogues **2a**−**e** were designed to act as selective, irreversible inhibitors of CoADR by modification of its active site cysteine, it is also possible that the observed inhibition can occur by nonspecific reaction of the Michael acceptor moieties with other enzyme-derived nucleophiles. To demonstrate that these analogues bind specifically in the active site of CoADR, the analogues **2a**, **2b**, **2d**, and **2e** were therefore evaluated for their ability to compete with $CoAS_2$ in the CoADR reaction. This was done by determining the initial rates of the reaction (i.e., without preincubation) in the presence of increasing substrate concentrations and various set concentrations of the inhibitor.[7] The resulting rate profiles and corresponding double reciprocal plots (Figures S7−S10) indicated that the inhibition is indeed competitive in all cases. Moreover, with one exception the determined K_i values (Table 1) are all submicromolar, indicating that these CoA analogues are excellent substrate mimics. In fact, the K_i value of ~40 nM exhibited by the most potent inhibitor, the phenyl sulfone **2e**, is nearly 50-fold lower than the $K_m(CoAS_2)$ of ~2.0 μM. Based on this limited set of compounds, a structure−activity relationship analysis for binding of these analogues in the CoADR active site suggests that sulfone-based analogues are better suited than their carboxylic acid ester counterparts and that small substituents (e.g., OEt, Me) are preferred over sterically bulky ones (O*t*Bu). In light of this analysis the excellent inhibition seen for the phenyl sulfone **2e** is therefore surprising, although it is possible that this analogue is uniquely able to form π-stacking interactions with the side chain of aromatic residues located nearby, such as Tyr_{361}.[3] Nonetheless, these results show that the CoADR active site can accommodate a variety of substituents on the Michael acceptor and suggest that their scope and diversity may be expanded in future studies.

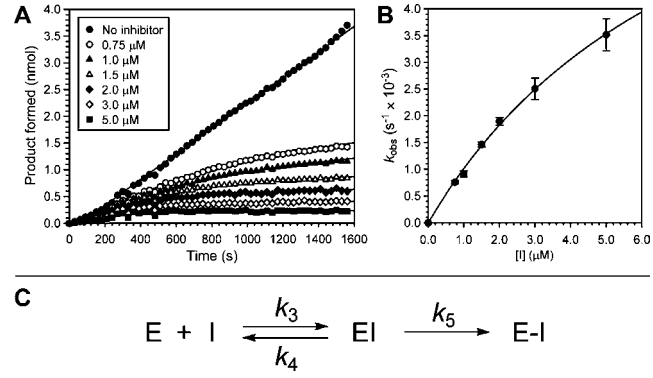


Figure 1. Inhibition of CoADR by CoA analogue **2e**. (A) Reaction progress in the presence of increasing inhibitor concentrations, showing time-dependent inactivation of CoADR. (B) Plot of the observed rate of inactivation constants (k_{obs}) versus the concentration of **2e**, from which the second-order rate of inactivation constant was determined. (C) Scheme showing the two-step mechanism of irreversible inactivation of CoADR that is in operation in the case of **2e**. Results for inhibition by analogues **2a** and **2d** are shown in Figures S11 and S12 respectively.

The finding that the CoA analogues **2a**, **2b**, **2d**, and **2e** bind in the active site of CoADR suggests that their Michael acceptor moieties should be ideally positioned to inactivate the enzyme by conjugate addition as envisaged (Scheme 1B). To determine whether the formed enzyme−inhibitor complexes result in the irreversible inhibition, time-dependence analyses were performed on the most potent inhibitors (i.e., **2a**, **2d**, and **2e**) by using the progress curve method (Figures 1A, S11A, and S12A).[8] Gratifyingly, the progress curves of all three analogues showed time-dependent irreversible inactivation of CoADR, with the observed rate of inactivation (k_{obs}) also increasing with increasing inhibitor concentration (Figure 1B). In addition, the hyperbolic shape of this plot confirms that a two-step inactivation mechanism (Figure 1C) is at play in the case of **2e**. This provides further explanation for the competitive inhibition that is observed in the previous experiment, as the determined K_i values would refer to the dissociation constant ($K_i = k_4/k_3$) of the enzyme−inhibitor (EI) encounter complex formed in the reversible first step. The second-order rate of inactivation constants (Table 1) determined from these plots of k_{obs} vs [I] showed that, while analogues **2a** and **2d** exhibited modest rates of inactivation, the phenyl sulfone **2e** is a much more potent inhibitor with a rate constant of ~40 000 $s^{-1} \cdot M^{-1}$. This relative order of inhibition activity is in agreement with the results of a previous study of the relative rates of the conjugate addition of 2′-(phenethyl)thiol to various Michael acceptors, which found the reactivity of a phenyl vinyl sulfone to be higher than that of the corresponding α,β-unsaturated methyl ester.[9]

To further confirm the irreversibility of inhibition, a CoADR sample was incubated in the presence of analogue **2e**, followed by gel filtration to remove all unbound small molecules. As expected, the inhibitor-treated enzyme showed no activity in comparison to a negative control sample treated in the same manner. Interestingly, the inhibitor-treated, gel-filtered enzyme did show a very slow return of activity after ~10 min of incubation (Figure S14). This suggests that the enzyme−inhibitor linkage can be broken over time, most probably by an elimination reaction that regenerates Cys_{43} and the inhibitor. However, in light of the progress curve data the rate of regeneration is seemingly negligible relative to that of inactivation.

In spite of the good CoADR inhibition shown by CoA analogues such as **2e**, these highly polar compounds cannot be used as *in vivo* growth inhibitors since they are not be able to cross the bacterial cell membrane. However, previous studies have shown

that pantothenamides similar to the CoA analogue precursors **8a**−**e** are able to enter bacterial cells, where they are converted to the corresponding CoA analogues.[10] Pantothenamides **8a**−**e** were therefore tested as inhibitors of *S. aureus* growth in a nutrient-rich medium (1% tryptone). Only the ester-containing **8a** and **8b** showed inhibition at concentrations below 200 μM, with observed minimum inhibitory concentration (MIC) values of 55 and 52 μM respectively. The disappointing lack of inhibition seen for pantothenamides **8c**−**e** may be due to a reduced cell permeability or to an inability of the native *S. aureus* CoA biosynthetic enzymes to convert them to the corresponding CoA analogues.[6,11] These factors clearly will have to be considered in the future development of inhibitors that target CoADR in the manner described here.

In conclusion, this report of Michael acceptor-containing CoA analogues is the first, to our knowledge, that describes CoADR inhibitors of any kind. While the potent inhibition reported here may be ascribed to the unique mechanism of CoADR, we believe that this study has demonstrated that Michael acceptor-based substrate analogues may present a new avenue for the development of inhibitors of other medicinally relevant PNDORs.

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Supporting Information Available: Detailed descriptions of all synthetic, assay, and data analysis procedures, Figures S1−S14, Tables S1−S3, and additional discussion of the inhibition analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) For **8c** we found that purified *S. aureus* PanK was not able to perform the first step in the biotransformation reaction used to prepare **2c**. See Supporting Information for details.

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