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A Hammett Study of *Clostridium acetobutylicum* Alcohol Dehydrogenase (CaADH): An Enzyme with Remarkable Substrate Promiscuity and Utility for Organic Synthesis

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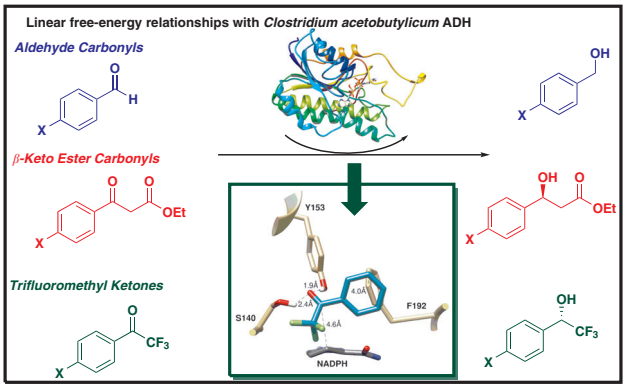

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Abstract Described is a physical organic study of the reduction of three sets of carbonyl compounds by the NADPH-dependent enzyme *Clostridium acetobutylicum* alcohol dehydrogenase (CaADH). Previous studies in our group have shown this enzyme to display broad substrate promiscuity, yet remarkable stereochemical fidelity, in the reduction of carbonyl compounds, including α-, β- and γ-keto esters (D-stereochemistry), as well as α,α-difluorinated-β-keto phosphonate esters (L-stereochemistry). To better mechanistically characterize this promising dehydrogenase enzyme, we report here the results of a Hammett linear free-energy relationship (LFER) study across three distinct classes of carbonyl substrates; namely aryl aldehydes, aryl β-keto esters and aryl trifluoromethyl ketones. Rates are measured by monitoring the decrease in NADPH fluorescence at 460 nm with time across a range of substrate concentrations for each member of each carbonyl compound class. The resulting v_0 versus [S] data are subjected to least-squares hyperbolic fitting to the Michaelis–Menton equation. Hammett plots of log(V_{max}) versus σ_X yield the following Hammett parameters: (i) for *p*-substituted aldehydes, ρ = 0.99 ± 0.10, ρ = 0.40 ± 0.09; two domains observed, (ii) for *p*-substituted β-keto esters ρ = 1.02 ± 0.31, and (iii) for *p*-substituted aryl trifluoromethyl ketones ρ = –0.97 ± 0.12. The positive sign of ρ indicated for the first two compound classes suggests that the hydride transfer from the nicotinamide cofactor is at least partially rate-limiting, whereas the negative sign of ρ for the aryl trifluoromethyl ketone class suggests that dehydration of the ketone hydrate may be rate-limiting for this compound class. Consistent with this notion, examination of the ^{13}C NMR spectra for the set of *p*-substituted aryl trifluoromethyl ketones in 2% aqueous DMSO reveals significant formation of the hydrate (*gem*-diol) for this compound family, with compounds bearing the more electron-withdrawing groups showing greater degrees of hydration. This work also presents the first examples of the CaADH-mediated reduction of aryl trifluoromethyl ketones, and chiral HPLC analysis indicates that the parent compound α,α,α-trifluoroacetophenone is enzymatically reduced in 99% ee and 95% yield, providing the (*S*)-stereoisomer, suggesting yet another compound class for which this enzyme displays high enantioselectivity.

Key words linear free-energy relationships, Hammett study, *Clostridium acetobutylicum* ADH, stereochemical fidelity, enzyme promiscuity, trifluoromethyl ketones

Biocatalysis is emerging as a key element in the synthesis of both chiral building blocks and advanced synthetic intermediates, particularly in process chemistry laboratories.[1] This includes the use of individual enzymes, enzyme cascades[2] and in indeed, most recently, entire retrosynthetic pathways[3] designed around evolved enzymes,[4] not to mention whole cell processes.[5] Such biocatalytic approaches play a pivotal role in the catalysis of a large number of industrially relevant reactions such as the production of biofuels by fermentation of glucose, the synthesis of polymer monomers by engineered enzymatic pathways and the specialized application of enzymes for key steps in pharmaceutical process chemistry. Landmark achievements in the latter area include (i) the remarkable engineering of a transaminase to efficiently set a key stereocenter in the synthesis of sitagliptin by the collaborative Codexis/Merck team,[6] and (ii) the spectacular biocatalytic total synthesis [9 enzymes (5 evolved)/2 pots] of islatravir by a team from the same two companies.[7]

Transaminases (pyridoxal phosphate) utilized for the sitagliptin case, and the dehydrogenases/ketoreductases (nicotinamide adenine dinucleotide(s)) discussed here, require active molecular cofactors stoichiometric with enzyme for catalytic turnover. Thus cofactor regeneration becomes a key component of such applications of enzymes in synthesis or process chemistry, with isopropylamine serving as a terminal reductant in the engineered transaminase case noted and, for example, isopropyl alcohol, formate, glucose, phosphite or ethanol serving as the terminal reductant for alcohol dehydrogenases, depending on the system, recycling enzyme(s) chosen.[8] With dehydrogenases, there have also been creative examples of either formal substrate disproportionation[9] approaches or 'self-sufficient hydride transfer'[10] processes that allow for internal recycling of the cofactor. The focus of this article will be upon

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alcohol dehydrogenase (ADH) enzymology, specifically the physical organic characterization of a 'privileged' ADH enzyme that we have uncovered in our laboratory.

In hybrid biocatalytic ventures, dehydrogenases have become powerful tools to set the absolute and relative stereochemistry for value-added targets of use in medicinal chemistry or chemical biology.[11] Indeed, in the Merck process group 'isolated (ADH) enzymes have clearly supplanted whole cell bioreductions and, in most instances, chemocatalytic ketone reductions.'[12] Our group has utilized a SsADH-10, a dehydrogenase from an archaeal hyperthermophile, to access a wide range of (*S*)-profen scaffolds[13] from racemic precursors via dynamic reductive kinetic resolution (DYRKR)[14] at elevated temperature. The Brenna group has used a set of ADH enzymes to dial in each of the four possible stereoisomers in a 4-methyl-3-heptanol insect pheromone system.[15] Others have developed one-pot enzyme cascade reactions in which ADH enzymes play key roles to access stereochemically homogeneous, functionalized building blocks for synthesis and chemical biology.[2d,16]

The Berkowitz group has a longstanding interest in the creative use of enzymes in synthesis, including the use of lipases in unnatural amino acid synthesis[17] and in lignan natural product total synthesis.[18] Alcohol dehydrogenase and alcohol oxidase enzymes have become the key workhorse enzymes in our efforts to develop enzyme-based screens for the discovery of new organic/organometallic reaction manifolds,[19] new catalytic combinations[20] and new types of chiral ligands.[21] In this effort, these 'reporting enzymes' serve as analytical tools to report back to the experimentalist, in real time, on the relative rates, and where possible, enantioselectivity of the reactions being screened. Therefore, we label this approach in situ enzymatic screening (ISES).[22] Important for this effort is the identification and characterization of new reporting enzymes and this motivation was a big driver for the exploration of the CaADH enzyme that is the subject of the studies described herein.

CaADH: Substrate Promiscuity/Stereochemical Fidelity

Our group expressed, purified and characterized the CaADH enzyme in 2011; the *Clostridium acetobutylicum* species to which it is native is unusual in that it naturally

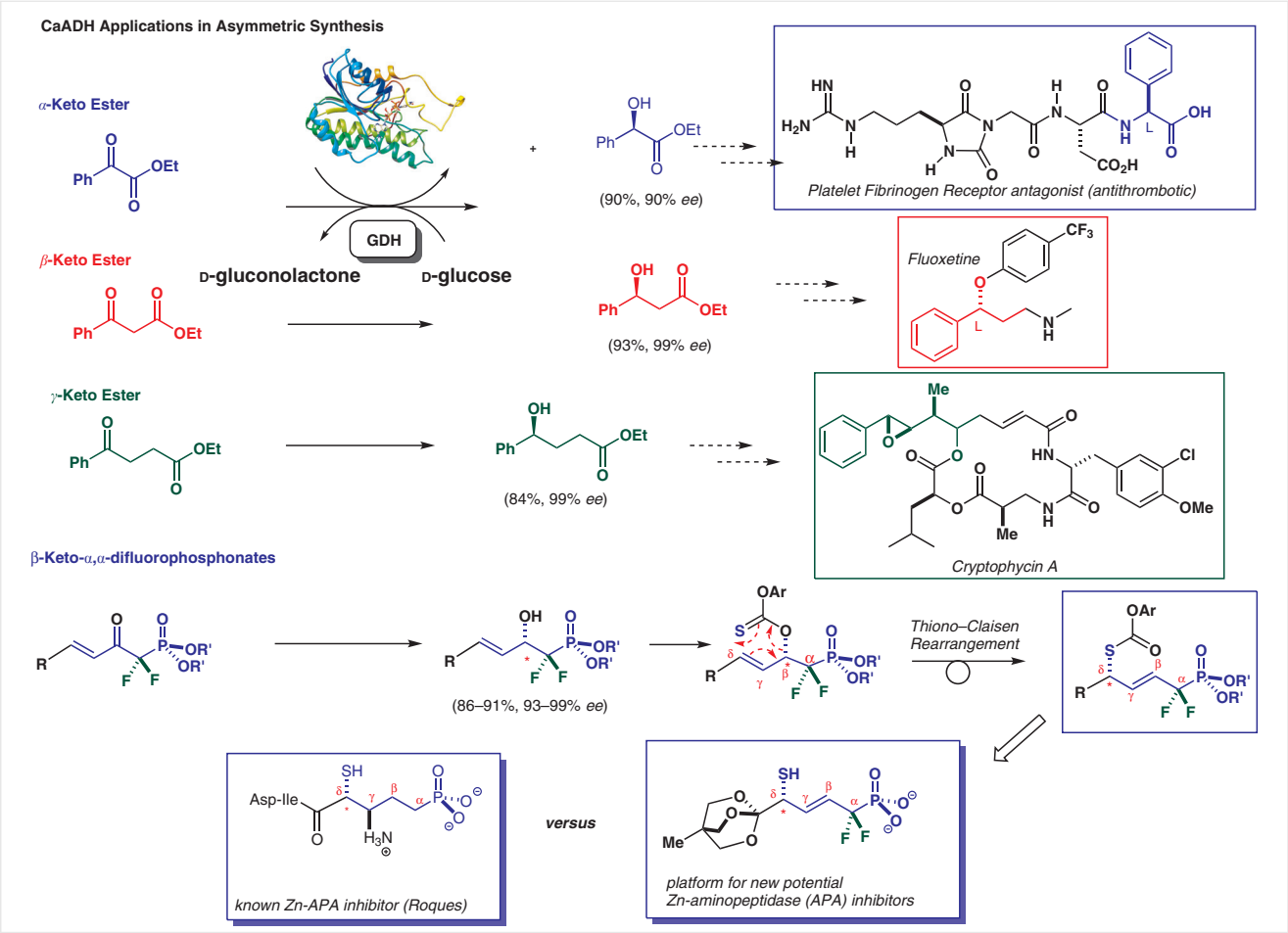


Figure 1 Use of CaADH for the enantioselective reduction of a broad range of carbonyl compounds to provide access to building blocks for the synthesis of important target molecules in medicinal chemistry and chemical biology

undergoes a solventogenic phase in its life cycle in which the microorganism produces acetone, butanol and ethanol (ABE phase).[23] For this reason, *Clostridial* enzymes and metabolic pathways have been of interest to bioengineers seeking to build viable biobutanol refinery technology.[24] From our point of view, this seemed a promising microbial source to pan for organic-solvent-compatible ADHs. Indeed, the CaADH enzyme is well expressed heterologously in *E. coli* and catalyzes the enantioselective reduction of α-, β- and γ-keto esters into the corresponding alcohols with good yield and excellent enantioselectivity, giving the D-enantiomers as key value-added building blocks for pharmaceutical and chemical biology applications (Figure 1).[25]

In more recent work, CaADH was found to enantioselectively reduce another distinct class of substrates, namely β-keto-α,α-difluoroalkyl phosphonates of real significance because α,α-difluorinated phosphonates are phosphatase-inert phosphate surrogates of considerable value in chemical biology and medicinal chemistry.[26] This enzymatic reaction proceeds with the opposite sense of facial selectivity, as compared to β-keto-carboxylate ester reduction, giving rise to the corresponding L-β-hydroxy-α,α-difluorophosphonates. Molecular modeling suggests that CaADH exhibits considerable active-site plasticity to accommodate these structurally distinct substrate classes.[27] Conversion of the enzymatic products into β-pentafluorophenyl allylic thionocarbonates leads to an exceptionally facial [3,3]-sigmatropic rearrangement in which the CaADH-imprinted β-hydroxy stereocenter is parlayed into a δ-thio stereocenter in the product. This sequence provides for a hybrid biocatalytic/sigmatropic rearrangement route that is valuable for the synthesis of potential new Zn-aminopeptidase A inhibitors, as is illustrated in Figure 1. Given the utility of CaADH in asymmetric synthesis, and its ability to reduce varied classes of carbonyl compounds, we set out to examine the electronics of CaADH enzymology more quantitatively, using physical organic chemical tools.

Hammett Analysis: A Physical Organic Tool in Enzymology

Experimental probes for linear free-energy relationships (LFERs) are established tools of physical organic chemistry based upon transition-state theory that can, in principle, also provide insight into enzyme catalysis. The Hammett LFER tool, in particular, examines how the rate or equilibrium constant of a reaction under study responds to substituent effects, provided that the reaction supports an aromatic substrate or educt platform.[28] Of course, there is the further assumption that, across a broad range of aromatic substituents, the series of compounds under study all react via the same rate-determining elementary steps. If this holds, then one expects an LFER between the logarithm of the rate constants for that series of reactions, plotted as a function of substituent and the logarithm of the associated equilibrium constants for benzoic acid dissociation, for the same substituents (i.e., the σ_X values).[29]

Experimentally determined LFERs have been a staple in mechanistic organic chemistry for years, and have found renewed application in reaction and catalyst optimization with, for example, the use of sterimol parameters.[30] The use of such tools to study enzyme mechanism was first proposed and explored several decades ago,[31] but is not without challenges. Indeed, Sinnott, Greig and others have noted that the quantitative analysis of the free-energy relationships for enzyme-catalyzed reactions presents some inherent challenges.[32] There are several criteria that enzyme active site environments may not strictly obey that could lead to deviations from LFER behavior in a Hammett study. On the one hand, aromatic substrates must be well tolerated for such an enzymatic LFER study to be possible. Beyond this, if steric and/or electronic variations in the aromatic substitutents lead to significant diffferences in binding orientation or affinity (perhaps leading to a change in the rate-limiting step) or simply lead to a change in mechanism due to inherent chemical differences in reactivity with the enzyme, one expects to see deviations from linearity.

Illuminating Examples of Hammett LFERs in Enzymology

Despite these challenges, Hammett-type LFER tools have been utilized to help elucidate active-site reaction pathways in enzymology.[33] For example, in a classic study, Kanerva and Klibanov examined how the workhorse hydrolase Carlsberg subtilisin performed in organic solvents.[33n] They were able to measure the enzymatic acylation rates of a series of substituted phenyl acetates in THF, acetone, acetonitrile, *tert*-amyl alcohol and butyl ether.

Because the enzymatic acylation step was being studied, the authors were able to substitute hexanol for water as the nucleophile in these organic solvents. These results demonstrated remarkably similar values for the Hammett reaction constant, $\rho = 0.83–0.93$ across this panel of solvents, similar to the value of 0.87 measured in water,[33n] suggesting that the acylation transition state for subtilisin does not significantly change upon immersion in organic solvents. These mechanistic fingerprinting results obtained by Hammett LFERs agree nicely with subsequent crystallographic studies showing that the enzyme retains key waters of hydration, even upon immersion in organic solvents, presumably preserving key active site structural, dipolar and H-bonding elements.[34]

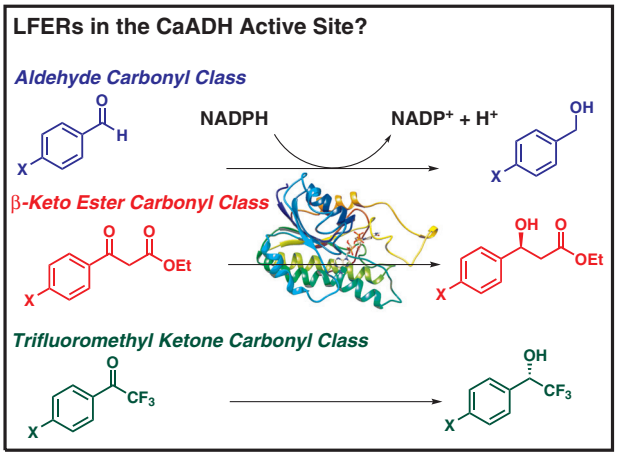
The Hammett LFER tool has also been used to study myeloperoxidase-mediated sulfoxidation of aryl sulfides [porphyrin-Fe(IV)=O active site species], suggesting the intermediacy of a sulfenium radical cation intermediate.[33j] Davidson and co-workers used Hammett studies to glean evidence for a carbanionic intermediate in the methylamine-dehydrogenase-mediated oxidation [tryptophan tryptophyl-*o*-quinone (TTQ) cofactor] of a series of *p*-substituted benzylamines.[33m] Recently, the Guo group performed a Hammett study of a promiscuous C–C bond-form-

ing reaction (Henry reaction) catalyzed by an acyl peptide releasing enzyme from the archaeal thermophile *Sulfolobus tokodaii* (ST0779).[33b] The Guo team studied the condensation of nitromethane with a series of substituted benzaldehydes and obtained a ρ value of 1.33, indicative of nitronate anion condensation with the aldehyde in the rate-determining step. For *o*-, *m*- and *p*-NO_2-substituted aldehydes, as well as the *p*-CN-benzaldehyde coupling partners, high (*S*)-enantioselectivity and good catalytic efficiency were seen. In another case, Bugg and co-workers obtained a Hammett ρ value of –0.71 for the retro-Claisenase-type enzyme, BpHD.[33h] That electron-rich substituents so significantly favor this reaction suggests that the C–C bond cleavage proceeds out of a *gem*-diol intermediate leading to an oxocarbenium ion species in the rate-determining step.

Hammett LFERs with Other NAD(P)H-Dependent Dehydrogenases

There have been several Hammett LFER studies with ADH enzymes as summarized in Table 1. To our knowledge, these have all examined ADH behavior across a panel of substituted benzaldehyde substrates in the active sites of *Oryctolagus cuniculus* glyceraldehyde DH,[31c] *Solanum tuberosum* aromatic alcohol dehydrogenase,[35] *Candida tenuis* xylose reductase[36] and *Saccharomyces cerevisiae* ethanol dehydrogenase.[37] All the observed ρ values were in the 1.2–1.7 range, apart for the latter enzyme in which a ρ value of >2 was observed by Klinman.[37] These results are consistent with hydride transfer from the nicotinamide cofactor to the benzaldehyde carbonyl center being at least partially rate-limiting. The elevated ρ value in the case of the yeast enzyme has been attributed to the fact that this ADH is a zinc-metalloenzyme, with the expectation that Zn^{2+}-mediated polarization of the substrate carbonyl leads to a greater partial positive charge at the benzylic center in the benzaldehyde educt and therefore a greater change in charge density along the hydride transfer reaction coordinate.

Given the aforementioned utility of the *C. acetobutylicum* alcohol dehydrogenase (CaADH) in asymmetric synthesis, we set out to examine the electronic dependence of its carbonyl reduction chemistry using the Hammett LFER tool. Moreover, given the substrate promiscuity that we have observed with CaADH (Figure 1), this ADH seemed to be an excellent candidate to explore the possibility of measuring ρ values for more than one carbonyl class in the same active site (Scheme 1).



Scheme 1 Hammett study of CaADH-catalyzed carbonyl reduction LFERs as a function of compound class

RESULTS: CaADH-Mediated Reduction of *p*-Substituted Benzaldehydes

In line with this objective, we experimentally measured the rate of NADPH-mediated reduction of eleven *p*-substituted benzaldehydes as substrates for the CaADH enzyme. These assays were run in a plate-reading fluorimeter with enzyme expressed and purified, in house, as described earlier.[25,27] Each well was irradiated at 340 nm (NADPH λ_{max}), with fluorescence emission being monitored at 460 nm. Each aldehyde was monitored for reduction rate across a range of a half-dozen concentrations, with each initial velocity measurement being run at least in duplicate. The v_o versus [S] data were worked up by performing a least-squares hyperbolic fit to the Michaelis–Menton equation. The fastest rates were observed for the reduction of *p*-nitrobenzaldehyde and the slowest for *p*-methoxybenzaldehyde. A Hammett plot of log k_{cat} versus σ_X showed pseudo-biphasic behavior as can be seen in Figure 2a. That is, the fastest substrates appear to bifurcate, with those containing the most polarizable ('soft') electron-withdrawing substituents

Table 1 Prior Hammett Studies of Alcohol Dehydrogenase Enzymes: All Performed with Substituted Benzaldehydes

Entry	Enzyme	σ/σ⁺	ρ	*V*max/(*V*max/*K*m)	Ref.
1	ethanol (alcohol) dehydrogenase (*Saccharomyces cerevisiae*)	σ⁺	2.17	*V*max	37
2	glyceraldehyde 3-phosphate DH (*Oryctolagus cuniculus*)	σ	1.27, 1.24	*V*max, *V*max/*K*m	31c
3	aromatic alcohol dehydrogenase (*Solanum tuberosum*)	σ	0.42, 1.38	*V*max (*m*-), *V*max (*p*-)	35
4	xylose reductase (*Candida tenuis*)	σ	1.4–1.7	both	36

(*p*-I, *p*-Br, *p*-SCF_3) tracking along a nice linear free-energy relationship with those bearing electron-donating groups. These substrates provide for an LFER corresponding to ρ = 0.99 ± 0.13 (Figure 2a, red trace). However, another set of four fast substrates bearing less polarizable ('hard') electron-withdrawing substituents with significant dipoles (*p*-F, *p*-Cl, *p*-OCF_3, *p*-CF_3) project out a second LFER with a significantly less positive slope, but a very good correlation with ρ = 0.40 ± 0.06 (Figure 2a, blue trace). The best fit appears to occur with bifurcation of the data at the OMe substituent.

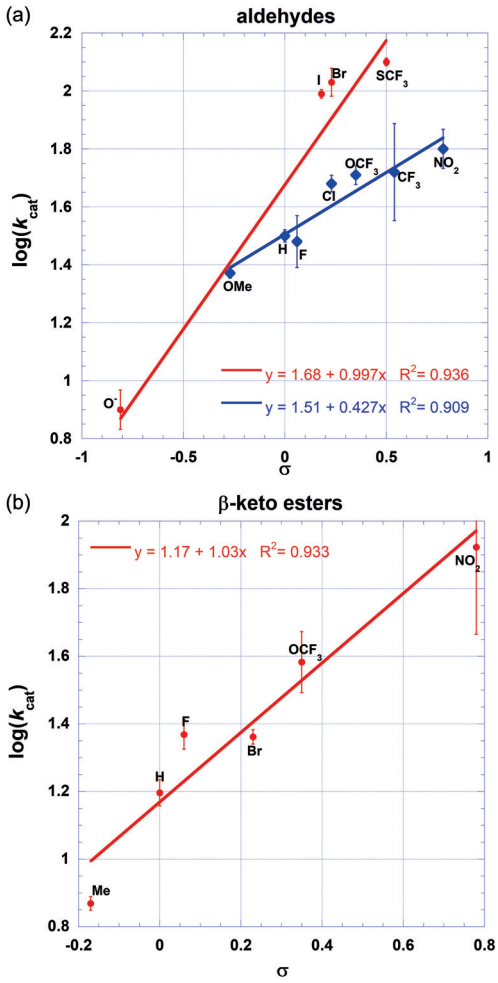


Figure 2 Correlations between the logarithm of turnover number of CaADH-catalyzed reduction of (a) aldehydes, and (b) β-keto esters

This Hammett plot is essentially a biphasic, concave-downward LFER plot and this pattern of kinetic behavior normally indicates a change in the rate-limiting step.[32a] That is to say, in moving from substrates with more polarizable EWG substituents to those with harder, less polarizable substituents, there may be a change in the rate-limiting kinetic profile. One possibility, for example, would be that these two sets of substituents interact differently in the enzyme active site, with the more dipolar substituents engaging in favorable dipole–dipole or charge–dipole interactions with active-site pocket residue(s) that lead to a slower dissociation rate for this substrate subclass. In such a case, the 0.99 ρ value associated with the faster substrate subclass would reflect a kinetic profile in which NADPH-mediated hydride delivery is largely, if not fully, rate-limiting. The 0.40 ρ value associated with the subclass of substrates bearing dipolar EWGs would then be indicative of partially rate-limiting hydride transfer that is punctuated by partially rate-limiting product dissociation. We are currently working to obtain an X-ray crystallographic structure of the title enzyme; such structural biological data would help us to evaluate these postulates, in the longer term. In the meantime, we have used homology model building and molecular docking to examine possible substrate binding modes in light of the discussion above (*vide infra*).

We are aware of only one other report of a biphasic Hammett plot for alcohol dehydrogenase based chemistry. As in another example of ADH LFER, Towers and co-workers examined the *Solanum tuberosum* ADH-mediated reduction of *m*- and *p*-substituted benzaldehydes.[35] The Hammett plot of log(V_{max}) versus σ_X reported in their work appears, in the first analysis, to show concave upward biphasic kinetic behavior with two ρ values of ~0.4 for the more electron-donating groups and of ~1.4 for the more electron-withdrawing groups. This upward concavity would suggest a change in mechanism.[32a] However, as the authors note, the lower value of ρ generally correlates with the kinetic behavior of the *m*-substituted substrates, whereas the larger ρ generally correlates with the *p*-substituted substrates. The authors argue for a difference in binding mode for the *m*- versus *p*-substituted substrates, perhaps suggesting the *p*-substitution allows for better positioning of the aldehyde carbonyl with respect to typical Bronsted acid proton-donating residues in the active site. This could give rise to a larger δ^+ in the ground state and the carbonyl carbon and a larger accumulation in negative charge with NADH-mediated hydride transfer for the *p*-configured substrates.

A classic case of a concave-upward biphasic Hammett plot that more clearly translates into a likely change in mechanism as a function of substituent can be found in the pioneering work on lysozyme by Tsai and co-workers.[38] These workers studied the enzymatic hydrolysis of a set of *p*-substituted aryl β-di-*N*-acetylchitobiosides. The biphasic Hammett plot obtained gave a reaction constant ρ value of –2.96 for electron-donating substituents ($\sigma_X \leq 0$) and a ρ value of +0.55 for electron-withdrawing substituents ($\sigma_X \geq 0$). These data and supporting evidence suggest that for electron-donating substituents an oxocarbenium ion mechanism is followed, whereas for electron-withdrawing substituents a direct displacement ensues, whereby an active-site carboxylate residue directly displaces an anomeric *p*-substituted phenolate leaving group in the rate-determin-

ing step. Several other instances of biphasic kinetic behavior for enzymatic Hammett LFER plots have been reported through the years.[32a,39]

CaADH-Mediated Reduction of *p*-Substituted β-Keto Esters

Next, we examined electronic substituent effects on the CaADH-mediated reduction of a second carbonyl class, namely a set of *p*-substituted aryl β-keto esters. The Hammett plot of log k_{cat} versus σ_X shows a linear correlation with $\rho = 1.02 \pm 0.31$ (Figure 2b). The positive value of ρ shows that in this substrate class electron-withdrawing substituents also speed the enzymatic reduction catalyzed by CaADH, and this result also suggests an increase in negative charge at the carbonyl center in the rate-limiting transition state, implying that for this less electronegative carbonyl class, hydride delivery from NADPH is largely rate-determining. This is the first example of which we are aware of the examination of this substrate class in an ADH active site (see Table 1 for reported ρ values seen for aldehyde substrates across a range of ADH active sites).

CaADH-Mediated Reduction of *p*-Substituted Aryl Trifluoromethyl Ketones

Given the notable substrate promiscuity exhibited by CaADH, we next set out to examine yet another substrate class in this active site, namely aryl trifluoromethyl ketones bearing a range of *p*-substituents. Pleasingly, we observed that CaADH also readily accepts this new substrate class and preliminary indications are that these reductions also proceed with high facial selectivity. Namely, for the parent compound, phenyl trifluoromethyl ketone (X = H), chiral HPLC analysis indicates that the CaADH-mediated reduction proceeds with very high enantioselectivity for the product α-trifluoromethyl benzyl alcohol.

This result is significant as such organofluorine compounds have value for pharmaceutical, agrochemical, and materials science applications.[40] Earlier, several groups had reported such enzymatic reductions of aryl trifluoromethyl ketones under yeast whole cell conditions[41] and with purified ADH enzymes,[11b–d,42] giving the enantioenriched (*R*)- or (*S*)-α-trifluoromethyl benzyl alcohols, depending on the choice of enzyme. The CaADH-catalyzed reduction of phenyl trifluoromethyl ketone observed here is among the most efficient (95% yield) and the most enantioselective yet seen (99% ee). The absolute stereochemistry of the product α-trifluoromethyl benzyl alcohol was established as (*S*) based upon retention time on chiral HPLC for the reported compound (see the Supporting Information).

These promising results encouraged us to synthesize a complete set of aryl trifluoromethyl ketones and to perform a Hammett study across this new CaADH carbonyl substrate class. In contrast to the kinetic studies with the aryl aldehyde and β-keto ester substrate classes for which it was found advantageous to measure NADPH-fluorescence owing to significant substrate absorbance, for the aryl trifluoromethyl ketone substrate class, we were able to follow the kinetics by monitoring the decrease in absorbance at 340 nm with NADPH consumption ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$). As can be seen from Figure 3a, we observed a negative sign for the reaction constant $\rho = -0.97 \pm 0.12$ for this class of substrates. This is the first enzymatic Hammett LFER study with aryl trifluoromethyl ketones of which we aware.

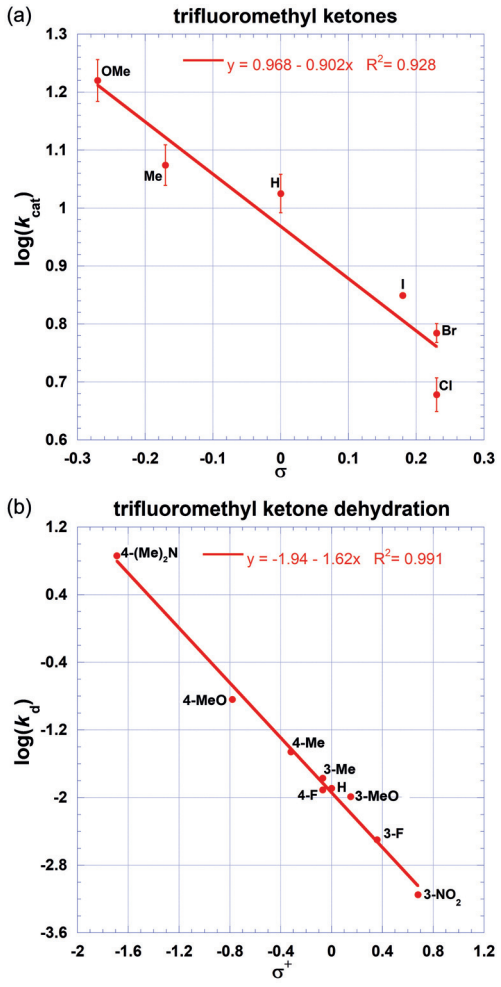


Figure 3 (a) Relation between the logarithm of turnover number of CaADH-catalyzed reduction of α,α,α-trifluoroacetophenone vs σ_X (b) Data replotted from Stewart and Van Dyke[45] for the log(dehydration equilibrium constant) for trifluoroacetophenone hydrates vs σ_X^+

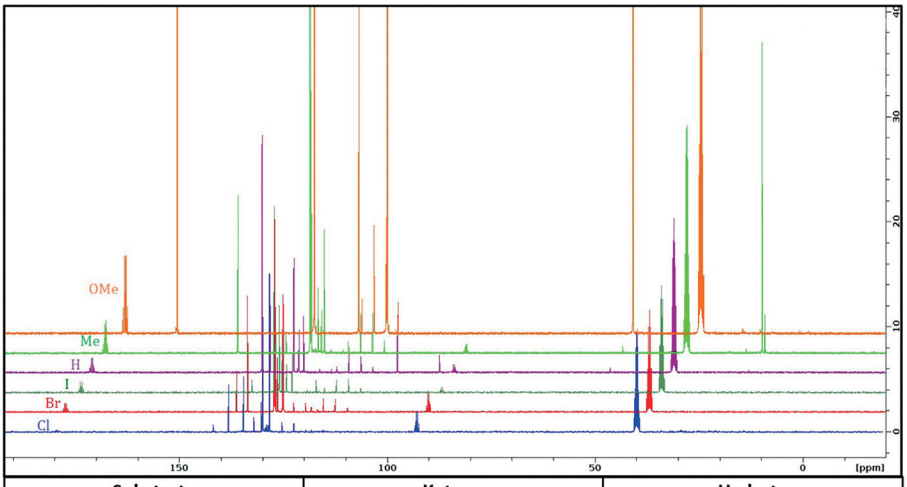
The negative ρ value seen in these studies stands in contrast to the ρ value of 3.14 observed by Teo and Stewart for sodium borohydride mediated ketone reduction of aryl trifluoromethyl ketones in an organic medium.[43a] Another non-enzymatic Hammett LFER study examines the electrochemical reduction of aryl trifluoromethyl ketones in acetonitrile with an organic-soluble electrolyte (NBu_4ClO_4), for which Yang and co-workers[43b] reported ρ = 0.52–0.61. Perhaps more germane to our studies, Ohno and co-workers have examined model Hantzsch ester reductions of aryl trifluoromethyl ketones in anhydrous acetonitrile and report

ρ values ranging from 1.3–3, depending upon whether a divalent cation is present.[44] The net positive ρ values observed in these model studies are consistent with either rate-limiting single-electron transfer (in the electrochemical reduction, in particular) or hydride transfer (Hantzsch ester studies) to the electrophilic carbonyl center in the aryl trifluoromethyl ketone substrates.

However, our experimental data for the CaADH-mediated reduction chemistry suggest that a net positive charge builds up in the rate-limiting transition state and in turn, this suggested to us that perhaps the true substrates are actually the trifluoromethyl ketone hydrates, under the conditions of these enzymatic experiments. Indeed, the Van Dyke group has systematically studied the effect of the substituents on the hydration/dehydration equilibrium constant for aryl trifluoromethyl ketones. In Figure 3b, we have replotted their equilibrium Hammett data; on the ordinate is the logarithm of the equilibrium constant for dehydration of the ketone hydrate, log(K_d), plotted versus σ^+, on the abscissa. This gives a nice equilibrium linear free-energy relationship with ρ = –1.62 indicating that electron-donating groups on the aryl trifluoromethyl ketones favor dehydration.[45]

To examine this further, we conducted a ^{13}C NMR experiment to examine the tendency of our own set of aryl trifluoromethyl ketones to hydrate in solution. The substrates were each dissolved in 2% (v/v) aqueous DMSO-d_6 and the NMR spectra acquired. The results are displayed in Figure 4, in stack plot form, from most electron-withdrawing to most electron-donating *p*-substituent, front to back. The results clearly show that in our hands, as well, the carbonyl hydration equilibrium for this class of aryl trifluoromethyl ketones shows a strong dependence upon the *p*-substituent, with electron-withdrawing substituents favoring hydration. Thus, the *p*-Cl-α,α,α-trifluoroacetophenone substrate is almost completely hydrated under the conditions of this NMR experiment (2 vol% H_2O) whereas the *p*-MeO-substituted counterpart exists largely as the free ketone. Accordingly, it is expected that under the conditions of our enzyme kinetic studies [10% (v/v) DMSO in aqueous buffer], our aryl trifluoromethyl ketone substrates exist largely in hydrated form.

All that said, it is expected that NADPH reduction in the CaADH active site will require dehydration of the geminal-diol (ketone hydrate) such that hydride transfer from the bound nicotinamide cofactor can ensue. It is therefore possible that dehydration of the *gem*-diol or carbonyl hydrate is largely rate-determining for this class of substrates. This would be consistent with both the negative ρ-value seen in the Hammett plot (Figure 3a) and the propensity for these aryl trifluoromethyl ketones to exist in hydrated form (Figure 4). So, the mechanism for enzymatic reduction may first involve dehydration of the *gem*-diol in the CaADH active site, followed by NAPDH-mediated hydride transfer.



Substrate	Ketone	Hydrate
OMe	100	0
Me	81	19
H	60	40
I	38	62
Br	11	89
Cl	3	97

Figure 4 Hydration of aryl trifluoromethyl ketones as a function of the *p*-substituent in 2% aqueous DMSO-d_6 as monitored by ^{13}C NMR (175 MHz). The results are displayed from electron-withdrawing group to electron-donating group (front to back). One sees the quartet diagnostic of the hydrate at ~95 ppm fade out and the corresponding quartet for the free ketone at ~180–185 ppm fade in, front to back

Such a mechanism is reminiscent of the proposed mechanism for lysozyme-catalyzed hydrolysis of *p*-substituted aryl β-di-*N*-acetylchitobiosides discusssed above.[38]

There, the negative value of ρ = –2.96 for electron-donating substituents was interpreted as evidence that enzyme-mediated acetal cleavage is rate-limiting, presumably yielding an oxocarbenium-ion-like intermediate, prior to addition of water. Here, a very similar acetal-like substrate would undergo partially rate-limiting cleavage to an oxocarbenium-like species (protonated carbonyl) in the active site, followed by highly favorable hydride transfer. Below we discuss this and the mechanisms for the other two substrate classes in the context of molecular modeling studies.

Molecular Modeling

In order to examine how substrates of all three carbonyl classes studied here bind to the CaADH active site, molecular modeling was undertaken. Given that no three dimensional structure of CaADH has yet been published, we constructed a homology model using Clustal W,[46] and relaxed this structure (GROMACS[47]) as described previously.[27] Docking was carried out using Autodock Vina[48] (Yasara package[49]). Carbonyl substrates were prepared and minimized in Spartan and exported as protein databand (pdb) files. Dockings were performed so as to generate a total of 25 bound poses for each ligand studied. Poses were evaluated based upon both Autodock-estimated binding energies for each pose and the reasonableness of the docking algorithm-generated structure as judged by inspection of substrate positioning, particularly with regard to hydride transfer from the NADPH cofactor to the substrate carbonyl.

The resulting models of the ternary CaADH-NADPH-carbonyl complexes for each substrate class are presented in Figure 5. Notice the important substrate binding and hydrogen bond donor roles played by the S140 and Y153 residues in all docked structures. These residues are part and parcel of the canonical carbonyl binding pocket in the family of short-chain dehydrogenases of which CaADH is a member.[50] Docking suggests that these residues may also interact with the hydroxy groups in the *gem*-diols derived from the aryl trifluoromethyl ketone substrates and may facilitate their dehydration (see Figure 5d) as described above. The arene rings of the aryl β-keto ester substrates (Figure 5b) and of the aryl trifluoromethyl ketone substrates (Figure 5c) are postulated to enjoy favorable edge-to-face π-π interactions with F192 and F241, respectively. This results in proper positioning of these carbonyl substrates so as to enantioselectively give the D-β-hydroxy ester and (*S*)-α-trifluoromethyl benzyl alcohol products, as observed.

In summary, we report here what to our knowledge is the first example of a Hammett LFER study of more than one carbonyl substrate class in a single dehydrogenase active site. The dehydrogenase under study, CaADH from *Clostridium acetobutylicum*, is an enzyme that has shown enormous potential in asymmetric synthesis, acting to enantioselectively reduce substrates of the α-keto ester, β-keto ester, γ-keto ester, β-keto-α,α-difluoroalkyl phosphonate

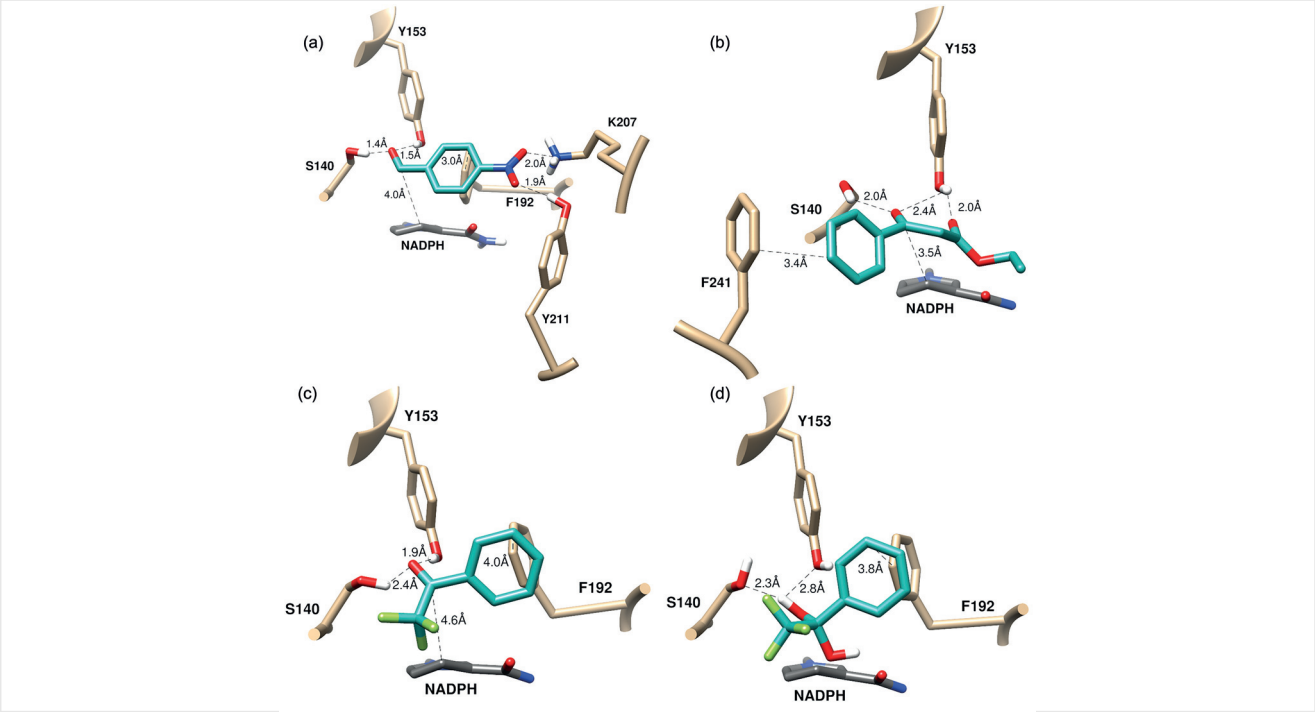


Figure 5 Molecular modeling of NADPH cofactor in the CaADH active site (homology model) with the following substrates: (a) *p*-nitrobenzaldehyde, (b) ethyl benzoyl acetate (β-keto ester), (c) phenyl trifluoromethyl ketone, and (d) the hydrate (*gem*-diol) form of phenyl trifluoromethyl ketone

and aryl trifluoromethyl ketone substrate classes, the latter example having been first demonstrated in this work. The Hammett ρ values obtained for the aryl aldehyde (Figure 2a: ρ = 0.99 ± 0.10 and ρ = 0.40 ± 0.09) and aryl β-keto ester (Figure 2b: ρ = 1.02 ± 0.31) substrate classes are, in these cases, consistent with at least partially rate-limiting hydride transfer from NADPH to the substrate carbonyl. It should be noted that rate-limiting single-electron transfer cannot be ruled out solely on the LFER data that we present. This would lead to a transient substrate ketyl radical anion and a nicotinamide radical cation. Though such mechanisms are not generally considered likely for NAD(P)H-mediated dehydrogenase chemistry, the recent work of Hyster[51] shows that under appropriate conditions (e.g., photoinitiation, haloketone substrates), nicotinamide enzymes can undergo one-electron chemistry.

For our studies described here with CaADH, the pseudo-biphasic, concave-downward nature of the Hammett plot observed for the *p*-substituted benzaldehyde substrate class (Figure 2a) suggests that there may be a change in the rate-determining step in moving from substrates bearing 'softer' highly polarizable substituents (I, SCF_3, Br) to those bearing 'harder' substituents with a significant dipole (F, Cl, OCF_3, CF_3, NO_2). One interpretation of this bifurcating kinetic pattern would be that for the former class, hydride transfer is rate-limiting, whereas for the latter substrate class, product dissociation becomes partially rate-limiting, owing to specific interactions of these dipolar substituents with the enzyme, giving rise to the lower value of ρ seen for this substrate subclass. Consistent with this notion, molecular docking of the *p*-nitrobenzaldehyde substrate with the CaADH homology model shows the possibility for significant ion–dipole and dipole–dipole interactions between the polar *p*-NO_2 group and the ammonium group of K207 and the hydroxy group of Y211, respectively (Figure 5a).

The negative ρ value observed for the aryl trifluoromethyl ketone (α,α,α,trifluoroacetophenone) substrate class together with model NMR studies suggests that these substrates are largely hydrated and that substrate dehydration, favored for substrates bearing electron-donating groups, may be rate-limiting for this interesting substrate class. Importantly, the efficient and highly enantioselective manner in which CaADH processes this new substrate class [95% yield, 99% ee, favoring the (*S*)-enantiomer of α-trifluoromethyl benzyl alcohol)[52] augers well for future applications of the CaADH enzyme in asymmetric synthesis and process chemistry.

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Supporting Information

Supporting information for this article is available online at https://doi.org/10.1055/s-0039-1691576.

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(52) **Enzymatic Reduction/Preparation of (*S*)-2,2,2-Trifluoro-1-phenylethanol; Typical Procedure**

To a solution of NADPH (21 mg, 0.0287 mmol, 0.01 equiv), D-glucose (3.09 g, 17.2 mmol, 6 equiv), CaADH (50 units) and $NADP^+$-dependent glucose dehydrogenase (2% w/w) in KPO_4 buffer (100 mM, pH 8.0) was added trifluoromethylphenyl ketone (500 mg, 2.87 mmol) in DMSO so that the final DMSO concentration was 10% (v/v). The reaction mixture was allowed to stir at rt for 8–10 h and the reaction progress was monitored by TLC. The product was extracted with EtOAc and dried over Na_2SO_4. Following vacuum filtration and concentration, the crude residue was purified by flash column chromatography on silica gel (EtOAc/hexane, 1:4) to give the title alcohol as a colorless oil (484 mg, 96%) in homogeneous form. The ee was determined to be 99.6% (*S*) by HPLC with a chiral stationary phase (see the Supporting Information).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.55–7.47 (m, 2 H), 7.47–7.41 (m, 3 H), 5.11–4.97 (m, 1 H), 2.66 (d, *J* = 4.5 Hz, 1 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 180.88 (q, *J* = 35 Hz), 135.85, 130.47 (q, *J* = 2.8 Hz), 128.91, 107.01 (q, *J* = 294 Hz). ^{19}F NMR (376 MHz, $CDCl_3$): δ = –78.36 (d, *J* = 6.7 Hz).