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Article

Taft linear free-energy relationships in the biocatalytic hydrolysis of sterically hindered nitrophenyl ester substrates

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SUMMARY

Linear free-energy relationships (LFERs) have been commonly used to uncover reaction mechanisms in organic chemistry by correlating trends in reactivity to reactant properties. However, the applications of LFERs have largely been limited to traditional organic synthesis and have much less frequently been applied toward enzyme-catalyzed reactions. In this study, we used the Taft LFER, which correlates reaction rates with steric properties of reactants, to study kinetic trends in the enzymatic hydrolysis of sterically hindered substrates. We synthesized 4-nitrophenyl ester compounds with substituents of varying degrees of steric hindrance, and then subjected these compounds to hydrolysis by the enzymes lipase, trypsin, and nattokinase. Kinetic data was obtained by using a spectrophotometer to monitor the formation of 4-nitrophenol, a bright yellow product of the ester hydrolysis with an optical readout at 413 nm. Contrary to initial hypotheses, Taft plots did not exhibit linear relationships and further analysis yielded mechanistic insight into the nature of the Taft steric parameter, the relative sensitivity of each enzyme to steric effects, and potential enzyme-substrate binding interactions. This analysis was paralleled with computational calculations to determine local charge density of the reaction center, which supported that the unexpected trends were largely a function of the aforementioned factors rather than electronic effects. Ultimately, we demonstrate the unconventional application of the Taft LFER toward biocatalytic transformations and open avenues toward the broader use of biocatalysts in synthetic organic chemistry.

INTRODUCTION

Since the start of the 20th century, linear free-energy relationships (LFERs) have attracted much attention from physical organic chemists interested in studying kinetic trends using physical parameters. By applying LFERs, chemists have been able to uncover underlying trends in reactivity and deduce reaction mechanisms while gaining insight into the factors that influence chemical reactivity (1,2). Additionally, by harnessing these mechanistic insights, chemists have been able to optimize the time, yield, and distribution of products generated through a chemical reaction, enabling more efficient syntheses and opening new synthetic avenues (3). The Hammett LFER, for example, has frequently been used to relate differences in reactivity of substituted aromatic substrates to electronic effects conferred by substituents (1,4). This mathematical relationship, $\log(k/k_H) = \sigma\rho$, states that the reaction rate of a substituted constant, k, and un-substituted constant, k_H, are a linear function of the reaction constant, ρ, and the Hammett substituent constant, σ. When the Hammett equation failed to explain reactive trends in aliphatic and *ortho*-substituted benzene derivatives, the Taft equation was published as a modification that accounted for steric effects in addition to electronic effects (2,3,5,6). The Taft equation, $\log(k/k_{CH3}) = \sigma^*\rho^* + \delta E_s$, states that the reaction rate of a substituted constant, k, and an un-substituted constant, k_{CH3}, are a linear function of the sensitivity factor to electronic effects, ρ*, and the Taft substituent constant, σ*, as well as the sensitivity factor to steric effects, δ, and the steric substituent constant, E_s (7,8). Thus, in the Taft equation, the $\rho^*\sigma^*$ term models electronic effects and the δE_s term models steric effects, together accounting for overall trends in reactivity.

The Hammett and Taft LFERs have both had diverse applications in physical organic chemistry. Free energy landscapes have been used to elucidate reaction mechanisms and reactive intermediates, such as in determining reaction orders of methanolysis reactions and changes in rate-determining steps of S_N2-type reactions (9,10). LFERs have also been applied toward reaction methodology development, namely in designing and evaluating inorganic ligands and identifying optimal reaction conditions (11–13). Finally, these two equations have had considerable—but comparatively less—impact on studies of biocatalysis and have been used to probe enzymatic active sites and elucidate transition states and rate-determining steps (14–18).

Previously, we reported the utilization of a library of 4-substituted nitrophenyl benzoate esters as colorimetric substrates for probing Hammett LFERs in enzymatically catalyzed ester hydrolysis events (19). In this study, we expanded the applications of the Taft LFER to investigate the impact of steric hindrance, which refers to the arrangement of atoms that impacts a molecule's ability to react, on the enzymatic efficiency of three serine proteases and esterases: pre-gastric lipase, bovine trypsin, and nattokinase (**Figure 1**). Each of these enzymes is able to hydrolyze ester bonds using a catalytic triad in the enzyme active site, in which a residue that is oriented by the other triad members acts as a nucleophile to covalently cleave the substrate. In general, enzymes present

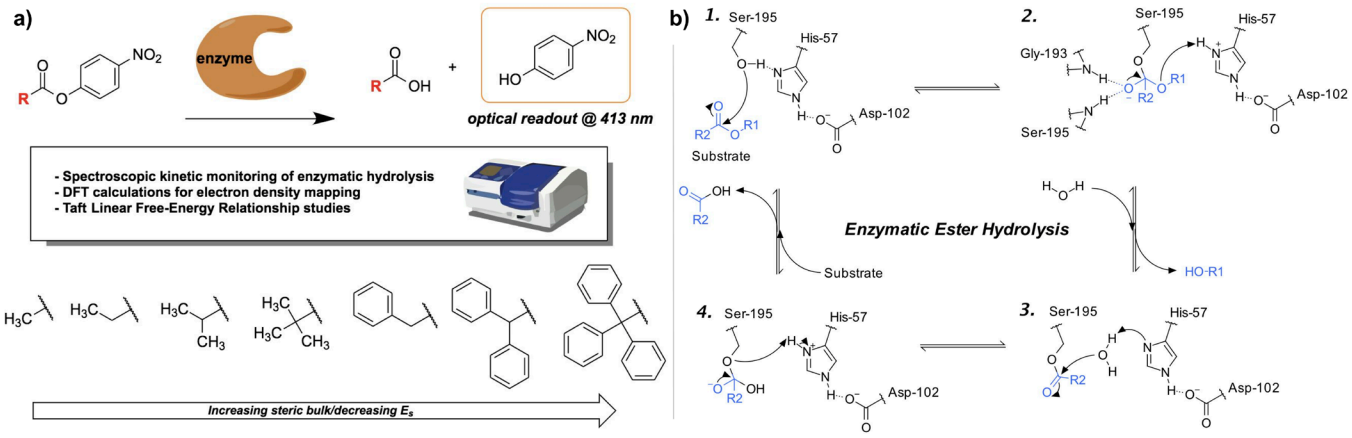


Figure 1: Graphical abstract, depicting the use of 4-nitrophenyl ester substrates as colorimetric probes to study enzyme kinetics, as well as the mechanism of biocatalysis. a) The kinetics of enzymatic hydrolysis of ester bonds is largely governed by stereoelectronic factors, and trends in the relative effects of sterically-hindered substituents can be modeled using the Taft LFER. Kinetic data can be obtained by spectroscopically monitoring the release of 4-nitrophenol, a product of hydrolysis with an optical readout. b) Enzymatic hydrolysis of ester bonds. The catalytic triad found in serine proteases and esterases consists of three residues: serine-195, histidine-57, and aspartate-102. The histidine engages the serine residue in a hydrogen bonding network, which enhances its nucleophilicity. Upon nucleophilic addition, the serine forms a tetrahedral intermediate with the substrate, and collapse of this intermediate yields an alcohol and a carboxylic acid.

several advantages to organic chemists as they can be applied as biocatalysts—biological catalysts of chemical reactions. Because enzymes depend on several factors including active site positioning and substrate electronic properties, this allows chemists to optimize enzymes for chemoselective reactions (20). In industrial settings, biocatalysts have presented new methods of streamlining pharmaceutical drug production through the development of rapid sequences of enzyme-catalyzed reactions called biocatalytic cascades (21,22). By understanding the physical organic principles that govern the reactivity of these biocatalysts, we may be able to support efforts in applying enzymes more broadly in chemical synthesis.

In this study, five 4-nitrophenyl ester substrates exhibiting varying degrees of steric hindrance were synthesized and subjected to hydrolysis by each of the three studied enzymes. Kinetic data was obtained by measuring visual readouts using a spectrophotometer since progression of the hydrolysis reaction yields 4-nitrophenol, a bright yellow compound with an optical readout at 413 nm. Kinetic data was then modeled using the Taft relationship. We hypothesized that increased steric hindrance would correlate with a reduced rate of enzymatic hydrolysis, as increased steric bulk surrounding the carbonyl carbon would impede the initial nucleophilic attack by the enzyme. This hypothesis was consistent with previous studies that have demonstrated that increased steric hindrance impedes nucleophilic attacks (23,24).

Moreover, computational calculations were employed to confirm whether changes in reactivity were due largely to steric or electronic effects. Each substrate was optimized via density-functional theory (DFT), a quantum mechanical method that uses an electron density function to predict the energy of a system, and then Mulliken charges, which represent local charge density, were extracted at the carbonyl carbon of the geometry-optimized structures. We hypothesized that although 4-nitrophenyl ester aliphatic substituents would have an effect on the charge density at the carbonyl carbon, these effects would be minimal and changes in reaction kinetics would be primarily governed by steric effects (25).

Contrary to initial hypotheses, Taft σ* constants did not inform enzymatic kinetics, and, consistent with initial hypotheses, DFT calculations confirmed that these differences were predominantly due to steric differences. Ultimately, our results enabled us to identify relative differences in enzymatic sensitivity to steric bulk and potential differences in binding pocket interactions. These results suggest that there may be other factors at play in enzymatic hydrolysis of our studied compounds beyond sheer steric bulk in proximity to the reaction center, including α-substituent effects on enzyme-substrate binding, substrate positioning in the active site, and competition between the active site and other enzymatic residues. The extent to which these factors determine sheer steric effects in the observed reaction kinetics may be enzyme-dependent. More broadly, our results demonstrate the applicability of the Taft LFER to model kinetic trends of biocatalytic transformations and probe differences in enzymatic active sites.

RESULTS

We synthesized five 4-nitrophenyl ester substrates with either acetate (incurs the least steric hindrance), propanoate, isobutyrate, pivalate, or phenyl (incurs the most steric hindrance) substituents. Substituents were strategically chosen, as each substituent incurs a different amount of steric hindrance. We then subjected these compounds to enzymatic hydrolysis by either lipase, trypsin, or nattokinase, as well as non-enzymatic hydrolysis in aqueous solution, through which we obtained kinetic data by monitoring the absorbance at 413 nm. Non-enzymatic blank readings were subtracted from samples undergoing enzymatic hydrolysis.

Since the Taft equation contains both electronic and steric parameters, we decided that electronic parameters could be disregarded as all aliphatic substituents likely did not create a significant electron-donating or -withdrawing effect. To confirm whether this was a valid assumption, we

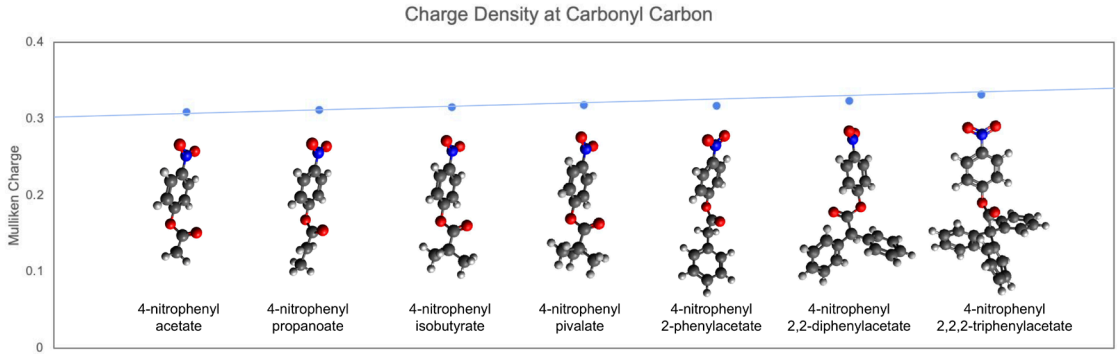


Figure 2: Carbonyl carbon Mulliken charges were calculated to compare whether bulky substituents significantly affected charge density. On the whole, Mulliken charges were consistent for all substrates and only had a range of 0.023 units.

performed DFT calculations to determine the Mulliken charge at each substrate's carbonyl carbon—the reaction center (**Figure 2**). When performing DFT calculations, we included two additional substrates with extremely bulky diphenyl and triphenyl substituents in order to draw more conclusive results, even though E_s steric parameters do not exist for these substituents. Consistent with our expectations, Mulliken charges were highly uniform and we were able to eliminate the electronic parameter from the Taft equation in order to isolate steric effects.

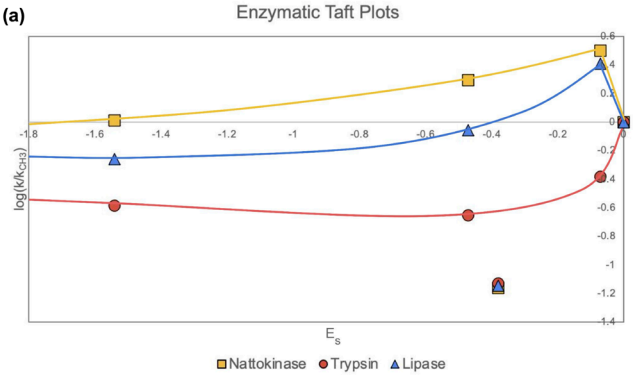
The Taft equation was then used to model the kinetic data along with previously reported Taft E_s constants (**Figure 3**) (4). All data were highly reproducible, as the standard deviations for all $\log(k/k_{CH3})$ values were between 0.000351 and 0.000227. Surprisingly, no distinct linear trends were evident in the Taft plots. For all enzymes, the data point corresponding to the hydrolysis of 4-nitrophenyl 2-phenylacetate (E_s = -0.38) was an outlier. Even though E_s constants have not been reported for diphenyl and triphenyl substituents, it was evident that for all three enzymes, 4-nitrophenyl 2,2-diphenylacetate (average V_0 = 0.0548 mol/min) cleaved at a slower rate than 4-nitrophenyl 2-phenylacetate (average V_0 = 0.305 mol/min), and 4-nitrophenyl 2,2,2-triphenylacetate (average V_0 = 0.00000373 mol/min) was likely unable to be enzymatically hydrolyzed.

DISCUSSION

Unlike prior applications of the Taft equation, our enzymatic Taft plots did not exhibit linear trends, which provides interesting insight into the nature of the Taft steric parameter and into enzymatic active sites, where reaction kinetics are governed by additional parameters such as enzyme-substrate binding, positioning of the reaction center in the active site, and interactions with residues near the active site that outcompete the active site (**Figure 3**). Previous studies have indicated that the Taft E_s parameter, the steric substituent constant originally derived through the acid-catalyzed hydrolysis of methyl esters, is not always directly proportional to physical measurements such as Van der Waals radii or Van der Waals surface area, especially for substituents related to phenyl groups (26,27). Our study corroborated this, as although Van der Waals measurements of phenyl substituents are greater than pivalate substituents, the Taft E_s parameter states that phenyl groups create less steric hindrance. As a result, substrates with a phenyl substituent created an outlier in our Taft plots, which wouldn't have been the case had Taft E_s parameters more consistently paralleled Van der Waals measurements. Moreover, we had also considered deriving Taft E_s constants for diphenyl and triphenyl substituents using linear trends between E_s values and Van der Waals radii or surface area, but the absence of these trends prevented us from being able to include these points on our Taft plots.

These results provide further insight into differences in the active sites of lipase, trypsin, and nattokinase. Mulliken charge calculations informed us that there was a slight increase in carbonyl carbon charge density as the bulkiness of the substituent increased, but this proportional relationship did not correlate to the trends observed in the Taft plots, indicating that other factors were at play (**Figure 2**). Relative to hydrolysis kinetics of 4-nitrophenyl acetate, lipase and nattokinase were able to hydrolyze 4-nitrophenyl propanoate at a quicker rate, suggesting that binding pocket interactions with the aliphatic substituent may have improved

(a)



(b)

Substituent	E_s constant	Nattokinase $\log(k/k_{CH3})$	Trypsin $\log(k/k_{CH3})$	Lipase $\log(k/k_{CH3})$
Acetate	0.00	0.00	0.00	0.00
Propanoate	-0.07	0.500	-0.384	0.410
Isobutyrate	-0.47	0.295	-0.654	-0.0569
Pivalate	-1.54	0.0124	-0.586	-0.259
Phenyl	-0.38	-1.16	-1.13	-1.15

Figure 3: Evaluation of kinetic trends in the hydrolysis of substituted 4-nitrophenyl ester substrates. a) Taft plots for the enzymatic hydrolysis of substituted 4-nitrophenyl esters by lipase, trypsin, and nattokinase. b) Values used in the generation of Taft plots, including E_s constants and $\log(k/k_{CH3})$ values.

the stability of the enzyme-substrate complex. Generally, as the substituent ranged from propanoate to pivalate, hydrolytic rates decreased, which is consistent with initial hypotheses. As an exception, however, trypsin was able to cleave pivalate-substituted substrates slightly faster than isobutyrate-substituted substrates, which may similarly be due to differences in the intrinsic stability of the enzyme-substrate complex. Trypsin also seems to be most affected by sheer steric bulk, for which binding affinity does not always make up for; unlike lipase and nattokinase, the addition of the propanoate substituent did not improve reaction kinetics to be faster than that of the acetate-substituted substrate.

One additional difference between the Taft plots of all three enzymes was the shape created by the data points. While the line of best fit for nattokinase was fairly linear, the line of best fit for trypsin was more parabolic. This indicates the enzymatic sensitivity to steric effects relative to a substituent that is one degree less bulky; as trypsin hydrolyzes increasingly bulky substituents, it becomes less sensitive to changes in steric hindrance, whereas nattokinase's efficiency is more proportionally influenced by the sheer change in steric hindrance. With regards to diphenyl and triphenyl substituents, results were consistent with initial hypotheses as the addition of phenyl rings hindered reaction kinetics, so much so that neither of the three enzymes were able to hydrolyze 4-nitrophenyl 2,2,2-triphenylacetate, either because it did not fit into any of enzymes' active sites, because its positioning in the enzymatic active site was not conducive to reactivity, or because it instead favored interactions with residues near the active site. This may hint at the similarities in the size of the active sites of lipase, trypsin, and nattokinase, and the appropriate substrate size that is necessary to preserve enzymatic efficiency.

The nonconformity of the kinetic data to trends traditionally observed in LFER plots reflects the challenges of defining physical organic parameters in enzymatic systems, where reactivity is no longer solely bound by sterics and electronics, but rather by the physical structure of the enzymatic active site in addition to stereoelectronics. It is also difficult to define a ubiquitous set of parameters that can account for kinetic trends across all enzymatic systems, as each enzyme will exhibit differing sensitivity to various parameters. Still, LFERs provide useful tools for identifying these relative differences and in investigating enzymatic selectivity under these unique parameters. In the future, we may synthesize 4-nitrophenyl substrates with broader classes of substituents, such as those with hydrogen-bonding abilities, and determine which classes of substituents exhibit the greatest conformity to LFERs, allowing us to gain further mechanistic insight into the laws that govern the reactivity of biocatalysts. In the long term, the unique reactivity of enzymes can provide further opportunities for developing chemoselective reaction methodology.

Thus, in this study we applied the Taft LFER to model kinetic trends in biocatalytic transformations, allowing us to gain insight into the mechanisms of lipase, trypsin, and nattokinase, their relative sensitivity to steric effects, and potential binding interactions that may occur in the enzymatic active site. As biocatalysts gain increasing popularity in synthetic routes for their chemoselectivity, high catalytic efficiency, and reduced hazardous byproducts, our efforts bridge the versatile linear free-energy relationships that captivated the minds of many chemists in the 1900s to the current state of the field.

MATERIALS AND METHODS

Synthesis of 4-nitrophenyl propanoate, 4-nitrophenyl 2-phenylacetate, & 4-nitrophenyl 2,2-diphenylacetate

4-nitrophenyl esters were synthesized via acylation of 4-nitrophenol by an acid chloride (**Figure 4**). 4-nitrophenol (0.50 g, 1 eq., 3.6 mmol) was dissolved in methylene chloride (DCM) and added to a round-bottom flask equipped with a magnetic stir bar, along with 1 eq. triethylamine. The flask was septum-sealed and stirred until 4-nitrophenol dissolved completely. Next, 1.2 eq. of the respective acid chloride was added to the reaction mixture and the reaction was monitored to completion via thin-layer chromatography (TLC). Crude product was concentrated *in vacuo* and purified on silica gel flash chromatography with a gradient of 0-20% ethyl acetate in hexanes, yielding crystals of 4-nitrophenyl esters. 4-nitrophenyl propanoate (94% yield), 4-nitrophenyl 2-phenylacetate (77% yield), and 4-nitrophenyl 2,2-diphenylacetate (68% yield) were synthesized in this manner.

Synthesis of 4-nitrophenyl acetate & 4-nitrophenyl isobutyrate

4-nitrophenyl esters were synthesized via acylation of 4-nitrophenol by an anhydride (**Figure 5**). 4-nitrophenol (1.00 g, 1 eq., 7.2 mmol) was dissolved in ethyl acetate and the respective anhydride (15 eq., 107.8 mmol) was added, along with triethylamine (1.002 mL, 1 eq., 7.2 mmol). The reaction was monitored to completion via TLC. Unreacted anhydride was then quenched with methanol and the crude material was concentrated *in vacuo*. The crude product was extracted 3 times in ethyl acetate over saturated sodium bicarbonate to remove excess acetic acid. The combined organic layers were dried over anhydrous magnesium sulfate, concentrated *in vacuo*, and purified on silica gel flash chromatography with

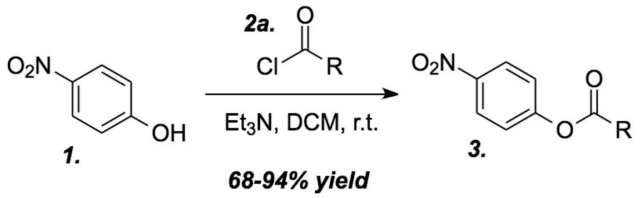


Figure 4: General procedure for the acylation of 4-nitrophenol by an acid chloride. 4-nitrophenyl esters (3) were synthesized via reaction between 4-nitrophenol (1) and the respective acid chloride (2a) in the presence of triethylamine.

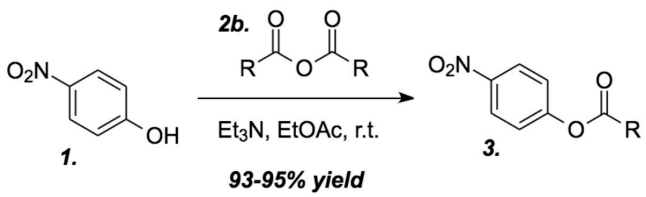


Figure 5: General procedure for the acylation of 4-nitrophenol by an anhydride. 4-nitrophenyl esters (3) were synthesized via acylation of 4-nitrophenol (1) by an anhydride (2b) in the presence of triethylamine.

a gradient of 0-20% ethyl acetate in hexanes to yield crystals of 4-nitrophenyl esters. 4-nitrophenyl acetate (95% yield) and 4-nitrophenyl isobutyrate (93% yield) were synthesized in this manner.

Synthesis of 4-nitrophenyl pivalate & 4-nitrophenyl 2,2,2-triphenylacetate

4-nitrophenyl esters were synthesized via a Steglich esterification between 4-nitrophenol and a carboxylic acid (**Figure 6**). Two eq. of the respective carboxylic acid was dissolved in DCM, to which EDC [1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride; 2.76 g, 2 eq. 20 mmol] and DMAP (4-dimethylaminopyridine; 0.26 g, 0.3 eq., 0.3 mmol) were added. After stirring for 5 minutes, 4-nitrophenol (1.00 g, 1 eq., 10 mmol) was added to the solution, and the reaction was monitored to completion via TLC. The crude product was extracted 3 times in ethyl acetate over saturated sodium bicarbonate to remove unreacted acid. The combined organic layers were then dried over anhydrous magnesium sulfate, concentrated *in vacuo*, and purified on silica gel flash chromatography with a gradient of 0-20% ethyl acetate in hexanes to yield crystals of 4-nitrophenyl esters. 4-nitrophenyl pivalate (85% yield) and 4-nitrophenyl 2,2,2-triphenylacetate (79% yield) were synthesized in this manner.

UV-visible spectroscopy

A Beer's Law plot of 4-nitrophenol was produced by collecting spectroscopic data at various micromolar concentrations using a Spectronic Genesys 5 UV-Vis spectrophotometer. 4-nitrophenol was prepared in 10% dimethyl sulfoxide (DMSO; DMSO Store, 99.995%) and 90% 10 mM Tris buffer at concentrations of 1.25 μM, 2.5 μM, 5 μM, and 10 μM. The peak absorbance of 4-nitrophenol was determined to be at 413 nm, which did not overlap with the absorption spectra of any of the substrates.

Enzyme and substrate solutions were prepared separately and then added into a glass cuvette together to monitor the rate of enzymatic hydrolysis. One millimolar substrate solutions of each of the seven compounds were prepared in DMSO, and enzyme solutions at pH 8 were prepared with 1 mM Tris base in deionized water and 0.5 mg enzyme per 1 mL of solution. Trypsin was purchased from Bio-Rad, lipase was purchased from Carolina Biological, and nattokinase was purchased from Belle Chemical. Once 1800 μL enzyme solution was added to 200 μL substrate solution in a glass cuvette, the absorbance at 413 nm was measured every minute for 6 minutes. Blank spectra were also taken for each of the substrates with 1 mM Tris buffer instead of enzyme or pH solution to account for hydrolysis of the substrate by deionized water. Blank readings

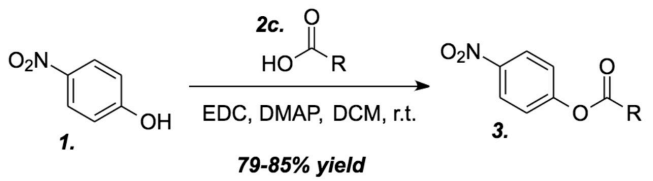


Figure 6: General procedure for Steglich esterification of 4-nitrophenol by a carboxylic acid. 4-nitrophenyl esters (3) were synthesized via esterification of 4-nitrophenol (1) by a carboxylic acid (2c) in the presence of EDC and DMAP.

were subtracted from readings collected from samples undergoing enzymatic hydrolysis and all experiments were repeated in triplicate.

The rate of hydrolysis of each substrate by each enzyme was determined by calculating the average rate of increase of the concentration of 4-nitrophenol over the first 6 minutes of hydrolysis. Average rate data were then inputted into the Taft equation and graphed alongside the Taft E_s constant (28).

Computational modeling

Each substrate was constructed virtually in Avogadro and optimized using DFT by ORCA (29,30). In all DFT calculations, CPCM implicit solvation with the dielectric constant of water was used as the solvation model, B3LYP was used as the functional, and def2-SVP was used as the basis set (31). Mulliken charges at the carbonyl carbon were then identified using these DFT calculations. Computational simulations and DFT calculations were performed on a Dell Poweredge 710 server with a 24 core Intel Xeon X5660 processor @ 2.80 GHz and 32 GB RAM.

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