Advanced Chemistry Classroom and Laboratory

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96-Well Plate Colorimetric Assay for K_i Determination of (±)-2-Benzylsuccinic Acid, an Inhibitor of Carboxypeptidase A

A Laboratory Experiment in Drug Discovery

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The course Drug Discovery (CHEM-4330) was initiated at Rensselaer Polytechnic Institute in the 2001 spring semester as part of a Howard Hughes Medical Institute-funded curriculum development of an undergraduate bioinformatics–molecular biology major. Drug Discovery (DD) is a required course for that major and serves as an elective for chemistry, biochemistry–biophysics, biology, chemical engineering, and related majors. Undergraduates take DD in their junior or senior years; two semesters of sophomore organic chemistry are prerequisites. Graduate students in chemistry, biology, and other disciplines also participate.

The goal of the course is to study applications of bioinformatics and genomics to the discovery of new drugs. DD is divided into a lecture component (1 hour 20 minutes, twice weekly) and a weekly three-hour laboratory. The objective of the lectures is to examine how modern biotechnologies are used in research to speed the discovery of new drugs, especially those small molecules to treat diseases with large unmet therapeutic need (e.g., cancer). Topics include high-throughput screening (HTS), combinatorial chemistry and molecular target (MT) validation using proteomics, and DNA and protein microarrays. Case studies such as the discovery of captopril *(1–5)* and imatinib *(6)* are interspersed through these lectures to illustrate successful approaches.

The objective of the lab component is to reduce to practice some of the HTS procedures used by researchers to discover drugs. Criteria used to select a MT for this lab were the following:

1. High relevance to human disease or to the discovery of useful drugs.
2. MT is amenable to 96-well format bioassay (e.g., no wash or filter steps) using absorbance or fluorescence microplate readers and can be accomplished in the three hours allotted for the lab.
3. MT is commercially available and relatively stable.
4. Inhibitors of the MT are commercially available or easily made.
5. X-ray crystal structures of MT-inhibitor complexes are available to students to rationalize the elements of molecular recognition and to develop a pharmacophore hypothesis.

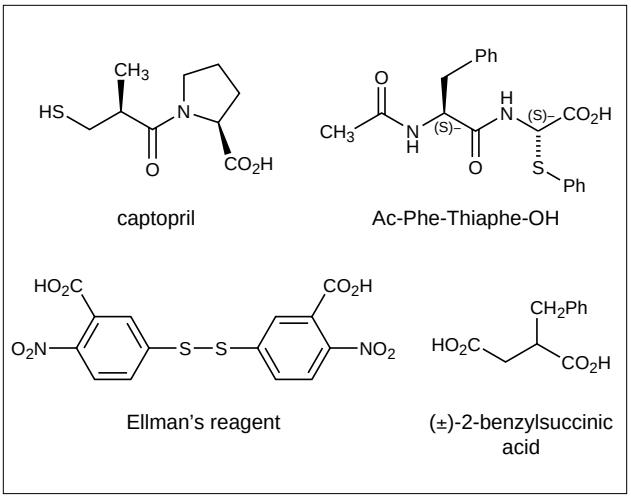

Figure 1. Structures of compounds.

The enzyme, carboxypeptidase A (CPA; EC 3.4.17.1; ref *7*), is ideally suited for our purpose. CPA fulfilled the criteria noted above including the fact that Squibb scientists used CPA-related biochemistry and inhibition in the 1970s to discover captopril (Figure 1), the first clinically useful inhibitor of angiotensin converting enzyme (ACE; refs *1–5*). Captopril and successor agents are important components of the physician's war chest to treat hypertension *(1)*. The case study of captopril is considered a "classic" because it is one of the first successful applications of rational drug design. In this approach, the structure of the natural substrate and the molecular mechanism of catalysis were used to aid the design of inhibitors. The Squibb team also recognized the close structural and biochemical relationship between ACE and the well-studied enzyme CPA; both are Zn-metalloproteinases that cleave an amide bond releasing a dipeptide (ACE) or single amino acid (CPA) from the C-terminus of substrate peptide. Using 2-benzylsuccinic acid (Figure 1), a byproduct inhibitor of CPA *(8)*, the structure of angiotensin I (decapeptide substrate for ACE), and a snake venom nonapeptide ACE inhibitor screening hit, Squibb scientists discovered captopril in rapid fashion.

With a strong rationale in hand for using 2-benzylsuccinic acid inhibition of CPA as a DD lecture tool, we developed an appropriate assay to determine its inhibition

potency in 96-well format to illustrate how HTS is used in modern drug discovery to identify bioactive molecules. Numerous absorbance *(8, 9)* and fluorescence *(10)* assays for CPA inhibition have been reported; however, we could not find reference to any performed in 96-well format. We report our efforts in developing a colorimetric 96-well plate assay for determination of the K_i for inhibition of CPA by (±)-2-benzylsuccinic acid using Ac-Phe-Thiaphe-OH (Figure 1) as substrate.

Reagents and Equipment

The reagents used in this experiment are commercially available and described in the Supplemental Material.[W] Solutions of reagents were dispensed into Corning 96-well polystyrene flat bottom clear plates using a variable pipetter (Labsystems Finnpipette), repeater pipetter (Eppendorf Repeater Plus Pipette), or a multichannel pipetter (Eppendorf Titermate). Kinetic data were generated using a Molecular Devices Corporation SpectraMax 340PC or SPECTRAmax PLUS^{384} plate reader with associated Windows-based SOFTmax PRO Version 3.1.2 software.

Experimental Procedure

A known procedure *(11)* performed in cuvettes (final volume 1 mL) for determination of the Michaelis constant (K_m) of Ac-Phe-Thiaphe-OH was first modified for 96-well plate format. We then developed a new 96-well assay to determine the K_i for inhibition of CPA by performing kinetic experiments in the presence of various concentrations of (±)-2-benzylsuccinic acid.

Following grading of the prelab, students worked individually or in groups of two or three per plate, depending on the size of that lab section. A multichannel pipetter was used for addition of enzyme; standard single channel or repeater pipetter was used at the student's discretion. Because adding enzyme in the last step initiated the reaction, students had to be very organized (i.e., plate reader reserved and software ready to go) before this last addition step. Assays were run in duplicate with the total volume of each well equal to 200 μL. The layout of wells, instrument settings, and order of addition are found in the Supplemental Material.[W] Formulas used by the software to generate K_m and K_i values were preprogrammed into each group table; however, students had to understand how each formula was derived.

After 25 min of plate reader time, students removed their raw data *.pda file to allow the next group to use the plate reader. Students then analyzed and refined the data (e.g., deleting an outlier well from the analysis), saving the *.pda file under a different name if it was modified in any way. Printouts of raw and refined (if needed) data *.pda file(s) were included in their final report along with discussion.

Hazards

Since CPA is a pancreatic-derived mammalian enzyme used in digestion and (±)-2-benzylsuccinic acid is a potent inhibitor of CPA, students use a hood and wear gloves and goggles when handling these and other reagents. MSDS sheets on all reagents are made available to students.

Results and Discussion

The Lineweaver–Burk plots generated by the software from raw data of an actual student-run experiment are shown in Figure 2. As would be expected from an enzyme–substrate–inhibitor system that displays Michaelis–Menten kinetics *(11)* and competitive inhibition *(8)*, all plots in Figure 2 converge on the *y* axis at the nearly the same point ($x = 0$). The intersection of the plots on the *x* axis (1/[S]) is related to the $K_{m(app)}$ (see Supplemental Material[W] for all formulas). For plot 1, $K_{m(app)}$ is the same as the K_m of substrate since [I] = 0 μM (Table 1). For plots 2–4, each K_i was calculated from the corresponding $K_{m(app)}$ and [I]. Results are shown in Table 1. Despite the fact that this experiment was performed only once by this student, low variability (R^2 = .946–.995) was observed and these data are in excellent agreement with literature values of K_m = 0.22 mM *(11)* and K_i = 1.1 μM *(8)*.

Besides being commercially available and stable, a major advantage of using Ac-Phe-Thiaphe-OH as substrate is the fascinating organic chemistry involved in the indirect detection of hydrolysis products at 405 nm *(11)*. Detection in the visible region is preferred because inexpensive polystyrene 96-well plates (versus UV transparent plates) can be used. As part of the prelab (see Supplemental Material[W]), students are asked to provide a scheme for all reactions involved in substrate hydrolysis, including release and breakdown of the colorless "unstable amine" product and subsequent detection with Ellman's reagent (Figure 1) to give yellow Ellman's anion.

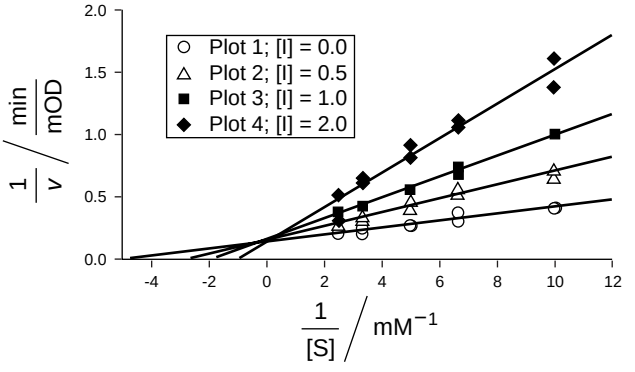

Figure 2. Lineweaver–Burk plots for the CPA catalyzed hydrolysis of Ac-Phe-Thiaphe-OH (initial concentrations vary from 0.1 mM to 0.4 mM) in the absence or presence of (±)-2-benzylsuccinic acid (inhibitor concentrations of 0, 0.5, 1.0, and 2.0 μM).

Table 1. Linear Fit[a] of the Lineweaver–Burk Plots Shown in Figure 2

Plot	[I]/μM	*A*	*B*	R^2	*K*/μM
1	0.0	0.134	0.028	.946	210[b]
2	0.5	0.144	0.055	.975	0.61[c]
3	1.0	0.150	0.085	.995	0.59[c]
4	2.0	0.134	0.139	.951	0.51[c]

[a] Plots fitted to $y = A + Bx$

[b] K_m

[c] K_i

Conclusions

In this experiment, students learn principles and techniques used by drug discovery HTS researchers including enzyme inhibition, Michaelis–Menten kinetics, Lineweaver–Burk plots, use of microplate readers and associated software, layout of bioassays, and multichannel and repeater pipetting. Because of the close ties to the lecture and the use of modern equipment, we find that experiments in DD genuinely ignite students' interest in biomedical science and enable them to make more informed decisions about their career paths. When working in groups and sharing common goals, they learn firsthand the benefits of teamwork, something that is a necessity in drug discovery research. We also observe a healthy sense of competition among groups to generate the best plots, values, et cetera. We plan to implement experiments where students resolve (±)-2-benzylsuccinic acid into its enantiomers and conduct inhibition assays to assess enantioselectivity of binding and inhibition.

Acknowledgments

We gratefully acknowledge the Howard Hughes Medical Institute for generous financial support, and Arno Spatola and DeAnna Long of Peptides International, Inc. for a gift of Ac-Phe-Thiaphe-OH used for assay development. We also thank the following people for their assistance: Hector Martinez at the University at Albany; John Salerno, Susan Smith, Donna Crone, Sandra Nierzwicki-Bauer, Karen Coonrad, Neal Gehani, and Xufeng Sun at Rensselaer Polytechnic Institute; and Evelyn McGown at Molecular Devices Corporation.

WSupplemental Material

Prelab instructions, guidelines for lab reports, questions for students, notes for instructors, and printouts of sample *.pda files are available in this issue of *JCE Online*.

Literature Cited

1. Zaman, M. A.; Oparil. S.; Calhoun, D. A. *Nat. Rev. Drug Disc.* 2002, *1*, 621–636.
2. Cushman, D. W.; Ondetti, M. A. *Nat. Med.* 1999, *5*, 1110–1112.
3. Cushman, D. W.; Cheung, H. S.; Sabo, E. F.; Ondetti, M. A. *Biochemistry* 1977, *16*, 5484–5491.
4. Ondetti, M. A.; Rubin, B.; Cushman, D. W. *Science* 1977, *196*, 441–446.
5. Wyvratt, M. J.; Patchett, A. A. *Med. Res. Rev.* 1985, *5*, 483–531.
6. Capdeville, R.; Buchdunger, E.; Zimmermann, J.; Matter, A. *Nat. Rev. Drug Disc.* 2002, *1*, 493–502.
7. (a) Vendrell, J.; Querol, E.; Avilés, F. X. *Biochem. Biophys. Acta*. 2000, *1477*, 284–298. (b) *Handbook of Proteolytic Enzymes;* Barrett, A. J., Rawlings, N. D., Woessner, J. F., Eds.; Academic Press: London, 1998; pp 1321–1326. (c) Lipscomb, W. N.; Sträter, N. *Chem. Rev*. 1996, *96*, 2375–2433. (d) Christianson, D. W.; Lipscomb, W. N. *Acc. Chem. Res.* 1989, *22*, 62–69. (e) *Enzyme Handbook;* Schomburg, D., Salzmann, M., Stephan, D., Eds.; Springer-Verlag: Berlin, 1991; Vol. 5, pp 1–6.
8. Byers, L. D.; Wolfenden, R. *Biochemistry* 1973, *12*, 2070–2078.
9. (a) Folk, J. E.; Schirmer, E. W. *J. Biol. Chem.* 1963, *238*, 3884–3894. (b) Auld, D. S.; Vallee, B. L. *Biochemistry* 1970, *9*, 602–609. (c) Vallee, B. L.; Riordan, J. F.; Bethune, J. L.; Coombs, T. L.; Auld, D. S.; Sokolovsky, M. *Biochemistry* 1968, *7*, 3547–3556. (d) Jacobsen, N. E.; Bartlett, P. A. *J. Am. Chem. Soc.* 1981, *103*, 654–657. (e) Mock, W. L.; Wang, L. *Biochem. Biophys. Res. Commun.* 1999, *257*, 239–243. (f) Mock, W. L.; Liu, Y.; Stanford, D. J. *Anal. Biochem.* 1996, *239*, 218–222. (g) Lee, K. J.; Kim, D. H. *Bioorgan. Med. Chem. Lett.* 1998, *6*, 1613–1622.
10. Latt, S. A.; Auld, D. S.; Vallee, B. L. *Anal. Biochem.* 1972, *50*, 56–62.
11. Brown, K. S.; Kingsbury, W. D.; Hall, N. M.; Dunn, G. L.; Gilvarg, C. *Anal. Biochem.* 1987, *161*, 219–225.

Supplementary Materials

96-Well Plate Colorimetric Assay for K_i Determination of (±)-2-Benzyl-succinic Acid, an Inhibitor of Carboxypeptidase A

A Laboratory Experiment in Drug Discovery

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Lab Documentation for Drug Discovery (CHEM-4330): Prelab Instructions

The objective of this experiment is to determine the K_i value of (±)-2-benzylsuccinic acid and the K_m value of Ac-Phe-Thiaphe-OH for carboxypeptidase A in kinetic mode.

Prelab:

1. Begin your prelab by drawing the structures involved in the CPA-catalyzed hydrolysis of Ac-Phe-Thiaphe-OH to Ac-Phe-OH and the corresponding unstable "amine byproduct", both of which are colorless. Since this assay is an absorbance-based assay done at 405 nm, we will use Ellman's reagent to convert the decomposition product of the "amine byproduct" to yellow Ellman's anion. Show the structures involved in these reactions as well. Draw these in the usual "Zig-Zag" [N- to C-terminus, left to right] representation and pay close attention to stereochemistry and ionization. Also draw the structure of the CPA inhibitor, (±)-2-benzylsuccinic acid (BS).

2. What are the literature values for the K_m of Ac-Phe-Thiaphe-OH for carboxypeptidase A and the K_i value of (±)-2-benzylsuccinic acid? Please refer to these papers:
   Brown, K. S.; Kingsbury, W. D.; Hall, N. M.; Dunn, G. L.; Gilvarg, C. *Anal. Biochem.* **1987**, *161*, 219-225.
   Byers, L. D.; Wolfenden, R. *Biochemistry* **1973**, *12*, 2070-2078.

3. Perform calculations to prepare the following:
   a. 250 mL of 0.2 M Tris-HCl buffer
   b. 10 mL of 5.0 M NaCl in distilled water
   c. 10 mL of 10.0 mM Ellman's Reagent in 0.2M Tris-HCl buffer
   d. 10 mL of 4.0 mM of Ac-Phe-Thiaphe-OH in 0.2M Tris-HCl buffer
   e. 20 mL of 4.2 nM CPA in 0.2M Tris-HCl buffer that comes from:
      - 1.0 mL a 210 nM "stock-210" solution in buffer that comes from:
      - 1.0 mL of a 0.5 mg CPA/mL "BSA-stock" solution (5% bovine serum albumin is required to stabilize protein) that comes from:
      - a 21 mg protein/mL "Sigma-stock" solution (use MW of CPA = 35,268)
   f. 10 mL of 2.0 mM solution of (±)-benzylsuccinic acid in 0.2M Tris-HCl buffer.
   g. 1.0 mL of 10.0 µM (±)-benzylsuccinic acid in 0.2M Tris-HCl buffer that comes from a 2.0 mM solution of (±)-benzylsuccinic acid in 0.2M Tris-HCl buffer.

Reagents and stock solutions needed for this experiment (Raw Material Source; CAS registry #) are the following: Use the hood and wear gloves and goggles when handling these reagents.

- 0.2 M pH 7.5 Tris-HCl buffer (Sigma T3253; 1185-53-1) - **supplied**
- 5.0 M NaCl in distilled water (Aldrich 22,351-4; 14762-51-7) - **supplied**
- 10 mM Ellman's Reagent (Aldrich D21,820-0; 69-78-3) in 0.2M Tris-HCl buffer - **supplied**
- 4.0 mM of Ac-Phe-Thiaphe-OH (Peptides International-http://www.pepnet.com/; 108906-59-8) in 0.2M Tris-HCl buffer [a.k.a. N-Acetyl-L-phenylalanyl-L-thiaphenylalanine or N-Acetyl-L-phenylalanyl-2-(2*S*)-(phenylthio)-glycine] - **supplied**
- 4.2 nM CPA (EC 3.4.17.1; Sigma C0261; 11075-17-5) in 0.2M Tris-HCl buffer stabilized with bovine serum albumin (Sigma A2153; 9048-46-8) - **supplied**
- 2.0 mM (±)-2-benzylsuccinic acid (Sigma B8011; 884-33-3) in 0.2M Tris-HCl buffer. - **supplied**
- 1.0 mL of 10.0 µM (±)-benzylsuccinic acid in 0.2M Tris-HCl buffer (**prepare** from 2.0 mM stock (±)-benzylsuccinic acid)

4. Download the *.pda file for this experiment from the Drug Discovery Lab website and fill in the Template using the information found in the Introduction. Also create a Layout for wells using the next page of these instructions. Formulas used by the software to perform calculations are pre-programmed in this *.pda file and can be found in the "Formulas" section. Save your new *.pda file with some unique (to you) filename and bring it to the lab. In the prelab section of your notebook, paste in the completed Layout and Template.

Lab report:

In addition to the usual items, please address the following points:

1. In the graph section of your *.pda file, the lines in Plots#1-4 should intersect the X-axis at different points and the Y-axis at the same point - Why? If they do not, explain why not. For comparison, see attached printout of a student-generated *.pda file containing very good results.
2. Compare your K_m values with literature values and explain any discrepancies. For the three K_i values you generated, compare them to each other and to the literature value and explain any discrepancies.

Lab Documentation for Drug Discovery (CHEM-4330): Prelab Instructions

[I]= 0 µM	[I]= 0.5 µM	[I]= 1.0 µM	[I]= 2.0 µM
1/2	3/4	5/6	7/8

Buffer - µL 0.2 M pH 7.5 Tris-HCl in well:

A
B
C
D
E
F

NaCl - µL of 5.0 M stock solution to give final [0.5 M]:

A
B
C
D
E
F

Ellman's Reagent - µL of 10 mM stock solution to give final [0.5 mM]:

A
B
C
D
E
F

Ac-Phe-Thiaphe-OH (substrate) - µL of 4.0 mM stock solution:

A
B
C
D
E
F

(±)-Benzylsuccinic acid - µL of 10 µM stock solution:

A
B
C
D
E
F

Carboxypeptidase A (enzyme) - µL of 4.2 nM stock solution to give final [0.84 nM]:

A
B
C
D
E
F

Lab Documentation for Drug Discovery (CHEM-4330): Guidelines for Lab Reports

Each student must hand in his/her own type-written lab report for grading following the completion of each Experiment. Unless stated otherwise, the report is due at the beginning of the next lab session. The penalty for late reports is 2 points for each day overdue. This is your own report and must be prepared independently even though you may have generated certain data as a group. What this means is that members of the same group might get quite different lab report grades. There is one exception - if you generated plate reader data as a group effort (e.g., Student A filled certain wells, Student B did others), you may (in fact, encouraged) work in groups to "refine" the data (see Results and Discussion below for what this means) to get the best line-fit.

The format for the lab report is as follows:

Title Page
- Title and number of Experiment
- Name of experimentalist
- Date report handed in (same as due date in most cases)
- Date(s) data were collected
- Name(s) of other group member(s) if applicable.

Abstract - A short description of the purpose of the experiment. Restate (from the prelab) the objective of the experiment in the first one or two sentences. Briefly (a few sentences) describe any techniques that will be studied. Try to inform the reader of the essence of the experiment in as concise a manner as you feel possible.

Background - Provide a short paragraph on the potential importance of generating this or similar data on the drug discovery process. Include other pertinent background information and theory you feel is important.

Procedure - Give a concise procedure that you used in this Experiment. Reference any pertinent literature.

Results and Discussion - Summarize the results of the Experiment but don't recopy the plate reader data; rather annotate the printout of the SOFTmax® PRO *.pda file with relevant information. Also discuss your results. Compare your results to published standards when available. Did you get the right value (e.g., K_m)? Why not? If you "refined" your data to get a better curve fit, explain why this was a valid thing to do (e.g., "due to a suspected pipetting error, I deleted the data from one well because it was so different than its duplicate). Elaborate on any difficulties. Is there more than one possible outcome? If your results were significantly different from published data, describe what might have caused the discrepancy. What would you do differently if you were to repeat the Experiment?

Conclusions - Summarize the completed experiment in one paragraph or less. Discuss what you learned from this lab and how it is relevant to your future studies.

Reference Section - Cite pertinent references for protocols, procedures, values, etc. in this section. For books and primary literature, respectively, use the following format:

Fink, M.; Freedman, A. M.; Resnick, R.; Zaks, A. In *Agonist and Antagonist Actions of Narcotic Analgesic Drugs;* Kosterlitz, H.W.; Collier, H.O.J.; and Villareal, J.G., Eds.; Macmillan, 1971; pp 266-276.

Newman, A.H.; Bevan, K.; Bowery, N.; Tortella, F.C. "Synthesis and Evaluation of 3-Substituted 17-Methylmorphinan Analogs as Potential Anticonvulsant Agents." *J. Med. Chem.* **1992**, *35*, 4135-4142.

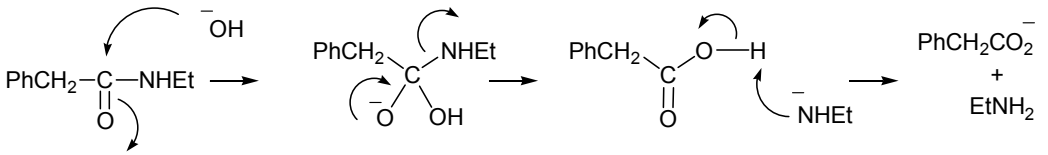
Appendix 1 - Hard copies (i.e., printouts) of:

a. SOFTmax® PRO *.pda file containing the raw data of the Experiment. Color printout is preferable but not mandatory.
b. SOFTmax® PRO *.pda file containing the "refined" data of the Experiment if you, in fact, did such a refinement.

Appendix 2 - Photocopy of the prelab part of your notebook.

Lab Documentation for Drug Discovery (CHEM-4330): Questions Related to Experiment

1. A detailed arrow-pushing type mechanism for the general base catalyzed hydrolysis of an amide is shown below. Draw a similar detailed arrow-pushing type mechanism for the general acid (i.e., 1N HCl) catalyzed hydrolysis of this amide.



2. Please refer to the simplified energy profile diagram shown in Figure 1 that illustrates the energetics of the two key steps (addition-elimination) involved in the CPA-catalyzed hydrolysis of a peptide substrate. Draw a detailed structure (e.g., stereochemistry, partial charges) for the second transition state (TS_2) in this process. Also illustrate the key molecular recognition elements used by the enzyme to lower its free energy.

3. Show the derivation of the following equation used by the SOFTmax® PRO Version 3.1.2 software to calculate the K_i of (±)-2-benzylsuccinic acid at a concentration of 1 µM. The software utilizes the data in the Lineweaver-Burk plots, Plot#1 and Plot#3, to perform this calculation.

K_i (in mM) = 0.001/((((ParmB('Plot#3@Lineweaver-Burk'))*(ParmA('Plot#1@Lineweaver-Burk')))/
((ParmA('Plot#3 @Lineweaver-Burk'))*(ParmB('Plot#1@Lineweaver-Burk'))))-1)

4. Analyze the non-covalent interactions that stabilize the binding of L-benzylsuccinic acid to the active site of CPA in the crystal structure 1CBX.pdb using rasmol. Highlight/label the groups that anchor the Zn ion in the active site of the enzyme in the same format used in Figure 2 to illustrate the interactions between inhibitor and Arg145. Note: To clarify this illustration, most of the protein was "removed" using the command: "restrict within (12.,ligand)" This commands stands for keeping only those amino acids of CPA within 12 Angstroms of the inhibitor in view. The distance between the two complementary H-bonds between the CO_2^- of inhibitor and protonated guanidine of Arg145 is illustrated by using the command "set picking monitor" followed by clicking the HBD and HBA groups. The distances (2.70 and 2.74) are barely visible. Submit your answer in color in the usual way.

Figure 1

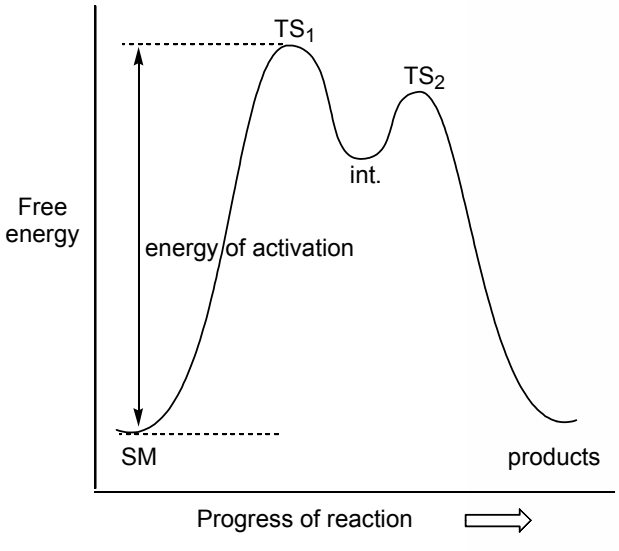
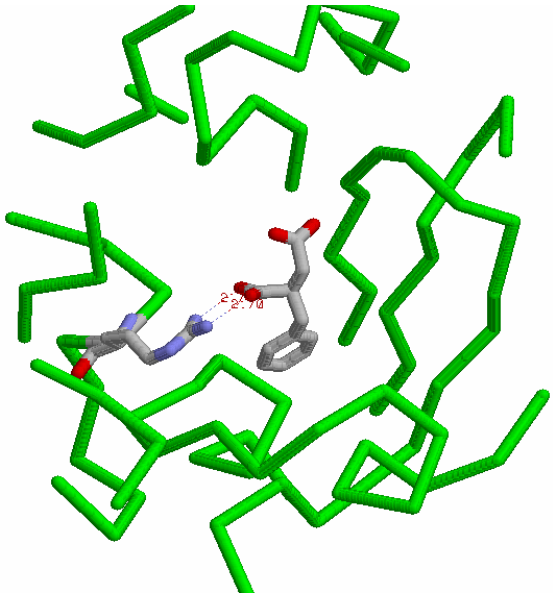


Figure 2



Lab Documentation for Drug Discovery (CHEM-4330): Notes for Instructors

Timing: At Rensselaer, there are two sections of DD lab held on Monday and Tuesday afternoons; each is at or near the maximum capacity of 12 of students. It takes about one hour to grade prelabs, load software, and organize, prepare and dispense reagents into 96-well plates. During the remaining 2 hours, each plate reader can generate kinetic data on three plates in sequence. This translates into roughly 6 groups of two students each using two plate readers simultaneously. We have also used a "flex-time" arrangement with some success where some groups come earlier and others come later to avoid periods of waiting for plate reader time. Of course, more students can be accommodated for this experiment if there are more than two plate readers, the lab is longer than 3 hours, there are more sections, and/or there are more than two students per group.

Results: Some but not all results came out as well as the Sample. As one would predict, careful planning and technique by the experimentalist(s) positively correlated with the quality of the results. If results were not acceptable to the group, students were encouraged to repeat the experiment in that or another section, if time allowed.

Stability of reagent solutions:

- Substrate - From experimentation, we found that 4.0 mM Ac-Phe-Thiaphe-OH in 0.2M Tris-HCl refrigerated for 9 days (spans 4 labs) gave the same results as freshly prepared solution. When stored in the refrigerator for one year, solid Ac-Phe-Thiaphe-OH worked as well as newly-purchased material.
- Enzyme - 4.2 nM CPA in 0.2M Tris-HCl buffer stabilized with bovine serum albumin was freshly prepared each Monday for that and the next day's lab from 0.5 mg CPA/mL "BSA-stock" solution. This later solution was stored in the refrigerator and the same solution was used for three weeks without any evidence of decomposition.
- Ellman's Reagent: 10 mM Ellman's Reagent in 0.2M Tris-HCl buffer was freshly prepared each Monday for that and the next day's lab. We noted variable results using a solution that had been stored for one week which we believe is due to decomposition.
- Other reagents: 0.2 M pH 7.5 Tris-HCl buffer, 5.0 M NaCl in distilled water, and 2.0 mM (±)-2-benzylsuccinic acid in 0.2M Tris-HCl buffer were stored for several weeks at 25 ^{o}C with no evidence of decomposition.

Formulas used by the software to generate kinetic parameters (e.g., K_m): The *.pda file type and the format of formulas the software use, are, to our understanding, unique to the SOFTmax® PRO software. In this course, however, we detail the derivation of the formulas the software utilizes to generate kinetic data such as K_m, $K_{m(app)}$ and K_i based on knowledge of the Lineweaver-Burk double reciprocal plot. Derivation of these formulas (see first page of the attached Sample *.pda) is fair game for exam and/or homework questions (see page 4, question 3 in the "Questions related to Experiment" section). Using K_m, the following is an example of one such derivation:

From standard biochemistry doctrine:

- The intercept of the Lineweaver-Burk plot with the 1/[S] axis = - $1/K_m$ (i.e., x when y = 0)
- The general equation for the straight line of a Lineweaver-Burk double reciprocal plot (1/v versus 1/[S]) is:
  y = A + Bx; where A is the y intercept at x = 0 and B is the slope
- Solving for x when y = 0; x = - A/B which is the same as - $1/K_m$

K_m equation used by software:[†]

- Km = (ParmB('Plot#1@Lineweaver-Burk'))/(ParmA('Plot#1@Lineweaver-Burk')); where Plot#1 is the graph of the computer generated Lineweaver-Burk double reciprocal plot with no inhibitor present; ParmB is parameter B of the computer-generated equation of for the straight line; ParmA is the corresponding parameter A.

Other equations used by software for this experiemnt:[‡]

- Km(app) = (ParmB('Plot#X@Lineweaver-Burk'))/(ParmA('Plot#X@Lineweaver-Burk')); where Plot#X is the double reciprocal plot when inhibitor at a specified concentration is added.
- Ki = ([I] in mM)/(((((ParmB('Plot#X@Lineweaver-Burk'))*(ParmA('Plot#1@Lineweaver-Burk')))/ ((ParmA('Plot#X@Lineweaver-Burk'))*(ParmB('Plot#1@Lineweaver-Burk'))))-1))); this is derived from the following standard biochemistry equation: $K_i = [I]/(B_x \cdot A/A_x \cdot B)-1$

[†]Equation came packaged with the software product. [‡]Equations were derived by one of authors (S.R.).

V_{max}: This Michaelis-Menten kinetic parameter is generally reported in units of [concentration]/time and can't be directly quantified from the data generated in this experiment as described. The plate reader measures the change in OD (optical density) at 405 nm over time and records the velocity in units of milliOD/min. Therefore, without relating milliOD to a particular concentration of Ellman's anion (i.e., generating a standard curve), the software-generated Lineweaver-Burk plot, as it is programmed in this experiment, can't be used to directly give the Michaelis-Menten kinetic parameter, V_{max}, in units of [concentration]/time.

Lab Documentation: Printout of Sample *.pda File

The objective of this experiment is to determine the Ki value of (±)-2-benzylsuccinic acid and the Km value of Ac-Phe-Thiaphe-OH for carboxypeptidase A in kinetic mode.

General Directions:

- The number of students per plate will be determined on the day of the lab depending on the size of the class and availability of equipment. Most likely there will be two students per plate.
- Results will be found in each Group Table (e.g., RxnW-[I]=0). Formulas used by the software to generate these values are found in each Group Table and can be visualized by clicking "Group" from the drop down menu followed by "Show Formulas". Generic versions of the formulas are also shown in the section "Formulas".
- Use any colorimetric plate reader (sample data was generated using SpectraMax® 340PC plate reader).
- The order of addition will be buffer, NaCl, Ellman's, substrate, inhibitor, and lastly enzyme. Use the multichannel pipetter for enzyme and repeater pipetter as appropriate.
- **Adding enzyme starts the reaction. If done too soon before plate reading, your data will be compromised. Be very, very organized at this step and make sure plate reader and software are ready for your use.**

Template/layout:

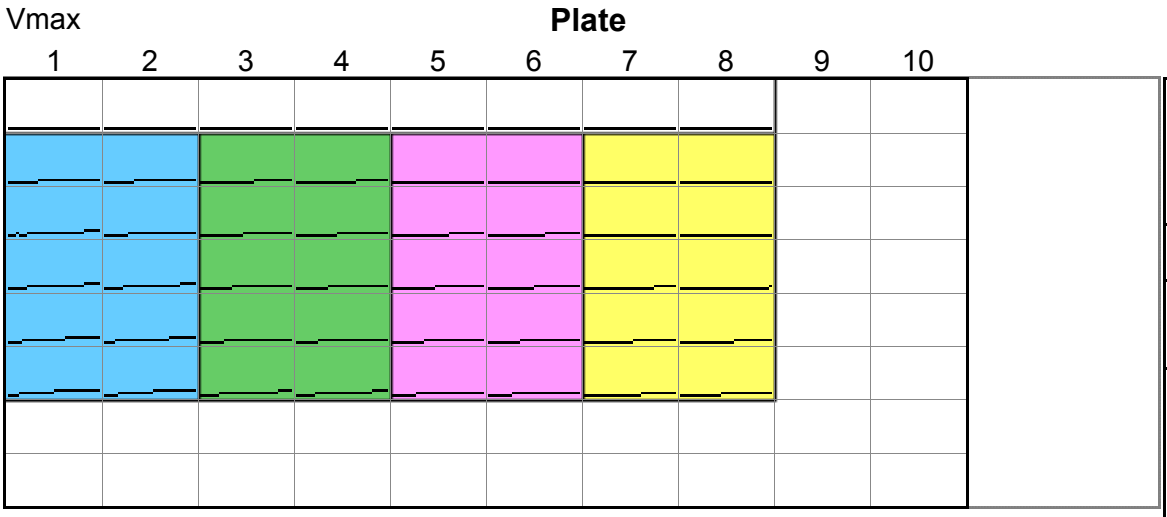
- Total volume of each well = 200 uL
- Run assays in duplicate
- A1-8 = BL (Blank) contains Tris-HCl buffer, NaCl in final [0.5 M];Ellman's Reagent in final [0.5 mM]
- B1-8 through F1-8 are Reaction Wells (Rx0*) containing contents of Blank plus enzyme in final [0.84 nM]
- Reaction wells labeled B, C, D, E & F contain final [S] of 0.10, 0.15, 0.20, 0.30 & 0.40 mM, respectively
- Reaction wells in lanes 1/2, 3/4, 5/6 & 7/8 contain final [I] of 0, 0.5, 1.0 & 2.0 uM, respectively.

Instruments settings in "Setup" are as follows: Kinetic Mode; Wavelength = 405 nm; Readings at one minute intervals for 25 minutes; Automix = 10 seconds before first reading; Blanking and Pre-read - off; AutoCalibrate = on; and Read strips = 1-8; Temperature = 25 oC.

Formulas used to generate various values using results from Plots #1-4 can be easily viewed by highlighting and expanding the desired Group Table followed by Group (drop down menu) - Show Formulas. A summary of generic formulas are as follows (please note that all use mM units [comments are shown in brackets]):

- R Squared = RSquared('Plot#X@Lineweaver-Burk') [**R Squared** indicates the "Goodness of fit" of the data to the linear regression equation]
- Km = (ParmB('Plot#1@Lineweaver-Burk'))/(ParmA('Plot#1@Lineweaver-Burk'))
- Km(app) = (ParmB('Plot#X@Lineweaver-Burk'))/(ParmA('Plot#X@Lineweaver-Burk'))
- v = (!WellValues) [shows each duplicate; "v = Average(!WellValues)" shows the average of the duplicates]["v" in the this equation is Softmax PRO's Vmax , i.e. the initial velocity of the reaction at various substrate concentrations]
- Ki = ([I] in mM)/((((ParmB('Plot#X@Lineweaver-Burk'))*(ParmA('Plot#1@Lineweaver-Burk')))/ ((ParmA('Plot#X@Lineweaver-Burk'))*(ParmB('Plot#1@Lineweaver-Burk'))))-1)

Lab Documentation: Printout of Sample *.pda File



Kinetic	
Time:	25:00
Interval:	1:00
Reads:	26

Lm1 405

Automix: Once
Calibrate: On

Lag Time: 0:00
End Time: 25:00
OD Min: 0
OD Max: 1
Vmax Pts: 26/26

Wavelength Combination: !Lm1
Data Mode: Absorbance
Plate Blank Used

RxnW-[I]=0 (uM)

Sample	[S] - mM	1/[S]	Wells	v (mOD/min)	1/v	v/[S]	[S]/v
Rx01	0.100	10.000	B1	2.414	0.414	24.136	0.041
			B2	2.483	0.403	24.829	0.040
Rx02	0.150	6.667	C1	3.330	0.300	22.203	0.045
			C2	2.735	0.366	18.237	0.055
Rx03	0.200	5.000	D1	3.729	0.268	18.646	0.054
			D2	3.709	0.270	18.547	0.054
Rx04	0.300	3.333	E1	4.609	0.217	15.365	0.065
			E2	4.054	0.247	13.514	0.074
RX05	0.400	2.500	F1	5.128	0.195	12.821	0.078
			F2	4.970	0.201	12.425	0.080

R Squared = 0.946
Km(app) = Km (in mM) = 0.21

Lab Documentation: Printout of Sample *.pda File

RxnW-[I]=0.5 (uM)

Sample	[S] - mM	1/[S]	Wells	v (mOD/min)	1/v	v/[S]	[S]/v
Rx01	0.100	10.000	B3	1.528	0.654	15.284	0.065
			B4	1.422	0.703	14.222	0.070
Rx02	0.150	6.667	C3	1.828	0.547	12.188	0.082
			C4	1.922	0.520	12.816	0.078
Rx03	0.200	5.000	D3	2.426	0.412	12.130	0.082
			D4	2.190	0.457	10.949	0.091
Rx04	0.300	3.333	E3	2.962	0.338	9.874	0.101
			E4	3.283	0.305	10.944	0.091
Rx05	0.400	2.500	F3	3.674	0.272	9.186	0.109
			F4	3.786	0.264	9.464	0.106

R Squared = 0.975
Km (app)(in mM) = 0.382
Ki (in mM) = 0.00060

RxnW-[I]=1.0 (uM)

Sample	[S] - mM	1/[S]	Wells	v (mOD/min)	1/v	v/[S]	[S]/v
Rx01	0.100	10.000	B5	1.001	0.999	10.011	0.100
			B6	0.992	1.008	9.925	0.101
Rx02	0.150	6.667	C5	1.367	0.731	9.116	0.110
			C6	1.448	0.690	9.656	0.104
Rx03	0.200	5.000	D5	1.819	0.550	9.096	0.110
			D6	1.790	0.559	8.949	0.112
Rx04	0.300	3.333	E5	2.338	0.428	7.793	0.128
			E6	2.359	0.424	7.862	0.127
Rx05	0.400	2.500	F5	2.660	0.376	6.650	0.150
			F6	2.608	0.384	6.519	0.153

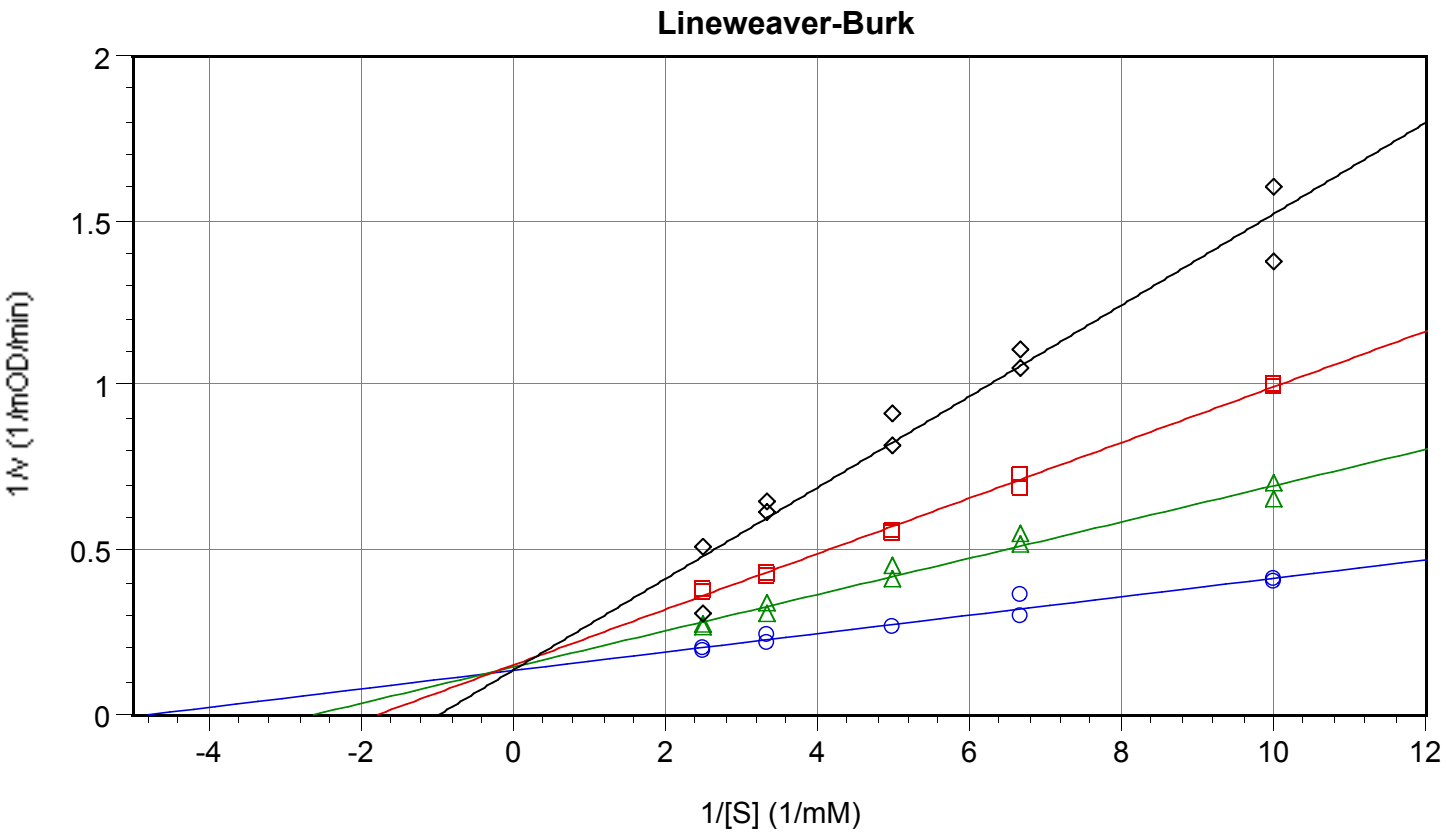
R Squared = 0.995
Km (app)(in mM) = 0.564
Ki (in mM) = 0.00059

RxnW-[I]=2.0 (uM)

Sample	[S] - mM	1/[S]	Wells	v (mOD/min)	1/v	v/[S]	[S]/v
Rx01	0.100	10.000	B7	0.726	1.377	7.261	0.138
			B8	0.623	1.605	6.230	0.161
Rx02	0.150	6.667	C7	0.954	1.049	6.357	0.157
			C8	0.903	1.107	6.020	0.166
Rx03	0.200	5.000	D7	1.221	0.819	6.106	0.164
			D8	1.093	0.915	5.467	0.183
Rx04	0.300	3.333	E7	1.617	0.618	5.390	0.186
			E8	1.544	0.648	5.145	0.194
Rx05	0.400	2.500	F7	3.213	0.311	8.032	0.125
			F8	1.949	0.513	4.873	0.205

R Squared = 0.951
Km (app)(in mM) = 1.032
Ki (in mM) = 0.00051

Lab Documentation: Printout of Sample *.pda File



	y = A + Bx:	A	B	R^2
○ Plot#1 (RxnW-[I]=0: 1/[S] vs 1/v)		0.134	0.028	0.946
△ Plot#2 (RxnW-[I]=0.5: 1/[S] vs 1/v)		0.144	0.055	0.975
□ Plot#3 (RxnW-[I]=1.0: 1/[S] vs 1/v)		0.15	0.085	0.995
◇ Plot#4 (RxnW-[I]=2.0: 1/[S] vs 1/v)		0.134	0.139	0.951