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Quantitative Analyses of Biochemical Kinetic Resolutions of Enantiomers†

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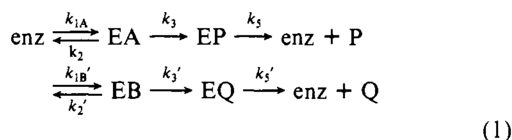
Abstract: Equations and useful graphs for the quantitative treatment of biochemical kinetic resolution data have been developed. These expressions have been verified experimentally, and they possess predictive values in relating the parameters of the extent of conversion of racemic substrate (c), the optical purity expressed as enantiomeric excess (ee), and the enantiomeric ratio (E).

Recently, Sharpless and his co-workers[1] introduced an elegant format for the organization of chemical kinetic resolution data. Although kinetic resolutions of racemic substances using enzymes and microorganisms have been extensively used,[2] there is a need of methods for the quantitative treatment of biochemical data that will allow synthetic chemists to make useful predictions. We herein describe the formulation of effective expressions to relate the three key parameters: the extent of conversion of racemic substrate (c), the optical purity, expressed as enantiomeric excess (ee) of the product or the remaining substrate, and the enantiomeric ratio (E).

Suppose that A and B are the fast and slow reacting enantiomers that compete for the same site on the enzyme. For a simple three-step kinetic mechanism assuming the reaction is virtually irreversible and there is no product inhibition (eq 1), the



$$\mathrm{enz} \underset{k_2}{\overset{k_{1A}}{\rightleftharpoons}} \mathrm{EA} \xrightarrow{k_3} \mathrm{EP} \xrightarrow{k_5} \mathrm{enz} + \mathrm{P}$$
$$\underset{k_2'}{\overset{k_{1B}'}{\rightleftharpoons}} \mathrm{EB} \xrightarrow{k_3'} \mathrm{EQ} \xrightarrow{k_5'} \mathrm{enz} + \mathrm{Q} \qquad (1)$$

ratio of the two partial reaction rates (v_A and v_B) may be shown by steady-state kinetics to be

$$\frac{v_A}{v_B} = \frac{V_A}{V_B}\frac{K_B}{K_A}\frac{A}{B} \qquad (2)$$

where V_A, K_A and V_B, K_B denote maximal velocities and Michaelis constants of the fast- and slow-reacting enantiomers, respectively.

Integration of eq 2 affords the homocompetitive equation 3, which reveals that *the discrimination between two competing enantiomers (A and B) by enzymes is dictated by E, the ratio of the specificity constants,*[3] *V/K.*

$$\frac{\ln (A/A_0)}{\ln (B/B_0)} = \frac{V_A/K_A}{V_B/K_B} = E \qquad (3)$$

In instances where kinetic resolution experiments are conducted by the selective destruction of one of the antipodes e.g., D-amino acid oxidases[4] have been used to oxidize D-α-amino acids preferentially, leaving most of the L acids unchanged, the relationship between the extent of conversion (c) and the enantiomeric excess of the recovered substrate fraction (ee(S)) for various values of the enantiomeric ratio (E) is governed by

$$\frac{\ln [(1-c)(1-\mathrm{ee(S)})]}{\ln [(1-c)(1+\mathrm{ee(S)})]} = \frac{V_A/K_A}{V_B/K_B} = E \qquad (4)$$

where

$$c = 1 - \frac{A+B}{A_0+B_0} \qquad \mathrm{ee(S)} = \frac{B-A}{A+B}$$

This expression is similar to that adopted by Sharpless[1] for the chemical resolution of allylic alcohols via enantioselective epoxidation. Although eq 4 yields an identical graph (Figure 1A) correlating the three interrelated variables (ee(S), c, and E), it should be borne in mind that the biochemical constant E has a vastly different connotation than the corresponding chemical constant K. In chemical kinetic resolutions, the chiral reagent is always in excess and is not recycled. Hence, K is simply the ratio of the two first-order or pseudo-first-order rate constants k_A/k_B. In contrast, under steady-state conditions, substrates A and B compete for free enzyme, which is being regenerated by enzymatic turnover. However, not all of the steps of regeneration contribute to the competition. For example, in mechanism 1, $[\mathrm{EP}]k_5 = [\mathrm{EQ}]k_5'$ at steady state,[5] and k_5 vs. k_5' does not influence the relative rates of disappearance of A and B. The remaining portion of turnover is governed solely by V/K. Consequently, E *is dependent on the ratio of the specificity constants (V/K) and is independent of substrate concentrations.*

In kinetic resolution experiments using hydrolytic enzymes such as acylases[6a] and carboxyesterases,[6b] it is also desirable to relate c, the extent of conversion, and ee(P), enantiomeric excess of the product fraction, to various values of E, for reasons which will become apparent. This correlation may be achieved by converting eq 3 into a form (eq 5) more compatible with experimental data and more suitable for graphical representation[7]

$$\frac{\ln [1 - c(1+\mathrm{ee(P)})]}{\ln [1 - c(1-\mathrm{ee(P)})]} = \frac{V_A/K_A}{V_B/K_B} = E \qquad (5)$$

where

$$c = 1 - \frac{A+B}{A_0+B_0} \qquad \mathrm{ee(P)} = \frac{P-Q}{P+Q}$$

It is evident that knowledge of any two of the variables allows the definition of the third. This graph (Figure 1B) shows the

† This paper is dedicated to Professor Henry A. Lardy on the occasion of his 65th birthday.

(1) Martin, V. S.; Woodard, S. S.; Katsuki, T.; Yamada, Y.; Ikeda, M.; Sharpless, K. B. *J. Am. Chem. Soc.* **1981**, *103*, 6237.

(2) For examples of biochemical kinetic resolution, see: (a) Bentley, R. In "Molecular Asymmetry in Biology"; Academic Press: New York, 1969; Vol. 1, Chapter 6. (b) Jones, J. B.; Beck, J. F. In "Techniques in Chemistry"; Jones, J. B., Sih, C. J., Perlman, D., Eds.; Wiley: New York, 1976; Vol. 10, Chapter 4.

(3) For a definition of V/K, see: (a) Fersht, A. In "Enzyme Structure and Mechanism"; Freeman: San Francisco, 1977; Chapters 3 and 10. (b) Walsh, C. In "Enzymatic Reaction Mechanisms"; Freeman: San Francisco, 1979; p 120.

(4) Meister, A.; Levintow, L.; Kingsley, R. B.; Greenstein, J. P. *J. Biol. Chem.* **1951**, *192*, 535.

(5) A comprehensive treatment of V/K effects with competitive substrates is beyond the scope of this paper. For a lucid explanation, see: Northrop, D. *J. Am. Chem. Soc.* **1981**, *103*, 1208.

(6) (a) Greenstein, J. P.; Winitz, M. In "Chemistry of the Amino Acids"; Wiley: New York, 1961; Vol. 2, p 1734. (b) Krisch, K. In "The Enzymes", 3rd ed.; Academic Press: New York, 1971; Vol. 5, Chapter 3.

(7) Figure 1B was computer generated by relating the variables c and ee(P) in eq 5 to a function of x for values of $0 \le x \le 1$; $c = 1 - x/2 - x^E/2$; and $\mathrm{ee(P)} = (x - x^E)/(2 - x - x^E)$. Implicit functions (eq 5 and 6) were solved by parametric representations. See: Buck, R. C. In "Advanced Calculus", 2nd ed.; McGraw Hill: New York, 1965; p 313. Copies of the computer programs are available upon request.

(8) (a) Jones, J. B.; Marr, P. W. *Tetrahedron Lett.* **1973**, 3165. (b) Chen, C. S.; Fujimoto, Y.; Sih, C. J. *J. Am. Chem. Soc.* **1981**, *103*, 3580.

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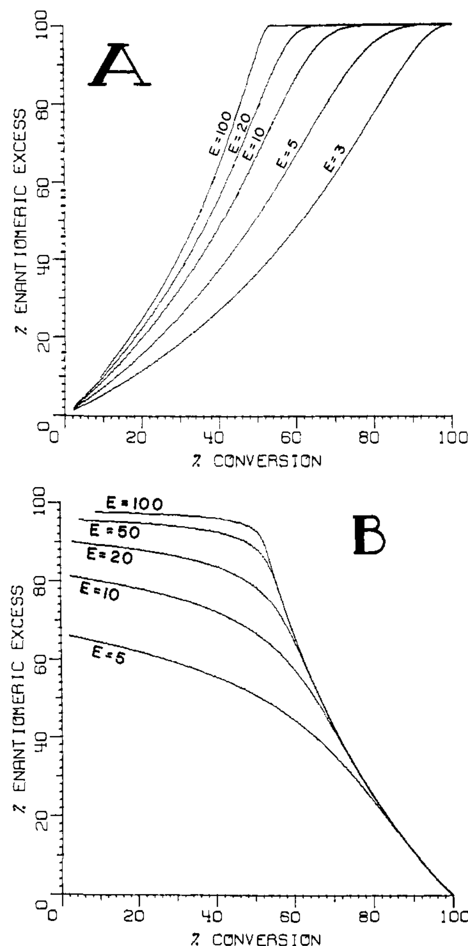


Figure 1. Plot of percent enantiomeric excess (ee) as a function of the percent conversion for various enantiomeric ratios (E): (A) substrate remaining, ee(S) = $(B - A)/(A + B)$; (B) product, ee(P) = $(P - Q)/P + Q)$.

abrupt decrease in ee(P) for values of c beyond 0.5. Consequently, one should not carry out the conversion beyond 50%, irrespective of the value of E.

There are numerous advantages to using hydrolytic enzymes for kinetic resolutions of enantiomers. These enzymes possess broad substrate specificities, favorable equilibria, and require no coenzymes for catalyses. But more importantly, the resulting products, P and Q ($P > Q$), derived from the first-resolution experiment, may be reesterified to regenerate A and B, respectively. The optical purity of this enriched fraction ($A > B$) may be further enhanced by its reincubation with the same biochemical system. In recycling studies, ee_0 (the enantiomeric excess of the initial antipodal mixture $[(A_0 - B_0)/(A_0 + B_0)]$), is always greater than zero. Hence, a new expression is needed to relate the variables: c, the extent of conversion $[1 - (A + B)/(A_0 + B_0)]$; E, the enantiomeric ratio; ee_0; and ee′, the enantiomeric excess of the recycled product fraction $[(P' - Q'/(P' + Q')]$.

If we consider 1 mol of an antipodal mixture with an initial enantiomeric excess of ee_0, this would contain $(1 + ee_0)/2$ moles of A_0 (fast reacting) and $(1 - ee_0)/2$ moles of B_0 (slow reacting). Hence, $A = (1 + ee_0)/2 - c[(1 + ee')/2]$ and $B = (1 - ee_0)/2 - c[(1 - ee')/2]$. Substitution of these terms into eq 3 affords eq 6. A plot[9] of this equation gives an array of useful theoretical

$$\left[1 - c\left(\frac{1 + ee'}{1 + ee_0}\right)\right] = \left[1 - c\left(\frac{1 - ee'}{1 - ee_0}\right)\right]^E \quad (6)$$

curves (Figure 2). It provides an overview of the interrelationships

(9) Figure 2 was computer generated by equating c and ee_0 to a function of x for values $0 \le x \le 1$, with ee′ fixed at 0.98; $c = [100(1 - x)(1 - x^E)]/(100 - 99x - x^E)$; and $ee_0 = (98 - 99x + x^E)/(100 - 99x - x^E)$.

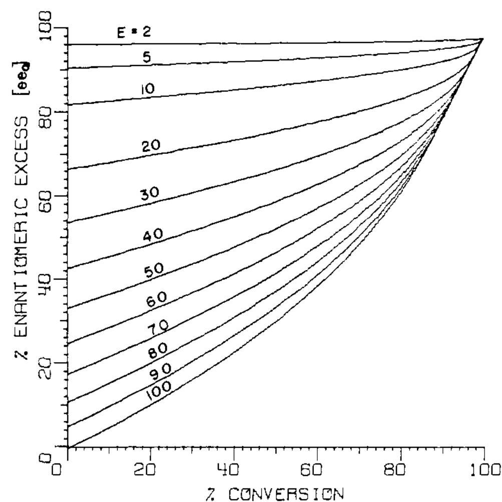


Figure 2. Plot showing the interrelationships of the initial percent enantiomeric excess (ee_0), the percent conversion (c), and the enantiomeric ratios (E) when the final enantiomeric excess (ee′) was fixed at 0.98.

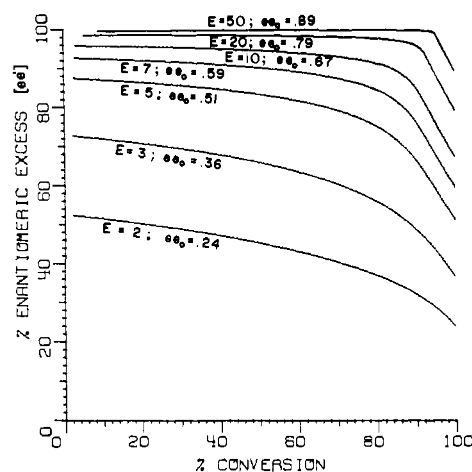


Figure 3. Expression of the final percent enantiomeric excess (ee′) as a function of the percent conversion for various values of initial enantiomeric excess (ee_0) and enantiomeric ratio (E).

between the variables ee_0, E, and c for a fixed value of ee′, which was set at 0.98.[10] For example, let us suppose that an antipodal mixture with an ee_0 of 0.40 is exposed to a biochemical system (enzyme or microorganism) possessing an E value of 50. To obtain the product fraction with a ee′ of ≥0.98, it is necessary to terminate the reaction when $c \le 0.21$. For a biochemical system with an E value of 60 in the above incubation, the reaction may be extended until $c \le 0.38$. In recycling work, the values of E and ee_0 are known and ee′ is usually fixed at 0.98.[10] Figure 2 enables one to predict the maximum conversion allowed to obtain a product fraction with an ee′ value of 0.98. Thus, the graph indicates when the biochemical reaction should be terminated.

When enzymes with modest E (5–10) values are used, it is perhaps more advantageous to arrange the aforementioned variables into the format shown in Figure 3. This graph[11] allows one to estimate the relationship between ee′ and c at various fixed values of E and ee_0. For example, starting with an antipodal mixture with an ee_0 of 0.67 and E of 10, the ee′ obtainable after 80% conversion is 0.91. When $c = 1$, ee′ = ee_0. In principle, the product could be recycled an infinite number of times to achieve

(10) The ee′ was fixed at 0.98 because the conventional methods used for ee determinations have accuracies of ±2%. For ee′ at 0.99, $c = [200(1 - x)(1 - x^E)]/(200 - 199x - x^E)$ and $ee_0 = (198 - 199x + x^E)/(200 - 199x - x^E)$.

(11) Figure 3 was generated by the use of ee and E of Figure 1B at $c = 0.50$. These values were substituted into eq 6 and the values of ee′ and c were related to a function of x for values $0 \le x \le 1$; $c = [2 - (1 - ee_0)x - (1 + ee_0)x^E]/2$ and $ee' = [2ee_0 + (1 - ee_0)x - (1 + ee_0)x^E]/[2 - (1 - ee_0)x - (1 + ee_0)x^E]$.

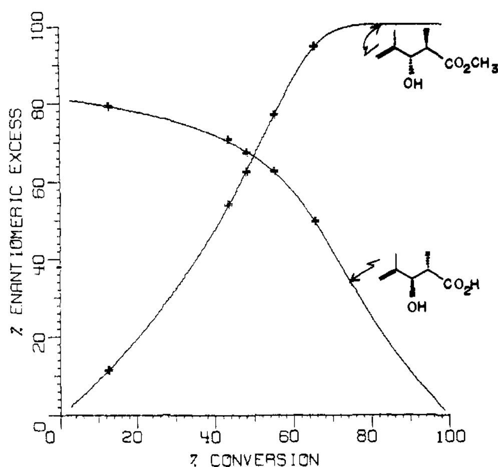


Figure 4. Dependence of percent enantiomeric excess (ee) on the percent conversion. The curves for $E = 9.7$ were computer generated from eq 4 (ester) and 5 (acid). # = Experimentally determined values with PLE.

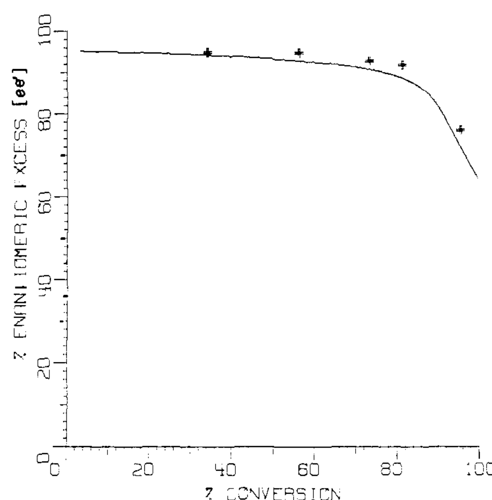
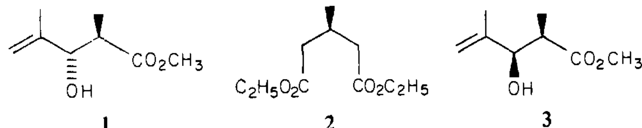


Figure 5. Final percent enantiomeric excess (ee′) as a function of the percent conversion. The curves for $E = 9.7$ and $ee_0 = 0.64$ were computer generated (eq 6). # = Experimentally determined values (PLE).

the ultimate goal of absolute optical purity (99.99...%). In reality, to prepare enantiomers with ee′ values of >0.98, it would be more convenient to select biochemical systems with E values of ≥10 and subject the product of recycling not more than two times.

Unfortunately, no suitable published biochemical data are available[12] to test the validity of our basic formulations. We therefore decided to collect the desired data by conducting kinetic resolution experiments using three different substrates with a purified enzyme preparation and an intact microorganism with different E values to provide the diversity needed to fully scrutinize the fidelity of our theoretical equations. These substrates were chosen because in their chiral forms, they are useful bifunctional synthons for our synthetic applications.[13]



Pig Liver Esterase (PLE). This carboxyesterase[6b] was selected for our investigations because it is commercially available in a highly purified form. Further, it has already been successfully applied to a number of synthetic problems.[8b,14]

(a) Resolution of ±1. The (±)-threo ester[15] (**1**) was incubated with PLE in Tris buffer, pH 8.0 at 25 °C. At various intervals, the values of ee(S) and ee(P) were determined,[16] from which c may be calculated by using the relationship[17] $c = (\mathrm{ee(S)} + \mathrm{ee}_0)/(\mathrm{ee(S)} + \mathrm{ee(P)})$. The remaining (+) ester (62.5% ee) was transformed into *threo*-2,4-dimethyl-3-hydroxypentanoic acid methyl ester ($[\alpha]_D^{25}$ −9.1° ($CHCl_3$)), and its absolute configuration was established by comparison with the known (2*R*,3*R*)-2,4-dimethyl-3-hydroxypentanoic acid methyl ester (85% ee), ($[\alpha]_D$ −12.5° ($CHCl_3$)).[18] This result clearly demonstrates that the 2*S*,3*R* isomer of **1** was preferentially hydrolyzed by PLE. The enantiomeric ratio (E) for the hydrolysis of (±)-**1** was calculated to be 9.7 ± 0.2 from the experimental values of c and ee by using either eq 4 (ester) or 5 (acid). It is gratifying to note that these experimental values coincided nicely with the computer-generated curves with an E value of 9.7 for the remaining ester and acid fractions (Figure 4).

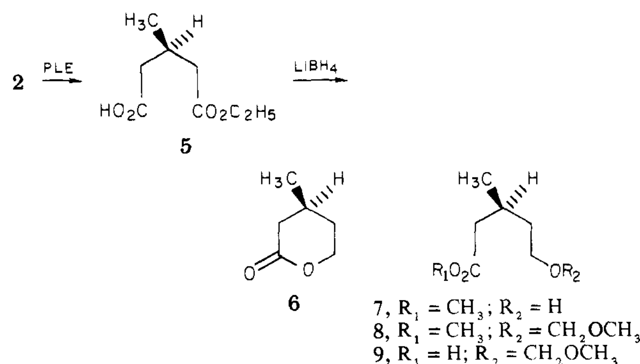
The resulting (−) acid (64% ee) was reesterified with CH_2N_2 and reincubated with PLE under the same conditions with a view toward enhancing the ee of the acid by recycling and testing the validity of eq 6. It is apparent that the relationship of ee′ of the product to c for $E = 9.7$ is consistent with the prediction of eq 6 (Figure 5).

(b) Enantiotopically Selective Hydrolysis of 2. Enantiotopically selective hydrolyses of symmetrical substrates such as **2** proceed as parallel reactions. It is intuitively obvious that the concentration

$$\mathrm{A} \xrightarrow{k_a} \mathrm{P}, \quad \mathrm{A} \xrightarrow{k_b} \mathrm{Q} \tag{7}$$

of A decreases with a rate constant that is the sum of k_a and k_b. Thus, the enantiomeric ratio, E, is simply the ratio of the rate constants ($P/Q = k_a/k_b = E$). The hydrolysis can therefore be carried to completion, since ee of the product fraction is independent of c.

Exposure of diethyl β-methylglutarate (**2**) to PLE at pH 8.0 under the usual conditions afforded the half-ester **5** ($[\alpha]^{25}_D$ −0.65°



(12) Most of the published *kinetic* resolution data were conducted by using crude liver homogenates that contained one or more of the same type of enzymes as well as proteolytic enzymes destroying one of these preferentially. Hence, the results are somewhat tentative. See: Ammon, R.; Jaarma, M. In "The Enzymes", 1st ed.; Sumner, J. B., Myrback, K., Eds.; Academic Press: New York, 1950; Vol. 1, p 390.

(13) (a) Sih, C. J. "Abstracts of Papers", 100th Anniversary Meeting of the Japanese Pharmaceutical Society, Tokyo, 1980; p 52. (b) Oishi, T.; Nakata, T. *J. Synth. Org. Chem., Jpn.* **1981**, *39*, 587.

(14) (a) Huang, F. C.; Lee, L. F. H.; Mittal, R. S. D.; Ravikumar, P. R.; Chan, J. A.; Sih, C. J.; Caspi, E.; Eck, C. R. *J. Am. Chem. Soc.* **1975**, *97*, 4144. (b) Ohno, M.; Kobayashi, S.; Iimori, T.; Wang, Y. F.; Izawa, T. *Ibid.* **1981**, *103*, 2405.

(15) Nakata, T.; Oishi, T. *Tetrahedron Lett.* **1980**, *21*, 1641.

(16) Values of ee(S) and ee(P) were determined by ^1H NMR spectroscopy (CCl_4) in the presence of Eu(hfc)$_3$. For compound **6**, see: Jakovac, I. J.; Jones, J. B. *J. Org. Chem.* **1979**, *44*, 2165.

(17) Two methods were used to determine c: (a) The amount of residual ester was estimated by quantitative GLC analyses (5% QF-1 on Chromosorb G HP column, 5 ft, 105 °C). (b) Since $A_0 = A + P$, the value of c is related to the ee of the substrate [ee(S)], ee of the product [ee(P)], and the initial enantiomeric excess (ee_0) as follows:

$$(1-c)\frac{1-\mathrm{ee(S)}}{2} + c\frac{1+\mathrm{ee(P)}}{2} = \frac{1+\mathrm{ee}_0}{2}$$

Hence, c may be calculated from the equation $c = (\mathrm{ee(S)} + \mathrm{ee}_0)/(\mathrm{ee(S)} + \mathrm{ee(P)})$.

(18) Meyers, A. I.; Yamamoto, Y. *J. Am. Chem. Soc.* **1981**, *103*, 4278.

($CHCl_3$) (81%)), whose absolute configuration was determined by reduction of **5** with $LiBH_4$ to give (−)-(3*S*)-3-methylvalerolactone (**6**, $[\alpha]^{25}_D$ −18.5°, reported $[\alpha]^{27}_D$ −24.5° ($CHCl_3$), 90% ee]).[19] The ee[16] of **6** was found to be 0.69. In order to enhance the optical purity of **6**, it was treated with methanol and triethylamine[20] to yield **7**. Since **7** has a strong tendency to lactonize back into **6**, it was converted into the methoxy methyl ether[21] **8** (55% from **5**). In an independent experiment, the value of E for racemic **8** was determined to be 7.0 ± 0.5. Hence, we predicted from eq 6 that the ee′ value after 80% conversion should be 0.89, which is in good agreement with the experimental value of 0.91 obtained after exposure of **8** to PLE. It is readily apparent that the combination of enantiotoically selective hydrolysis of symmetrical substrates followed by a kinetic resolution step has distinct advantages. This approach not only enhances the optical purity of the product but also raises the ceiling of theoretical yield from 50% to a number dependent on the two independent E values.

Resolution of (±)-3 with *Gliocladium roseum*. Although there are a number of potential factors[22] that may contribute to significant variations in intact cell experiments, all of these are directly or indirectly associated with growth rates of microorganisms and substrate concentrations. Since the enantiomeric ratio, E, is not dependent on these variables, good correlations of eq 4 and 5 with kinetic resolution data should be obtained when pH and temperature are kept constant. Another factor that requires consideration is that many microorganisms possess one or more of the same type of enzymes with different E values. However, the E value of each enzyme for a specified substrate is manifested in the experimentally determined E_{app}[23] value, where α is a proportionality constant for the different concentration of each enzyme present.

$$E_{app} = \frac{\sum \alpha_1(V/K)_1 + \alpha_2(V/K)_2 ... \alpha_i(V/K)_{n-1}}{\sum \alpha_1(V'/K')_1 + \alpha_2(V'/K')_2 ... \alpha_i(V'/K')_{n-1}} \quad (8)$$

To confirm our assumptions, we exposed the erythro ester (±)-**3** (2 g/L) to *Gliocladium roseum* in the soybean dextrose medium.[8b] An E value of 20 ± 1 was determined for this system, and the experimental c and ee values are in good agreement with the predicted curves (Figure 6). The remaining ester (95% ee) was converted (hydrogenation and hydrolysis) into *erythro*-2,4-dimethyl-3-hydroxypentanoic acid ($[\alpha]^{25}_D$ +13.57° ($CHCl_3$)) and correlated with (2*R*,3*S*)-2,4-dimethyl-3-hydroxypentanoic acid ($[\alpha]_D$ +10.54,[24a] +7.7[24b] ($CHCl_3$)), thereby establishing that the 2*S*,3*S* isomer of **3** was preferentially attacked.

In conclusion, we have developed basic equations and useful graphs for the unified treatment of biochemical kinetic resolution data. These expressions have now been verified experimentally and possess predictive value in relating the important components of c, ee, and E. The biochemical significance of the enantiomeric ratio (E) cannot be overemphasized. It is a constant independent of time and substrate concentrations and is governed by the ratio of the specificity constants, V/K, which is an intrinsic property of that biochemical system.

Experimental Section

^{1}H NMR spectra were recorded on a Varian EM-390 spectrometer in deuteriochloroform solution with tetramethylsilane as the internal standard. Chemical shifts are reported in the form of δ value of signal (peak multiplicities, coupling constant (if appropriate), number of protons). When peak multiplicities are reported, the following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broadened. Low resolution EI mass spectra were obtained on a Finnigan 4000 spectrometer at 70 eV. High-resolution EI mass spectra were obtained on an AEI MS-9 double-focusing mass spectrometer. Optical rotations were measured with a Perkin-Elmer Model 241C instrument in chloroform unless otherwise stated. The designation c refers to concentration in g/100 mL. Gas chromatography (GC) was performed with a Varian Aerograph Model 2400 instrument. Elemental analyses were performed by Galbraith Laboratories.

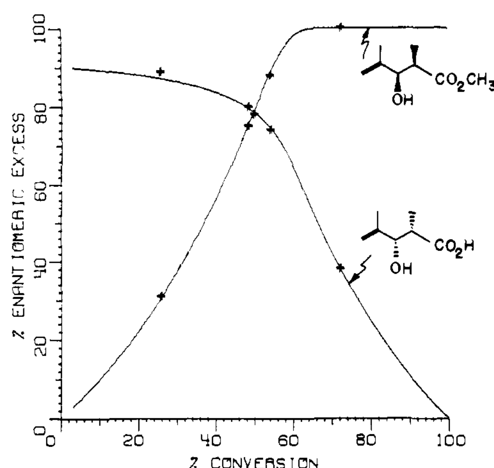


Figure 6. Dependence of percent enantiomeric excess (ee) on the percent conversion. The curves for E = 20 were calculated from eq 4 (ester) and 5 (acid). # = experimental values of *G. roseum*.

Column chromatography was performed with MN-Kieselgel 60 (0.05–0.2 mm; 70–270 mesh, Brinkmann). All solvents were glass distilled prior to use. Pig liver esterase (type I) and 3-methylglutaric acid were products of Sigma.

Racemic *threo*-Methyl 2,4-Dimethyl-3-hydroxypent-4-enoate ((±)-1) and *erythro*-Methyl 2,4-Dimethyl-3-hydroxypent-4-enoate ((±)-3). Freshly distilled methacrylaldehyde (11.9 g, 0.15 mol) and methyl 2-bromopropionate (25.1 g, 0.15 mol) were dissolved in 30 mL of benzene and 5 mL of ether. To 12 g of freshly activated zinc powder was added 5 mL of the above solution, and the suspension was heated to 70 °C. When the reaction was initiated, the remainder of the solution was slowly added over a period of 60 min with constant stirring. After the solution was refluxed for 30 min, the mixture was cooled and 90 mL of cold 10% H_2SO_4 was added with vigorous stirring. The organic layer was then separated and washed successively with 75 mL of 5% H_2SO_4, 40 mL of 10% $NaHCO_3$, 40 mL of 5% H_2SO_4, and 50 mL of H_2O. After the solution was dried over Na_2SO_4, it was concentrated to dryness in vacuo to yield 21.4 g (90%) of a 1:1 mixture of (±)-**1** and (±)-**3**. (This ratio was based on GC analysis using 10% AT-1000 on Chromosorb W AW, 6 ft, 160 °C.) This mixture was chromatographed over 630 g of silica gel. Elution of the column with hexane–ethyl acetate (13:1) afforded pure fractions of (±)-**1** and (±)-**3**, which were monitored by GC analyses. The absolute configuration of the less polar compound was established to be (±)-**3** on the basis of published NMR data:[15] δ 5.02 (d, J = 11.4 Hz, 2 H), 4.39 (dd, J = 5.4, 3.3 Hz, 1 H), 3.70 (s, 3 H), 2.73 (d, J = 3.3 Hz, 1 H), 2.9–2.5 (m, 1 H), 1.72 (s, 3 H), 1.16 (d, J = 6.6 Hz, 3 H). Anal. Calcd for $C_8H_{14}O_3$: C, 60.76; H, 8.86. Found: C, 60.68; H, 8.85. The more polar compound, (±)-**1**, exhibited signals at δ 4.90 (br s, 2 H), 4.12 (d, J = 9 Hz, 1 H), 3.70 (s, 3 H), 2.80 (s, 1 H), 2.9–2.5 (m, 1 H), 1.72 (s, 3 H), 1.07 (d, J = 6.6 Hz, 3 H). Anal. Calcd for $C_8H_{14}O_3$: C, 60.76; H, 8.86. Found: C, 60.66; H, 8.81. It was reported[15] that the chemical shift of the C-3 protons in threo compounds always appear at higher field (δ 4.12) than those of corresponding C-3 protons in erythro compounds (δ 4.39). Further, $J_{2,3}$(threo) is larger than $J_{2,3}$(erythro).

Kinetic Resolution of (±)-1 by PLE. A solution of 300 mg of the racemic threo ester (**1**) and 170 units of pig liver esterase in 30 mL of 0.05 M Tris buffer, pH 8.0, was incubated at 25 °C with stirring. A total of five separate incubations for each time interval were conducted. At the indicated time intervals (30, 90, 150, 460, and 720 min), the reaction was terminated by adjusting the pH of the solution to 2 with 1 N HCl, and the mixture was extracted with ethyl acetate. After drying over Na_2SO_4, the solvent was concentrated to dryness in vacuo. A small portion of the residue was used for quantitative GC analyses (5% QF-1 on Chromosorb W HP, 6 ft, 105 °C) of the residual ester. The residue was then dissolved in hexane–ethyl acetate (10:1) and chromatographed over 20 g of silica gel. Elution of the column with hexane–ethyl acetate

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(10:1 and 4:1) afforded pure residual ester (**1**) and the acid, respectively, which were used for enantiomeric excess determination by NMR spectroscopy using CCl_4 in the presence of 0.2 equiv of Eu(hfc)$_3$ and observing the splitting of the CH_3 at C-4 (δ 1.73, 1.63) (Figure 4).

Enhancement of Optical Purity of the Threo Acid by Recycling with PLE. The above threo acid samples, obtained at the indicated time intervals, were reesterified with CH_2N_2. The resulting ester (100 mg) from each time interval was exposed to 60 units of PLE in 10 mL of 0.05 M Tris buffer, pH 8.0, at 25 °C. At the indicated intervals (45, 60, 70, 90, and 120 min), the reaction mixture was brought to pH 2 with 1 N HCl. The residual ester was quantitatively analyzed by GC and the enantiomeric excesses of the acids were determined via the methods already described (Figure 5).

Transformation of 1 into *threo*-Methyl 2,4-Dimethyl-3-hydroxypentanoate. To the recovered ester (methyl 2,4-dimethyl-3-hydroxy-*threo*-4-pentenoate) (100 mg, 0.633 mmol, $[\alpha]^{25}_D$ = +0.7, 62.5% ee) in 0.5 mL of pyridine under N_2 was added 99mg (0.950 mmol) of trimethylsilyl chloride at 25 °C. After stirring for 20 min, the reaction was terminated by passing the reaction mixture over 2 g of silica gel. Elution of the column with hexane–ethyl acetate (4:1) afforded 130 mg (87%) of crude *threo*-methyl 2,4-dimethyl-3-((trimethylsilyl)oxy)pent-4-enoate; ^{1}H NMR δ 4.89 (s, 1 H), 4.18 (d, *J* = 9 Hz, 1 H), 3.70 (s, 3 H), 2.80–2.40 (m, 1 H), 1.67 (s, 3 H), 0.96 (d, *J* = 6.4 Hz, 3 H), 0.05 (s, 9 H). To this crude silyl ether (130 mg, 0.551 mmol) in 1 mL of ethyl acetate was added 13 mg of 10% palladium on carbon. The reaction mixture was stirred under H_2 for 150 min, and 8.4 mL of H_2 was absorbed. After filtration, the filtrate was evaporated in vacuo to yield crude *threo*-methyl 2,4-dimethyl-3-((trimethylsilyl)oxy)pentanoate (128 mg); ^{1}H NMR δ 3.67 (s, 3 H), 3.87–3.60 (m, 1 H), 2.85–2.42 (m, 1 H), 2.00–1.42 (m, 1 H), 1.07 (d, *J* = 7.5 Hz, 3 H), 0.82 (dd, *J* = 6.3 Hz, 6 H), 0.1 (s, 9 H). A mixture consisting of 128 mg of crude *threo*-methyl 2,4-dimethyl-3-((trimethylsilyl)oxy)pentanoate, 3 mL of H_2O–THF (1:1) solution, and 1 mL of 0.5 N HCl was stirred at room temperature for 5 h. The contents were then extracted with ether. The extract was washed with brine, dried, and evaporated to dryness. The crude residue (110 mg) was chromatographed over silica gel (5 g). Elution of the column with hexane–ethyl acetate (7:3) gave 12 mg of pure *threo*-methyl 2,4-dimethyl-3-hydroxypentanoate: $[\alpha]^{25}_D$ –9.1° (*c* 1.2); ^{1}H NMR δ 3.70 (s, 3 H), 3.40 (dd, *J* = 6, 12 Hz, 1 H), 2.87–2.40 (m, 1 H), 2.56 (s, 1 H), 2.00–1.47 (m, 1 H), 1.20 (d, *J* = 7.5 Hz), 0.92 (dd, *J* = 3 Hz, 6 H).

Enantiotopically Selective Hydrolysis of Diethyl β-Methylglutarate (2). To a suspension of **2** (800 mg) in 80 mL of 0.1 M phosphate buffer pH 8.0 was added 400 units of PLE. The mixture was stirred at 25 °C until the starting material completely disappeared (90 min), as monitored by GC (1.5% OV-101 on Chromosorb W HP, 6 ft, 200 °C). The reaction was terminated by the addition of 2 N HCl until the pH was lowered to 2.0. The contents were extracted with ethyl acetate three times. The solvent was then dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The crude half-ester was chromatographed over a silica gel (30 g) column. Elution of the column with hexane–ethyl acetate (4:1) afforded the pure half-ester **5** (560 mg, 81%): $[\alpha]^{25}_D$ –0.65° (*c* 5.6); ^{1}H NMR δ 3.68 (s, 3 H), 2.36 (br s, 4 H), 1.05 (d, *J* = 6 Hz, 3 H).

(–)-(3*S*)-Methylvalerolactone (6). The half-ester **5** (540 mg) was dissolved in 15.5 mL of 0.2 N LiOH. After standing at 25 °C for a few minutes, the solution was evaporated under reduced pressure, and the residue was dried in vacuo. The resulting lithium salt was suspended in 5 mL of dry THF under a blanket of N_2. To this suspension was added 150 mg of $LiBH_4$ in 5 mL of dry ether, and the mixture was heated for 120 min at 50 °C. Excess hydride was quenched by the careful addition of methanol (1 mL), and the mixture was heated for 30 min at 50 °C. After evaporation of solvent under reduced pressure, the contents were diluted with water, brought to pH 2.0, and extracted with ethyl acetate three times. The organic phase was dried over $MgSO_4$ and then concentrated to dryness to yield an oily residue, which was dissolved in dry benzene containing a catalytic quantity of *p*-toluenesulfonic acid. After evaporation of the solvent in vacuo, the residual oil was chromatographed over 15 g of silica gel. Elution of the column with hexane–ethyl acetate (3:1) yielded **6** (265 mg): $[\alpha]^{25}_D$ –18.5° (*c* 6.4) (reported $[\alpha]^{27}_D$ –24.5° for 90% ee);[19] ^{1}H NMR δ 4.1–4.54 (m, 2 H), 1.07 (d, *J* = 6 Hz, 3 H).

Methyl (–)-(3*S*)-5-((Methoxymethyl)oxy)pentanoate (8). A solution containing 215 mg of the lactone **6**, 1.9 mL of triethylamine, and 3.5 mL of methanol was stirred at 25 °C for 60 min. Concentration of the solvent in vacuo afforded virtually the pure (–)-hydroxy methyl ester **7** (230 mg); ^{1}H NMR δ 3.86 (2, 3 H), 3.68 (t, *J* = 6 Hz, 2 H), 0.97 (d, *J* = 6 Hz, 3 H).

To 230 mg of (–)-**7** dissolved in 4 mL of CH_2Cl_2 was added 558 μL (3.20 mM) of EtN(*i*-Pr)$_2$ and 245 μL (3.22 mM) of $ClCH_2OCH_3$. After the mixture was stirred for 16 h at 25 °C, the solvent was removed under reduced pressure. The residue was dissolved in ether, and the ethereal solution was successively washed with dilute HCl, saturated $NaHCO_3$, and brine. After the ethereal extract was dried over $MgSO_4$, the solvent was concentrated in vacuo to give an oily residue, which was chromatographed over 8 g of silica gel. Elution of the column with hexane–ethyl acetate (10:1) gave 258 mg of **8** (73% from **6**): $[\alpha]^{25}_D$ –2.36° (*c* 7.6); ^{1}H NMR δ 4.61 (s, 2 H), 3.68 (s, 3 H), 3.57 (t, *J* = 6 Hz, 2 H), 3.36 (s, 3 H), 0.98 (d, *J* = 6 Hz, 3H).

Hydrolysis of 8 with PLE. A solution containing 205 mg of **8** (69% ee) and 70 units of PLE in 20 mL of 0.1 M phosphate buffer pH 8.0 was incubated at 25 °C. The reaction was monitored by GC (1.5%, OV-101 on Chromosorb W HP, 6 ft, 170 °C) and terminated at 80% conversion by addition of 2 N HCl until the pH of the mixture reached 2.0. After extraction of the contents with ethyl acetate, the solvent was dried over $MgSO_4$ and concentrated to dryness in vacuo. The oily residue was chromatographed over 7 g of silica gel. Elution of the column with hexane–ethyl acetate (4:1) afforded 145 mg (71%) of **9**: $[\alpha]^{25}_D$ –3.15° (*c* 7.3); ^{1}H NMR δ 4.63 (s, 2 H), 3.59 (t, *J* = 6 Hz, 2 H), 3.35 (s, 3 H), 1.03 (d, *J* = 6 Hz, 3 H). After treatment of **9** with CH_2N_2, the resulting ester was transformed back to the lactone **6** by using a mixture consisting of 1 mL of concentrated HCl and 4 mL of methanol. The optical purity of the resulting **6** was estimated to be 91% ee, $[\alpha]^{25}_D$ = –24.8° (*c* 2.7). The value was further confirmed by converting **6** into 3,5-dimethyl-1,5-hexanediol, which was analyzed by NMR spectroscopy as described.[16]

Incubation of (±)-8 with PLE and *E*-Value Determination. Racemic **8** was prepared from 3-methylpentane-1,5-diol in 40% overall yield via a three-step reaction sequence: partial methoxymethyl ether formation, Jones oxidation, and CH_2N_2 treatment.

Racemic **8** (700 mg) was exposed to 300 units of PLE in 70 mL of 0.1 M phosphate buffer pH 8.0. The reaction was monitored by GC analyses of aliquots of reaction mixture taken at various intervals. At 50% conversion (2 h), the reaction was terminated by the addition of 2 N HCl to lower the pH of the mixture to 2.0. The contents were worked up as previously described. The oily residue was chromatographed over 15 g of silica gel. Elution of the column with hexane–ethyl acetate (10:1) afforded 240 mg of recovered ester (**8**), $[\alpha]^{25}_D$ +1.89° (*c* 8.9). Elution of the column with hexane–ethyl acetate (3:1) gave 300 mg of the acid (**9**), which was treated with CH_2N_2 to yield the methyl ester, $[\alpha]^{25}_D$ –1.87° (*c* 9.0). The enantiomeric excesses of these samples were determined by NMR spectroscopy as described.[16] An *E* value of 7.0 ± 0.5 was calculated from the results obtained.

Kinetic Resolution of (±)-3 with *Gliocladium roseum*. The surface growth from a 1-week-old agar slant of *Gliocladium roseum* was suspended in 5 mL of saline (0.85%) solution. Portions (2 mL) of this suspension were used to inoculate 50 mL of the soybean dextrose medium (soybean meal, 5 g; glucose, 20 g; yeast extract, 5 g; K_2HPO_4, 5 g; NaCl, 5 g; distilled water to 1 L; pH adjusted to 6.5 with HCl) held in 250-mL Erlenmeyer flasks (F-1 stage). The flasks were incubated at 25 °C on a rotary shaker (250 cycles/min; 2-in. radius) for 24 h, after which a 10% by volume transfer was made to each of five 1-L Erlenmeyer flasks (F-2 stage) containing 300 mL of the soybean dextrose medium. After 24 h of incubation on a rotary shaker, 600 mg of (±)-**3** was added to each flask. The F-2-stage flasks were then incubated under the condition used in the incubation of the F-1-stage flasks. At various time intervals (5, 15, 20, 25, and 40 h), the reaction was terminated by bringing the pH to 2, and the mixture was extracted with ethyl acetate. The method of isolation and GC analyses of residual erythro ester (**3**) were the same as those used for the threo ester (**1**). The enantiomeric excesses of the ester and acid fractions were determined by NMR spectroscopy using 0.4 equiv of Eu(hfc)$_3$ and noting the splitting of the CH_3 at C-4 (δ 2.58, 2.51) (Figure 6).

Conversion of 3 into *erythro*-2,4-Dimethyl-3-hydroxypentanoic Acid. The recovered ester (*erythro*-methyl 2,4-dimethyl-3-hydroxy-4-pentenoate) (150 mg, 0.95 mmol, $[\alpha]^{25}_D$ +19°, ee = 0.95) was converted into *erythro*-methyl 2,4-dimethyl-3-hydroxypentanoate (28 mg, $[\alpha]^{25}_D$ +8.20°) by using the same procedure as that for the threo compound. To the methyl ester (25 mg, 0.156 mmol) in 1 mL of dimethyl ether and 0.5 mL of H_2O was added 37.5 mg (1.56 mmol) of LiOH at room temperature. After stirring for 45 min, the reaction mixture was diluted with 10 mL of water and extracted with ether to remove unreacted starting material. The aqueous layer was acidified to pH 2.5 and extracted with ethyl acetate. The extract was washed with brine, dried, and evaporated to dryness. The residue (25 mg) was chromatographed over 10 g of silica gel. Elution of the column with $CHCl_3$–acetone (4:1) gave 15 mg of *erythro*-2,4-dimethyl-3-hydroxypentanoic acid: $[\alpha]^{25}_D$ +13.57° (*c* 1.5); ^{1}H NMR δ 3.79–3.5 (m, 1 H), 2.86–2.51 (m, 1 H), 2.0–1.50 (m, 1 H), 1.20 (d, *J* = 7.5 Hz, 3 H), 0.97 (dd, *J* = 8.4 Hz, 6 H).

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Registry No. (±)-**1**, 83588-18-5; (+)-**1**, 83648-33-3; (2*S*,3*R*)-**1**, 83648-34-4; **2**, 6829-42-1; (±)-**3**, 83588-19-6; (2*S*,3*S*)-**3**, 83648-35-5; (2*R*,3*R*)-**3**, 83648-36-6; **5**, 72594-19-5; **6**, 61898-56-4; **7**, 68702-74-9; (±)-**8**, 83648-37-7; (−)-**8**, 83588-20-9; **9**, 83588-21-0; PLE, 9016-18-6; (−)-*threo*-2,4-dimethyl-3-hydroxypentanoic acid, 78655-80-8; *erythro*-2,4-dimethyl-3-hydroxypentanoic acid, 77341-63-0; methacrylaldehyde, 78-85-3; methyl 2-bromopropionate, 5445-17-0; (−)-*threo*-2,4-dimethyl-3-hydroxypent-4-enoic acid, 83588-22-1.

p-Guanidinobenzoic Acid Esters of Fluorescein as Active-Site Titrants of Serine Proteases

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Abstract: Two active-site titrants of serine proteases, fluorescein mono-*p*-guanidinobenzoate hydrochloride (FMGB·HCl) and fluorescein di-*p*-guanidinobenzoate dihydrochloride (FDGB·2HCl), have been synthesized, purified, and chemically and enzymatically characterized. Electronic absorption and fluorescence emission spectra, fluorescence lifetimes and quantum yields, solubilities, and rates of spontaneous hydrolysis at pH 7–10 are reported. Macroscopic and microscopic kinetic constants for interaction of FMGB·HCl with trypsin, urokinase, plasmin, and thrombin have been determined. FMGB·HCl, which rapidly releases fluorescein upon formation of a stable acyl-enzyme intermediate with trypsin and other trypsin-like enzymes, is the most sensitive active-site titrant for serine proteases yet described.

Serine proteases are involved in a wide variety of physiological processes,[1] including ovulation,[2-4] blood coagulation,[5] embryogenesis,[6] processing of hormones,[7,8] inactivation of the λ repressor,[9] and activation of digestive enzymes.[10] Sensitive substrates are essential for accurate assay of serine proteases because such enzymes are often present in very small amounts and because, as proteases capable of self-degradation, they must be assayed at low concentrations. Active-site titrants are especially useful substrates for serine proteases because they form enzymatically inactive acyl-enzyme intermediates, thus causing the rate of self-proteolysis to decrease during assay. However, since the acyl-enzyme intermediate of an active-site titration does not turn over, the detectable product must be unusually easy to monitor. An ideal active-site titrant would react rapidly to form a stable, enzymatically inactive intermediate as it released a product capable of detection at extremely low concentration.

Recently, we reported the synthesis and biochemical characterization of FDE[11] (fluorescein diester[12]), an active-site titrant for serine proteases designed to fulfill these criteria. Efforts to purify this compound and to establish its structure by chemical and physical methods, especially to determine whether it is a mono- or a diester, have not yet been rewarded. Part of the difficulty stems from the controversy over the relationship between the electronic and fluorescence spectra of fluorescein (**1**) and its derivatives and their molecular structures[13-18] and part appears to arise from the presence of the thioureido bridge and the terminal carboxyl group in FDE. Because FDE has nevertheless proven quite useful,[19,20] we decided to make simpler analogues and to establish their structures unequivocally. Here we report the synthesis, purification, and chemical, physical, and enzymatic characterization of two such analogues,[21] fluorescein mono-*p*-guanidinobenzoate hydrochloride (FMGB·HCl, **2**) and fluorescein di-*p*-guanidinobenzoate dihydrochloride (FDGB·2HCl, **3**). These substrates are the most sensitive active titrants for serine proteases yet described.

Experimental Section

Melting points were determined on a Büchi melting point apparatus and are corrected. Electronic absorption spectra were measured on a Beckman Acta Model VI spectrophotometer with matched silica cells having 1-cm pathlength. Field desorption mass spectra (FDMS) were obtained on a Varian-MAT 731 spectrometer equipped with a Varian-MAT combination electron impact–field desorption ion source. Microanalyses were performed by Mr. Josef Nemeth and his staff at the University of Illinois, who also weighed samples for quantitative electronic absorption spectra and for determination of the purities of FMGB·HCl (**2**) and FDGB·2HCl (**3**) by hydrolysis with base and with trypsin.

Materials. *p*-Guanidinobenzoic acid hydrochloride (PGBA·HCl, **4**) was purchased from Sigma. Fluorescein (**1**), dicyclohexylcarbodiimide (DDC), and benzamidine hydrochloride hydrate (**5**) were purchased from

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(21) Systematic names for these compounds are 3′-(4-guanidinobenzoyloxy)-6′-hydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one (**2**) and 3′,6′-bis(4-guanidinobenzoyloxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]-xanthen]-3-one (**3**).

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