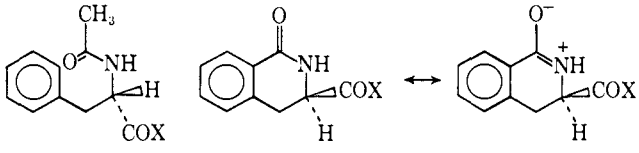
α-Chymotrypsin. The Use of Substrates of Restricted Geometry to Define the Reactive Conformation of Methyl N-Acetyl-L-phenylalaninate[1]

Marc S. Silver, Mai Stoddard, Tyo Sone, and Michael S. Matta

Contribution from the Department of Chemistry, Amherst College, Amherst, Massachusetts 01002. *Received November 14, 1969*

Abstract: The *p*-nitrophenyl esters (NPE's) of a series of 2-napthoic acid derivatives, N-acetyl-D- and -L-phenylalanine (D- and L-APNPE) and D- and L-3-carboxydihydroisocarbostyril (D- and L-CDNPE) have been synthesized. Determination of the rates of hydrolysis by α-chymotrypsin (CT) and hydroxide ion of these esters, and some of the corresponding methyl esters (D- and L-APME, D- and L-CDME), coupled with data of earlier studies, leads to the following conclusions: (1) structure–reactivity correlations for CT with methyl and *p*-nitrophenyl esters exhibit similar patterns, but the magnitude of effects observed with the latter is less; (2) the observation that the NPE of *trans*-decalin-2α-carboxylic acid (equatorial ester) undergoes hydrolysis by CT much more rapidly than the 2β-isomer (axial ester) confirms that D-CDNPE or D-CDME is cleaved by the enzyme when its ester group lies in the equatorial position; (3) the rapid and stereoselective hydrolysis by CT of the NPE of (*S*)-1,2-dihydro-2-naphthoic acid establishes that hydrogen-bond interactions of the amide group of the D-dihydroisocarbostyril substrates are of negligible importance for enzymatic reactivity; such interactions, although generally overestimated, appear significant for L-APME. These conclusions guide formulation of a "best" set of rules governing the use of D-CDME to define the reactive conformation of L-APME, but the available experimental data do not really substantiate the legitimacy of this model-building procedure.

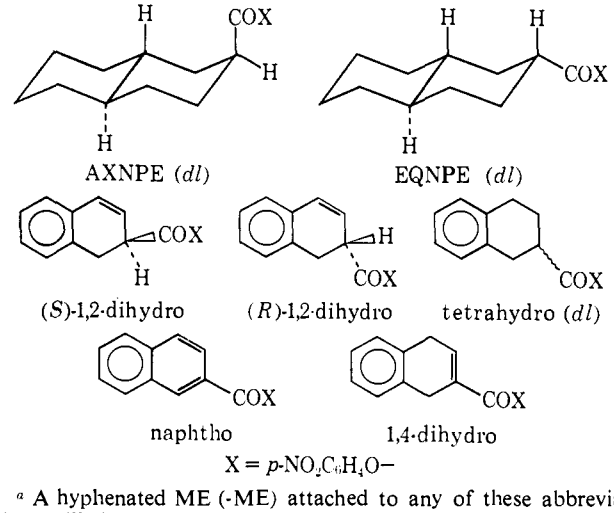
Methyl N-acetyl-L-phenylalaninate (L-APME) and similar specific substrates of α-chymotrypsin (CT) possess many degrees of internal rotational freedom. Establishing by chemical methods which conformation of these substrates most readily undergoes hydrolysis by the enzyme (the "reactive conformation") consequently presents a formidable challenge. The similarity[2,3] in gross kinetic behavior toward CT of D-3-carbomethoxydihydroisocarbostyril (D-CDME) and L-APME has suggested that the relatively fixed geometry of the former may assist in defining the reactive conformation of the latter.[2–9] This paper attempts to evaluate critically this model-building



L-APME X = CH_3O– D-CDME
L-APNPE X = *p*-$NO_2C_6H_4O$– D-CDNPE

procedure with the aid of experiments designed to answer three important questions: (1) do studies with methyl and *p*-nitrophenyl esters reveal the same pattern of structural- and stereoselectivity[10] by CT? (2) Which conformation of D-CDME undergoes hydrolysis? The flexibility of the 1,3-cyclohexadiene-like ring[11] permits the ester group to assume an axial- or equatorial-like position relative to the amide-containing ring.[12] Esters of *trans*-decalin-2β- (AXNPE, axial ester group) and *trans*-decalin-2α-carboxylic acids (EQNPE, equatorial ester group) possessing conformations rigorously defined[11] by the geometry of the

Chart I[a]



[a] A hyphenated ME (-ME) attached to any of these abbreviations will signify the corresponding methyl ester; thus AXNPE-ME.

(1) Supported by Grant No. AM 08005 of the U. S. Public Health Service.

(2) G. Hein and C. Niemann, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1341 (1961).

(3) G. Hein and C. Niemann, *J. Amer. Chem. Soc.*, **84**, 4487, 4495 (1962).

(4) E. S. Awad, H. Neurath, and B. S. Hartley, *J. Biol. Chem.*, **235**, PC 35 (1960).

(5) I. B. Wilson and B. F. Erlanger, *J. Amer. Chem. Soc.*, **82**, 6422 (1960).

(6) B. F. Erlanger, *Proc. Natl. Acad. Sci. U. S.*, **58**, 703 (1967).

(7) (a) S. G. Cohen and R. M. Schultz, *J. Biol. Chem.*, **243**, 2607 (1968); (b) S. G. Cohen, A. Milovanovic, R. M. Schultz, and S. Y. Weinstein, *ibid.*, **244**, 2664 (1969).

(8) (a) W. B. Lawson, *ibid.*, **242**, 3397 (1967); (b) Y. Hayashi and W. B. Lawson, *ibid.*, **244**, 4158 (1969).

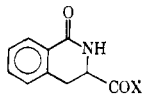
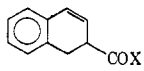
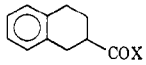
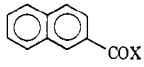
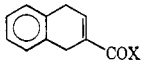
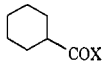
(9) For preliminary communications of part of this work see (a) M. S. Silver and T. Sone, *J. Amer. Chem. Soc.*, **89**, 457 (1967); (b) M. S. Matta and M. S. Silver, paper presented at the 156th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1968, Biol. 169.

(10) A more detailed investigation is in progress.

(11) The stereochemistry of 1,3-cyclohexadiene and of *trans*-decalin is discussed in E. L. Eliel, N. L. Allinger, S. J. Angyal, and G. A. Morrison, "Conformational Analysis," Interscience Publishers, Inc., New York, N. Y., 1965, pp 71, 125.

(12) The following shorthand notations will be used: (a) a conformation with an axial ester group = axial conformation; the proposition that this is the reactive conformation of D-CDME = axial ester hypothesis; (b) "equatorial conformation" and "equatorial ester hypothesis" are defined analogously.

Table I. Hydrolysis by CT of Several Substrates of Restricted Geometry[a]

Compd[b]		k_c, sec^{-1}	10^6K_m, M	Relative[c] k_{OH}	Relative $(k_c)_n$	Relative $(k_c/K_m)_n$
	D	146	124	102	9.7	0.84
	L	11.8	750	102	0.78	0.011
	(*S*)	3.3	4.5	4.4	5.1	12
	(*R*)	0.072	3.3	4.4	0.11	0.39
		0.15[d]	10.7[d]	1.0	1.0	1.0
		0.027[d]	10.7[d]	0.71	0.25	0.26
		0.021[d]	10.2[d]	0.29	0.48	0.51
AXNPE[e,f]				0.024		0.036
EQNPE		0.16[f]	9.4[f]	0.33	3.3	3.8
		0.066[d]	16.4[d]	0.36	1.2	0.80
CH_3COX		0.076[d]	166[d]	1.76	0.29	0.017

[a] At pH 7.0, 20% methanol–3% acetonitrile, 25°. Data for the last two esters from ref 15. [b] X = p-$NO_2C_6H_4O$. [c] On this scale k_{OH} for APNPE is 8.2. [d] Obtained from data at pH 8.0 by taking 0.65 × k_c and 1.02 × K_m. [e] See ref 14. [f] Obtained from data at pH 8.0, 30% methanol–3% acetonitrile, by taking 0.24 × k_c and 0.255 K_m.

trans-decalin ring system undergo hydrolysis by CT and appear to resemble the CDME substrates. (3) What is the function of the amide group of D-CDME? Does it participate in hydrogen bonding or merely impart a proper stereochemistry to the amide-containing ring, perhaps *via* the resonance form shown? The rates of hydrolysis of 2-naphthoic acid derivatives (Chart I) by CT have supplied answers to these questions.

Results

The seven p-nitrophenyl esters of Chart I and both enantiomers of APNPE and CDNPE were prepared. The acetylphenylalanine and dihydroisocarbostyril substrates were optically pure; (*S*)- and (*R*)-1,2-Dihydro contained approximately 82 and 78% of the desired optical isomer, respectively, and the observed kinetics were corrected for the contaminating enantiomer.[13] Absolute configurations are shown for these six compounds. Tables V–VII of the Experimental Section record the observed rates of enzymatic hydrolysis of these substrates under a variety of reaction conditions. Tables I–III present data corrected to a common set of reaction conditions to facilitate comparisons of substrate reactivities.

Kinetic Treatment. Equation 1 describes the kinetic behavior of these substrates under turnover conditions ($K_m \simeq [S]_0 \gg [E]_0$).[14] A plot of $1/v_0$ *vs.* $1/[S]_0$ determined[16] the values of k_c and K_m reported in Tables V–VI of the Experimental Section. If eq 2 represents the mechanism of chymotryptic hydrolysis, then eq 3 and 4 express the algebraic relationship between the

$$v = k_c[E]_0[S]/(K_m + [S]) \quad (1)$$

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2 \quad (2)$$

$$K_s = (k_{-1} + k_2)/k_1 \simeq k_{-1}/k_1 \quad (3)$$

$$K_m = (k_3/(k_2 + k_3))K_s;\ k_c = k_2k_3/(k_2 + k_3);\ k_c/K_m = k_2/K_s \quad (4)$$

experimental and mechanistic kinetic parameters.[17] Table I collects those data pertinent to evaluation of the role of the amide group of D-CDME and to examination of the axial–equatorial question. Table II is more useful for a detailed comparison of L-APME and D-CDME.

The following considerations[17–19] have determined our use of $(k_3)_n$ and $(k_c/K_m)_n$ in discussing structure–reactivity relationships. (1) Generally k_c is a function of both k_2 and k_3 while K_m depends upon k_2, k_3, and K_s (eq 4). In an extreme case, k_c measures k_2 for one substrate, k_3 for another. We shall assume throughout our discussion that eq 5 pertains for p-nitrophenyl esters. Consequently, for these esters, comparisons of k_c, which reflect solely k_3, are justified.[17,18] Suggestive evidence that eq 5 holds for both a methyl

$$K_m = (k_3/k_2)K_s \text{ and } k_c = k_3 \text{ if } k_2 \gg k_3 \quad (5)$$

ester and its corresponding p-nitrophenyl ester is the observation of equal k_c's for the two.[17] Table III shows that L-APNPE–L-APME, D-CDNPE–D-CDME, and (*S*)-1,2-Dihydro–(*S*)-1,2-Dihydro-ME do and L-CDNPE–L-CDME may *not* satisfy this criterion.[20]

(13) Details of syntheses, kinetic procedures, and analyses, etc., are in the Experimental Section.

(14) The enzymatic reactivity of AXNPE and D-APNPE was so low that only the ratio k_c/K_m could be measured.[15]

(15) M. S. Silver, *J. Amer. Chem. Soc.*, **88**, 4247 (1966).

(16) G. N. Wilkinson, *Biochem. J.*, 80, 324 (1961).

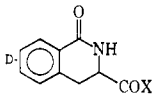
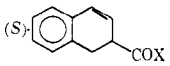
(17) M. L. Bender and coworkers, *J. Amer. Chem. Soc.*, **86**, 3669 (1964).

(18) D. W. Ingles and J. R. Knowles, *Biochem. J.*, 104, 369 (1967).

(19) M. L. Bender and F. J. Kezdy, *Ann. Rev. Biochem.*, **34**, 49 (1965).

(20) V. K. Antony and L. D. Rumsh (*Dokl. Akad. Nauk SSSR*, **185**, 821 (1969)) have reported kinetic data for the hydrolysis of D-CDNPE in quantitative disagreement with ours. We do not know whether the apparently low optical purity of their ester causes these discrepancies.

Table II. Hydrolysis by CT of a Series of Methyl and *p*-Nitrophenyl Esters[a,b]

Compd[c]	10^3K_i, *M* X = NH_2[d]	Relative $(k_c/K_m)_n$ X = ME	Relative $(k_c/K_m)_n$ X = NPE	Relative $(k/_cK_m)_nK_i$ X = ME	Relative $(k/_cK_m)_nK_i$ X = NPE	Relative $(k_3)_n$	$(k_c/K_m)_{fast}/(k_c/K_m)_{slow}$[g] X = ME[e]	$(k_c/K_m)_{fast}/(k_c/K_m)_{slow}$[g] X = NPE[f]	$(k_3)_{fast}/(k_3)_{slow}$[g]
		36	14			23	3.3×10^3	7.5×10	2.2×10
		220	190			12		3.4×10	4.5×10
L-AcTrpCOX	1.5	15,000	1,300	2,100	180	4,100	$>3 \times 10^6$	$<1 \times 10^4$	1.8×10^4
L-AcPheCOX	10.6	1,000	1,000	1,000	1,000	1,000	$>6.6 \times 10^5$	3.1×10^3	8×10^3
L-AcLeuCOX	140	26	46	340	610	170	$>1.1 \times 10^4$	$<6 \times 10^2$	1.5×10^2
AcGlyCOX	690	0.001	0.21	0.068	13.6	6.6			
Column	1	2	3	4	5	6	7	8	9

[a] All data corrected to pH 7.0, 25°, 0–3% acetonitrile. A reaction in 3% acetonitrile would have k_c identical with and K_m twice that of the same reaction in water (G. E. Clement and M. L Bender, *Biochemistry*, **2**, 836 (1963)). Original data are found in Tables I, III, V, and VII or ref 18 or J. R. Knowles, *J. Theoret. Biol.*, **9**, 213 (1965). [b] Effects of an order of magnitude or greater are significant and N-acetylphenylalanine is the standard reference compound. [c] Usual abbreviation for amino acids plus Ac = N-acetyl, and $CONH_2$, COME, and CONPE for an amide, methyl (or ethyl) ester, and *p*-nitrophenyl ester, respectively. [d] For D isomer if such exists. [e] Estimated for bottom three entries by assuming D-ester has k_c/K_m less than that of AcGlyCOME. J. P. Wolf and C. Niemann (*Biochemistry*, **2**, 493 (1963)) report D-AcAlaCOME too slow to measure. See also D. W. Ingles and J. R. Knowles, *Biochem. J.*, **108**, 561 (1968). [f] Estimated for Trp and Leu by assuming K_m for D isomer is one-fifth lowest $[S]_0$ reported in ref 18 to give zero-order kinetics. A similar estimate for Phe gives $<2 \times 10^4$ *vs.* 3×10^3 observed [g] For the dihydroisocarbostyril compounds the ratio refers to the ratio D/L, for the dihydronaphthoates, (*S*)/(*R*), and for the rest, L/D.

Table III. Comparison of k_c's of Methyl and *p*-Nitrophenyl Esters[a]

Compd	k_c, sec^{-1} X = ME	k_c, sec^{-1} X = NPE
L-APX	38 ± 2.4	45 ± 3
D-CDX	17 ± 2[b]	13 ± 1.6
L-CDX	0.024 ± 0.003	0.59 ± 0.02
(*S*)-1,2-Dihydro	0.30[c]	0.29 ± 0.02

[a] At 25°, pH 7.0, 0.3% acetonitrile. This work unless noted otherwise. [b] Average of data at pH 7.8 (ref 7) and 7.9 (ref 2, 3) corrected to pH 7.0 by taking $0.7 \times k_c$. [c] Obtained from data for (*S*)-1,2-Dihydro-ME at pH 7.8, 10% methanol (ref 7) by taking $0.0167 \times k_c$.

Comparison of k_c for D- and L-CDME could therefore be meaningless.

(2) A substrate may combine with CT in "nonproductive" modes. If such binding is relatively favorable, both K_m and k_c will be less than eq 4 specifies.[2,3] Since k_c/K_m reduces to k_2/K_s even under these circumstances, either k_c/K_m or k_3 is preferred for discussion of structure–reactivity relationships.[18,19]

(3) Comparisons of the relative ease of hydrolysis of substrates using k_c/K_m or k_3 reflect contributions of both inherent structural features (primarily stereoelectronic) and specific enzyme–substrate interactions. If the former effect determines the relative rates of reaction of the substrates with hydroxide ion,[21] then division of k_c/K_m or k_3 by these relative hydroxide-ion rates provides normalized data [$(k_c/K_m)_n$ and $(k_3)_n$] which isolate the desired contribution of enzyme specificity.[18,22] The treatment assumes that attack on *p*-nitrophenyl and alkyl esters by CT possesses negligible electrophilic character.[19,23]

(21) For APNPE a slightly different procedure is necessary.[13,18]

(22) Some of our conclusions differ from those of Cohen[7] because he has not employed normalized rate constants.

(23) J. H. Wang (*Science*, **161**, 328 (1968)) has discussed the role of proton transfers in reactions catalyzed by CT and other enzymes.

Discussion

We first consider the three questions posed earlier.

I. Do Methyl and *p*-Nitrophenyl Esters Give the Same Answers?[24] We reluctantly reopen[19] this question because of concern for how reactivity correlations among methyl esters and among *p*-nitrophenyl esters compare. Analysis of the data of Table II (see the Appendix for details) suggests the following. For the substrates named at the left of Table II, relative reactivities (structural selectivity) determined with either type of ester are essentially identical (columns 2 and 3). As to stereoselectivity, the ratio of the rate of hydrolysis of each of these substrates to that of its enantiomer is always much lower when *p*-nitrophenyl is the leaving group (columns 7 and 8). We dub this the "NPE-effect."

II. Which Conformation of D-CDME Undergoes Reaction?[25] Observation that the rate of hydrolysis of EQNPE by CT is rapid in an absolute sense (point 1 below) and exceeds that of AXNPE by 10^2 ($(k_c/K_m)_n$) to $>10^3$ (k_c/K_m) provides strong confirmation of the equatorial ester hypothesis if EQNPE, AXNPE, and the associated 2-naphthoic acid derivatives are acceptable models for the dihydroisocarbostyril esters. Certainly the present series of substrates resembles the dihydroisocarbostyril family in gross appearance more than have the other model compounds which have been employed in exploration of the equatorial–axial question: esters of 3- and 4-*t*-butylcyclohexanecarboxylic acids,[15,26] diethyl fumarate and maleate,[27] bridged biphenyl **1**,[28] and hydrocoumarilic derivative D-**2** and related compounds.[8] This subjective con-

(24) The query is only meaningful for arguments employed $(k_c/K_m)_n$.

(25) The discussion utilizes the data of Table I and rate comparisons refer to $(k_c/K_m)_n$ unless otherwise stated.

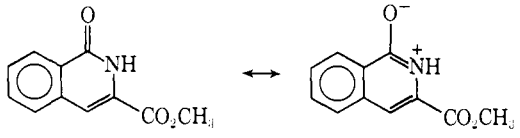
(26) M. S. Silver and T. Sone, *J. Amer. Chem. Soc.*, **90**, 6193 (1968).

(27) S. G. Cohen, L. H. Klee, and S. Y. Weinstein, *ibid.*, **88**, 5302 (1966).

(28) B. Belleau and R. Chevalier, *ibid.*, **90**, 6864 (1968). Evaluation of the authors' claim that their results are only compatible with the equatorial ester hypothesis is impossible in the absence of a detailed explanation of how this conclusion was reached.

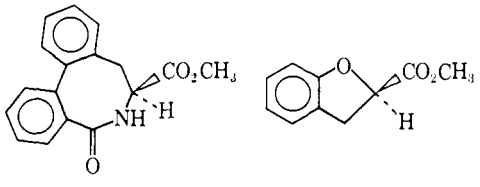
clusion that the 2-naphthoic acid substrates are acceptable models for the dihydroisocarbostyril derivatives is supported by the following facts.

(1) Cancellation of electronic effects by the normalization process yields rate constants for D-CDNPE which do not differ significantly from those for (*S*)-1,2-Dihydro, EQNPE, and Tetrahydro. (2) Stereoselectivity in the hydrolysis of D- and L-CDNPE and (*S*)- and (*R*)-1,2-Dihydro is the same.[29] (3) Introduction of an additional double bond into (*S*)-1,2-Dihydro produces Naphtho and reduces k_c/K_m by 290 times. The analogous procedure transforms D-CDME into CID and reduces k_c/K_m by ~450 times.[2,3] (4) Indole com-



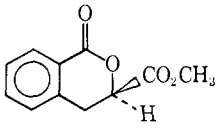
CID

petitively inhibits the hydrolysis of (*S*)- and (*R*)-1,2-Dihydro (Table VI). Indole inhibition of D-CDME is competitive while that of L-CDME is noncompetitive.[2,3] (5) The fact that K_m for D-CDNPE is much larger than K_m for (*S*)-1,2-Dihydro and that the K_m's for D-CDME, **3**, and **4** greatly exceed[7] K_m for (*S*)-1,2-Dihydro-ME does not militate against the appropriateness of the models. The 3,4-dihydro-2-naphthoate derivatives apparently exhibit a high affinity for CT in a *productive* binding mode, since their $(k_c/K_m)_n$'s are also more favorable. This high affinity is characteristic of naphthalene,[30] some naphthalene derivatives,[31] and the other compounds of Chart I and is apparently diminished by the keto oxygen adjacent to the aromatic ring.



1

D-**2**



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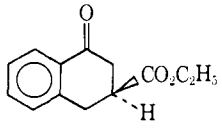

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The sole divergence between experimental results and expectations based on the equatorial ester hypothesis lies in the failure to detect appreciable stereoselectivity in the hydrolysis of racemic EQNPE.[32] To account for the stereoselectivity in the hydrolysis of D- and L-CDNPE, the hypothesis requires that equatorial D-CDNPE be at least 80 times more reactive than both equatorial and axial L-CDNPE. The ability of CT to distinguish between enantiomeric equatorial ester groups of substrates is established; the rates of hydrolysis of the *p*-nitrophenyl esters of the optically active *cis*-3-*t*-butylcyclohexanecarboxylic acids differ by a factor of 30.[26,29]

The axial ester hypothesis postulates that D-CDNPE undergoes hydrolysis in the axial conformation. An advocate of this position requires that axial D-CDNPE be much more reactive than equatorial D-CDNPE and axial and equatorial L-CDNPE. In terms of the data of Table I, he must then explain why axial D-CDNPE is 28 times (0.84/0.036) more reactive than AXNPE but EQNPE is at least 50–350 times [(3.8)/(0.84/10)–(3.8/0.011)] more reactive than equatorial D- and L-CDNPE.[33]

Only the reactions of **1** and **2** with CT have been cited as providing direct experimental evidence for the axial ester hypothesis. Compound **1** seems to us so complex that its behavior can be reconciled to any theory (see ref 7). As to D-**2**, it is 80 times more reactive than its enantiomer (for D-CDME the figure is >3000) and only one-eighth as reactive as D-CDME.[8] Certainly D-**2**, with its pseudoaxial ester group, is not a perfect model for D-CDME, but the behavior of D-**2** and related substrates[8] still remains difficult to rationalize with the equatorial ester hypothesis.

III. What Is the Function of the Amide Group of D-CDME?[25] The amide group of D-CDNPE (and D-CDME) is not essential for rapid hydrolysis by CT, since D-CDNPE, (*S*)-1,2-Dihydro, EQNPE, and Tetrahydro exhibit nearly equivalent reactivities.[34] Like the ester group of **3** and the carbon–carbon double bond of (*S*)-1,2-Dihydro, the amide group permits hydrolysis of appreciable stereoselectivity, in contrast to the results with racemic EQNPE or Tetrahydro. The source of this stereoselectivity is obscure. It could originate in the stereochemistry of the 1,3-cyclohexadiene-like ring[11] or in the lesser number of hydrogen atoms at the 3 and 4 positions of the ester-bearing ring. It surely does not arise from specific hydrogen bond interactions. Hydrogen bond donation by the olefinic hydrogens of (*S*)-1,2-Dihydro should be insignificant[35] (and for **3**, impossible), while donation *to* the olefinic bond of (*S*)-1,2-Dihydro must be much weaker than and have a stereochemical requirement different from that associated with the unshared electron pairs of the amide oxygen of D-CDNPE.[35]

IV. The Role of the Amide Group of L-APME.[36] The previous paragraph argues that strong hydrogen-bond interactions between the amide group of D-CDME and CT are nonexistent. If this is very different from the case of L-APME then a similar alignment of the amide group of equatorial D-CDME and of the reactive conformation of L-APME is unnecessary and perhaps even unlikely.

Ingles and Knowles[37] have summarized the evidence for strong hydrogen-bond interactions of the amide

(29) Because of the NPE effect, the newly acquired data on D- and L-CDNPE make the stereoselectivity observed in previous model studies with *p*-nitrophenyl esters (ref 9 and 26) much more impressive.

(30) J. L. Miles, D. A. Robinson, and W. J. Canady, *J. Biol. Chem.*, 238, 2932 (1963).

(31) R. A. Wallace, A. N. Kurtz, and C. Niemann, *Biochemistry*, 2, 824 (1963).

(32) Since neither Tetrahydro nor Tetrahydro-ME[7] undergoes stereoselective hydrolysis, the NPE effect will not assist here.

(33) The axial conformation of D-CDNPE lacks 1,3-diaxial hydrogens and could be somewhat more reactive than AXNPE.[8] No comparable explanation for the depressed reactivity of the equatorial conformations of D- and L-CDNPE is apparent. The estimate of 50 results from assuming that "preferred conformation" amplies that axial D-CDNPE is at least 10 times more reactive than its equatorial conformer.

(34) The methyl esters D-CDME, (*S*)-1,2-Dihydro-ME, and Tetrahydro-ME mirror the behavior of the corresponding *p*-nitrophenyl esters.[7,22]

(35) G. C. Pimentel and A. L. McClellan, "The Hydrogen Bond," W. H. Freeman & Co., San Francisco, Calif., 1960, especially Chapter 6.

(36) The discussion is based primarily on Table IV.

(37) D. W. Ingles and J. R. Knowles, *Biochem. J.*, 108, 561 (1968). Several comparisons in this paper unfortunately rely on unnormalized data.

group of L-APME which are postulated to introduce a distortion of substrate that is manifested by rapid rates (k_2, k_3) rather than tight binding (K_s). We find the experimental evidence summarized below more in accord with assigning much of the effect of the acetylamino group to its electronic character (accounted for by utilizing normalized rate constants) and with concluding that its specific hydrogen-bonding contributions have been generally overestimated.[38] Model studies[39] support this view since they suggest that transfer of the amide group of L-APME from an aqueous environment to a different hydrogen-bonding situation should be thermodynamically unfavorable. It appears a gross oversimplification to visualize binding of the amide group *per se* to the protein as contributing 4 kcal/mol,[37] and if some interaction of this energy is involved[38] then the binding of the amide must be intimately and essentially linked to other processes such as conformational changes of the enzyme.[40]

Comparison of $(k_c/K_m)_n$ and $(k_3)_n$ for a series of L substrates should best demonstrate the contribution of the acetylamino group to the enzymatic reactivity of L-APME. The data of Table IV show that α-acetylamino is only 20–80 times more effective than α-hydrogen, and even these figures may inflate the importance of the amide function since the methyl group of acetylamino may provide some favorable hydrophobic[19] binding (N-acetyl-L-tyrosine amide and hydrazide have K_m's about 3 times less favorable, but k_c's 6–20 times more favorable than for the corresponding N-formyl substrates[2,3]). Of the other L esters in Table IV, the N-methyl derivative may introduce bad steric repulsions, while X = OH shows no special reactivity because of H-bond donation to CT, and X = CH_3COCH_2, CH_3COO show none originating in H-bond donation by the enzyme.

Table IV. Relative Rates of Hydrolysis by CT of L-$C_6H_5CH_2CHXCO_2CH_3$

X =	Relative[a] $(k_c/K_m)_n$	Relative[b] $(k_3)_n$ L	D	Relative[b,c] k_{OH}	pK_a of CH_2X-COOH[d]
H	1.0	1.0	1.0	1.0	4.76
CH_3	0.4			(1.0)	4.88
Cl[e]	<0.09			(>45)	2.86
HO	2			(24)	3.83
CH_3COCH_2[e]	1			(1.0)	4.60
CH_3CONH	24	83	0.013	24	3.67
HCONH	~58			(24)	
CH_3CONCH_3[f]	<0.002			(24)	
CH_3COO	0.1	2.3	0.25	86	

[a] The original data refer to pH 7.8–7.9, 25°, pure water or 10–20% methanol or ethanol, methyl or ethyl esters and are in (a) J. E. Snoke and H. Neurath, *J. Biol. Chem.*, **182**, 577 (1950), (b) S. G. Cohen and S. Y. Weinstein, *J. Amer. Chem. Soc.*, **86**, 5326 (1964); (c) R. L. Peterson, K. W. Hubele, and C. Niemann, *Biochemistry*, **2**, 942 (1963); (d) ref 2, 3, and 7. Relative reactivities could be estimated because of the number of esters common to several studies, provided that these relative reactivities are independent of reaction conditions and that a methyl ester in X% methanol and the corresponding ethyl ester in X% ethanol undergo enzymatic hydrolysis at about the same rate (see ref 17). [b] Reference 37. [c] Parenthesized numbers to use in the normalization process were estimated with the pK_a data, chemical intuition, and the measured values as guides. [d] H. A. Sober, Ed., "Handbook of Biochemistry," The Chemical Rubber Co., Cleveland, Ohio, 1968, pp J-154 and 161. [e] DL ester was studied. [f] The L-tyrosine N-methyl ester was actually employed.

The strongest evidence for a sizable net H-bond interaction by the NH of L-APME is the enhanced reactivity of L-APME relative to the L-acetoxy ester. The factor of 40 whereby $(k_3)_n$ for the former exceeds that for the latter may define the maximum extent of this interaction. Table IV shows that the increased difference in reactivity between the two substrates in acylation ($(k_c/K_m)_n$) arises from the depressed rate of the L-acetoxy ester and not from a more enhanced rate for L-APME, as the strong H-bond hypothesis would suggest. The cause of the low $(k_c/K_m)_n$ for the L-acetoxy substrate is unclear; it may arise somehow from the relatively great hydrophobicity[41] of the acetoxy group.[44]

To us, employment[37] of the stereospecificity ratio $(k_3)_L/(k_3)_D$ to investigate H-bond interactions of L-APME seems undesirable, since this ratio depends upon the relative reactivities of D isomers. If a D substrate undergoes hydrolysis with its α-hydrogen and hydrolyzable group at their proper binding sites, but its aromatic ring and α substituent at the normal acylamino and hydrophobic binding sites of CT, respectively,[44,45] then these D-isomer reactivities may primarily be determined by the hydrophobic and not the H-bond capabilities of the α substituents. The rapid deacylation of the D-acetoxy ester relative to D-APME accords perfectly with this view.[41–43]

V. D-CDME as a Model for L-APME. Chemical evidence and intuition favor the following set of rules for utilizing D-CDME to analyze the geometry of the reactive conformation of L-APME. (1) Use equatorial D-CDME as the model, since the equatorial ester hypothesis is correct. (2) Make the aromatic ring and ester group of the reactive conformation of L-APME and the corresponding parts of D-CDME approximately coincide spatially. (3) Do not necessarily bring the amide functions of the two esters into juxtaposition, for the amide of D-CDME participates in no H-bond interactions, while that of L-APME probably does. Guided by these rules, we can add little to Cohen's persuasive arguments for **5a** (refer to **5c** for orientation) as a likely description of the reactive conformation of L-APME, with a complementary model for the configuration of the active site of CT.[7] Instead, we shall

(38) We primarily wish to stress the ambiguity of the present experimental chemical data. Interestingly, the *experimental* X-Ray diffraction data for the complex of formyl-L-tryptophan with CT show the "amido hydrogen points towards the carbonyl oxygen of Ser-214 but is too far away to be forming a hydrogen bond" (T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.* **46**, 337 (1969)).

(39) G. C. Kresheck and I. M. Klotz, *Biochemistry*, 8, 8 (1969).

(40) R. Biltonen and R. Lumry, *J. Amer. Chem. Soc.*, **91**, 4251, 4256 (1969), and references therein.

(41) To which both thermodynamic[42] and structure–reactivity studies[43] attest.

(42) For transfer from water to CCl_4 in dilute solutions, $\Delta H°$ is +7.8 kcal/mol for $CH_3CONHCH_3$ and −3.1 kcal/mol for $CH_3CO_2CH_2CH_3$. See E. W. Washburn, Ed., "International Critical Tables," Vol. V, McGraw-Hill Book Co., Inc., New York, N Y., 1929, pp 148–151.

(43) Much of Cohen's data has been rationalized by postulating binding of an ester group to the hydrophobic site of CT. For example, $C_2H_5O_2CCH_2CH_2CO_2C_2H_5$ is hydrolyzed, but $CH_3CONHCHCH_3CH_2$-$CO_2C_2H_5$ is not. See S. G. Cohen, *et al.*, *J. Amer. Chem. Soc.*, 83, 4225 (1961); 86, 4999 (1964); **88**, 5306 (1966).

(44) Innumerable questions about the rigidity of the acyl group in the acyl-enzyme, nonproductive binding, multiple productive modes of binding, etc., complicate this idyllic picture.

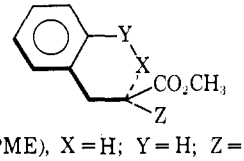
(45) C. L. Hamilton, C. Niemann, and G. S. Hammond, *Proc. Natl. Acad. Sci. U. S.*, **55**, 664 (1966).

examine how justifiable this model-building procedure is and discuss alternatives to it.

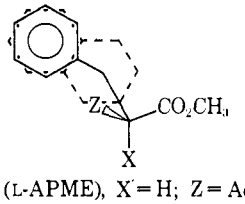
The present experimental data diminish our enthusiasm[9] for D-CDME as a model for L-APME because: (4) all *normalized* rate parameters for the D-dihydroisocarbostyril substrates and $(k_c)_n$ for (*S*)-1,2-Dihydro are seriously inferior to the corresponding quantities for the appropriate N-acetyl-L-phenylalanine esters (Table II). (5) Stereoselectivity in hydrolyses of the bicyclic semirigid substrates is universally less than with acetylphenylalanine derivatives (Tables I and II, ref 7 and 8). (6) Hydrolyses of these bicyclic substrates exhibit a whimsical pattern of stereoselectivity. It is relatively high for CDME, CDNPE, 1,2-Dihydro, and *p*-nitrophenyl *cis*-3-*t*-butylcyclohexanecarboxylate but almost negligible with EQNPE, Tetrahydro, Tetrahydro-ME, and 3-carboethoxy-α-tetralone.[7,8] (7) Of most concern, no significant increase in the rate of enzymatic hydrolysis results from fusion of a six-membered ring to *p*-nitrophenyl cyclohexanecarboxylate, producing either Tetrahydro or EQNPE (Table I). An analogous comparison for nonrigid substrates might be methyl α-N-acetyl-L-aminovalerate to L-APME or methyl N-acetyl-L-hexahydrophenylalaninate. The latter compounds are over 100 times more reactive than the first.[46]

We find the evidence that 2-naphthoic acid derivatives are suitable models for D-CDME and D-CDNPE convincing. Consequently point 7 makes rule 2 extremely suspect, and 4–7 *in toto* make deduction of the reactive conformation of L-APME from equatorial D-CDME logically indefensible since no chemical experiment has unambiguously established how the two substrates should be compared. The reactive geometries of the two substrates may truly "closely" resemble each other. Unfortunately the experiments available do not prove it and in fact leave the term "closely" ill-defined. Furthermore, no experiment has shown that gross geometric differences, such as those implied by Chart II, and not more subtle considerations

Chart II



5a (L-APME), X = H; Y = H; Z = AcNH
5b (D-APME), X = AcNH; Y = H; Z = H
5c (D-CDME), X = NH; Y = C=O; Z = H
5d X = CH_2; Y = CH_2; Z = AcNH



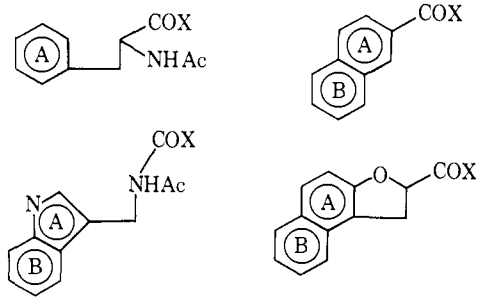
6a (L-APME), X = H; Z = AcNH
6b (D-APME), X = AcNH; Z = H

(as the angular orientation of the reactive ester group[18]) are primarily responsible for the distinction between good and poor substrates.

(46) (a) J. B. Jones, T. Kunitake, C. Niemann, and G. E. Hein, *J. Amer. Chem. Soc.*, 87, 1777 (1965); (b) J. B. Jones and C. Niemann, *Biochemistry*, 2, 498 (1963).

Of the models proposed for the reactive conformation of L-APME, those which are derived from the false axial ester hypothesis cannot be dismissed on the basis of rigid substrate studies, if the reservations expressed about D-CDME are valid. Likewise point 7 and the discussion of sections III and IV weaken many of the arguments[2,3,7,26] against Erlanger's model, which does not superimpose the aromatic rings of D-CDME and L-APME. Cohen's model **5a** is the one most simply derived from rules 1–3 but does not appear to adequately explain the necessarily slow hydrolysis of D-APME in conformation **6b**.[47] We proposed **6a** as the reactive conformation of L-APME, since D-APME appeared unable to achieve a similar reactive conformation *via* either **5b** or **6b**.[9]

Is a completely different analysis of the behavior of the D-CDME substrates possible, aside from that[6] of Erlanger? The recent data of Hayashi and Lawson[8b] suggest an alternative worth exploring. There is a strongly hydrophobic binding site (A site) of CT with which the aromatic ring of L-APME associates.[2,3,45,48] The lack of a significant difference in enzymatic reactivity between the cyclohexyl ester and Tetrahydro or EQNPE could indicate that the *ester-bearing* ring of these substrates (and D-CDME) should be correlated with this A site. The second ring of these bicyclic substrates then better approximates the homocyclic ring of an L-tryptophan substrate (B site). Only with the tricyclic substrate shown do the ester groups of rigid and nonrigid substrates finally achieve close correspondence, and does the former approach the latter in ease and stereoselectivity of hydrolysis.[8b]



VI. Finale. Conclusions based upon carefully limited variations in substrate structure appear to us reasonably persuasive. Examples are the exploration of the factors governing the reactivity of D-CDME, of Lawson's substrates, or of the N-acetyl-L-amino acid derivatives.[2,3,48] The assumptions required for analysis of structure–reactivity effects in studies employing a wide variety of substrates make any resultant detailed models[49] of substrate conformation difficult to justify. In the case of L-APME and CT, the enzyme is obviously tolerant of major deviations by some parts of the substrate from their optimum binding sites and intolerant of minor deviations in other instances. Similarity or dissimilarity in the ease of hydrolysis of two substrates therefore has no necessary geometric corollary. As a further complication, the enzyme's response to a particular variation in substrate structure depends upon

(47) The slow enzymatic hydrolysis of racemic **5d** (preliminary experiments) is also difficult to reconcile to a literal interpretation of the model.
(48) See J. R. Knowles, Table II, footnote *a*.
(49) We refer to specifications of bond angles and distances, not to enumerations of effects (ref 19, 45).

Table V. Kinetic Parameters for Some CT-Catalyzed Turnover Reactions[a]

Substrate	Runs[b]	$10^8[E]_0$, *M*	$10^6[S]_0$, *M*	pH	% MeOH	k_c, sec^{-1}	10^6K_m, *M*
(*S*)-1,2-Dihydro	24	11.1	2.7–13.5	5.4	20	0.16 ± 0.00	1.8 ± 0.1
	13	0.95	5.5–26.6	7.0	20	3.3 ± 0.1	4.5 ± 0.4
	23	1.09	2.7–13.5	8.0	20	7.0 ±0.3	4.1 ± 0.5
(*R*)-1,2-Dihydro	28	55	2.4–12.7	5.4	20	0.0042 ± 0.007	2.5 ± 0.1
	15	45	1.9–12.4	7.0	20	0.072 ± 0.002	3.3 ± 0.2
	17	11.5	2.4–12.7	8.0	20	0.12 ± 0.007	3.5 ± 0.5
Tetrahydro	24	6.9	2.8–15.7	8.0	20	0.23 ± 0.01	10.5 ± 1.1
	17	25.7	4.9–12.4	8.0	30	0.62 ± 0.07	42 ± 6
Naphtho	15	55.7	2.9–7.0	8.0	20	0.042 ± 0.000	10.5 ± 1.0
1,4-Dihydro	22	55.7	3.1–16.6	8.0	20	0.032 ± 0.002	10.0 ± 0.8
EQNPE	14	9.7	2.5–12.4	8.0	30	0.68 ± 0.15	37 ± 10
L-APNPE	5[c]	3.55–39	190	5.4	0	4.6 ± 0.2	
	22	1.75	12.5–200	5.4	20	53 ± 2	78 ± 7
	7[c]	1.86–3.56	190	7.0	0	45 ± 3	
D-APNPE	6[c]	5560	125–249	5.4	0	0.00059 ± 0.00008	
D-CDNPE	5[c]	7.8	71.6	5.4	0	1.7 ± 0.09	
	15	12.3	11.1–150	5.4	20	27 ± 2	240 ± 22
	16[c]	1.4	4.7–145	7.0	0	13 ± 1.6	
	14	0.83	12.2–208	7.0	20	146 ± 4	124 ± 6
L-CDNPE	19	37	4.5–45	7.0	0	0.59 ± 0.02	9.5 ± 0.1
	18	45	12.5–200	7.0	20	11.8 ± 2.3	750 ± 170
L-APME	20	14	357–7580	7.0	0	38.3 ± 2.4	580 ± 75
L-CDME	16	1800	600–3000	7.0	0	0.024 ± 0.003	2690 ± 470

[a] All reactions at 25° and contain 2.5–3% acetonitrile. [b] Number of points in the Lineweaver–Burk plot. [c] Variation in $[S]_0$ did not alter v_0, which presumably means $v_0 = k_c[E]_0$.

other features present in the substrate (as in the erratic stereoselectivity observed in the hydrolysis of a carboalkoxy group attached to a six-membered ring (point 6 above)). This coupling effect may sometimes arise from nonbonded interactions within the substrate which limit the number of conformations the substrate can achieve.[4]

Experimental Section

Kinetic Methods.[50] Worthington Lots CDI 16121-2, 16150-1, and 6JF of α-chymotrypsin were employed. Buffer ingredients were reagent grade, distilled water was redistilled through an all-glass apparatus, and methanol and acetonitrile were Matheson Coleman and Bell Spectroquality. The following buffers[51] were employed: (a) 0.1 *M* acetate, pH 5; (b) 0.067 *M* phosphate, pH 5.4, 6.7, 7.0, and 7.7; (c) 20% methanol buffers of pH 5.4, 7.0, and 8.0, obtained by diluting 20.0 ml of methanol to 100 ml with the pH 5, 6.7, and 7.7 buffers, respectively; (d) 30% methanol, pH 8, obtained by dilution of 30.0 ml of methanol to 100 ml with the pH 7.7 buffer. All volumes were measured at 25°. In addition, 20% methanolic buffers of pH 9 (borate) and pH 10 and 11 (borate–carbonate) were required in the determination of the rates of the hydroxide ion promoted hydrolyses.

In a typical kinetic run with a *p*-nitrophenyl ester, 3.0 ml of buffer plus 0.100 ml of enzyme solution were equilibrated at 25.3 ± 0.5 in a cuvette installed in the thermostated cell holder of a Cary Model 14 or 16 (equipped with a Model 1626 interface and Sargent SRL recorder) spectrophotometer. Addition of 0.100 ml of a solution of substrate in acetonitrile initiated reaction and the optical density at 400 nm (pH ≥7) or 330 nm (pH 5.4) recorded as a function of time.[52] The following variations were employed: (a) for pH 5.4 runs, the addition of the enzyme solution to a mixture of buffer plus substrate initiated reaction; (b) for runs with indole, 0.050 ml of a solution of indole in acetonitrile plus 0.050 ml of substrate solution substituted for the usual substrate solution; (c) runs to determine the rate of hydrolysis caused by hydroxide ion omitted the enzyme solution. The pH's of sample reaction mixtures were determined with Radiometer PHM 4C or TTT1 meters.

The rates of hydrolysis of L-APME and L-CDME were measured in 0.01 *M* phosphate buffer containing 0.2 *M* NaCl and 2.5% acetonitrile with a Radiometer TTT1/SBR2/SBU1 apparatus.[54]

Analysis of Kinetic Data. Most enzymatic reactions were studied under turnover conditions ($K_m \approx [S]_0 \gg [E]_0$). Initial velocities, v_0, of turnover reactions, evaluated by the polynomial method of Booman and Niemann,[55] eventually provided[16] k_c and K_m. Doubling $[E]_0$ resulted in a doubling of v_0 for several runs employing $[E]_0 \approx 10^{-8}$ *M*, thus establishing the absence of wall effects[56,57] for those runs with dilute enzyme solutions. For L-APNPE and D-CDNPE in aqueous pH 5.4 and 7.0 buffer, v_0 was essentially constant over the experimentally accessible range of $[S]_0$. Under these conditions, v_0 defined $k_c[E]_0$ and hence k_c at high $[S]_0$, and values for k_c so obtained were found to be identical with those measured when $[S]_0$ was halved.

The enzymatic reactivities of AXNPE and D-APNPE were so low that they could only be evaluated *via* acylation reactions ($K_s \gg [E]_0 \geq [S]_0$) which provide a second-order rate constant k_2' which is equal to k_c/K_m.[15] Data for Naphtho and 1,4-Dihydro from both acylation and turnover experiments reaffirm this equivalence.

The hydrolyses of DL-AXNPE and Tetrahydro proceeded with no detectable stereoselectivity, while it was estimated that the two stereoisomers of DL-EQNPE might differ in enzymatic reactivity by a factor of 3–4. These estimates were made from acylation runs, which for Tetrahydro and EQNPE were of poor quality because of the relatively high substrate reactivity. Cohen[7] estimated a stereoselectivity factor of 6 for DL-Tetrahydro-ME.

Corrections for the spontaneous hydrolysis of substrates were applied wherever needed. In the case of maximum correction, the spontaneous rate was 15% of that observed in the presence of enzyme. All calculations by the method of least squares employed an IBM 1130 computer. Tables V–VII summarize the experimental data. Indole also inhibited the hydrolysis of AXNPE and EQNPE, but the inhibition was not quantified.

Normalization Procedures. Measurement of the hydroxide ion promoted hydrolysis of the *p*-nitrophenyl esters employed permitted normalization as described in the Results. Data scattered through the literature support the assumption made that the relative reactivity toward hydroxide ion of a series of methyl, serine (≈CT), or *p*-nitrophenyl esters will be essentially identical. Since azlactone

(50) Procedures were essentially those detailed in ref 15 and 26.

(51) W. M. Clark, "The Determination of Hydrogen Ions," The Williams and Wilkins Co., Baltimore, Md., 1928.

(52) Addition of substrate to enzyme and lowering of the pH minimize complications resulting from azlactone formation in studies with D- and L-APNPE.[53]

(53) J. de Jersey and B. Zerner, *Biochemistry*, **8**, 1967 (1969).

(54) We are indebted to Mr. Douglas L. Jacobs for performing these measurements.

(55) K. A. Booman and C. Niemann, *J. Amer. Chem. Soc.*, 78, 3642 (1956).

(56) R. L. Bixler and C. Niemann, *ibid.*, 81, 1412 (1959).

(57) Reference 18 claims the absence of wall effects with $[E]_0 = 6.7 \times 10^{-9}$ *M*.

Table VI. Indole Inhibition of Some CT-Catalyzed Turnover Reactions[a]

Substrate	Runs	$10^3[I]_0$, M	k_c, sec^{-1}	10^6K_m, M	10^3K_i, M[b]
(*S*)-1,2-Dihydro	17	4.35	7.1 ± 0.5	8.1 ± 0.9	4.7
	14	6.53	9.9 ± 1.2	14.9 ± 2.4	4.4
(*R*)-1,2-Dihydro	13	4.35	0.15 ± 0.02	10.1 ± 1.6	3.2
	11	6.53	0.16 ± 0.02	13.2 ± 2.4	3.2
Tetrahydro	14	4.35	0.24 ± 0.0	23.1 ± 2.7	3.8
	14	6.53	0.51 ± 0.12	69 ± 17	3.4
Naphtho	10	4.35	0.026 ± 0.004	15.5 ± 3.6	3.3
	10	6.53	0.031 ± 0.001	26 ± 6	2.9

[a] At 25°, pH 8, 3% acetonitrile–20% methanol, with $[E]_0$ and $[S]_0$ as for the corresponding uninhibited runs listed in Table V. [b] Evaluated from the expression $K_i = [I]_0/\{[(k_c/K_m, [I] = 0)/(k_c/K_m, [I] = [I]_0)] - 1\}$.

Table VII. Four Acylation Reactions of CT[a]

Substrate	Runs	$10^6[S]_0$, M	$10^6[E]_0$, M	k_2', M^{-1} sec^{-1}	k_c/K_m, M^{-1} sec^{-1}
Naphtho[b]	17	2.9–7.0	5.1–9.4	4,050 ± 180	3,960[c]
1,4-Dihydro[b]	12	3.1–8.3	5.1–9.4	3,520 ± 150	3,180[c]
AXNPE[d]	12	3.7–9.2	25–49	13.1 ± 0.8	(18,000[e])
D-APNPE[f]	12	8.0–13.3	25.5–50.9	220 ± 10	

[a] At 25°, pH 8, 3% acetonitrile. All uncertainties are standard deviations. [b] 20% MeOH. [c] From turnover data of Table V under the same conditions. [d] 30% MeOH. [e] For EQNPE under the same conditions, from Table V. [f] pH 5.4, 20% MeOH.

formation is an important pathway in the hydroxide ion promoted hydrolysis of APNPE, the rate of this reaction does not serve as a proper basis of normalization.[18,58] Hydrolysis by hydroxide ion of the corresponding CBZ compound is uncomplicated by azlactone formation[18,58] and the rate of this reaction can serve as a guide to the desired normalization if the relative stereoelectronic effects on hydrolysis of the acetyl and CBZ groups are known. Two pieces of information suggested that these effects of the two groups are essentially identical and the normalization procedure justified. First, within experimental error the pK_a's of N-acetyl-L-tryptophan and N-CBZ-L-tryptophan, determined spectrophotometrially, are the same. Second and more convincingly, hydrolyses of methyl N-acetyl- and N-CBZ-L-tryptophanate[59] by hydroxide ion, which should not involve azlactone formation,[58] occur at the same rate under identical experimental conditions.

Chemicals Purchased. CBZ-L-tryptophan, methyl acetyl-L-tryptophanate, and *p*-nitrophenyl CBZ-D- and -L-phenylalaninate were purchased from Cyclo Chemical Corporation and methyl acetyl-L-phenylalaninate from Mann Research Laboratories; these materials were not further purified. Recrystallization from ethanol–benzene of Matheson Coleman and Bell 2-naphthoic acid gave acid of mp 183–185° (lit.[61] mp 185.5°).

DL-1,2-Dihydro-2-naphthoic acid, from the reduction of 2-naphthoic acid with sodium amalgam, had mp 100–101° (lit.[62] mp 101°).

1,4-Dihydro-2-naphthoic acid, from the base-catalyzed isomerization of the 1,2-dihydro isomer, had mp 159–160° (lit.[63] mp 161°).

3,4-Dihydro-2-naphthoic acid, supposedly obtainable by heating the 1,4-dihydro isomer with 30% potassium hydroxide, had mp 115.5–116.5° (lit.[64] mp 118°). The product was probably a mixture of the 1,4 and 3,4 isomers (see below).

1,2,3,4-Tetrahydro-2-naphthoic acid, from the reduction of 2-naphthoic acid with lithium in ammonia, had mp 95.3–99° (lit.[65] mp 95.5–96°).

Decalin Acids. The method of Chapman, Shorter, and Toyne[66] yielded *trans*-decalin-2β-carboxylic acid, mp 80–81° (lit.[66] mp 80–81°) and the 2α isomer, mp 105–106° (lit.[66] mp 105–106°).

DL-3-Carboxydihydroisocarbostyril and related compounds were synthesized according to the procedures of Hein and Niemann.[2,3] The title acid had mp 235–238° (lit.[2,3] mp 235–237°) and its methyl ester had mp 112° (lit.[2,3] mp 115–116.5°). Enzymatic resolution of the racemic methyl ester gave D acid with mp 232–234.5°, $[\alpha]^{25}D$ −47° (*c* 2, methanol) (lit.[2,3] mp 232.5–234°, $[\alpha]$ D −45°), and unreacted L ester with mp 87.5–88.5°, $[\alpha]^{25}D$ +80° (*c* 2, methanol) (lit.[2,3] mp 88–89°, $[\alpha]D$ +82°). Hydrolysis of the L ester in the manner described for methyl (+)-1,2-dihydro-2-naphthoate yielded L acid with mp 234–236°, $[\alpha]^{25}D$ +46° (*c* 2.5, methanol).

Resolution of 1,2-Dihydro-2-naphthoic Acid. We provide details of our work with this acid since it and its derivatives readily undergo racemization and/or isomerization. The racemic acid (1.74 g) was dissolved in 0.75 ml of aqueous ammonia and its silver salt precipitated by the addition of a solution of 1.72 g of silver nitrate in 4 ml of water. Treatment of the dried silver salt (2.72 g, 97%) with a solution of 1.56 g of methyl iodide in 80 ml of ether according to Vogel[67] gave a 54% yield of oily methyl ester, which was not carefully characterized. Unreacted acid can be recovered from the material which fails to dissolved in the ether solution.

The actual resolution was accomplished with CT. The pH of 600 ml of a dilute phosphate buffer containing 20% isopropyl alcohol and 4.0 g of racemic methyl ester was adjusted to pH 7.8 and a solution of 0.5 g of CT in 5 ml of water added. Periodic additions of 0.267 *N* sodium hydroxide to the slowly stirred solution kept the pH approximately constant. After 4 hr, a solution of 0.41 g of CT in 4 ml of water was added. At the end of ~8 hr 96% of the theoretical amount of base (calculated for only one enantiomer undergoing hydrolysis) had been consumed. A chloroform extract of this solution, when separated, washed with water, dried and concentrated, afforded 1.33 g (67%) of (+)-methyl ester. A second chloroform extract of the acidified residual aqueous layer, when separated, washed with water, dried and concentrated, yielded 1.57 g (85%) of crude (−)-acid, mp 97–102°. Recrystallization from ethanol–water of the combined (−)-acid from several such resolutions gave a best sample of (−)-1,2-dihydro-2-naphthoic acid: mp 106–107°; $[\alpha]^{25}D$ −282.5° (*c* 0.8, chloroform) (lit.[62] $[\alpha]^{25}D$ −197.5°).

Oily (+)-methyl ester (1.6 g) was hydrolyzed in a solution of 12 ml of concentrated hydrochloric acid and 40 ml of acetic acid for 48 hr at room temperature.[68] Addition of 120 ml of water was

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(59) The CBZ ester, prepared from the corresponding acid by the method of Wieland and Sarges,[60] was an oil It possessed the expected infrared and nmr spectra and upon complete hydrolysis gave a solution whose optical density agreed with that calculated for the appropriate solution of CBZ-L-tryptophan. The rates of hydrolysis of the two methyl esters were measured spectrophotometrically at 300 nm.

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Table VIII. Physical Properties of *p*-Nitrophenyl Esters

Compd	Mp, °C	Formula	Analysis[a] Calcd, % C	Calcd, % H	Found, % C	Found, % H	$[\alpha]^{25}D$	% purity[b]
AXNPE	74.5–75	$C_{17}H_{21}NO_4$	67.31	6.98	67.41	6.90		108 ± 3
EQNPE	94–95	$C_{17}H_{21}NO_4$	67.31	6.98	67.66	6.97		104 ± 4
(*S*)-1,2-Dihydro	97.5–99.5	$C_{17}H_{13}NO_4$	69.14	4.43	69.07	4.71	−197.5[c]	99 ± 3
(*R*)-1,2-Dihydro	93–96	$C_{17}H_{13}NO_4$	69.14	4.43	69.43	4.52	+171.4[c]	100 ± 1
Tetrahydro	101–104	$C_{17}H_{15}NO_4$	68.67	5.09	68.84	5.22		101 ± 2
Naphtho	142–146[d]	$C_{17}H_{11}NO_4$	69.62	3.78	69.57	4.02		96 ± 1
1,4-Dihydro	132–134	$C_{17}H_{13}NO_4$	69.14	4.43	69.29	4.36		97 ± 2
D-CDNPE	188–190	$C_{16}H_{12}N_2O_5$	61.54	3.87	61.58	3.87	−84.0[e]	100 ± 1
L-CDNPE	187–190	$C_{16}H_{12}N_2O_5$	61.54	3.87	61.40	3.92	+86.0[e]	100 ± 1
D-APNPE[f]	133						+17.6	100 ± 2
L-APNPE[g]	139.5–140						−18.5	95 ± 1

[a] Analyses by Micro-Tech Laboratories, Skokie, Ill. [b] Average per cent of theoretical amount of *p*-nitrophenol liberated in several total hydrolyses. [c] 0.4% in $CHCl_3$. [d] Reference 61, p 559 gives mp 142–143°. [e] 0.7% in acetone. For D-CDNPE, ref 20 gives −19.6°. [f] Reference 18 gives mp 135–137°, $[\alpha]^{20}D$ +17.4° (*c* 2, $CHCl_3$). [g] Reference 18 gives mp 140–140.5°, $[\alpha]^{20}D$ −18.6°.

followed by extraction with four 30-ml portions of benzene. The combined benzene extracts were shaken with ice cold 5% sodium bicarbonate, and the aqueous phase separated, acidified, and extracted with chloroform. This chloroform solution, treated as described for the enantiomeric acid, gave 0.46 g (31%) of crude acid, mp 100–104°, and eventually, a relatively pure sample of 0.38 g of (+)-1,2-dihydro-2-naphthoic acid, mp 102–104°; $[\alpha]^{25}D$ +260° (*c* 0.8, chloroform).

Cohen[7] has established the absolute configuration of the 1,2-dihydro-2-naphthoic acids. His proof relies upon the following reaction. Catalytic reduction at atmospheric pressure of 0.91 g of 1,2-dihydro acid, $[\alpha]^{25}D$ +93.5° (*c* 0.8, chloroform), $[\alpha]^{29}D$ +89° (*c* 3.6, acetic acid), in 25 ml of acetic acid plus 0.2 g of platinum oxide resulted in the uptake of 96% of the theoretical amount of hydrogen in 1 hr. Removal of the catalyst by filtration gave a solution with $[\alpha]^{29}D$ −18.7° (*c* 3.6, acetic acid). Addition of water to the acetic acid solution precipitated an acid which was collected, washed with water, and dried. This sample of 1,2,3,4-tetrahydro-2-naphthoic acid had mp 91–93.5°, $[\alpha]^{25}D$ −18° (*c* 2.3, chloroform), and an infrared spectrum identical with that of the pure racemic acid. Pickard and Yates[62] describe a sample of possibly optically pure tetrahydro acid of $[\alpha]D$ −51.8° (chloroform). Based on this figure, we calculate that the reduction product is 35% optically pure, and that optically pure 1,2-dihydro-2-naphthoic acid should have $[\alpha]D$ ±267°, as compared to our best value of −282.5°. The data suggest that complete resolution of the 1,2-dihydro-2-naphthoic acids was achieved, but the *p*-nitrophenyl esters prepared from these acids clearly were *not* optically pure (see below).

Synthesis and Properties of *p*-Nitrophenyl Esters. The N-acetyl-D- and -L-phenylalanine esters were prepared from the corresponding CBZ esters.[18] Silica gel chromatography of the crude products with ethyl acetate as eluent, followed by two recrystallizations from ethyl acetate–hexane, gave the substrates whose properties, together with those of all the other *p*-nitrophenyl esters, are presented in Table VIII. Both esters behaved in kinetic runs as though optically pure.

The *p*-nitrophenyl esters of 2-naphthoic, 1,2,3,4-tetrahydro-2-naphthoic, *trans*-decalin-2α-carboxylic, and *trans*-decalin-2β-carboxylic acids were simply prepared by isolating the product from the reaction of the appropriate acid chloride with *p*-nitrophenol in pyridine–ether solution at −5°.

Preparation of the *p*-nitrophenyl esters of D- and L-3-carboxydihydroisocarbostyril was most difficult. The procedure finally adopted[69] involved the reaction of 0.96 g (5.0 mmol) of L acid, 0.84 g (6.0 mmol) of *p*-nitrophenol, and 1.08 g (5.2 mmol) of dicyclohexylcarbodiimide (DCC) in 10 ml of dimethylformamide at 0° for 2 hr and then overnight at room temperature. Addition of 0.5 ml of acetic acid was followed by filtration, dilution of the filtrate with 50 ml of water, and extraction of this solution with two 50-ml portions of ethyl acetate. The combined ethyl acetate extracts were washed with dilute hydrochloric acid and water, dried over anhydrous magnesium sulfate, filtered, and partially concentrated. The collected precipitate (188 mg, mp 184–188°) upon recrystallization from acetone–hexane gave 94 mg of the product described. Further concentration of the ethyl acetate extracts gave additional precipitates, but these could not be purified by recrystallization. The *p*-nitrophenyl ester of the D acid was prepared in an identical manner. Kinetic data indicated both esters were optically pure. We were unable to synthesize the *p*-nitrophenyl ester of carboxyisocarbostyril by this or any other procedure.

The *p*-nitrophenyl esters of 1,4-dihydro-2-naphthoic, 3,4-dihydro-2-naphthoic, and the optically active 1,2-dihydro-2-naphthoic acids were routinely prepared *via* the DCCD method in ethyl acetate solvent.[70] The nmr spectrum of the 3,4-dihydro ester was interpretable if it was assumed that it contained about 30% of the 1,4 isomer. Kinetic curves from acylation reactions showed no deviations from that expected for a homogeneous substance, so it appears that the rates of hydrolysis of the two esters by CT are approximately equal. Unfortunately, (*R*)- and (*S*)-1,2-Dihydro were not optically pure, as judged by their kinetic behavior; either the acid precursors were not completely resolved, or racemization took place in the DCC reaction. The optical purity of the two samples was estimated as follows. The (*S*) isomer (more reactive one) released only 83.5% of the theoretical amount of *p*-nitrophenol in a series of reactions with very dilute enzyme ($[E]_0 \approx 1 \times 10^{-8}$ *M*), under conditions where the (*R*) isomer is essentially unreactive. The (*R*) ester, in 23 runs with $[E]_0 \approx 1 \times 10^{-7}$ *M*, gave a burst of *p*-nitrophenol corresponding to the presence of 22 ± 1% of (*S*) isomer, for under these conditions the latter was essentially completely hydrolyzed by the time our observations began. A check on these two estimates can be made by calculating for each isomer what the optical rotation of an optically pure sample should be. The (*S*) ester with $[\alpha]D$ −197.5°, if 67% optically pure, extrapolates to $[\alpha]D$ −295°. The (*R*) ester with $[\alpha]D$ +171.4°, if 56% optically pure, extrapolates to $[\alpha]D$ +306°. The estimates are internally consistent. In calculating $[S]_0$ for the Lineweaver–Burk plots, allowance was made for the presence of 20% contaminating enantiomer in each of these substrates.

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Appendix

The NPE Effect.[24,71] The data of columns 2–3 show that *p*-nitrophenyl esters display the same general pattern of structure–reactivity effects as methyl esters but on a slightly compressed scale. If the K_i's of column 1 define the "normal" binding specificity of CT, then the product $(k_c/K_m)_n K_i$ (columns 4 and 5)[72] represents an *ad hoc* approximation to the kinetic specificity of acylation if in the acylation step all substrates manifest their normal binding specificity.

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(70) M. Bodanszky and V. duVigneaud, *Biochemical Prep.*, **9**, 110 (1962).

(71) The discussion utilizes the data of Table II.

(72) Essentially the procedure of ref 17.

Comparison of columns 4 and 5 to $(k_3)_n$, which we assume defines the "normal" kinetic specificity of CT, suggests that in acylation with *p*-nitrophenylates the kinetic and/or binding partiality of CT for acetyl-L-tryptophan is lower than under "normal" circumstances.[73] To isolate the cause requires a separation of k_2 and K_s.[74]

The available stereochemical data (columns 7 and 8) provide stronger evidence for a diminished selectivity in acylation reactions with *p*-nitrophenyl esters.[75] If $(k_3)_{fast}/(k_3)_{slow}$ measures the kinetic stereoselectivity of CT, then $(k_c/K_m)_{fast}/(k_c/K_m)_{slow}$ should be much larger, since it reflects kinetic stereoselectivity plus the preferential productive binding of the more rapidly hydrolyzed isomer. The methyl esters fullfil this expectation but the *p*-nitrophenyl esters do not. With APME, for example, the predicted[76] total stereoselectivity is $\sim 2 \times 10^6$ while the observed figure is $>7 \times 10^5$. For APNPE, the prediction is the same and the discrepancy between prediction and observation is at least 10^3. It appears that this discrepancy arises from an unexpectedly high reactivity for D-APNPE with CT.

Among possible explanations for the NPE effect are the following two. First, methyl and *p*-nitrophenyl esters may have different hydrogen-bonding requirements.[23] The generality of this statement is limited by the observation that amides and methyl esters show the same structural selectivity[19] although their hydrogen-bond requirements should be quite dissimilar. Second, *p*-nitrophenyl esters may be capable of entering into multiple productive binding modes, contrary to the usual assumptions.[19,45,73]

(73) The small $(k_c/K_m)_n K_i$ for AcGlyCOME probably illustrates the relatively great tendency for it and $AcGlyCONH_2$ (but not AcGlyCONPE?) to enter into nonproductive binding. See the population analysis of ref 45.

(74) Hess and coworkers recently reported k_2 and K_s for L-AcTrpCOEE and L-AcPheCOME and estimated them for L-AcTrpCONPE. Their data accord with the analysis of Table II but do not permit resolution of the question we raise. See (a) K. G. Brandt, A. Himoe, and G. P. Hess, *J. Biol. Chem.*, **242**, 3973 (1967); (b) A. Himoe, K. G. Brandt, R. J. DeSa, and G. P. Hess, *ibid.*, **244**, 3483 (1969).

(75) Reference 53 reports stereoselectivity in acylation with the enantiomeric azlactones of N-benzoyltyrosine less than in deacylation of the resultant acylenzymes.

(76) Population analysis[45] shows productive binding for L-AcPheCONH$_2$ 300 times more favorable than for the D isomer. Couple 300 to the 8×10^4 of column 9 and 2×10^6 results.

The Synthesis and Proof of Structure of Perosamine (4-Amino-4,6-dideoxy-D-mannose) Derivatives[1]

C. L. Stevens, R. P. Glinski, K. G. Taylor, P. Blumbergs, and S. K. Gupta

Contribution from the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received October 23, 1969*

Abstract: The synthesis and structure of proof of derivatives of 4-amino-4,6-dideoxy-D-mannose are described. These compounds are shown to be identical with the corresponding derivatives of perosamine, the carbohydrate moiety of the antibiotic perimycin. Most critical to the synthetic sequence was the ability to change the direction of the epoxide opening of methyl 3:4-anhydro-6-deoxy-α-D-talopyranoside (**3a**) by structure modification, so as to favor attack of azide ion at C-4 rather than at C-3. The ratio of C-3:C-4 attack on **3a** was ~1:3 but could be altered to a 4:1 ratio by benzoylating the C-2 hydroxyl prior to azide opening of the epoxide.

Efforts in this laboratory have led to recent disclosures of the synthesis and chemistry of six of a possible eight members of a new class of carbohydrates, the 4-amino-4,6-dideoxy-D-hexoses having the glucose,[2a] galactose,[2b] idose,[3] altrose,[3] gulose,[4] and talose[5] configurations. The importance of these new aminosugars is underscored by numerous reports of their occurrence and isolation from a variety of natural sources.[6a–i] As an extension of these efforts, routes to 4-amino-4,6-dideoxy-D-mannose (**1**) were investigated. As this work was nearing completion, Schaffner and Lee reported[6i] the isolation of a basic carbohydrate moiety, perosamine, from the acid hydrolysis of the

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