α-Chymotrypsin and Rigid Substrates. Reactivity of Some *p*-Nitrophenyl 1,2,3,4-Tetrahydro-2-naphthoates and Indan-2-carboxylates[1]

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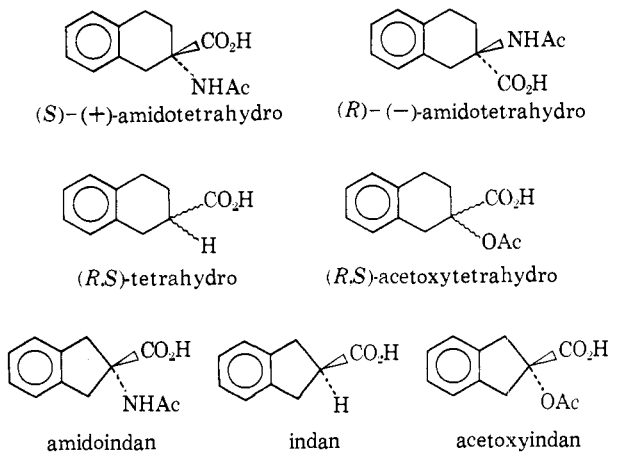
Abstract: The *p*-nitrophenyl esters of some semirigid 1,2,3,4-tetrahydro-2-naphthoic and indan-2-carboxylic acids (α substituents, NHAc, H, OAc) were prepared and their α-chymotrypsin-catalyzed hydrolysis rates were measured at pH 5.4, in 20% methanol. The *S* isomer of *p*-nitrophenyl 2-acetamido-1,2,3,4-tetrahydro-2-naphthoate was hydrolyzed 22 times faster than its antipode. No stereoselectivity was observed in hydrolysis of the other 2-naphthoates. Relative reactivities of the α-substituted 2-naphthoates as a function of the α substituent were NHAc (*S* isomer) > H > NHAc (*R* isomer) ≫ OAc. Chymotryptic hydrolysis of the indan esters appears to be less sensitive to the nature of the α substituent. Attempts to normalize the enzymatic rates of the substrates using second-order rate constants determined for the hydroxide ion catalyzed hydrolysis of their methyl esters (ME's) or *p*-nitrophenyl esters (NPE's) were stymied when it was found that the relative sensitivity of the compounds to alkaline hydrolysis is a function of the type of leaving group displaced. This effect was attributed to the greater steric demands of the NPE's than the ME's, but it is not clear whether acylation and deacylation of the enzyme are affected similarly by steric factors. This dilemma, intensified by the possibility of the existence of other less easily identified steric interactions of the rigid substrates with the active site of the protein, tempers our enthusiasm for their use as facsimilies of the reactive conformation of analogously α-substituted β-phenylpropionates at the active site of α-chymotrypsin.

The hydrolysis of methyl *N*-acetyl-(*S*)-phenylalaninate ((*S*)-APME) is catalyzed rapidly and with high stereoselectivity by α-chymotrypsin (CT).[2,3] High reactivity results when the β-aryl, α-hydrogen, and α-acetamido groups of this specific substrate are situated at the active site of CT in a conformation which leaves the ester function proximate to Ser-195 and His-57 of the protein.[4] Cohen has proposed that (*S*)-APME binds to CT in the conformation of Figure 1 during CT-catalyzed hydrolysis.[5] As an experimental test of the validity of this model, we have examined trends in the reactivity of CT toward the *p*-nitrophenyl esters (NPE's) of several semirigid acids in which the spatial relationship of the phenyl ring to the carboxylate ester and an α substituent is similar to that postulated for (*S*)-APME.[6]

Results

The NPE's and methyl esters (ME's) synthesized for use in this study are shown in Chart I.[10] Optically

Chart I



pure (+)- and (−)-amidotetrahydro *p*-nitrophenyl esters were prepared from the acids isolated following the stereospecific hydrolysis of (*R,S*)-amidotetrahydro methyl ester by CT. We have failed to design a stereospecific synthetic or degradative scheme to determine the absolute configuration of the (+) or (−) esters. On the basis of the known stereochemical preference of CT for one optical isomer of other (±)-2-naphthoic acid-like esters,[5b,7] the more rapidly hydrolyzed (+) isomer has been tentatively assigned the *S* configuration. The circular dichroism spectra of (*S*)-(+)-APME and (−)-amidotetrahydro methyl ester may support this assignment.[11] Both compounds exhibited multiple

(1) Presented in part at the Southeast–Southwest Regional Meeting of the American Chemical Society, New Orleans, La., Dec 2–4, 1970, Abstracts of Papers, p 31.

(2) J. B. Jones and C. Niemann, *Biochemistry*, 2, 498 (1963).

(3) M. L. Bender and W. A. Glasson, *J. Amer. Chem. Soc.*, 82, 3336 (1960).

(4) For reviews see (a) T. G. Bruice and S. Benkovic, "Bioorganic Chemistry," Vol. I, W. A. Benjamin, New York, N. Y., 1966, Chapter 2; (b) M. L. Bender and F. J. Kezdy, *Annu. Rev. Biochem.*, 34, 49 (1965); (c) D. M. Blow in "The Enzymes," Vol. 3, 3rd ed, P. D. Boyer, Ed., Academic Press, New York, N. Y., 1971, Chapter 6; (d) G. P. Hess in ref 4c, Chapter 7.

(5) (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); (c) S. G. Cohen, L. H. Klee, and S. Y. Weinstein, *J. Amer. Chem. Soc.*, 88, 5302 (1966); (d) S. G. Cohen, Z. Neuwirth, and S. Y. Weinstein, *ibid.*, 88, 5306 (1966); (e) S. G. Cohen and L. W. Lo, *J. Biol. Chem.*, 245, 5718 (1970).

(6) Throughout the discussions in this paper it is assumed that the preferred conformation of the ester group of the 1,2,3,4-tetrahydro-2-naphthoic acid esters when bound to CT is equatorial. Data in ref 7 convincingly refute recent arguments[8,9] in favor of an axial conformation.

(7) M. S. Silver, M. Stoddard, T. Sone, and M. S. Matta, *J. Amer. Chem. Soc.*, 92, 3151 (1970).

(8) W. B. Lawson, *J. Biol. Chem.*, 242, 3397 (1967).

(9) B. Belleau and R. Chevalier, *J. Amer. Chem. Soc.*, 90, 6864 (1968).

(10) The Experimental Section gives a detailed description of the syntheses, resolutions, and kinetic analyses mentioned here.

(11) We are indebted to Drs. S. I. Weissman of Washington University and J. A. Spencer of this department for their cooperation in obtaining the requisite spectra.

Table I. Kinetic Parameters for the α-Chymotrypsin-Catalyzed Hydrolysis of Some *p*-Nitrophenyl Esters[a]

Acid	$10^7[E]_0$, M	$10^6[S]_0$, M	10^2k_c, sec^{-1}	k_c/K_m, M^{-1} sec^{-1}
(*S*)-Amidotetrahydro	6.14	2.92–19.5	3.3 ± 0.1[b]	7082
(*R*)-Amidotetrahydro	206–412	4.22–8.44		320 ± 23
(*R,S*)-Tetrahydro	3.80	3.70–17.2	0.97 ± 0.09[c]	1066
(*R,S*)-Acetoxytetrahydro	445–1090	20.1–36.0		9.4 ± 2.4
Amidoindan	9.85–19.7	9.15–16.1	0.50 ± 0.05	
Indan	6.22–61.8	12.4–24.8	1.1 ± 0.1	
Acetoxyindan	12.8–25.6	66.9–134	1.0 ± 0.1	
(*S*)-AP			5300	680,000

[a] At pH 5.4, 20% methanol, 25°. Data for last ester from ref 7. Lineweaver–Burk plots contained 12–18 points. Values of k_c obtained at enzyme saturation utilized data from at least six experiments. [b] $K_m = 4.66 \pm 0.37 \times 10^{-6}$ M. [c] $K_m = 9.20 \pm 2.0 \times 10^{-6}$ M.

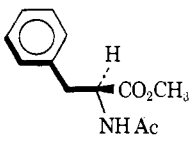


Figure 1. Proposed reactive conformation of (*S*)-APME at the active site of CT.

bands in the region 250–275 nm and a single absorption near 225 nm. Cotton effects for all (−)-amidotetrahydro methyl ester transitions were negative. The sign of the 225-nm band of (*S*)-APME was reversed and both positive and negative Cotton effects were observed in the 250–275-nm transitions. This could mean that (−)-amidotetrahydro methyl ester and (*S*)-APME are of opposite absolute configuration, but the proof is equivocal since it is not clear whether open-chain and cyclized compounds are directly comparable.

Enzymatic rate determinations were carried out using the Chart I NPE's as substrates. The conditions and observed kinetic parameters are recorded in Table I. The alkaline hydrolysis rates of the NPE's and ME's were measured for use in considering potential normalization procedures. Table II contains relative values of the experimental second-order rate constants.

Table II. Relative Hydroxide Ion Catalyzed Hydrolysis Rates of Some *p*-Nitrophenyl and Methyl Esters[a]

Acyl group	Rel $(k_{OH})_{NPE}$	Rel $(k_{OH})_{ME}$
(*S*)-AP	48,800	620
Amidotetrahydro	33,500	5.6
Tetrahydro	100	100
Acetoxytetrahydro	0.2	16.8[b]
Amidoindan	67,000	58.8
Indan	180	140
Acetoxyindan	5.6	176[b]

[a] Relative second-order rate constant[10] for the hydrolysis of the *p*-nitrophenyl or methyl ester; the values of the tetrahydro esters are arbitrarily set at 100 to facilitate comparisons. [b] Estimated value. From data contained in ref 7 and 27, it may be calculated that an ester of *N*-acetyl-(*S*)-phenylalanine is hydrolyzed about three times more slowly than the same ester of *O*-acetyl β-phenyllactate. Since steric effects probably influence the hydrolysis rates of the α-acetamido- and α-acetoxytetrahydro-2-naphthoic acid methyl esters equally the reactivity of the latter compound was assumed to be three times the former. The value for acetoxyindan methyl ester was obtained in the same way.

Analysis of Enzyme Kinetics. The minimal mechanism describing hydrolysis of ester substrates by CT, for which abundant experimental evidence exists,[4] was assumed to hold (eq 1). The experimental steady-state parameters k_c and K_m obtained from initial velocity measurements (eq 2) are related to the mechanistic parameters by the relationships of eq 3–5.

$$\mathrm{RCOX} + \mathrm{E} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (\mathrm{RCOX};\ \mathrm{E}) \xrightarrow{k_2} \underset{+\ \mathrm{X}}{\mathrm{RCO{-}E}} \xrightarrow{k_3} \mathrm{RCOOH} + \mathrm{E} \quad (1)$$

$$v_0 = k_c[E]_0[S]_0/K_m + [S]_0 \quad (2)$$

$$K_s = (k_{-1} + k_2)/k_1 \qquad K_m = (k_3/(k_2 + k_3))K_s \quad (3)$$

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

If $k_2 \gg k_3$

$$K_m = (k_3/k_2)K_s \qquad k_c = k_3 \quad (5)$$

Since at pH values >7 nonenzymatic hydrolysis of substrates containing an α-acetamido function was quite rapid, all enzymatic rates were measured at pH 5.4 where spontaneous contributions to the observed rates were less pronounced. Corrections were applied if the spontaneous rate was >2% of the enzymatic rate. CT-catalyzed hydrolysis of (*S*)-amidotetrahydro and tetrahydro nitrophenyl esters was sufficiently rapid to permit evaluation of the kinetic parameters under turnover conditions ($K_m \simeq [S]_0 \gg [E]_0$). Initial velocities of *p*-nitrophenol release over a range of $[S]_0$ values provided data for the construction of double reciprocal plots from which k_c and K_m were obtained.[12] No stereoselectivity was detected in the enzymatic hydrolysis of tetrahydro nitrophenyl ester and (*S*)-amidotetrahydro nitrophenyl ester appeared to be free of contamination by the slow isomer.

The relatively large spontaneous hydrolysis rate of (*R*)-amidotetrahydro nitrophenyl ester at pH 5 coupled with its low enzymatic rate precluded the possibility of turnover measurements. Chymotryptic hydrolysis of acetoxytetrahydro nitrophenyl ester was so slow no measurable rates were obtained under turnover conditions. A second-order rate constant equal to k_c/K_m was measured for these two substrates using an acylation procedure ($[E]_0 \geq [S]_0 \ll K_m$).[13] When the acylation data were subjected to a first-order treatment, linear plots were obtained which indicated the absence of any (*S*)-amidotetrahydro nitrophenyl ester contaminant in

(12) H. Lineweaver and D. Burk, *J. Amer. Chem. Soc.*, 56, 658 (1934).
(13) M. S. Silver, *ibid.*, 88, 4247 (1966).

the (*R*)-NPE and a lack of stereoselectivity in the hydrolysis of acetoxytetrahydro nitrophenyl ester.

The three indan esters of Chart I had K_m values too low to allow the individual determination of k_c and K_m and their reactivity was too high to determine k_c/K_m by acylation. The k_c values of these substrates were obtained at enzyme saturation ($K_m \ll [S]_0 \gg [E]_0$). Saturation was considered experimentally achieved when doubling $[S]_0$ did not affect v_0. When $[E]_0$ was sufficiently high a burst of *p*-nitrophenol equivalent to the enzyme concentration was produced in each case, proving that for the indan NPE's $k_2 \gg k_3$ and therefore $k_c = k_3$.[14] In this paper interpretations of reactivity changes with substrate structural variation are made using k_c or k_c/K_m. The specificity ratio k_c/K_m invariably reduces to k_2/K_s even in the presence of nonproductive binding modes[4b] as long as only one productive mode exists. In NPE hydrolysis k_2 is usually $\gg k_3$,[4b] the relationship of eq 5 likely holds, and k_c is considered identical with the deacylation rate constant k_3 for our substrates whereas k_c/K_m defines specificity in acylation.

Discussion

Consideration of Normalization Procedures. This study was prompted by the desire to assess the potential utility of amidotetrahydro and amidoindan nitrophenyl esters as probes of the active site of CT. The ester function, α-acetamido group, and phenyl rings of these semirigid molecules are constrained in positions similar to those deduced for the reactive conformation of (*S*)-APME in previous chemical studies[5] and X-ray crystallographic evidence.[15,16] One early working hypothesis was that if the proposed conformation is correct, after suitable cancellation of inherent reactivity differences in the substrates owing to stereoelectronic effects, the rate of the more rapidly hydrolyzed isomer of amidotetrahydro nitrophenyl ester should be equal to that observed for (*S*)-APNPE. Separation of CT specificity contributions to observed rates from stereoelectronic effects inherent to the substrate has been attempted previously by normalizing with respect to a reference nucleophilic reaction. The hydroxide ion catalyzed hydrolysis of the substrate or a closely related derivative[5e,7,14,17] is often used. In this work alkaline hydrolysis of the NPE's as an indicator of inherent reactivity was rejected. The much higher reactivity of the α-acetamido-containing NPE's compared with the other NPE's of Table II supports azlactone formation rather than nucleophilic attack as the primary mechanism for their decomposition.[14,18] Since nonactivated α-acylamino esters do not hydrolyze *via* azlactone formation,[18] the alkaline hydrolysis of the Chart I ME's was examined. The data of Table II reveal the role of steric effects in base-catalyzed hydrolysis of the ME's and NPE's.

(1) Amidotetrahydro methyl ester is 18 times less reactive than tetrahydro methyl ester indicating steric effects in going from a tertiary to a fully substituted α carbon are more than sufficient to overcome the rate enhancement expected to result from the addition of an α-acetamido function.[19] The magnitude of the effect corresponds to that found for other open-chain and bicyclic ME's.[5e]

(2) Comparison of the tetrahydro methyl ester/acetoxytetrahydro methyl ester reactivity ratio to that of the corresponding NPE's shows that steric hindrance associated with the leaving group influences the alkaline hydrolysis rates. The first ratio is 6, the second is 500. The bulkier *p*-nitrophenyl group must severely reduce the susceptibility of the ester function to nucleophilic attack.

(3) The corresponding indan derivatives display similar but less drastic reactivity variations than the tetrahydronaphthoates. This decreased steric requirement may represent a manifestation of the Thorpe–Ingold effect.[20–22]

Establishing that the relative rates of alkaline hydrolysis of the Chart I ME's and NPE's are a function of the type of leaving group casts doubt on how appropriate corrections to the enzymatic rates should be made. Use of saponification rates probably succeeds in cancelling variations in electronic character among substrates of low steric demand. However, the use of the methyl ester rates to normalize the enzymatic rate parameters obtained with sterically hindered substrates so that they may be compared to the corresponding values of analogous flexible substrates, in this case the identically α-substituted β-phenylpropionates, requires two assumptions. First, nucleophilic attack by the enzyme and by hydroxide ion on all substrates must be equally sensitive to steric effects. The fact that the relative nonenzymatic rates of the Chart I ME's and NPE's are dependent upon the nature of the leaving group renders the reliability of this supposition doubtful. In enzymatic hydrolysis of a Chart I substrate, steric effects due to the leaving group might differ in acylation of the NPE and hydrolysis of the acylated serine residue. Second, it must be clear that sterically caused rate diminutions in the models spring only from effects associated with nucleophilic attack by the crucial serine at the active site of CT, not from unfavorable interactions of the presumedly crude facsimilies with some other portion of the reactive center. Such interactions evidently do occur in CT-catalyzed hydrolysis of bicyclic and α-alkylated flexible substrates. Cohen has rationalized the low reactivity of α-Me-(*S*)-APME by invoking steric hindrance between the substrate α-methyl group and the protein. After normalization α-Me-(*S*)-APME is 10^3 times less reactive than the natural substrate.[5e]

In view of these reservations, we have elected to interpret the data of Table I without normalization, a course of action which limits the possibility of achieving our original goal, but avoids potentially specious comparisons between the rigid models and their β-phenylpropionic analogs.

(14) D. W. Ingles and J. R. Knowles, *Biochem. J.*, 104, 369 (1967).
(15) T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, 46, 337 (1969).
(16) R. Henderson, C. S. Wright, G. P. Hess, and D. M. Blow, *Cold Spring Harbor Symp. Quant. Biol.*, 36, 63 (1971).
(17) T. H. Marshall and A. Akgün, *J. Biol. Chem.*, 246, 6019 (1971).
(18) J. deJersey, A. A. Kortt, and B. Zerner, *Biochem. Biophys. Res. Commun.*, 23, 745 (1966).

(19) The sluggish alkaline hydrolysis of N-methylated amidotetrahydro methyl ester attests to the influence of steric effects on reactivity of the 2-naphthoates. In preliminary experiments carried out under the same conditions used to measure the hydroxide ion catalyzed rate of amidotetrahydro methyl ester,[10] there was no detectable production of N-methylated acid after 48 hr.
(20) E. L. Eliel, "Stereochemistry of Carbon Compounds," McGraw-Hill, New York, N. Y., 1962, pp 197–202.
(21) R. M. Beesley, C. K. Ingold, and J. F. Thorpe, *J. Chem. Soc.*, 108, 1080 (1915).
(22) C. K. Ingold, *ibid.*, 119, 305 (1921).

Structural Specificity of CT toward the Rigid Substrates. Table I shows that the k_c values and k_c/K_m values of the hydroaromatic NPE's are seriously inferior to those of (*S*)-APNPE. The effects described above may lead in part to the low reactivity of the models, but quantitative measurement of the extent of such diminution is impossible. An enzyme's structural specificity is often manifested in high rates, yet high observed reactivity in a rigid model substrate is insufficient to solve a reactive conformation problem. 3-Carbomethoxydihydroisocarbostyril, which does not achieve the same reactive conformation as (*S*)-APME,[5,7] is hydrolyzed by CT about as fast as the natural substrate.[23-25] In model studies, specificity differences owing to small variations in substrate structures also can be informative. In this work, the most compelling evidence for the interaction of CT with an α substituent of a model lies in the difference between (*S*)-amidotetrahydro and acetoxytetrahydro nitrophenyl esters in k_c/K_m. These substrates have quite similar stereoelectronic properties and their observed specificity parameters should be directly comparable. The dramatic reactivity drop of over 700 times upon replacement of amido N–H by oxygen could arise from enhanced reactivity of (*S*)-amidotetrahydro nitrophenyl ester, decreased reactivity of the acetoxy compound, or a combination of both effects. The fact that tetrahydro nitrophenyl ester is only four–seven times less reactive than (*S*)-amidotetrahydro appears to favor the second argument. On the other hand, this small difference could result from less demanding steric requirements in hydrolysis of tetrahydro nitrophenyl ester. Without a good normalization procedure, it is impossible to distinguish among the possibilities. The same reactivity pattern in k_c/K_m may not hold for the indan-2-carboxylic acid NPE's. Experimental difficulties limit the numerical data to k_c, but since CT-catalyzed hydrolysis of these substrates is too rapid to measure when $[E]_0 \geq [S]_0$, their k_c/K_m values undoubtedly exceed 1000 M^{-1} sec^{-1}. Pattabiraman and Lawson determined k_c/K_m for amidoindan and indan methyl esters.[26] Based on normalized data, they reported that the α-acetamido function does not contribute to the reactivity of these compounds. An extension of their experiments to include acetoxyindan methyl ester would be of interest.

Whatever its cause, the reactivity difference between (*S*)-amidotetrahydro nitrophenyl ester and acetoxytetrahydro nitrophenyl ester is reminiscent of the behavior of the analogous β-phenyl propionates.[27] The presence of a rate-enhancing α-amide–enzyme hydrogen bonding interaction in specific substrates is conceded,[4,27] although its importance may have been overestimated.[7] Even in natural substrates, the role played by hydrogen bonding in increasing kinetic specificity is unclear. Arguments have been presented relating this interaction in (*S*)-APME to a gain in free energy of binding which is used to lower the activation energy of the kinetic steps *via* induction of strain in the substrate.[27] Desolvation of enzyme and substrate[28] or freezing of bond rotations upon binding[29] may also lead to decreased activation energies.

Stereochemical Specificity of CT toward the Tetrahydro-2-naphthoates. No stereoselectivity is detectable in the CT-catalyzed hydrolysis of tetrahydro and acetoxytetrahydro nitrophenyl esters. The *S* isomer of acetamidotetrahydro nitrophenyl ester is hydrolyzed 22 times faster than its mirror image. The low stereospecificity CT displays toward the models is in marked contrast to the hydrolysis of APNPE, where the *S*/*R* ratio is 3000.[7,30] No chemical or physical experiment has unambiguously identified the reactive conformation of a slow isomer of a specific open-chain or nonspecific bicyclic substrate.[31] Compared to the multitude of conformations theoretically available to unreactive flexible isomers, the number of rigid substrate slow isomer conformations is limited. The stereochemical behavior of CT with amidotetrahydro nitrophenyl ester does not correspond to that of APNPE. If it did, the result would be at best a pleasant fortuity. In other words, if (*S*)-amidotetrahydro nitrophenyl ester is somehow shown to be an excellent model for the reactive conformation of (*S*)-APNPE, that its enantiomer will be a model for (*R*)-APNPE is not likely.

Conclusion

The goal of this research, to use a new series of rigid substrates to assist in defining the reactive conformation of (*S*)-APNPE at the active site of CT in solution, was thwarted in large measure by concerns about the dependence of the enzymatic reactivity of the models on various steric effects. The similarity in the relative specificity of CT toward the α-acetamido- and α-acetoxy-substituted tetrahydro-2-naphthoates and β-phenylpropionates warrants further investigation of substrates of this type. However, in our opinion, attempts to extrapolate the present results from the rigid models to the analogous flexible substrates on a one-to-one basis would be most improvident at this time.

Experimental Section[32]

2-Acetamido-1,2,3,4-tetrahydro-2-naphthoic Acid. Alkaline hydrolysis of Aldrich 7,8-benzo-1,3-diazaspiro[4.5]decane-2,4-dione[33] afforded 2-amino-1,2,3,4-tetrahydro-2-naphthoic acid, mp 300–303° (lit.[33] mp 297–303°). Acetylation of the hydrolysis product by a standard method[34] produced the title acid, mp 277°, after two recrystallizations from water.

Anal. Calcd for $C_{13}H_{15}NO_3$: C, 66.94; H, 6.48; N, 6.01. Found: C, 67.07; H, 66.6; N, 6.17

2-Acetamidoindan-2-carboxylic Acid. The conversion of 2-

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

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

(25) G. E. Hein, R. B. McGriff, and C. Niemann, *J. Amer. Chem. Soc.*, **82**, 1830 (1960).

(26) T. N. Pattabiraman and W. B. Lawson, Abstracts of Papers, 160th National Meeting of the American Chemical Society, Chicago, Ill., Sept 14–18, 1970.

(27) D. W. Ingles and J. R. Knowles, *Biochem. J.*, 108, 561 (1968). Unfortunately, the data which provoked the strain theory may not in fact support it.[7]

(28) S. G. Cohen, V. M. Vaidya, and R. M. Schultz, *Proc. Nat. Acad. Sci. U. S.*, 66, 249 (1970).

(29) M. L. Bender, F. J. Kezdy, and C. R. Gunter, *J. Amer. Chem. Soc.*, **86**, 3714 (1964). Which enzyme or substrate bonds are frozen are not specified.

(30) M. S. Silver and M. S. Matta, *Arch. Biochem. Biophys.*, **151**, 62 (1972).

(31) For speculation, see ref 4c, 5, 23, and 24.

(32) Melting point determinations were carried out in capillary tubes (<200°) or on a Fisher-Johns melting point block (>200°) and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn. Nmr and infrared measurements on most substrates and precursors were obtained and were in good agreement with the predicted spectra.

(33) J. A. Faust, L. H. Jules, L. Yee, and M. Sayun, *J. Amer. Pharm. Assoc., Sci. Ed.*, **46**, 118 (1957); *Chem. Abstr.*, 51, 12072 (1957).

(34) J. P. Greenstein, *Methods Enzymol.*, **3**, 555 (1957).

Table III. Physical Properties of *p*-Nitrophenyl Ester Substrates

Acyl group	Mp, °C	Formula	Analysis					
			Calcd, %			Found, %		
			C	H	N	C	H	N
(*S*)-Amidotetrahydro	148–149[a]	$C_{19}H_{18}N_2O_5$	64.40	5.12	7.91	64.64	5.15	7.91
(*R*)-Amidotetrahydro	150–151[b]	$C_{19}H_{18}N_2O_5$	64.40	5.12	7.91	64.21	5.05	7.89
(*R,S*)-Tetrahydro	101.5–102.5[c]	$C_{17}H_{15}NO_4$						
(*R,S*)-Acetoxytetrahydro	146–147	$C_{19}H_{17}NO_6$	64.20	4.82	3.95	63.96	4.59	3.70
Amidoindan	145–146	$C_{18}H_{16}N_2O_5$	63.52	4.74	8.23	63.36	4.81	8.27
Indan	103–104.5	$C_{16}H_{13}NO_4$	67.88	4.70	4.91	67.98	4.63	4.94
Acetoxyindan	150–151	$C_{18}H_{15}NO_6$	63.34	4.43	4.10	63.51	4.61	4.02
(*S*)-AP	139–140[d]	$C_{17}H_{16}N_2O_5$						

[a] $[\alpha]^{25}D$ +30.2° (*c* 2, ethyl acetate). [b] $[\alpha]^{25}D$ −29.8° (*c* 2, ethyl acetate). [c] Reference 7 gives mp 101–104°. [d] $[\alpha]^{25}D$ −18.0° (*c* 2, chloroform); ref 14 gives mp 140–140.5°, $[\alpha]^{20}D$ −18.6° (*c* 2, chloroform).

indanone[35] to 7,8-benzo-1,3-diazaspiro[4.4]nonane-2,4-dione, mp 270–272° (lit. mp 260–267°), was accomplished by the method of Mauger and Ross.[36] Hydrolysis of the hydantoin followed by acetylation[34] of the crude 2-aminoindan-2-carboxylic acid provided the 2-acetamido acid, mp 267–270°.

Anal. Calcd for $C_{12}H_{13}NO_3$: C, 65.74; H, 5.68; N, 6.39. Found: C, 65.67; H, 5.96; N, 6.24.

Indan-2-carboxylic Acid. This compound was synthesized by the method of Bergmann and Hoffmann[37] and had mp 125.5–127.5° (lit.[37] mp 130°).

1,2,3,4-Tetrahydro-2-naphthoic acid, prepared by the method of Eliel and Hoover,[38] had mp 96–98° (lit.[38] 95.5–96°).

2-Acetoxy-1,2,3,4-tetrahydro-2-naphthoic Acid. β-Decalone[39] was converted *via* its cyanohydrin to 2-hydroxy-1,2,3,4-tetrahydro-2-naphthoic acid, mp 135–136° (lit.[40] mp 143–145°), neut equiv 194.8 (theory 192.2), by the general method of Harnden.[41] Acetylation[41] of the hydroxy acid gave 2-acetoxy-1,2,3,4-tetrahydro-2-naphthoic acid, mp 124–125° (lit.[40] mp 135–136°).

2-Acetoxyindan-2-carboxylic Acid. The method described above was used to prepare 2-hydroxyindan-2-carboxylic acid, mp 179–181°, and the title acid, mp 163–165°.

Anal. Calcd for $C_{10}H_{10}O_3$: C, 67.41; H, 5.66. Found: C, 67.38; H, 5.43. For $C_{12}H_{12}O_4$: C, 65.45; H, 5.49. Found: C, 65.37; H, 5.62.

Preparation of Methyl Esters. Methyl esters of cyclized substrate acids containing an α-acetamido function were synthesized using the general method of Brenner and Huber.[42] Each solid methyl ester was recrystallized several times from chloroform–hexane.

Methyl *dl*-2-acetamido-1,2,3,4-tetrahydro-2-naphthoate had mp 131–132°.

Anal. Calcd for $C_{14}H_{17}NO_3$: C, 68.00; H, 6.93; N, 5.66. Found: C, 68.12; H, 7.02; N, 5.57.

Methyl 2-acetamidoindan-2-carboxylate melted at 146–146.5°.

Anal. Calcd for $C_{13}H_{15}NO_3$: C, 66.94; H, 6.48; N, 6.01. Found: C, 67.07; H, 6.32; N, 5.93.

Acetyl-(*S*)-phenylalanine methyl ester was obtained as previously described.[2] It had mp 89–90° (lit.[2] mp 90°). Since the optical purity was of no consequence for its intended use in normalization, it was not determined.

The procedure of Cohen[5b] was used to prepare methyl 1,2,3,4-tetrahydro-2-naphthoate, bp 95–97° (0.5 mm) (lit.[5b] bp 129° (5–6 mm)), and extended to the synthesis of methyl indan-2-carboxylate, bp 99–102° (6 mm) (lit.[43] bp 83–84° (0.3 mm)).

Methyl 2-Acetoxy-1,2,3,4-tetrahydro-2-naphthoate. A solution of the acetoxy acid in ether was treated with a slight excess of ethereal diazomethane. The oily residue was placed on a Florisil column and eluted with 1:5 ethyl acetate–pentane. The methyl ester was obtained as a viscous colorless liquid, bp 143° (2 mm), $n^{25}D$ 1.5198. The same procedure, omitting the chromatography step, yielded methyl 2-acetoxyindan-2-carboxylate, mp 81–82°.

Anal. Calcd for $C_{14}H_{16}O_4$: C, 67.73; H, 6.50. Found: C, 67.51; H, 6.72. For $C_{13}H_{14}O_4$: C, 66.66; H, 6.02. Found: C, 66.81; H, 5.92.

Resolution of Methyl 1,2,3,4-Tetrahydro-2-acetamido-2-naphthoate. To 3.0 g (12.2 mmol) of the methyl ester dissolved in 120 ml of methanol was added 480 ml of water and the pH was adjusted to 7.9 with 0.1 *M* sodium hydroxide. α-Chymotrypsin (400 mg) dissolved in 5 ml of water was added. During 12 hr 60 ml of base was consumed (98% of theoretical) and the reaction stopped. The reaction mixture was extracted with chloroform. The chloroform extracts were washed with water, dried over magnesium sulfate, filtered, and evaporated at reduced pressure to afford 1.5 g (100%) of (−) ester, mp 148–150°. Three recrystallizations from chloroform–hexane gave 1.4 g of (−)-methyl 2-acetamido-1,2,3,4-tetrahydro-2-naphthoate, mp 150.5–151°, $[\alpha]^{25}D$ −59.6° (*c* 2.75, ethyl acetate).

The aqueous layer was adjusted to pH 2 with concentrated hydrochloric acid and refrigerated overnight. Isolation of the precipitate by filtration gave 900 mg of (+) acid which was recrystallized twice from water. The final yield of (+)-2-acetamido-1,2,3,4-tetrahydro-2-naphthoic acid, mp 259–261°, $[\alpha]^{25}D$ +39.8° (*c* 2, dimethylformamide), was 400 mg.

(−)-2-Acetamido-1,2,3,4-tetrahydro-2-naphthoic acid was obtained by stirring 550 mg of the corresponding methyl ester overnight in 50 ml of 10% sodium hydroxide. The solution was acidified to pH 4 and the crystalline product was removed by filtration and dried at reduced pressure. The yield of (−) acid, mp 263–264°, $[\alpha]^{25}D$ −45.5° (*c* 2, dimethylformamide), was 400 mg.

Synthesis of *p*-Nitrophenyl Esters. *p*-Nitrophenyl esters were obtained by the reaction of equivalent amounts of *p*-nitrophenol, the appropriate acid, and dicyclohexylcarbodiimide in ethyl acetate at room temperature for 25 hr.[44] The oily yellow residue remaining after removal of dicyclohexylurea by filtration and evaporation of the solvent at reduced pressure was recrystallized several times from chloroform–hexane. *p*-Nitrophenyl 2-acetoxy-1,2,3,4-tetrahydro-2-naphthoate could be crystallized only after column chromatography on Florisil using 1:4 ethyl acetate–hexane as the eluent. The physical constants of all of the *p*-nitrophenyl esters are given in Table III.

Kinetics Measurements. α-Chymotrypsin was Worthington lots CDI 8LK and OBK. Active site concentration was determined by titration with *trans*-cinnamoyl imidazole.[45] Deionized water was distilled through a glass apparatus. All buffer components were reagent grade and acetonitrile and methanol were Matheson Coleman and Bell spectroquality. The pH 5.4 buffer used for the enzyme kinetics runs was prepared by diluting 20 ml of methanol to 100 ml with pH 5, 0.1 *M* acetate buffer. Enzymatic runs were carried out by addition of 0.1 ml of substrate solution in acetonitrile to 3.0 ml of buffer in a cuvette in the cell compartment of a Cary Model 14 spectrophotometer thermostated at 25.0 ± 0.5°. The enzyme solution, 0.1 ml, was added and the *p*-nitrophenol production monitored at 330 nm as a function of time. Initial velocities, v_0, of turnover reactions were calculated using a polynomial curve fitting method.[46] The v_0 values thus obtained were used in double

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reciprocal plots to provide k_c and K_m. Calculations of second-order rate constants k_2', equal to k_c/K_m, were by the method of least squares. Initial velocities of saturation kinetics runs were measured directly from the recorded optical density *vs.* time curves. All polynomial and least-squares computations employed an IBM 360/40 computer. Table I displays the k_c, K_m, and k_c/K_m values ultimately obtained. The rates of hydroxide ion catalyzed hydrolysis of most *p*-nitrophenyl esters were determined at 400 nm in 20% methanolic carbonate–bicarbonate buffers of pH 9–11.[47] *p*-Nitrophenyl esters of amidotetrahydro, amidoindan, and (*S*)-AP were more conveniently hydrolyzed at pH 8 in 20% methanolic phosphate buffer (0.067 *M*). Reaction mixture and buffer pH values were measured on a Brinkman E 300B meter. First-order rate constants obtained from the half-lives of the hydrolyses were divided by apparent hydroxide ion concentrations to give the second-order rate constants which were used to obtain the relative reactivities of Table II *p*-nitrophenyl esters.

Hydroxide ion catalyzed hydrolysis rates of (*S*)-APME, tetrahydro methyl ester, indan methyl ester, and amidoindan methyl ester were measured in a Brinkman Model 3D combititrator equipped with a 1-ml buret, thermostated reaction vessel, and type H electrode. Hydrolyses were carried out at 25° by adding to 20 ml of 30% DMSO–water, 0.1 *M* in sodium chloride, 1 ml of DMSO containing the appropriate esters. Duplicate runs were obtained at ester concentrations of 0.8 and 1.6 m*M*. The system was purged with nitrogen, the pH brought to 11.4 by addition of concentrated sodium hydroxide, and the uptake of 0.05 *M* sodium hydroxide was recorded as a function of time. The observed first-order rate constants were obtained by the half-lives method from plots of $V_\infty - V_t$ *vs.* time. The following first-order rate constants (in $sec^{-1} \times 10^4$) were obtained from the linear plots through 3 half-lives: (*S*)-APME, 7.01; tetrahydro methyl ester, 1.13; indan methyl ester, 1.59; amidoindan methyl ester, 0.66. Acetoxyindan methyl ester took up 2 equiv of base in a curve with no clean break; therefore, its reactivity and that of acetoxytetrahydro methyl ester were estimated. Amidotetrahydro methyl ester was hydrolyzed too slowly to be measured on the pH-stat and a manual titrimetric procedure was used.

To 200 ml of a 30% DMSO–water 0.1 *M* in sodium chloride and 0.0105 *M* in sodium hydroxide equilibrated at 25° under nitrogen was added 10 ml of DMSO containing 2.1 mmol of the substrate, making the final concentration of each reactant 0.01 *M*. At appropriate intervals 10-ml aliquots were withdrawn and added to flasks containing 10 ml of 0.015 *N* potassium acid phthalate to quench the reaction. The excess phthalate was titrated against 0.01 *N* sodium hydroxide to the phenolphthalein end point. A second-order treatment[48] gave a straight line and a rate constant of $7.22 \times 10^{-3}\ M^{-1}\ sec^{-1}$ was calculated from the slope. Using the same procedure the second-order rate constant for tetrahydro methyl ester was $1.25 \times 10^{-1}\ M^{-1}\ sec^{-1}$. A relative reactivity scale in which tetrahydro methyl ester was arbitrarily assigned a standard value of 100 was set up using the values obtained by the two methods (Table II).

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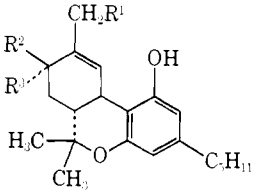
Communications to the Editor

Synthesis of 11-Hydroxy-Δ^9-tetrahydrocannabinol and Other Physiologically Active Metabolites of Δ^8- and Δ^9-Tetrahydrocannabinol

Sir:

Studies[1a-e] using laboratory animals have established that Δ^9-tetrahydrocannabinol (Δ^9-THC, **1a**), the principal psychotomimetic constituent of marihuana and hashish,[2] is readily metabolized to a number of hydroxylated metabolites. One of these, 11-hydroxy-Δ^9-THC (**1b**), has been identified in man[3a-d] and shown to have high physiological activity.[4a-c] Recent studies[3a,5] in our laboratories have revealed that Δ^9-THC is, in addition, partially metabolized in man to 8α- and 8β-hydroxy-Δ^9-THC (**1c**, **1d**), and these metabolites



1a, $R^1 = R^2 = R^3 = H$
b, $R^1 = OH$; $R^2 = R^3 = H$
c, $R^1 = R^2 = H$; $R^3 = OH$
d, $R^1 = R^3 = H$; $R^2 = OH$

also exhibit some degree of activity.[3a,6] These findings, and the urgent need for these metabolites in the further elaboration of the pharmacology of marihuana, prompt us to report a simple, essentially one-step synthesis which provided all three metabolites, and represents the first, albeit low yield, practical source of 11-hydroxy-Δ^9-THC.[7]

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(7) Previously 11-hydroxy-Δ^9-THC has only been available through microsomal hydroxylation,[1a] a tedious and expensive method when applied preparatively. Chemists have made many unsuccessful attempts to synthesize this compound, the only published report[8] involving selenium dioxide oxidation of Δ^9-THC. The low yield (1%) and