Specificity in the Alkylation of Methionine at the Active Site of α-Chymotrypsin by Aromatic α-Bromo Amides†

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ABSTRACT: α-Chymotrypsin is inactivated by affinity labeling with a number of aromatic α-bromo amides. At pH 5 the primary site of alkylation is Met-192. The widely differing rates of inactivation can be correlated neither with the reactivity of the bromine atom nor with the degree of binding (K_i) to the enzyme. Consideration of the stereochemisty of the inhibitors led to the conclusion that coplanarity of the amide group and the benzene ring in an inhibitor results in rapid alkylation of Met-192. Noncoplanarity of these groups results in slow alkylation of Met-192 and in some cases alkylation of Ser-195 as well [cf. Lawson, W. B. (1980) *Biochemistry*, following paper in this issue]. Locked, coplanar analogues of some compounds were synthesized, and their reactions with chymotrypsin substantiate this concept. Thus, bromoacetylaniline ($t_{1/2}$ 42 min) is only slightly less reactive than its locked analogue, (bromoacetyl)indoline ($t_{1/2}$ 16 min). In contrast, the noncoplanar (bromoacetyl)benzylamine ($t_{1/2}$ 3870 min) is far less reactive than its coplanar analogue, BA-isoindoline ($t_{1/2}$ 5 min). [The half-times given are for reactions carried out at pH 5 with a 20-fold molar excess of inhibitor over enzyme (6×10^{-5} M).] The stereochemistry of the interactions of the inhibitors with the enzyme was visualized using a color-coded-skeletal (CCS) model of the active site [Clarke, F. H. (1977) *J. Chem. Educ. 54*, 230–235] and Corey–Pauling–Koltun (CPK) models of the inhibitors. The rapidly reacting coplanar compounds fit well for attack by the sulfur atom of Met-192 on the CH_2Br group of the compounds. Unreactivity of the noncoplanar compounds is probably due to one or more of the following factors: (1) a poor orientation of the CH_2Br group for attack by Met-192 on binding of the noncoplanar compounds to the active site, (2) hydrogen bonding between the NH group of an inhibitor and the CO group of Ser-214, and (3) hydrogen bonding between the CO group of the inhibitor and the NH groups of Gly-193 and Ser-195. During the course of this work 1-aminoindan was resolved into its optical isomers, and their absolute configurations were determined.

Chymotrypsin specifically hydrolyzes esters and amides of aromatic amino acids and has been studied intensively for more than 40 years (Northrop, 1939; Green & Neurath, 1954; Desnuelle, 1960; Blow, 1976). A combination of chemical modification studies and kinetics led to the conclusion that a serine and a histidine residue are vital for enzymatic activity and that a methionine residue is near the active site but probably not involved in the catalytic mechanism (Koshland et al., 1962). With the advent of affinity labeling agents (Baker, 1967; Lawson, 1978), His-57 (Ong et al., 1965), Ser-195 (Gold & Fahrney, 1964; Matthews et al., 1967), and Met-192 (Lawson & Schramm, 1965) were conclusively identified in the amino acid sequence of Hartley (1964) and shown to be components of the active site. The steric relationships of these residues to one another and to the substrate binding pocket were clarified in the X-ray structure determination (Birktoft & Blow, 1972) and have been discussed in recent reviews, particularly in relation to the mechanism of action of chymotrypsin and related enzymes (Blow, 1976; Kraut, 1977).

Some years ago we investigated the specific alkylation of the residue that proved to be Met-192 with a series of five compounds that possessed a reactive bromine atom at increasing distances from a benzene ring. With one exception, the rate of inactivation of chymotrypsin decreased with increasing distance between the bromine and the ring. The exception was quite striking, however, as may be seen in the half-times for inactivation by the inhibitors: benzyl bromide (7 min), α-bromoacetophenone (15 min), BA-aniline[1] (30 min), BA-benzylamine (3000 min), and BA-β-phenethylamine (60 min) (Schramm & Lawson, 1963).[2] One or more of the following factors might play important roles in the alkylation of Met-192: (a) the binding affinity of the inhibitor to the active site, (b) the chemical reactivity of the bromine atom, and (c) the stereochemistry of the inhibitors in their interactions with the active site. In the present study we have examined these factors with a number of α-bromo amides that inhibit chymotrypsin irreversibly by alkylation of Met-192. The results show that the stereochemistry of the inhibitors is the most important factor in determining the rates of inhibition. Further, with an easily constructed model of the active site (Clarke, 1977), the interactions of these compounds with chymotrypsin may be readily visualized.

Materials and Methods

α-Chymotrypsin (3× recrystallized, salt free) was obtained from Worthington Biochemical Corp. Organic compounds were purchased from Eastman Organic Chemicals, except for fluoroanilines (Pierce), DL-1-aminoindan and 2-aminoindan (K & K), and D- and L-phenylalanine (Mann). Isoindoline (Bornstein et al., 1973) was a generous gift from Dr. Joseph Bornstein. They were used as supplied, except for *N*-methylbenzylamine, which was purified by conversion to the crystalline tosyl derivative and acid hydrolysis of the derivative back to the amine. D- and L-malic acids were obtained from Mann and Calbiochem. The optical isomers of indan-1-carboxylic acid were prepared by the method of Fredga (1956),

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[1] Abbreviations used: BA, bromoacetyl; CPK, Corey–Pauling–Koltun (models); CCS, color-coded-skeletal (models).

[2] A second exception not apparent from the half-times is α-bromoacetophenone, which might be expected to react more rapidly than it does because of the high reactivity of its bromine atom (see below).

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who also determined their absolute configurations. L-Tyrosine ethyl ester hydrochloride was prepared by Fischer esterification of L-tyrosine (Mann) and had mp 166–167 °C; the reported melting point is 166 °C (Röhmann, 1897). α-Bromoacetamide (**26**) was prepared by bubbling ammonia into a benzene solution of bromoacetyl bromide and was crystallized from ethyl acetate, mp 85–87 °C [lit. mp 88 °C (Bischoff, 1897)].

Melting points were uncorrected. Ultraviolet spectra were determined with a Cary Model 14 spectrophotometer. Optical rotations were determined in a Rudolph Model 80 polarimeter with a Model 200 photoelectric attachment or with a Rudolph Autopol III automatic polarimeter. Enzyme assays were carried out with a Zeiss PMQ II spectrophotometer coupled to a Zeiss TEW converter and a Leeds & Northrup recorder or with a Cary 118 spectrophotometer. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratories, Woodside, NY; by Dr. Carl Tiedcke, Laboratory of Microchemistry, Teaneck, NJ; or by Instranal Laboratory, Inc., Rensselaer, NY. Analytical results obtained for the elements listed were within ±0.3% of the theoretical values.

Resolution of 1-Aminoindan. A solution of 30.5 g (0.229 mol) of racemic 1-aminoindan and 15.4 g (0.115 mol) of L-malic acid in 250 mL of absolute ethanol gave on standing 37.9 g of white crystals. Four recrystallizations from 92% ethanol afforded 6.7 g of (−)-1-aminoindan L-malate as white needles, mp 182–189 °C dec, $[\alpha]^{27}_D$ −4.97° (*c* 5.09, water). A sample was converted to (−)-1-benzamidoindan, mp 167–168 °C, $[\alpha]^{27}_D$ −13.8° (*c* 6.32, chloroform), $[\alpha]^{27}_D$ −29.3° (*c* 1.93, ethyl acetate). Hückel & Bollig (1953) report a melting point of 149–150 °C for an impure sample of the benzoyl derivative.

Filtrates from the recrystallizations and from a similar small-scale resolution were evaporated under vacuum, and the free base was isolated by treatment with NaOH, extraction into ether, and distillation under vacuum. A solution of 29.1 g (0.219 mol) of optically impure (+)-1-aminoindan so obtained and 14.7 g (0.110 mol) of D-malic acid in 250 mL of absolute ethanol deposited 36.8 g of white crystals. Three recrystallizations from 92% ethanol gave 11.9 g of (+)-1-aminoindan D-malate, mp 182–189 °C dec, $[\alpha]^{27}_D$ +4.97° (*c* 5.33, water). A sample was converted to (+)-1-benzamidoindan, mp 167–168 °C, $[\alpha]^{27}_D$ +13.8° (*c* 6.05, chloroform), $[\alpha]^{27}_D$ +29.2 (*c* 2.14, ethyl acetate). Hückel & Bollig (1953) give mp 165–166 °C, $[\alpha]^{27}_D$ +7.53 (*c* 5.5, chloroform), for the benzoyl derivative. Correct elementary analyses and satisfactory rotations were obtained for the bromoacetyl derivatives of both (+)- and (−)-1-aminoindans (Table I).

Absolute Configuration of (+)-1-Aminoindan. Conversion of (R)-(+)-1-Indancarboxylic Acid to (R)-(+)-1-Benzamidoindan. (A) (R)-(+)-1-Indancarboxamide. A solution of 1.30 g (8.02 mmol) of (*R*)-(+)-1-indancarboxylic acid in 5 mL of thionyl chloride was refluxed 15 min and evaporated to an oil. Cooled (−10 °C), concentrated ammonium hydroxide (20 mL) was added, and the white precipitate was washed with ice–water and dried. Recrystallization from methanol gave 0.90 g (70%) of (*R*)-(+)-1-indancarboxamide as white needles, mp 200–201 °C, $[\alpha]^{27}_D$ +49° (*c* 1.54, ethanol). Anal. C, H, N.

(B) Hofmann Degradation of (R)-(+)-1-Indancarboxamide and Conversion of the Product to (R)-(+)-1-Benzamidoindan. (*R*)-(+)-1-Indancarboxamide (495 mg, 3.07 mmol) was added to a solution of 0.25 mL of bromine in 6.2 mL of 3 N NaOH, cooled in an ice–salt bath, and the mixture was warmed cautiously. At about 70 °C reaction took place and some charring occurred. The reaction mixture was treated with ether, and the amine was extracted into dilute HCl. The aqueous layer was made alkaline, and the amine was extracted into ether. After the extract was dried (Na_2SO_4), introduction of gaseous HCl precipitated the amine hydrochloride (118 mg, mp 233 °C) which was converted to the benzoyl derivative. Recrystallization from ethanol gave 62 mg of (*R*)-(+)-1-benzamidoindan, mp 167–168 °C, $[\alpha]^{27}_D$ +27.7° (*c* 2.08, ethyl acetate). This product did not depress the melting point of (+)-1-benzamidoindan prepared from resolved (+)-1-amidoindan (see above), which consequently is assigned to the *R* series; a mixture melting point with (*S*)-(−)-1-benzamidoindan was 144–146 °C.

α-Bromo Amides. Amines were bromoacetylated in nonaqueous media in the presence of excess amine (method 1) or pyridine (method 2). Bromoacetylation of phenylalanine was done by the general method of Abderhalden & Haase (1931; method 3). Table I lists the properties of the α-bromo amides.

Inactivation of Chymotrypsin. The rates of inactivation at 25 °C of the enzyme by bromo amides were routinely determined at pH 5 and 7. A concentrated (ca. 3 mg/mL) solution of chymotrypsin in 1 mM HCl (concentration measured as in Lawson & Schramm, 1965) was diluted 1:1 to 1.5 mg/mL (6×10^{-5} M) in 0.2 M sodium acetate buffer, pH 5.0, and also in 0.2 M Tris-HCl buffer, pH 7.0. To 10 mL of enzyme solution at each pH was added 1.0 mL of ethanol containing a 20-fold molar excess of inhibitor. At appropriate intervals 25-μL aliquots of the reaction mixtures were diluted into 3 mL of a 1 mM solution of L-tyrosine ethyl ester hydrochloride in 0.05 M phosphate buffer, pH 6.5, and the enzyme activity was followed at 233.5 nm (Schwert & Takenaka, 1955). Half-times for inhibition (Table I) were obtained from semilogarithmic plots. Blanks in 10% ethanol showed little loss of enzymic activity at pH 5 for 1 week or at pH 7 for 3 days. After the activity had reached a constant value, the solution was dialyzed against 1 mM HCl and lyophilized for amino acid analysis (Spackman et al., 1958; Lawson & Schramm, 1965; Lawson et al., 1968).

Kinetic constants (k_2 and K_i) for the inhibition of chymotrypsin by some inhibitors were determined by the method of Kitz & Wilson (1962). Unless solubility required lower concentrations of a compound, the enzyme inactivation by 10-, 20-, 50-, and 100-fold excess of inhibitor was determined as above. First-order rate constants (k) were obtained from semilogarithmic plots; a graph of $1/k$ vs. $1/I$ was then used to obtain k_2 and K_i.

Chemical Reactivity of the Inhibitors. The reactivity of the bromine atoms of several inhibitors was determined by their reaction with two nucleophiles, sodium thiosulfate (Baker et al., 1962) and 4-(4-nitrobenzyl)pyridine (Baker & Jordaan, 1965).

CCS Model of the Active Site of Chymotrypsin. The model was constructed essentially according to Clarke (1977). Minit centers, tubing, metal sleeves, and space fillers were purchased from Science Related Materials, Janesville, WI, and a $^1/_8$-in. round aluminum rod (for support rods) was obtained from Small Parts, Inc., Miami, FL. Clarke's coordinates are correct to ±0.2 cm with the following exceptions (F. H. Clarke, personal communication, 1977): (His-40) CB-Y 16.5, ND1-Z 15.3; (Ala-56) CB-X 20.4; (His-57) CA-Y 28.8, CG-X 19.5, CG-Z 15.3, ND1-X 19.3, ND1-Z 16.4, CE1-Z 15.6, NE2-Z 14.1, CD2-Z 13.9; (Asn-100) CG-Z 23.9; (Asn-101) OD1-Y 30.6; (Asp-194) the second CA listed is CB; (Ser-195) OG-X 22.5, OG-Y 20.3, OG-Z 14.6; (Val-213) CB-X 19.3; (Ser-214) CA-Y 20.1; (Trp-215) CE2-X 8.3, CE3-X 8.6; (Ser-217) OG-X 9.4; (Cys-220) CA-Z 7.3, CB-Z 7.3, SG-Z 6.9; (Thr-

Table I: Properties of Inhibitors and Their Reactions with Chymotrypsin

compound		properties			reaction with chymotrypsin[c]			
						half-time (min)		
no.	*N*-bromoacetyl derivative of	mp (bp) (°C)	*a*	anal. or lit.[b]	molar excess	pH 5	pH 7	reaction site(s)
1	aniline	134–135	1a	1	20	42	42	Met
2	indoline	126–127	1b	CHN	20	16	16	Met
3	*N*-methylaniline	48–49	1c	2	20	7380	4320	Met, Ser
4	2-methylaniline	114–115	1d	3	20	640	640	Met
5	3-methylaniline	87–88	1d	3	20	45	45	Met
6	4-methylaniline	167–168.5	1e	3	20	34	34	Met
7	2,6-dimethylaniline	154–155	1d	CHN	20	very slow	very slow	Met
8	2-fluoroaniline	72–73	2f	CHN	20	136	165	Met
9	3-fluoroaniline	112–113	2f	CHN	20	17	21	Met
10	4-fluoroaniline	138–139	1e	CHN	20	15	17	Met
11	2-methoxyaniline	65–66	1b	CHN	20	2700	2520	Met
12	3-methoxyaniline	97–98	1b	CHN	20	112	102	Met
13	4-methoxyaniline	130–131.5	1a	CHN	20	12	17	Met
14	4-nitroaniline	176–178.5	2i	CHN	20	12	13	Met
15	benzylamine	107–109	1b	4	20	3870	2640	Met, Ser
16	isoindoline	135–136	2e	CHN	20	5	5	Met
17	(*R*)-(+)-1-aminoindan[d]	157–158.5	2h	CHN	20	7620	7620	Met, Ser
18	(*S*)-(−)-1-aminoindan[e]	157.5–159	2a	CHN	20	2140	2240	Met, Ser
19	*N*-methylbenzylamine	(150 (2 mm))	2	CHN	20	1680	390	Met, Ser
20	β-phenethylamine	77–78	1b	5	20	56	56	Met
21	2-aminoindan	131–132	1c	CHN	20	70	70	Met
22	*N*-methyl-β-phenethylamine	42–43.5	2f	CHN	20	540	324	Met, Ser
23	D-phenylalanine	125–126	3i	6	100	5900	5900	Met
24	L-phenylalanine	123–125	3i	CHN	100	4480	4480	Met

[a] Method of preparation (see method 1, 2, or 3 in text) followed by recrystallization solvent: a, ethanol; b, ethanol-water; c, petroleum ether; d, methanol-water; e, methanol; f, ether-petroleum ether; g, acetonitrile; h, ethyl acetate-cyclohexane; or i, water. [b] Literature: (1) Abenius (1889) gives mp 130–131 °C; (2) Bischoff (1901) reports mp 69 °C; (3) Bittner & Gerig (1970) give mp 110–112 °C for 4, 89–90 °C for 5, and 165–166 °C for 6; (4) Kushner et al. (1951) gives mp 106–107.5 °C; (5) Schramm & Lawson (1963) report mp 77–78 °C; (6) Abderhalden & Haase (1931) give mp 116–119 °C. [c] In 0.1 M acetate buffer (pH 5.0) and in 0.1 M Tris buffer (pH 7.0) at the molar excess given over chymotrypsin (60 μM). The half-times for loss of enzyme activity were obtained from pseudo-first-order plots, and the reaction sites were obtained by amino acid analysis of the reaction products (see text). [d] $[\alpha]^{25}_D$ 101.3° (*c* 2.66, ethanol). [e] $[\alpha]^{25}_D$ −103.0° (*c* 2.88, ethanol).

222) OG2 is CG2; (Thr-224) CA-X 5.5, CB-X 5.6, OG1-X 4.5, CG2-X 5.0. The hydrogen bond from OG of Ser-214 is incorrectly given by Clarke; it goes to OD1 (not OD2) of Asp-102 (Birktoft & Blow, 1972).

Results and Discussion

Inhibition of Chymotrypsin. Half-times for the inactivation of chymotrypsin at pH 5 and 7 by various α-bromo amides are given in Table I. The final activity was quite low, usually less than 5% (Schramm & Lawson, 1963), although for practical reasons this could not be attained or determined accurately with the slowest reacting compounds. The sites of alkylation (Table I) were determined by amino acid analysis. All the compounds alkylated a methionine residue, which was shown to be Met-192 in the case of the reaction with BA-aniline (**1**) by Lawson & Schramm (1965). With compound **1** the rate of inactivation is the same between pH 4 and pH 9. Some compounds inactivated the enzyme more rapidly at pH 7 than at pH 5, and in these instances alkylation of a serine residue, presumably Ser-195, was established in addition to methionine alkylation. The sum of methionine and serine alkylation did not, however, exceed 1 mol/mol of enzyme.

The most interesting findings of our earlier work (Schramm & Lawson, 1963) have been substantiated. BA-benzylamine (**15**) inactivates chymotrypsin about 100 times slower than BA-aniline (**1**), and the next higher homologue of **15**, BA-phenethylamine (**20**), is nearly as good as inhibitor as **1**. At pH 5 the alkylation takes place mainly at a methionine residue as determined by amino acid analysis. Since this methionine has been identified as Met-192 with chymotrypsin modified by **1**, we may assume that the other inhibitors also react with Met-192. As with chymotrypsin modified at Met-192 by *p*-nitrophenyl (bromoacetyl)-α-aminoisobutyrate (Lawson & Schramm, 1965), the enzyme modified by **1**, **15**, and **20** at Met-192 is functionally intact but displays an increased K_m toward *N*-acetyl-L-tyrosine ethyl ester (Schramm & Lawson, 1963; also see Kézdy et al., 1967).

Chemical Reactivity of the Inhibitors and Their Affinity for the Enzyme. The rates of inactivation of chymotrypsin by the compounds in Table I vary considerably. One possible reason could be that the more active compounds are intrinsically more reactive toward nucleophiles than the less active compounds. The reactivities of a few compounds to sodium thiosulfate (Baker et al., 1962) and of a larger number to 4-(*p*-nitrobenzyl)pyridine (Baker & Jordaan, 1965) are rather similar and cannot explain the effects of the α-bromo amides on chymotrypsin.

Another reason for the varied rates of inactivation could be different affinities of the compounds for the binding site in chymotrypsin. The enzyme was treated with different concentrations of a number of inhibitors under pseudo-first-order conditions. From double reciprocal plots of the loss in activity according to the method of Kitz & Wilson (1962), binding constants (K_i) and reaction constants (k_2) were obtained (Table II). These K_i's represent real binding constants, since the reversible binding step is followed by an alkylation step that is irreversible under the conditions used.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_2} E\text{–}I \qquad K_i = k_{-1}/k_1$$

Consequently, the complications that surround the significance of K_m's in enzymatic hydrolyses (Bender & Kézdy, 1965) do not arise here. The binding constants of the α-bromo amides fall in a narrow range (about 10-fold), while the reaction

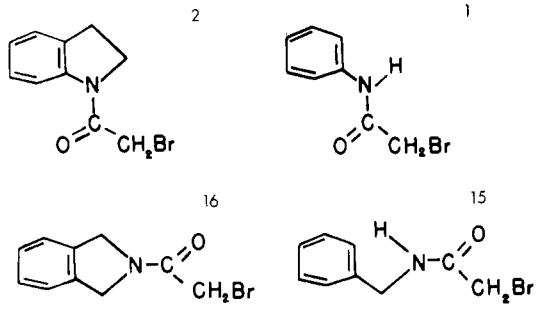
Table II: Binding (K_i) and Maximum Reaction (k_2) Constants in the Inactivation of Chymotrypsin by Some Inhibitors at pH 5.0[a]

compound	K_i (mM)	$10^4 k_2$ (min^{-1})
BA-aniline (1)	8.1	1330
BA-*N*-methylaniline (3)	13.5	13
BA-benzylamine (15)	6.7	13
BA-isoindoline (16)	4.0	5880
BA-*N*-methylbenzylamine (19)	7.8	33
BA-β-phenethylamine (20)	4.2	588
BA-D-phenylalanine (23)	1.0	1.3
BA-L-phenylalanine (24)	3.0	2.4
α-chloroacetophenone (27)	15	49
α-bromoacetophenone (28)	18	8000

[a] Method of Kitz & Wilson (1962).

constants vary widely (over 5000-fold).

Although there is no correlation between binding per se and the rate of inactivation, binding is necessary for rapid reaction of these compounds with Met-192 in chymotrypsin. α-Bromoacetamide (**26**) inactivates the enzyme very slowly in a second-order reaction (k = 57.5 min^{-1} M^{-1}); there is no detectable binding step (as expected) since **26** lacks an aromatic ring. Chemical reactivity is important when binding and structural features remain constant. α-Bromoacetophenone (**27**) inactivates chymotrypsin about 160 times faster than does α-chloroacetophenone (**28**) at pH 5, but their binding constants are about the same (Table II). In both cases methionine is attacked, and the rate ratio is in the range expected for the difference in reactivity of bromides and chlorides toward a soft nucleophile (Pearson & Songstad, 1967). Chemical reactivity and binding ability are similar for the compounds in Table I. The reasons for their considerable differences in rates of inactivation of chymotrypsin had to be sought in the stereochemistry of the compounds and in their interactions with the enzyme.



Stereochemistry of the Inactivation of Chymotrypsin by α-Bromo Amides. The compounds in Table I inactivate chymotrypsin at pH 5 primarily by alkylation of Met-192. Since the large difference in rate of inactivation (Schramm & Lawson, 1963) exhibited by BA-aniline (**1**, $t_{1/2}$ 42 min) and BA-benzylamine (**15**, $t_{1/2}$ 3870 min) cannot be explained on the basis of intrinsic reactivity or binding affinity, we investigated the stereochemistry of these compounds. Both compounds are secondary amides that should exist predominantly in the trans planar conformation (Pauling, 1960; Stewart & Siddall, 1970; Winkler & Dunitz, 1971). Examination of space-filling (CPK) models showed that the amide group of BA-aniline (**1**) should be coplanar (or nearly so) with the benzene ring, a conformation favored by resonance. This view is supported by the X-ray structure determination of acetanilide, in which the angle between the planes of the benzene ring and the amide group is 17.6° (Brown, 1966). In contrast, coplanarity of the amide group and benzene ring of BA-benzylamine (**15**) should be hindered by interactions between the NH group of the amide and the ortho hydrogens of the benzene ring. Various rotational conformers of **15** can exist, but coplanarity of the ring and amide group appears unlikely, and no resonance between the two groups is possible because of the methylene bridge. To our knowledge, no X-ray structure determination of **15** or acetylbenzylamine has been carried out. However, both in solution (Pedersen & Pedersen, 1965) and in the crystalline state (Pedersen, 1967), *N*-methylacetanilide exists in a conformation with the amide group perpendicular to the plane of the benzene ring. Its analogue in our series, BA-*N*-methylaniline (**3**), is a very slow inactivator of chymotrypsin ($t_{1/2}$ 7380 min). Evidence for the noncoplanarity of **3** was obtained by ultraviolet spectroscopy (Table III). While the spectrum of BA-aniline (**1**) shows a major peak at 252 nm, that of **3** displays only a shoulder at 225–230 nm with reduced intensity, compatible with steric inhibition of resonance (Wheland, 1955).

Table III: Ultraviolet Spectra of Some α-Bromoacetanilides[a]

compound	substituent	λ_{max} (nm)	ε
1	none	252	10800
3	*N*-methyl	225–230[b]	6480
4	2-methyl	238	5600
5	3-methyl	255	10900
6	4-methyl	257	11600
7	2,6-dimethyl	end absorption only[c]	

[a] In ethanol. [b] Shoulder. [c] ε is 5100 at 225 nm.

Ortho substitution has long been known to cause noncoplanarity in aromatic systems (Westheimer, 1956). While 3- and 4-substituted BA-anilines (**5**, **6**, **9**, **10**, **12**, **13**, and **14**) have about the same inhibitory activity toward chymotrypsin as BA-aniline (**1**) itself, the ortho (2-) compounds in each series (**4**, **8**, and **11**) are consistently less active than their meta (3-) and para (4-) counterparts[3] (Table I). Further, the activity decreases with increasing bulk of the substituent: fluoro (**8**) > methyl (**4**) > methoxy (**11**). The two *o*-methyl substituents in BA-2,6-dimethylaniline (**7**) practically abolish activity. Steric inhibition of resonance is apparent in the ultraviolet spectrum of BA-2-methylaniline (**4**) and especially in that of the *o*-dimethyl compound **7** (Table III). With BA-anilines, it is noteworthy that the polarity of the substituent has little if any effect on the rate of inhibition. For the para derivatives the rate varies roughly over a threefold range: hydrogen (**1**, $t_{1/2}$ 42 min), methyl (**6**, $t_{1/2}$ 34 min), fluorine (**10**, $t_{1/2}$ 15 min), methoxy (**13**, $t_{1/2}$ 12 min), and nitro (**14**, $t_{1/2}$ 12 min).

Both BA-*N*-methylaniline (**3**) and ortho-substituted BA-anilines, in particular BA-2,6-dimethylaniline (**7**), are poor chymotrypsin inhibitors. We ascribe this to noncoplanarity between the amide groups and the benzene rings of these compounds. The conformations of the amide groups themselves in **3** and **7** are not necessarily the same. A mixture of endo and exo conformations (carbonyl group cis and trans, respectively, to the benzene ring; Pedersen, 1967) is most likely with the tertiary amide, BA-*N*-methylaniline (**3**), while for the secondary amide, BA-2,6-dimethylaniline (**7**), the trans form (endo in Pedersen's terminology) probably predominates (also see Stewart and Siddall, 1970). Since both noncoplanar conformations of BA-*N*-methylaniline (**3**) are probably present in substantial amounts in equilibrium, neither is likely to be favorable for reaction with Met-192.

[3] Bittner & Gerig (1970) have also found that the ortho compound **4** is a slower inactivator of chymotrypsin than **1** or the meta and para compounds **5** and **6**. Their rate data are roughly comparable to ours. The somewhat (ca. twofold) higher reactivity of the para compound **5** as compared to **1** was attributed to better binding.

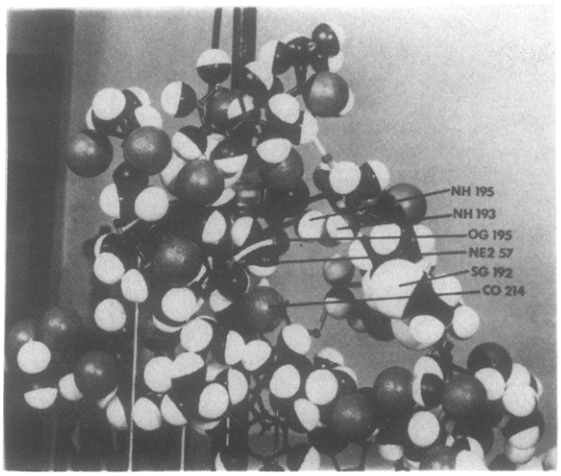


FIGURE 1: CCS model of the active site of chymotrypsin. In this and in subsequent figures, groups on the enzyme are followed by the sequence numbers of the residues to which they belong. Abbreviations used: CO 214, carbonyl group of Ser-214; NE2 57, nitrogen 2 of His-57; NH 193 and NH 195, backbone NH groups of Gly-193 and Ser-195; OG 195, hydroxyl oxygen atom of Ser-195; SG 192, sulfur atom of Met-192.

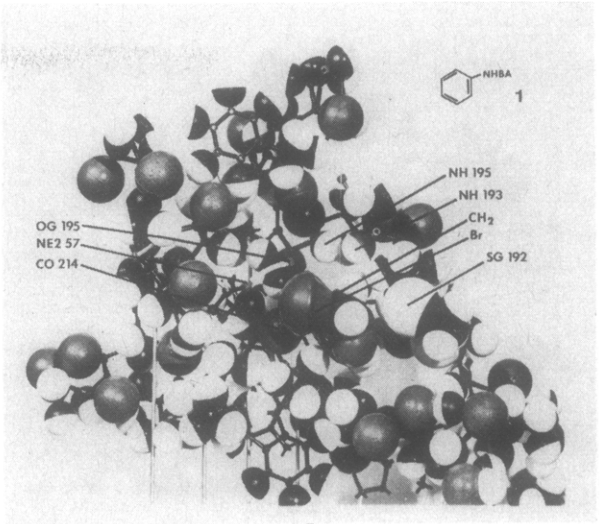


FIGURE 2: BA-aniline (**1**) in position to be attacked by SG of Met-192. In this and the following figures, unnumbered atoms belong to the molecule of the inhibitor.

Another conformational factor in these compounds (and those discussed below) is the steric relationship between the bromine atom and the carbonyl group of the amides. Two forms, in which the bromine is either cis or gauche to the carbonyl group, should be present in equilibrium. Studies on α-chloro amides indicate that the less polar gauche form predominates but that its proportion depends upon the polarity of the medium (Mizushima et al., 1956; Bellamy & Williams, 1957). By analogy with phenacyl compounds (Bartlett & Trachtenberg, 1958; Sisti & Lowell, 1964), we assume that the gauche form is by far the more reactive toward nucleophiles, and this assumption appears to be consistent with the correlations between fit and reactivity of the compounds in the active site of the enzyme (see below).

Because high reactivity toward Met-192 in the compounds discussed so far correlates well with near coplanarity of the amide group and the aromatic ring, while low reactivity is characteristic of compounds in which these groups are noncoplanar, we tested the behavior of two α-bromo amides that are "locked" structurally into coplanar configurations (Table I). BA-Indoline (**2**), a locked analogue of BA-aniline (**1**), is only slightly more effective as an inhibitor than **1**. In contrast, BA-isoindoline (**16**), a coplanar analogue of BA-benzylamine (**15**), is about 800 times more effective than **15** and is the most rapid chymotrypsin inactivator of all the α-bromo amides studied. These results support the concept that coplanarity of the amide group and the benzene ring in these compounds is necessary for rapid alkylation of Met-192 in chymotrypsin.

Correlation of the Stereochemistry of the Inhibitors with That of the Active Site of Chymotrypsin. A CCS model of the active site of chymotrypsin was constructed according to Clarke (1977; Figure 1) and used to examine the possible interactions of the inhibitors with the enzyme. The CCS model system allows the easy construction of accurate, sturdy, and lightweight skeletal models of considerable size to which space fillers may be added (and later removed if desired). Since the scale (1 Å = 12.5 mm) is the same as that of the widely used CPK models, the two may be used together. The use of models for predictive purposes should not be carried too far because rather small differences in conformation can lead to substantial differences in reactivity (Rodgers et al., 1976). However, this active-site model (Clarke, 1977) has proved quite useful in visualizing the published interactions of substrates and inhibitors with chymotrypsin (Henderson, 1970; Blow, 1971, 1976; Segal et al., 1971; Segal, 1972; Kraut, 1977) and by extrapolation with trypsin (Huber & Bode, 1978).

A model of BA-aniline (**1**) was constructed with the trans amide bond coplanar[4] to the ring and with the bromine atom gauche to the carbonyl group. When the benzene ring of **1** is fitted into the binding pocket much in the same way that the benzene ring of phenylalanine fits, the sulfur atom[5] of Met-192 is very nicely situated for an attack on the back side of the CH_2Br group (Figure 2). In this conformation, **1** can be fitted into the pocket in either of two planar orientations, depending on whether the NH group is pointed in the direction of the carbonyl group of Ser-214 or away from it. Both could easily lead to the alkylation of Met-192, but the closer the NH group is drawn to this carbonyl group, the farther away is the CH_2Br group from the sulfur of Met-192.

With slight variations, the same general fit is obtainable with all the inhibitors that react rapidly with Met-192: meta- and para-substituted BA-anilines **5**, **6**, **9**, **10**, **12**, **13**, and **14**, the locked bromo amides **2** and **16**, and BA-phenethylamine (**20**) and its locked analogue, **21**. For three substituents (methyl, fluoro, and methoxy) the rates of inactivation are uniformly in the order para > meta $\gg$ ortho (**4**–**6**, **8**–**10**, and **11**–**13**). We shall not attempt to explain the meta effect, which, except for the methoxy compound **12**, is slight. The much greater ortho effect will be discussed below, but it cannot be due to the presence of an ortho substituent per se if the amide group is coplanar to the benzene ring. BA-indoline (**2**) is such a compound and is one of the best inhibitors of the series. BA-β-phenethylamine (**20**) and its analogue, BA-2-aminoindan (**21**), probably react in extended and quasi-equatorial conformations, respectively, both having the amide groups roughly coplanar with the aromatic rings.

With the compounds that alkylate Met-192 very slowly, it is easier to see in the individual cases why they might be unreactive than to try to guess exactly how they bind to the

[4] The coplanarity (or noncoplanarity) of the inhibitors has no apparent relationship with their chemical (or intrinsic) reactivity. It does affect the orientation of the compounds when bound to the active site and, consequently, the access to the back side of the CH_2Br group for attack by Met-192.

[5] The side chain of Met-192 is doubtless mobile when the enzyme is in solution but must remain in the same general position relative to the rest of the active site.

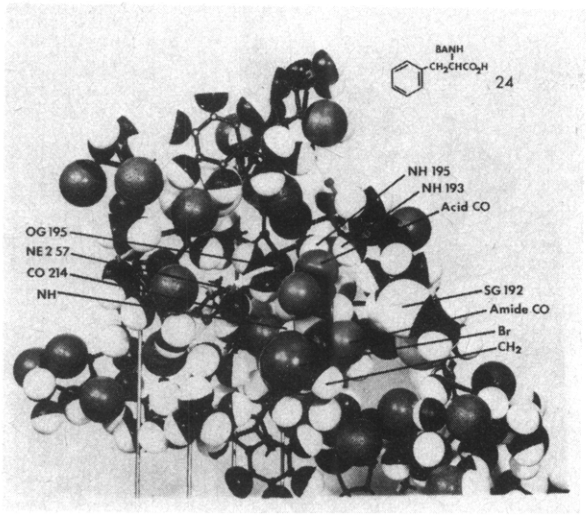


FIGURE 3: BA-L-phenylalanine (**24**) bound like a typical substrate in the active site of chymotrypsin.

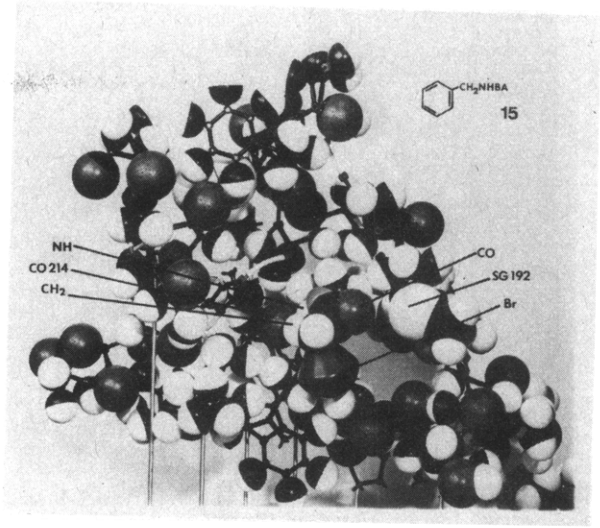


FIGURE 4: BA-benzylamine (**15**) bound such that its NH group makes a hydrogen bond with the CO group of Ser-214.

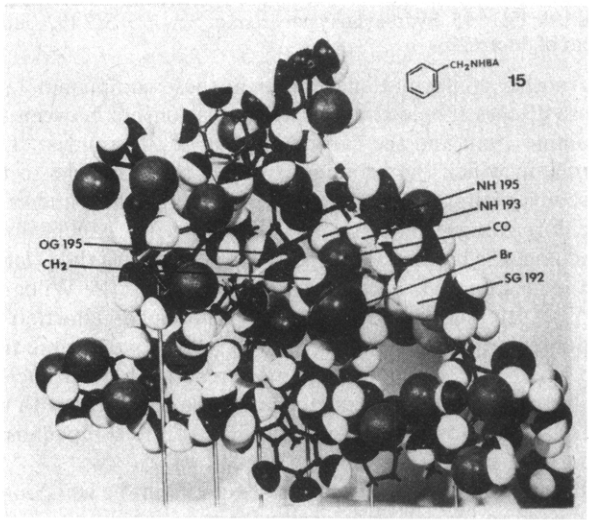


FIGURE 5: BA-benzylamine (**15**) with its CO group in position between the NH groups of Gly-193 and Ser-195.

enzyme. Exceptions to this are the BA derivatives of D- and L-phenylalanines (**23** and **24**). To the extent that these bind as typical substrates (Blow, 1971, 1976), the amide NH group will hydrogen bond with the CO group of Ser-214, the bromo amide group will be noncoplanar with the benzene ring that binds in the specificity pocket, and, in the case of the L compound **24**, the CO of the carboxyl group will hydrogen bond to the NH groups of Gly-193 and Ser-195. In this conformation (Figure 3) the CH_2Br group is not only located at some distance from the sulfur of Met-192 but is also incorrectly oriented for an attack by the latter. An approach of the sulfur would also be hindered by the CO group of the amide of **23** or **24**. Of course these phenylalanine derivatives do alkylate Met-192, though at a very slow rate. The actual reaction probably proceeds with **23** and **24** in other binding modes than that depicted in Figure 2, such as that described above for BA-β-phenethylamine (**20**).

We believe that two features of the "normal" binding of **23** and **24**, noncoplanarity of the amide group and the ring and hydrogen bonding of the amide NH group to the CO of Ser-214, contribute to the unreactivity toward Met-192 of the other slow inhibitors. These lack carboxyl groups that could hydrogen bond with the NH groups of Gly-193 and Ser-195. However, some of the slow inhibitors alkylate Ser-195 in addition to Met-192 (Table I), and a mode of binding that could lead to this involves hydrogen bonding of the amide CO group with the NH groups of Gly-193 and Ser-195. The subject of serine alkylation is considered in more detail in the following paper (Lawson, 1980).

BA-benzylamine (**15**), our first "slow" inhibitor, should exist mainly in a noncoplanar conformation. Figures 4 and 5 show **15** in two different modes of binding to the active site of chymotrypsin. In the first (Figure 4) there is hydrogen bonding of the NH group to the CO of Ser-214. The second (Figure 5) shows binding of the CO group to the NH groups of Gly-193 and Ser-195. A third mode is somewhat analogous to that depicted for BA-aniline (**1**) in Figure 2, but the energy required for an approximation to coplanarity of **15** should make alkylation of Met-192 difficult. Nevertheless, some variant of this is probably responsible for the slow reaction that occurs, because it brings the back side of the CH_2Br group much closer to Met-192 than the interactions shown in Figures 4 and 5.

The ortho-substituted BA-anilines **4**, **8**, and **11** alkylate Met-192 slower than their meta and para counterparts. Especially slow is BA-2,6-dimethylaniline (**7**). These compounds should exist to varying degrees (probably almost entirely in the case of **7**) in noncoplanar forms which do not orient well for Met alkylation. Hydrogen bonding of the amide NH to the CO of Ser-214 may play a role as well. Hydrogen bonding of the amide CO to the NH groups of Gly-193 and Ser-195 is probably not important, since these ortho-substituted compounds do not alkylate Ser-195 noticeably. The predominant cause of slow reactivity toward Met-192 in these compounds must be noncoplanarity and (when possible) hydrogen bonding to the CO of Ser-214.

The rest of the inhibitors, **17**–**19** and **22**, are discussed in more detail in the following paper (Lawson, 1980). These compounds must exist predominantly in noncoplanar conformations that favor serine alkylation with the exception of BA-*N*-methyl-β-phenethylamine (**22**), which can also exist in an extended conformation like **20** that allows a moderate rate of Met alkylation.

In conclusion, the rapid alkylation of Met-192 in chymotrypsin by site-specific α-bromo amides is favored by coplanarity of the bromo amide group with the benzene ring. When these two groups are noncoplanar, methionine alkylation is slow, and the rate may also be retarded either by hydrogen bonding of the NH of the amide group to the CO of Ser-214 or by hydrogen bonding of the CO of the amide to the NH

groups of Gly-193 and Ser-195.

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