Synthesis of Analogues of the Carboxyl Protease Inhibitor Pepstatin. Effect of Structure in Subsite P_3 on Inhibition of Pepsin[1]

Daniel H. Rich* and Michael S. Bernatowicz

School of Pharmacy, University of Wisconsin-Madison, Madison, Wisconsin 53706. *Received January 25, 1982*

A series of pepstatin analogues having minimum structural requirements for tight-binding inhibition has been synthesized and tested on porcine pepsin. Subtle changes in the geometry and size of side chains at the valine-1 position of pepstatin were found to dramatically affect inhibitor potency as well as the type of kinetic behavior observed. The inhibitors reported here can be grouped into two categories: the more potent inhibitors are slow-binding inhibitors, i.e., exhibit slow, time-dependent inhibition; the weaker inhibitors, with K_i values greater than 10^{-8} M, are not time-dependent inhibitors. A minimum kinetic mechanism is proposed to account for the observed kinetic behavior.

Pepstatin, isovaleryl-L-valyl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic acid (1), is a naturally occurring inhibitor of carboxyl (acid) proteases. Since its discovery by Umezawa,[2] pepstatin has been shown to bind to the active site[3-5] of carboxyl proteases with unusually small dissociation constants (4.57×10^{-11} M for pepsin).[6]

We have shown that the binding of pepstatin and certain pepstatin analogues to pepsin is characterized by a slow binding process leading to a lag-transient in the reaction progress curves.[7,8] Slow binding requires the presence of a 3(*S*)-hydroxy group in the central statine residue of the inhibitor plus structurally defined N-terminal and C-terminal units.[8] The P_3 subsite of pepstatin (Val[1]) (see Figure 1; the definition of P_1, P_2 follows Schecter and Berger[9]) appeared to be particularly important for the time-dependent process because analogues lacking a substituent in this position did not show the lag-transient.[8] We report here the results of a study to further characterize the factors affecting time-dependent inhibition. Our results show the importance of structural features contained in pepstatin for binding at the S_3 site of pepsin. Both steric bulk and side-chain geometry influence the ability of the inhibitor to induce slow changes in the enzyme–inhibitor complex, thereby giving rise to the time-dependent inhibition observed for the most tightly bound inhibitors.

Results

A variety of pepstatin analogues have been synthesized.[7-11] The peptide Iva-Val-(*S*,*S*)-Sta-Ala-Iaa (**5**)[8] was selected as the standard for this study because it has been shown to have the minimum structural features required to produce the slow-binding, time-dependent inhibition characteristic of pepstatin. Peptides **2**–**11** were derived from **5** by replacing the N-terminal isovaleryl group with substituents of various sizes and geometry and were selected in order to probe the specificity of the S_3 binding site of pepsin.

Inhibition constants (K_i) for pepstatin analogues **2**–**11** (Table I) were determined from IC_{50} values taken from plots of V_i/V_0 vs. inhibitor concentration, where V_i is the inhibited velocity and V_0 is the velocity in the absence of inhibitor. A typical plot is shown in Figure 2. The IC_{50} values were converted to K_i by the equation of Cha:[12]

$$K_i = (IC_{50} - E_t/2)(1 + S/K_m)^{-1}$$

where E_t is the total enzyme concentration, K_m is the Michaelis constant for the substrate (4.0×10^{-5} M), and S is the substrate concentration. In cases where inhibition was time dependent, the inhibitor was incubated with the enzyme for 10 min, and the reaction was initiated by addition of substrate. Velocities were taken at apparent steady state. Inhibitors that did not display time-dependent inhibition gave linear, steady-state initial velocities when the reactions were initiated by addition of enzyme. These initial velocities were the same as those produced when non-time-dependent inhibitors were incubated with enzyme 10 min prior to substrate addition.

The data in Table I show that relatively subtle differences in structure can affect K_i by as much as 1000-fold (**2** vs. **11**) and that two types of inhibitory kinetic behavior are apparent. Some inhibitors display a lag-transient or slow approach to apparent steady state and are, therefore, time dependent in their binding; on the other hand, less potent inhibitors do not exhibit this phenomenon. Thus, subtle structural variations at the valine-1 position of pepstatin (P_3) can dramatically influence both total binding and inhibitory kinetic mechanism.

The rate of binding can be influenced by geometry (urethane vs. amide bond) as well as steric bulk. Neither urethane derivative **2** nor **4** shows the time-dependent inhibition characteristic of the more tightly bound amides (**5**–**11**). Steric factors also are important because the *tert*-butylacetic acid derivative **3**, which is isosteric with the Boc group, is not a slow-binding inhibitor. Thus, relative to the Iva derivative **5**, the added methyl group suppresses the time-dependent process. Branching of the side chain at P_3 also affects K_i and the rate of binding. As the size of the branched-chain acyl group is increased from

(1) Abbreviations used follow IUPAC–IUB tentative rules as described in *J. Biol. Chem.*, **247**, 977 (1972). Additional abbreviations used are: DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DMF, dimethylformamide; Boc, *tert*-butyloxycarbonyl; Iva, isovaleryl; Sta, 4-amino-3-hydroxy-6-methylheptanoic acid (statine); Iaa, isoamylamide; *t*-BuAc, *tert*-butylacetyl; *i*-Poc, isopropyloxycarbonyl; *i*-bu, isobutyryl; 2-Me-bu, 2-methylbutyryl; Et_2Ac, diethylacetyl; PLC, preparative layer chromatography.

(2) Umezawa, H.; Aoyagi, T.; Morishima, H.; Matzusaki, M.; Hamada, H.; Takeuchi, T. *J. Antibiot.* **1970**, *23*, 259–262.
(3) Subramanian, E.; Swan, I. D. A.; Davies, D. R. *Biochem. Biophys. Res. Commun.* **1976**, *68*, 875–880.
(4) Kunimoto, S.; Aoyagi, T.; Nishizawa, R.; Komai, T.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1974**, *27*, 413–418.
(5) Tang, J. J. N. "Acid Proteases—Structure, Function and Biology"; Plenum Press: New York, 1979.
(6) Workman, R. J.; Burkitt, D. W. *Arch. Biochem. Biophys.* **1979**, *194*, 157–164.
(7) Rich, D. H.; Sun, E. T. In "Peptides—Proceedings of the Fifth American Peptide Symposium"; Goodman, M.; Meienhofer, J., Eds.; Wiley: New York, 1977; pp 209–212.
(8) Rich, D. H.; Sun, E. T. O. *Biochem. Pharmacol.* **1980**, *29*, 2205–2212.
(9) Schecter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157–162.
(10) Rich, D. H.; Sun, E. T. O.; Ulm, E. *J. Med. Chem.* **1980**, *23*, 27–33.
(11) Bernatowicz, M. S.; Boparai, A. S.; Rich, D. H. In "Peptides: Synthesis, Structure and Function"; Rich, D. H.; Gross, E., Eds.; Pierce Chemical Co.: Rockford, IL 1981; pp 439–442.
(12) Cha, S.; Agarwal, R. P.; Parks, R. E., Jr. *Biochem. Pharmacol.* **1975**, *24*, 2187–2197.

0022-2623/82/1825-0791$01.25/0

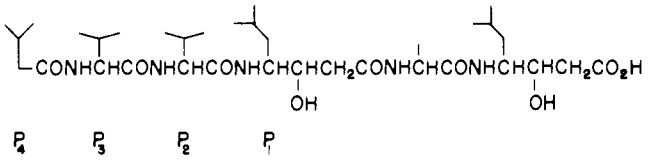


Figure 1. Structure of pepstatin (1) showing positions designated P_1, P_2, P_3, and P_4.

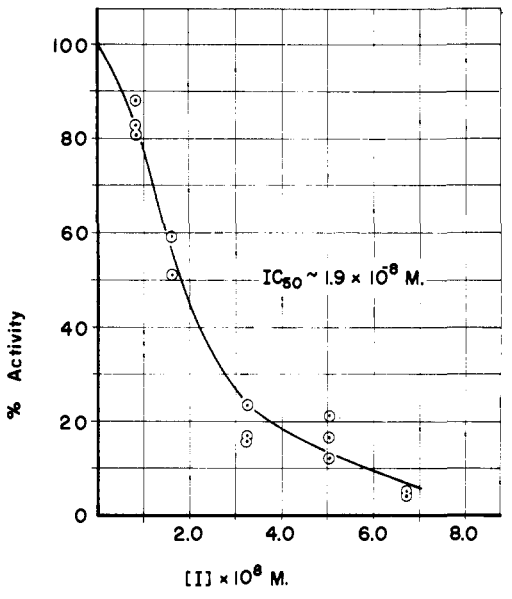


Figure 2. Inhibition of pepsin by analogue 6. Enzyme concentration was 2.8×10^{-8} M; substrate concentration was 8.8×10^{-5} M. The reactions were conducted at pH 4.0, in 0.04 M formate buffer, at 25 °C.

isobutyl (6) to 2-methylbutyryl (7) to diethylacetyl (8), binding to pepsin increases 10-fold. Analogue 8 is as potent in our assay as pepstatin or the Iaa analogue 11.

Steric factors in the P_4 position also were evaluated, although less extensively. Addition of the Iva group at P_4 (as in 11) gave an inhibitor which is about 30-fold better than analogue **5** in which the binding site is not occupied. Replacement of the Iva group with a Boc group diminished binding approximately 60-fold, but this K_i value may be too large. Determinations of K_i were only approximate because of the low solubility of Boc-derivative **9**.

Discussion

A minimum kinetic mechanism has been proposed[11,13] (eq 1) for the binding of pepstatin to pepsin, which can

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3,\ \text{fast}}{\rightleftharpoons}} EI^* \underset{k_6}{\overset{k_5,\ \text{slow}}{\rightleftharpoons}} EI^{**} \qquad (1)$$

be used to rationalize the inhibition data. A collision complex (EI) is formed in a diffusion-controlled associative process. Kitagishi et al. have estimated k_2/k_1 to be 8×10^{-4} M at pH 5.0 and k_3 to be about 600 s^{-1} for *N*-acetylpepstatin using stopped-flow techniques.[14] Thus, k_3 describes a fast first-order isomerization of a collision complex, EI, to an intermediate complex, EI*, to produce a more tightly bound form of the inhibitor. Using our assay system, the fast first-order process is not detectable because the 600 s^{-1} value for k_3 corresponds to a millisecond time scale; the rapid formation of the EI and EI* complexes from free enzyme and free inhibitor would ap-

Table I

no.	compd	K_i, M	lag-transient[a] (TDI)	mp, °C (uncorrected)	TLC R_f (solv syst)[b]	$[\alpha]^{24}_D$, deg	yield, %	formula	anal.
2	Boc-Val-(*S,S*)-Sta-Ala-Iaa	1.7×10^{-7}	no	173.5–174.5[c]	0.49 (A)	−43.4[c] (*c* 0.4, MeOH)	94[c]	$C_{26}H_{50}N_4O_6 \cdot H_2O$[c]	C, H, N
3	*t*-BuAc-Val-(*S,S*)-Sta-Ala-Iaa	7.5×10^{-9}	no	202–204	0.50 (A) 0.63 (B)	−57 (*c* 0.11, MeOH)	86	$C_{27}H_{52}N_4O_5$	C, H, N
4	Ipoc-Val-(*S,S*)-Sta-Ala-Iaa	8.6×10^{-9}	no	211–213	0.52 (A) 0.54 (B)	−52 (*c* 0.13, MeOH)	75	$C_{25}H_{48}N_4O_6$	C, H, N
5	Iva-Val-(*S,S*)-Sta-Ala-Iaa	2.9×10^{-9}	yes	237–238	0.41 (A) 0.55 (B)	−61.1[c] (*c* 0.5, MeOH)	77[c]	$C_{26}H_{50}N_4O_5$[c]	C, H, N
6	*i*-Bu-Val-(*S,S*)-Sta-Ala-Iaa	1.6×10^{-9}	yes	240–242	0.40 (A) 0.49 (B)	−57 (*c* 0.23, MeOH)	69	$C_{25}H_{48}N_4O_5$	C, H, N
7	2-Me-bu-Val-(*S,S*)-Sta-Ala-Iaa[f]	3.1×10^{-10}	yes	222 (sinters) 231–233	0.39 (A) 0.60 (B)	−64 (*c* 0.25, MeOH)	63	$C_{26}H_{50}N_4O_5$	C, H, N
8	Et_2Ac-Val-(*S,S*)-Sta-Ala-Iaa	1.8×10^{-10}	yes	230–232	0.42 (A) 0.55 (B)	−52 (*c* 0.05, MeOH)	63	$C_{27}H_{52}N_4O_5$	C, H, N
9	Boc-Val-Val-(*S,S*)-Sta-Ala-Iaa	$\sim 6 \times 10^{-9}$[d]	yes	222–224	0.38 (A) 0.63 (B)	−62 (*c* 0.31, MeOH)	88	$C_{31}H_{59}N_5O_7$	C, H, N
10	Boc-D-Val-Val-(*S,S*)-Sta-Ala-Iaa	1.1×10^{-8}	yes	221–223	0.43 (A) 0.58 (B)	−27 (*c* 0.23, MeOH)	75	$C_{31}H_{59}N_5O_7$	C, H, N
11	Iva-Val-Val-(*S,S*)-Sta-Ala-Iaa	$\sim 1 \times 10^{-10}$[e]	yes	>250	0.24 (A) 0.46 (B)	−62 (*c* 0.14, MeOH)	86	$C_{31}H_{59}N_5O_6$	C, H, N

[a] TDI = time-dependent inhibition. [b] A = 10% MeOH in $CHCl_3$ (v/v); B = 15% EtOH in EtOAc (v/v). [c] Previously reported.[10] [d] The low solubility of the inhibitor in the assay system presented difficulties in precise quantitation. [e] The kinetic behavior of this inhibitor was essentially the same as observed for pepstatin using the assay system described. [f] Prepared from racemic 2-methylbutyric acid.

(13) Schmidt, P. G.; Bernatowicz, M. S.; Rich, D. H., submitted for publication.
(14) Kitagishi, K.; Nakatani, H. H.; Hiromi, K. *J. Biochem. (Tokyo)* **1980**, *87*, 573–579.

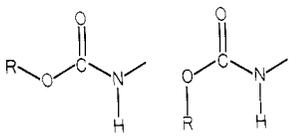
pear as a single step. Rich and Sun have determined that the dissociation constant for EI* is about 1×10^{-8} M for pepstatin.[8] This value is in remarkable agreement with the kinetic data in Table I where a value near 1×10^{-8} M separates the slow-binding pepstatin analogues from the less potent inhibitors ($K_i \geq 1 \times 10^{-8}$ M) that do not exhibit time-dependent binding. Thus, the time-dependent inhibitors undergo a slow conversion of the intermediate EI* complex to a tightened EI** complex, which accounts for their tighter binding. Evidently, all inhibitors studied here proceed beyond the EI collision complex to achieve the intermediate complex EI* to varying degrees because these inhibitors have K_i's significantly lower than 8×10^{-4} M reported for k_2/k_1 by Kitagishi.[14]

Independent evidence supports the existence of multiple EI complexes. Difference proton NMR studies of Schmidt et al.[13] reveal that the binding of pepstatin and pepstatin analogues **2**–**5**, **9**, 11 to pepsin produces a difference ^{1}H NMR spectrum characteristic for the binding as an EI* complex. Very weak inhibitors, E.g., Boc-Sta-OH or Boc-(3*R*,4*S*)-Sta-Ala-Iaa, do not produce the difference pattern. This establishes that there must be at least two enzyme–inhibitor complexes. Because pepstatin, as well as analogues **5** and **9**, undergoes the slow-binding process without changing the intensity or pattern of the difference ^{1}H NMR spectrum but compounds **2**–**4** are not slow-binding inhibitors, the time-dependent inhibitors must be partitioning into an additional complex, EI**.

Our results establish that the process leading to slow binding of pepstatin analogues to pepsin is influenced by subtle structural variants in the P_3 inhibitor site. This site is occupied by an N^{α}-acylated Val^1 residue in pepstatin. Replacement with the isovaleryl group, as in **5**, reduces inhibition, but the inhibitor remains a slow-binding inhibitor of pepsin. The decreased binding (increased K_i) appears to result from the lack of an α-substituent at P_3 because incorporation of branched acyl groups gave improved inhibitors. The isobutyryl analogue **6** is comparable to the isovaleryl analogue **5** even though it lacks the additional alkyl substituents. As the size of the branched alkyl group in this position is increased, tighter-binding analogues were obtained. The diethylacetyl derivative **8** was found to be indistinguishable from pepstatin in our assay. Further substitution at this site (e.g., 2-isopropylbutyryl-Val-Sta-Ala-Iaa) might lead to even better inhibitors.

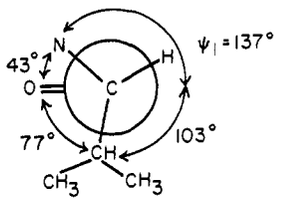
The kinetic properties of the urethane derivatives **2** and **4** may provide evidence for a preferred geometry for the P_3 group when it is bound to the enzyme. The weak inhibition for Boc-analogue **2** is surprising because the Boc group is larger than an isovaleryl group by only a methyl group. For this reason, the *tert*-butylacetyl and isopropyoxycarbonyl analogues **3** and **4** were synthesized to evaluate the relative effects of steric bulk vs. carbamate group on inhibition. The *tert*-butylacetyl derivative **3** is a substantially better inhibitor than the Boc analogue **2** but still does not bind slowly to pepsin. Clearly the presence of a urethane group at P_3 is unfavorable to the process leading to slow binding. This was confirmed when the Ipoc analogue **4**, which is isosteric with the Iva group, was found not to be a time-dependent inhibitor.

The preferred geometry of the Ipoc group in **4** may provide an indication for the conformation in the P_3 subsite of inhibitors in different enzyme–inhibitor complexes. X-ray diffraction[15] and solution conformation[16] studies have established that the preferred geometry for a Boc group is syn (**12**) rather than anti (**13**) and coplanar with



syn (12) anti (13)

the carbonyl group. However in the X-ray crystal structure of pepstatin complexed to the acid protease from *R chinensis*, derived by Bott et al.,[17] the side-chain isopropyl group is about 77° rotated from coplanarity with the carbonyl group ($\psi_1 \simeq 137°$) (see 14). It is clear that the



14

isopropyl group in Ipoc analogue **4** cannot adopt the same conformation as the isopropyl side chain in Val^1 of pepstatin in the crystal complex without rotating 77° from coplanarity with the carbonyl group. This rotation would be expected to be unfavorable because of the loss of electron overlap between the oxygen and the carbonyl. Amide bonds twisted this much are destabilized by 8 to 16 kcal, depending on whether the twist is compensated by solvation.[26] Our data indicate that inhibitors with planar constrained P_3 groups are capable of binding as an intermediate complex to pepsin but not as a tightened complex. If the conformation of pepstatin bound to porcine pepsin approximates the conformation bound to *R chinensis* pepsin, then the process leading to a tightened complex is favored by a P_3 substituent which can assume a torsion angle (ψ_1) of 137°. The X-ray diffraction studies of the *Rhizopus* pepsin–pepstatin complex[17] show the valine-1 side chain of pepstatin deeply buried in the cleft of the enzyme and surrounded by several hydrophobic enzyme residues comprising the S_3 binding site. It is interesting that X-ray diffraction studies of Blundell et al.[18] show that the S_3 site is very narrow in the related carboxyl protease isolated from *Endothia parasitica*.

Substituents in the P_4 position of pepstatin also contribute to binding because the Iva-Val-Val analogue **11** is a better inhibitor than the Iva-Val analogue **5**. The nature of the interaction with the enzyme seems to be different because even though replacement of the Iva group with a Boc group decreases binding as much as 60-fold (Table I), the analogue remains a slow binding inhibitor. Less stericly hindered substituents in this position are known to be compatible with good pepsin inhibition.[19]

It has been proposed that pepstatin is a transition-state analogue inhibitor of carboxyl proteases.[20,21] One mechanism proposed for the hydrolysis of pepsin substrates

(15) Benedetti, E.; Pedone, C.; Toniolo, C.; Nemethy, G.; Pottle, M. S.; Scheraga, H. A. *Int. J. Pept. Protein Res.* **1980**, *16*, 156–172.
(16) Branik, M.; Kessler, H. *Chem. Ber.* **1975**, *108*, 2176–2188.
(17) Bott, R. R.; Davies, D. R., personal communication.
(18) Blundell, T. L.; Jones, H. B.; Khan, G.; Taylor, G.; Sewell, B. T.; Pearl, L. H.; Wood, S. P. *Proc. FEBS Meet.* **1980**, *60*, 281–287.
(19) Aoyagi, T.; Yagisawa, Y.; Kumagi, M.; Hamada, M.; Morishima, H.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1973**, *26*, 539–541.
(20) Marshall, G. R. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1976**, *35*, 2494.
(21) Marciniszyn, J. P.; Hartsuck, J. A.; Tang, J. J. N *J. Biol. Chem.* **1976**, *251*, 7088–7094.

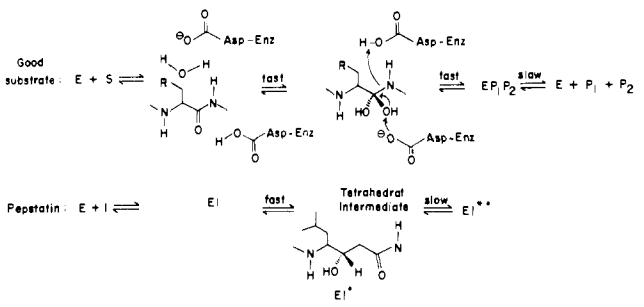


Figure 3. Top: Proposed mechanism for hydrolysis of an amide bond in a good substrate by pepsin. Bottom: Comparison of statine structure in pepstatin with the tetrahedral intermediate for amide bond hydrolysis.

(Figure 3) on the basis of X-ray diffraction studies[22,18] and ^{18}O exchange experiments[24] has a water molecule attacking the amide bond via general-base catalysis. Figure 3 illustrates the similarity of pepstatin to the proposed transition state or tetrahedral intermediate for this mechanism. If pepstatin mimics the transition state or tetrahedral intermediate, it is likely that this occurs during the rapidly formed intermediate EI* complex because this process occurs on the same time scale as the catalytic events for good pepsin substrates.[25] The slow conversion of EI* to the tightened EI** complex may mimic product release steps, found to be rate determining for good pepsin substrates,[25] if pepstatin is acting only in a mechanism-based fashion. In addition to mimicking the transition state, pepstatin may function as a collected-product inhibitor at the tightened complex EI**. Although the exact nature of the molecular events associated with the slow conversion of EI* to EI** is not understood, it is clear from the data presented here that the S_3 and S_4 binding sites influence this process, possibly by inducing conformational changes that stabilize a hydrogen bond between the statine 3(*S*)-hydroxy group and a catalytic aspartic acid. X-ray diffraction studies of enzyme–inhibitor complexes of a variety of the analogues reported here may provide a detailed understanding of the molecular events associated with the slow tightening process and remain a subject for future work.

Experimental Section

Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were determined on a Perkin-Elmer Model 241 automatic polarimeter (0.9999-dm cell). Proton nuclear magnetic resonance spectra were recorded at 270 MHz using a Fourier transform quadrature phase detection spectrometer with a Bruker instruments magnet and probe and a Nicolet 1180 computer (courtesy of P. G. Schmidt). Chemical shifts were reported as δ units (parts per million) relative to tetramethylsilane as internal standard.

TLC was performed on 0.25-mm thickness silica gel plates (Merck, silica gel 60 F-254). For column chromatography, Brinkman silica gel 60, 70–270 mesh, was used. TLC solvent systems used were: A, 10% methanol in chloroform (v/v); B, 15% ethanol in ethyl acetate. Compounds were visualized on the plates by reactions with ninhydrin, Chlorox–*o*-tolidine, 5% phosphomolybdic acid in ethanol, and ultraviolet light. All compounds used in the kinetic studies appeared as a single spot on TLC. The peptides reported here were synthesized from Boc-Val-Sta-Ala-Iaa (2) in a stepwise fashion using the appropriate N-acylating agents. Kinetic constants were measured using synthetic heptapeptide Phe-Gly-His-Phe(NO_2)-Phe-Ala-Phe-OMe as described and a Gilford Model 250 spectrophotometer connected to a Gilford 6051 recorder.[8]

General Procedure A. Removal of the *tert*-Butoxycarbonyl Group. Boc peptide (1 mmol) in a solution of 4 N HCl in dioxane (3–5 mL) was stirred at room temperature, and the reaction was monitored by TLC. Complete reaction was generally achieved in about 40 min. Excess reagent was removed under reduced pressure to give a solid residue. The residue was reevaporated from ether several times and dried in vacuo over KOH and P_2O_5 for several hours. The resulting hydrochlorides were used without further purification.

General Procedure B. Preparation of Symmetrical Anhydride. N-Protected amino acid (2 equiv, ~100 mg/mL in methylene chloride) and DCC (1 equiv) were stirred at 4 °C for about 1 h. The reaction mixture was cooled for about 20 min on dry ice and filtered to remove DCU. The filtrate was used immediately without further purification. Carboxylic acid anhydrides were prepared in a similar manner but were purified by vacuum distillation.

General Procedure C. Coupling Reactions and N-Acylation Reactions via Symmetrical Anhydride. A solution of peptide hydrochloride (~100 mg/mL in DMF) was cooled on an ice bath and neutralized with 1 equiv of triethylamine. After addition of the symmetrical anhydride (2 equiv), stirring was continued. The solution was allowed to warm to room temperature overnight. Reaction time totaled about 20 h. The solvent was removed under reduced pressure at about 35 °C to yield solid residues. The solid residues were purified by washing with several small portions of ether and drying in vacuo, followed by chromatography on silica gel using methanol–chloroform systems. For PLC, 10% methanol in chloroform (v/v) was used for development of the plate and extraction of the peptide from the gel. For column chromatography, 2% methanol in chloroform was used for elution of the purified peptide. The peptides reported here were crystallized from methanol–ether.

***N*-(*tert*-Butylacetyl)-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (3).** The title compound was prepared from HCl·Val-(*S,S*)-Sta-Ala-Iaa and *tert*-butylacetic anhydride by general procedure C: NMR (methanol-d_4) + 0.76–1.16 [m, 27 H, includes + 1.04 (s)], 1.22–1.80 [m, 10 H, includes + 1.34 (d) $J \simeq 7$ Hz], 2.19 (s, 2 H), 2.33 (d, 2 H, $J \simeq 7$ Hz), 3.47–4.31 (m, 4 H).

***N*-[(Isopropyloxy)carbonyl]-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (4).** The title compound was prepared from HCl·(*S,S*)-Sta-Ala-Iaa and the symmetrical anhydride of Ipoc-Val-OH by general procedure C: NMR (methanol-d_4) δ 0.70–1.05 (m, 18 H), 1.26 (d, 6 H, $J \simeq 7$ Hz), 1.33 (d, 3 H, $J \simeq 7$ Hz), 1.39 (m, 4 H), 1.60 (m, 2 H), 2.14 (m, 1 H), 2.35 (d, 2 H, $J \simeq 7$ Hz), 3.91 (m, 2 H), 4.03 (m, 1 H), 4.25 (q, 1 H, $J \simeq 7$ Hz).

***N*-Isobutyryl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (6).** The title compound was prepared from HCl·Val-(*S,S*)-Sta-Ala-Iaa and isobutyric anhydride by general procedure C: NMR (methanol-d_4) δ 0.74–1.02 (m, 18 H), 1.04–1.14 (m, 4 H), 1.31 (d, 6 H, $J \simeq 7$ Hz), 1.40 (m, 3 H), 1.48–1.68 (m, 3 H), 2.08 (sextet, 1 H, $J \simeq 7.6$ Hz), 2.28 (d, 2 H, $J \simeq 8$ Hz), 2.55 (quintet, 1 H, $J \simeq 7$ Hz), 3.2 (m, 4 H), 3.8–4.1 (m, 3 H), 4.22 (q, 1 H, $J \simeq 6.5$ Hz).

(±)-*N*-(2-Methylbutyryl)-L-valyl-4(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (7). The title compound was prepared from HCl·Val-(*S,S*)-Sta-Ala-Iaa and (±)-2-methylbutyric anhydride by general procedure C. No attempt was made to separate the resulting diastereomeric peptides: NMR (methanol-d_4) δ 0.74–0.95 (m, 18 H), 0.95–1.06 (m, 3 H), 1.12 (d, 2 H, $J \simeq 7$ Hz), 1.26–1.48 [m, 6 H, includes δ 1.32 (d), $J \simeq 7$ Hz], 1.48–1.70 (m, 2 H), 2.19 (m, 1 H), 2.26–2.41 (m, 3 H), 3.88 (m, 1 H), 3.99 (m, 1 H), 4.10 (t, 1 H, $J \simeq 8.5$ Hz), 4.21 (q, 1 H, $J \simeq 7$ Hz).

***N*-(Diethylacetyl)-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (8).** The title compound was prepared from HCl·Val-(*S,S*)-Sta-Ala-Iaa and di-

(22) Hsu, I. N.; Delbaere, L. T. J.; James, M. N. G.; Hofmann, T. *Nature (London)* **1977**, *266*, 140–145.
(23) James, M. N. G.; Hsu, I.; Delbaere, L. T. J. *Nature (London)* **1977**, *267*, 808–813.
(24) Antonov, V. K.; Ginodmon, L. M.; Rumsh, L. D.; Kapitannikov, Y. V.; Barshevskaya, T. N.; Yavashev, L. P.; Gurova, A. G.; Volkova, L. I. *Eur. J. Biochem.* **1981**, *117*, 195–200.
(25) Fruton, J. S. *Adv. Enzymol.* **1976**, *44*, 1–36.
(26) (a) Shipman, L. L.; Christoffersen, R. E. *J. Am. Chem. Soc.* **1973**, *95*, 1408–1416. (b) Christensen, D. H.; Kortzeborn, R. N.; Led, J. J. *J. Chem. Phys.* **1970**, *53*, 3912–3922.

ethylacetic anhydride by general procedure C: NMR (methanol-d_4) δ 0.76–1.0 (m, 24 H), 1.22–1.70 [m, 13 H includes δ 1.30 (d), $J \simeq 7$ Hz], 2.0–2.2 (m, 2 H), 2.31 (d, 2 H, $J \simeq 7$ Hz), 3.89 (br m, 1 H), 3.98 (m, 1 H), 4.14 (d, 1 H, $J \simeq 7$ Hz), 4.24 (q, 1 H, $J \simeq 7$ Hz).

***N*-(*tert*-Butoxycarbonyl)-L-valyl-L-valyl-4(*S*)-amino-3-(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (9).** The title compound was prepared from HCl·Val-(*S,S*)-Sta-Ala-Iaa and the symmetrical anhydride of Boc-L-Val-OH by general procedure C: NMR (methanol-d_4) δ 0.78–1.10 (m, 24 H), 1.24–1.78 [m, 18 H, includes δ 1.44], 1.87–2.20 (m, 2 H), 2.33 (d, 2 H, $J \simeq 7$ Hz), 3.78–4.38 (m, 5 H).

***N*-(*tert*-Butoxycarbonyl)-D-valyl-L-valyl-4(*S*)-amino-3-(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (10).** The title compound was prepared as described for **9** starting with Boc-D-Val-OH: NMR (methanol-d_4) δ 0.80–1.02 (m, 24 H), 1.25–1.47 [m, 16 H, includes δ 1.43], 1.50–1.66 (m, 2 H), 1.95–2.22 (m, 2 H), 2.31 (d, 2 H, $J \simeq 7$ Hz), 3.85–4.02 (m, 3 H), 4.33 (d, 1 H, $J \simeq 7.5$ Hz), 4.23 (q, 1 H, $J \simeq 7$ Hz).

***N*-Isovaleryl-L-valyl-L-valyl-4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoyl-L-alanyl Isoamylamide (11).** The title compound was prepared from HCl·Val-Val-(*S,S*)-Sta-Ala-Iaa and isovaleric anhydride by general procedure C: NMR (methanol-d_4) δ 0.75–1.0 (m, 30 H), 1.23–1.43 [m, 7 H, includes δ 1.30 (d), $J \simeq 7$ Hz], 1.49–1.68 (m, 3 H), 1.95–2.15 (m, 4 H), 2.31 (d, 2 H, $J \simeq 7$ Hz), 3.89 (broad, 1 H), 3.99 (m, 1 H), 4.14 (m, 2 H), 4.25 (q, 1 H, $J \simeq 7$ Hz).

Acknowledgment. This work was supported in part by grants from the National Institutes of Health (AM 20100) and Merck Sharp & Dohme. We thank Dr. Paul G. Schmidt for 270-MHz NMR spectra of the inhibitors. We thank Dr. R. Bott for the preliminary X-ray data of the *R. chinensis* pepsin–pepstatin complex.

Synthesis and Biological Activity of Some Very Hydrophobic Superagonist Analogues of Luteinizing Hormone-Releasing Hormone[1]

John J. Nestor, Jr.,*[†] Teresa L. Ho,[†] Richard A. Simpson,[†] Bonnie L. Horner,[†] Gordon H. Jones,[†] Georgia I. McRae,[‡] and Brian H. Vickery[‡]

Institute of Organic Chemistry and Institute of Biological Sciences, Syntex Research, Palo Alto, California 94304.
Received September 14, 1981

The effect of increased hydrophobicity at position 6 of luteinizing hormone-releasing hormone (LH-RH) has been investigated by the incorporation of a series of 15 very hydrophobic, unnatural D-amino acids at this position. The unnatural amino acids studied can be considered analogues of phenylalanine with carbocyclic aromatic side chains consisting of substituted phenyl (e.g., 2,4,6-trimethylphenyl, *p*-biphenyl) or polycyclic aromatic (e.g., naphthalene, anthracene) units. When enzymatic resolution (subtilisin Carlsberg) of the most hydrophobic amino acids failed, the racemic amino acids were incorporated, and the diastereomeric LH-RH analogues were resolved by preparative high-performance liquid chromatography. The analogues were synthesized by the solid-phase technique. All of the synthetic compounds were very potent LH-RH superagonists, but [6-(3-(2-naphthyl)-D-alanine)]LH-RH, [6-(3-(2-naphthyl)-D-alanine),7-(N^{α}-methylleucine)]LH-RH and [6-(3-(2,4,6-trimethylphenyl)-D-alanine)]LH-RH appear to be among the most potent LH-RH agonist analogues yet reported when tested in a rat estrus cyclicity suppression assay designed to show the paradoxical antifertility effects of these compounds [$ED_{50} \approx 7 \times 10^{-8}$ g; twice daily in saline]. These analogues are twice as potent as [D-Trp^6,ProNHEt^9]LH-RH in this assay system (i.e., ~200 times the potency of LH-RH).

The isolation and determination of the structure of luteinizing hormone-releasing hormone (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, LH-RH) from porcine[2] and ovine[3] hypothalami have led to the synthesis of a large series of analogues,[4,5] some of which have exhibited dramatically enhanced agonist ("superagonist") or antagonist potency. With further pharmacological study it has become clear that such compounds offer promising routes to the control of reproduction in both males and females. Of particular interest to us has been the demonstration that chronic treatment with pharmacological doses of long lived superagonist LH-RH analogues leads to "paradoxical" antifertility[6] and antisteroidal[7] effects which appear to be due to the desensitization of target cells in the anterior pituitary[8] and the gonads.[9] These results and their implications for fertility control led us to undertake a synthetic program designed to yield more potent superagonist LH-RH analogues with a better pharmacodynamic profile.

Previous analogue programs have demonstrated that the primary sites for successful modification of the LH-RH structure to yield superagonist analogues are at positions 6, 7, and 10. At position 6, D-amino acids led to major increases (3–40 times) in potency,[10] with an apparent preference for lipophilic amino acids. D-Trp gave the most potent analogues[11,12] at the start of these studies, and [D-Trp^6,ProNHEt^9]LH-RH was therefore chosen as the

[†] Institute of Organic Chemistry.
[‡] Institute of Biological Sciences.

(1) Contribution no. 610 from the Institute of Organic Chemistry, Syntex Research. Some of these data were presented in a preliminary form at the 7th American Peptide Symposium, Madison, WI, June 1981. The unnatural amino acids used in this study have been given the following abbreviations: Tmo, 3-(3,4,5-trimethoxyphenyl)alanine; Cha, 3-cyclohexylalanine; Pfp, 3-(pentafluorophenyl)alanine; Nal(1), 3-(1-naphthyl)alanine; Nal(2), 3-(2-naphthyl)alanine; Mtf, 3-[*m*-(trifluoromethyl)phenyl]alanine; Ptf, 3-[*p*-(trifluoromethyl)phenyl]alanine; Tmp, 3-(2,4,6-trimethylphenyl)alanine; Bna, 3-(1-bromo-2-naphthyl)alanine; Daa, 3-(9,10-dihydro-9-anthryl)-alanine; Bpa, 3-(*p*-biphenylyl)alanine; Fla, 3-(2-fluorenyl)alanine; Ana, 3-(9-anthryl)alanine; Dca, 3-(dicyclohexylmethyl)-alanine; Bha, 3-(benzhydryl)alanine. The abbreviations for natural amino acids and protecting groups follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature [*J. Biol. Chem.*, **247**, 977 (1971)].

(2) H. Matsuo, Y. Baba, R. M. Nair, A. Arimura, and A. V. Schally, *Biochem. Biophys. Res. Commun.*, **43**, 1334 (1971).
(3) R. Burgus, M. Butcher, M. Amoss, N. Ling, M. W. Monahan, J. Rivier, R. Fellows, R. Blackwell, W. Vale, and R. Guillemin, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 278 (1972).
(4) J. Rivier, M. Brown, C. Rivier, M. Ling, and W. Vale in "Peptides 1976", A. Loffett, Ed., Editions de l'Universite de Bruxelles, Brussels, 1976, pp 427–451, and references cited therein.
(5) A. V. Schally, D. H. Coy, and C. A. Meyers, *Annu. Rev. Biochem.*, **47**, 89 (1978), and references cited therein.

0022-2623/82/1825-0795$01.25/0