For bacteriostatic tests, inocula from master plates were grown overnight at 37 °C and then diluted 1000-fold in the same medium. Cultures were grown at 37 °C in *Erlenmeyer* flasks on a rotary shaker, and the growth was follwed by measuring the turbidity at 650 nm. Drugs were added after 2 h of growth.

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Registry No. 1, 58337-34-1; 4 (R = H), 89020-24-6; 4 (R = H)·acetate, 89683-28-3; 4 (R = CH_3), 89683-31-8; 4 (R = CH_3)·acetate, 89683-32-9; 4 (R = $CH(CH_3)_2$), 89683-26-1; 4 (R = $CH(CH_3)_2$)·acetate, 89683-27-2; 4 (R = $CH_2CH(CH_3)_2$), 89702-38-5; 4 (R = $CH_2CH(CH_3)_2$)·acetate, 89702-39-6; Gly, 56-40-6; Ala, 56-41-7; Val, 72-18-4; Leu, 61-90-5.

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Synthesis and Kinetic Studies of Protease Substrates Containing the 1-Methyl-6-aminoquinolinium Ion as a Fluorogenic Leaving Group

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Several sensitive substrates for porcine pancreatic elastase, chymotrypsin, and trypsin were prepared that utilize the permanently charged, fluorogenic cation 1-methyl-6-aminoquinoline (MAQ^+) as the leaving group. Kinetic rates for the hydrolysis of substrates were determined fluorimetrically and compared with analogues having 6-aminoquinoline (6-AQ) as an uncharged leaving group. It was found that substrates containing the quaternized leaving group generally have a higher k_{cat}/K_m ratio. An exception to this trend was noted with a trypsin substrate, Bz-DL-Arg-MAQ^+. During the course of this investigation, several significant advantages of the MAQ^+ ion as a fluorogenic leaving group in protease substrates were found: (a) its appearance can be measured fluorimetrically using wavelengths of light that result in its *maximal* fluorescence, while under these conditions, the unhydrolyzed substrate is essentially nonfluorescent, (b) it confers a high degree of water solubility to hydrophobic peptides, thereby eliminating the need for organic cosolvents to dissolve substrates, and (c) quaternized substrates can be prepared readily and in good yield from the corresponding 6-(peptidylamido)quinolines. These positively charged synthetic fluorogenic substrates are, therefore, useful probes for investigating the steric and electronic properties of the active-site environment of proteolytic enzymes.

Proteolytic enzymes play a key role not only in the regulation of cellular protein turnover but also in the control of many other physiological functions. Examples of these are digestion, blood coagulation, fibrinolysis, inflammation, control of blood pressure, maturation of peptide hormones, ovulation, fertilization,[1] and morphogenic changes during development.[2] Uncontrolled destruction of intra- or extracellular proteins by proteases is associated with many pathological conditions, such as the breakdown of articular cartilage by elastase[3] and collagenase[4,5] during rheumatoid arthritis, the destruction of pulmonary elastin by elastase during emphysema,[6] and the activation of plasminogen during the invasion of healthy tissues by tumor cells.[7] The information concerning the catalytic and regulatory mechanisms of proteases is, therefore, crucial to the understanding of these pathophysiological states. Moreover, such information will be useful for a rational design of medicinal agents that will modulate or prevent unwanted proteolysis.

Synthetic chromogenic or fluorogenic substrates composed of peptidyl amides of aromatic amines have been widely used to detect and quantify proteases and to define their amino acid specificities.[8] Because of the exceptional sensitivity of fluorimetry, fluorogenic substrates are particularly ideal as probes of enzyme structure and mechanism. Three problems, however, are generally encountered that limit the use of these agents. First, the fluorescence spectra of the substrate and product often overlap to a significant degree, and it becomes necessary, therefore, to use wavelengths longer than the excitation maximum to excite the product in order to avoid high levels of background emission from the excess unhydrolyzed substrate. This compromise decreases the fluorescence of the product, thereby lowering the sensitivity of the assay. Second, the aromatic amines are usually hydrophobic and thus require organic cosolvents (e.g., dimethylformamide or dimethyl sulfoxide) to solubilize them. The presence of these solvents produces unpredictable inhibitory or stimulatory effects on the enzyme.[9,10] In addition, such solvents are inappropriate for the in situ assay of proteases in cell culture. Third, the currently available chromogenic or fluorogenic groups are not readily amenable to derivatization. In order to study the steric and electronic properties of the portion of the active site on the enzyme that binds the amino acids of the substrate extending toward the C terminus from the scissile bond (the leaving group subsite), a leaving group of the substrate that can readily be derivatized into a series of congeners is required. Such studies have so far received only minor attention.[11,12]

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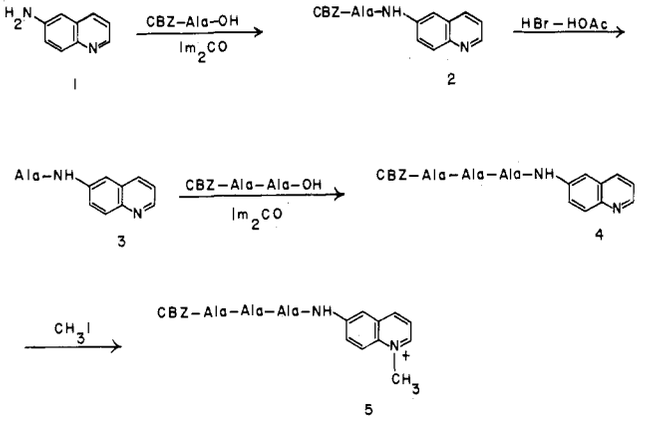
Scheme I



Recently, we have developed[13,14] a new class of synthetic protease substrates employing 6-aminoquinoline (6-AQ) as the fluorogenic leaving group. We demonstrated that this heterocyclic amine has unique spectroscopic properties. Owing to the large separation between the fluorescence spectra of the substrate and product, the 6-AQ liberated during catalysis can be excited selectively using its excitation wavelength maximum without causing the substrate to fluoresce. There is also an important synthetic advantage of using 6-AQ for the preparation of the peptide substrate, namely, it undergoes acylation in high yield because its amino group is more basic[15] than the resonance-deactivated amino groups of other frequently used chromophores such as *p*-nitroaniline and 4-methyl-7-aminocoumarin. Furthermore, by quaternizing 6-AQ at the N-1 position with a variety of alkylating agents, a series of sterically and electronically different substrates can be generated for various proteolytic enzymes.

In this article we demonstrate that substrates containing 6-AQ as the leaving group can be converted in a single reaction into MAQ^+ salts that retain the advantageous spectroscopic properties of 6-AQ. In all cases the quaternization reaction is complete within 24–48 h and both the organic solvent and the low boiling alkylating agent can be readily removed in vacuo. This simple procedure also generates highly water soluble substrates and provides a convenient alternative to the multistep sequences employed to transform the N-terminal amino acid of hydrophobic substrates into their more soluble succinylated or glutarylated derivatives. To evaluate the utility of MAQ^+ as a fluorogenic probe of enzyme structure, substrates for pancreatic elastase, chymotrypsin, and trypsin were synthesized, the rates of their hydrolysis were determined fluorimetrically, and these values were then compared with other substrates having unsubstituted neutral leaving groups.

Synthesis and Fluorescence Properties of Substrates

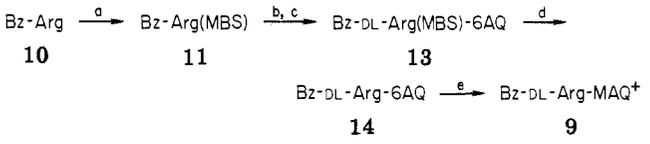
Scheme I outlines the preparation of a tripeptidyl substrate for pancreatic elastase, Cbz-Ala-Ala-Ala-MAQ^+ (**5**). Acylation of 6-AQ (**1**) with Cbz-alanine using 1,1′-carbonyldiimidazole[16,17] as the condensing agent afforded monoalanyl peptide **2**. The carbobenzoxy group of this compound was removed by brief exposure to HBr-saturated acetic acid, and the free amino group of **3** was then acylated with Cbz-alanylalanine (**3**) to produce Cbz-Ala-Ala-Ala-6AQ (**4**). Quaternization of the quinoline group proceeded smoothly by stirring **4** with methyl iodide for 24 h. A dipeptidyl substrate, Cbz-Ala-Ala-MAQ^+ (**6**), was synthesized in a similar manner by acylation of **1** with Cbz-alanylalanine, followed by alkylation of the heterocyclic nitrogen with methyl iodide. It should be pointed out that the use of strong alkylating agents such as methyl iodide would not be appropriate for substrates containing the amino acid histidine or methionine since it is likely that their side-chain functional groups will be alkylated.

In order to assess the influence of the charged fluorophore of **5** and **6** on the proteolysis reaction, a tripeptidyl substrate having a neutral leaving group, succinyl-Ala-Ala-Ala-6AQ (**7**), was prepared by first removing the carbobenzoxy group of **4** and then acylating the exposed N-terminal amine with succinic anhydride.

A quaternary substrate for chymotrypsin, Cbz-Phe-MAQ^+ (**8**), was synthesized by methylation of the previously described[13] Cbz-Phe-6-AQ.

Scheme II presents the route employed to prepare the trypsin substrates Bz-DL-Arg-MAQ^+ (**9**) and Bz-DL-Arg-6AQ (**13**). Protection of the reactive guanidino group of arginine was best accomplished by convertine *N*-benzoylarginine (**10**) to its *p*-methoxybenzenesulfonyl (MBS) derivative **11**, following the procedure introduced by Nishimura and Fujino.[18] The carboxyl group of this compound was then reacted as its mixed anhydride with **1** to give amidoquinoline **12**, and the MBS group was removed by methanesulfonic acid to afford substrate **13**. Finally, quaternization was effected with excess methyl iodide to produce the dicationic substrate **9**.

Scheme II[a]



[a] (a) *p*-Methoxybenzenesulfonyl chloride, (b) isobutyl chloroformate, (c) 6-aminoquinoline, (d) methanesulfonic acid, and (e) methyl iodide.

As we reported previously for 6-AQ,[13] the quinolinium ion also can be selectively excited, even in the presence of excess substrate, close to its excitation maximum. It is possible, therefore, to monitor the appearance of MAQ^+ using its fluorescence maxima (λ_{ex} = 410 nm, λ_{em} = 550 nm) while, under these conditions, emission from the unhydrolyzed substrate is reduced to 0.2% of its maximum. As a result, interferring background fluorescence is essentially eliminated.

Biological Results and Discussion

Table I lists kinetic constants determined for the enzyme-catalyzed hydrolysis of substrates containing the MAQ^+ group and the uncharged 6-AQ moiety. Unfortunately, some of the compounds having an N-terminal Cbz group were insoluble in water and it was necessary to convert them to succinyl or glutaryl derivatives in order to obtain rate data.

Pancreatic Elastase Substrates. The magnitude of the k_{cat}/K_m ratio of a substrate most closely reflects the

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Table I. Kinetic Constants for the Enzymatic Hydrolysis of Synthetic Fluorogenic Substrates[a]

substrate (concn range, μM)	enzyme (concn, μM)	K_m, mM	k_{cat}, s^{-1}	k_{cat}/K_m, M^{-1} s^{-1}
5, Cbz-Ala-Ala-Ala-MAQ$^+$ (50–200)	elastase (1.4)	0.23	1.09	4739
6, Cbz-Ala-Ala-MAQ$^+$ (130–1700)	elastase (1.4)	4.17	0.043	10
7, succinyl-Ala-Ala-Ala-6AQ (50–250)	elastase (1.4)	0.50	1.7	3400
8, Cbz-Phe-MAQ$^+$ (7.5–100)	chymotrypsin (2.0)	0.18	0.40	2222
14, glutaryl-Phe-6AQ[b] (200–1600)	chymotrypsin (2.0)	1.80	0.14	79
9, Bz-DL-Arg-MAQ$^+$ (50–200)	trypsin (1.0)	0.22	0.14	636
13, Bz-DL-Arg-6AQ (50–200)	trypsin (1.0)	0.39	0.60	1500

[a] Kinetic constants were determined from the initial rates of hydrolysis by the Lineweaver–Burk method. [b] Data taken from ref 16.

efficiency with which it undergoes proteolysis and is a parameter that can be used to compare the relative specificities of a series of enzyme substrates.[19] Examination of the kinetic constants determined for the hydrolysis of **5** and **6** confirms the observation of Bieth and others[20-23] as to the importance of an extended peptide chain on the rate of the proteolysis reaction. Addition of a third alanine at the P_3 subsite[24] confers to **5** a nearly 500-fold greater k_{cat}/K_m ratio that arises from approximately equal improvement in each of the kinetic constants.

For **5**, the k_{cat}/K_m ratio is about 1.5 times higher than that measured for the succinylated 6-AQ substrate **7**. This small effect, combined with the uncertain influence of the negatively charged succinyl group, make meaningful comparison and interpretation of the results tenuous. The synthesis of additional compounds and further kinetic studies are currently underway in order to gain meaningful information pertaining to the steric and electronic properties of the active site of this enzyme.

Chymotrypsin Substrates. The ability of a cationic leaving group to accelerate the hydrolysis of substrates by chymotrypsin is clearly seen by comparing the kinetic data presented for glutaryl-Phe-6AQ (**14**) and **8**. While the presence of the glutaryl group adds a complicating factor to the interpretation of the results for this enzyme, the large effect seen with these substrates as well as agreement with literature precedences makes interpretation of these results possible.

The 10-fold lower Michaelis constant of **8** could result from electrostatic interaction between the positively charged fluorophore and an anionic region near the active site of chymotrypsin. Fastrez and Fersht[25] studied a series of synthetic chymotrypsin substrates and found that introducing the cationic trimethylammonium group at the para position of *N*-acetyltyrosinanilide brought about an increase in the k_{cat} compared with substrates having more hydrophobic para substituents. The increase in the turnover number was attributed to destabilization of a specific mode of nonproductive binding: the trimethylammonium ion prevents the leaving group from entering the hydrophobic pocket that normally accepts the side chain of tyrosine, and as a result, a catalytically active enzyme–substrate complex is formed more rapidly. Similarly, the charged fluorophore of **8** will be unable to enter the S_1 subsite[24] and it too should achieve productive binding conformations more rapidly than **14**, which has the more hydrophobic 6-AQ leaving group.

Trypsin Substrates. Trypsin has a sharply defined specificity for hydrolyzing peptides at the carboxyl groups of lysine and arginine.[26] This preference is attributed to attraction between the substrate and a specific anionic region on the enzyme.[27] Eyl and co-workers,[28] using chemical methods, identified this site as the side-chain carboxylate moiety of Asp-177. Because **9** has two cationic centers that can compete for the binding pocket on the enzyme, one would predict that it will participate to a greater extent in nonproductive binding than the monocationic **13**.

The kinetic data presented in Table I show that the K_m and k_{cat} for **9** are indeed lower than those measured for **13**. Nevertheless, it is unlikely that this sufficiently explains the rate difference. When a substrate becomes involved in nonproductive binding, both its k_{cat} and K_m are decreased *to the same extent* and, therefore, the k_{cat}/K_m ratio remains unchanged.[25] Consequently, other factors must contribute to the poor substrate behavior of **9**. One possible explanation is that the high concentration of positive charge in the substrate induces subtle conformational changes in the protease that result in a less active catalytic site.

Conclusions

These studies have demonstrated that 6-AQ and MAQ$^+$ afford several important advantages that argue for their more extensive use in synthetic substrates for proteases: (a) alkyl groups can be introduced at the N-1 position of the heterocycle to generate a series of charged, fluorogenic substrates, (b) the unique spectroscopic properties of MAQ$^+$ enable it to be detected with use of wavelengths of light that result in its maximal fluorescence, (c) the ionic leaving group confers much higher solubility in water to hydrophobic peptides, thereby eliminating the undesirable use of organic cosolvents in assay media, and (d) peptidylquinolinium salts can be prepared easily and in good yield. Thus, synthetic fluorogenic substrates containing a 6-AQ or MAQ$^+$ leaving group are useful probes of the active sites of various proteases for their binding and catalytic activities.

Experimental Section

1,1′-Carbonyldiimidazole, *p*-methoxybenzenesulfonyl chloride, methanesulfonic acid, succinic anhydride, methyl iodide, and anisole were obtained from Aldrich Chemical Co., Milwaukee, WI. Carbobenzoxy-L-alanine, carbobenzoxy-L-alanylalanine, *N*-

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benzoyl-L-arginine, isobutyl chloroformate, Amberlite CG-50, quinine sulfate, orcein-elastin, porcine pancreatic elastase (type I), α-chymotrypsin (type III), 2-nitro-4-carboxyphenyl diphenylcarbamate, trypsin, and *p*-nitrophenyl *p*-guanidinobenzoate were obtained from Sigma Chemical Co., St. Louis, MO. Analytical and preparative thin-layer chromatography plates were obtained from Analtech, Newark, NJ.

Melting points were determined with a capillary melting point apparatus (Thomas-Hoover) and are uncorrected. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, and Schwarzkopf Microanalytical Laboratory, Woodside, NY. Prior to submission of samples for elemental analyses, they were dried for 24 h at 25 °C (0.1 torr). All analytical data for elements, unless otherwise indicated, were within ±0.4% of the theoretical values. In four cases, **5–8**, the elemental analyses differed from the calculated values by 1 or more equiv(s) of water. Since these compounds are hygroscopic and they are unstable to rigorous drying conditions, the presence of one or more waters of crystallization seems likely. Because these compounds are nonvolatile, standard high-resolution mass spectrometry cannot be used as an independent means of structure verification. Therefore we used fast atom bombardment mass spectrometry to confirm the identity of compounds **5–8**. This novel technique,[29] carried out by Dr. Charles Iden (Director of the Mass Spectrometry Facility, Department of Pharmacological Sciences, SUNY at Stony Brook) confirmed the molecular weights of the compounds. Likewise, the fragmentation patterns shown by these compounds are consistent with their proposed structures.

Fluorescence measurements were conducted with a Perkin-Elmer MPF-44A recording spectrofluorimeter that was standardized prior to each experiment such that a 6.7 μM solution of quinine sulfate in 0.1 N H_2SO_4 produced intensity equal to 1.0 relative fluorescence unit (RFU). Thin-layer chromatography (TLC) was performed on Analtech silica gel plates containing a fluorescent indicator. Peptides containing 6-AQ or MAQ^+ appeared as blue fluorescent zones, using long-wavelength UV light (366 nm). The following solvent systems were employed for TLC elution: A, chloroform/methanol (9:1); B, chloroform/methanol (20:1); C, chloroform/methanol (7:1); D, chloroform/methanol (5:1); E, methanol; F, methanol/acetic acid (10:1). Ion-exchange chromatography was conducted on Amberlite CG-50 resin (carboxylate form), using a stepwise gradient of ammonium carbonate from 0.1 to 1.0 M.

6-(*N*-Carbobenzoxy-L-alanylamido)quinoline (2). Into 15 mL of anhydrous tetrahydrofuran was added 3.03 g (18.7 mmol) of 1,1′-carbonyldiimidazole and 4.19 g (18.7 mmol) of carbobenzoxy-L-alanine. After stirring for 30 min at room temperature, 466 mg (3.23 mmol) of 6-aminoquinoline[13] was added and the solution allowed to stir for 72 h. The product was isolated by diluting the organic solution with water, adjusting the pH to 10 with concentrated NaOH, and extracting several times with chloroform. The combined organic phases were washed thoroughly with water, dried over $MgSO_4$, and evaporated under reduced pressure to afford a white solid. Recrystallization from acetone–petroleum ether produced 1.11 g, 98% yield, of white needles: mp 171–172 °C; R_f (A) 0.62. Anal. ($C_{20}H_{19}N_3O_3$) C, H, N.

6-(*N*-Carbobenzoxy-L-alanyl-L-alanyl-L-alanylamido)quinoline (4). To a solution of 230 mg (0.66 mmol) of **2** in 2 mL of glacial acetic acid was added 5 mL of 30% HBr in acetic acid. After 30 min at room temperature, the solution was diluted with 50 mL of anhydrous ether, which precipitated compound **3** as its dihydrobromide salt in the form of a fine white powder. These solids were washed several times with ether, dried overnight in vacuo, and used in the following reaction without further purification. To 1.00 g (3.40 mmol) of carbobenzoxy-L-alanyl-L-alanine in 15 mL of anhydrous tetrahydrofuran at room temperature was added 551 mg (3.40 mmol) of 1,1′-carbonyldiimidazole. After 30 min, the dihydrobromide salt of **3** (250 mg, 0.66 mmol) was added to the solution together with 2 equiv of triethylamine to ensure the formation in situ of its free base. The solution was then stirred for 72 h at room temperature and the crude product that had precipitated was isolated by filtration. Recrystallization from methanol afforded 111 mg, 35% overall yield from **2**: mp 263–265 °C; R_f (A) 0.50. Anal. ($C_{26}H_{29}N_5O_5$) C, H, N.

1-Methyl-6-(*N*-carbobenzoxy-L-alanyl-L-alanyl-L-alanylamido)quinolinium Iodide (5). To 93 mg (19 mmol) of 4 in 5 mL of anhydrous dimethylformamide was added 1 mL of methyl iodide. After the mixture was stirred at room temperature in the dark for 24 h, the solvent and alkylating agent were removed under reduced pressure to afford a thick orange oil. This was dissolved in a minimal volume of ethanol and precipitated as a solid by the dropwise addition of ether. After filtration, the product was washed several times with anhydrous ether and recrystallized from ethanol–ether to give 75 mg, 61% yield: mp 132 °C dec; R_f (E) 0.06. Anal. ($C_{27}H_{32}N_5O_5I$) H; C: calcd, 51.18; found, 48.31; N: calcd, 11.06; found, 10.26.

6-(*N*-Carbobenzoxy-L-alanyl-L-alanylamido)quinoline (15). This dialanyl peptide was prepared and isolated by using conditions similar to that described above for the synthesis of **2**. From 1.00 g (3.43 mmol) of carbobenzoxy-L-alanyl-L-alanine, 551 mg (3.43 mmol) of 1,1′-carbonyldiimidazole, and 82 mg (0.57 mmol) of 1 was prepared 177 mg of crude product. Recrystallization from acetone–petroleum ether afforded 167 mg, 70% yield: mp 223–225 °C; R_f (A) 0.52. Anal. ($C_{23}H_{24}N_4O_4$) C, H, N.

1-Methyl-6-(*N*-carbobenzoxy-L-alanyl-L-alanylamido)quinolinium Iodide (6). To 29 mg (0.06 mmol) of **15** in 5 mL of chloroform was added 1 mL of methyl iodide. After the mixture was stirred for 24 h at room temperature, the solvent and alkylating agent were removed in vacuo to afford a thick orange oil. Crystallization from ethanol–ether produced 36 mg, 90% yield, of a finely divided yellow powder: mp 135 °C dec; R_f (E) 0.06. Anal. ($C_{24}H_{27}N_4O_4I$) H, N; C: calcd, 51.26; found, 50.05.

6-[*N*-(Succinylamido)-L-alanyl-L-alanyl-L-alanylamido]quinoline (7). To a solution of 111 mg (0.226 mmol) of 4 in 1 mL of glacial acetic acid was added 3 mL of 30% HBr in acetic acid. After 30 min, the solution was diluted with 50 mL of ether, which resulted in the precipitation of white crystals. The solids were filtered, washed thoroughly with ether, and dried overnight under vacuum to produce a deliquescent dihydrobromide salt. This was dissolved in 5 mL of methylene chloride containing 2.2 equiv of triethylamine. Succinic anhydride (22 mg, 0.216 mmol) was added and the solution allowed to stand overnight at room temperature. At the end of this time, the white precipitate that had formed was collected and recrystallized from methanol to afford 88 mg, 97% yield: mp 193–194.5 °C; R_f (A) 0.28. Anal. ($C_{22}H_{27}N_5O_6 \cdot H_2O$) H; C: calcd, 57.77; found, 58.23; N: calcd, 15.32; found, 13.90.

1-Methyl-6-(*N*-carbobenzoxy-L-phenylalanylamido)quinolinium Iodide (8). To 2 mL of methyl iodide was added 20 mg (0.047 mmol) of 6-(*N*-carbobenzoxy-L-phenylalanylamido)quinoline.[13] After stirring at room temperature for 24 h, the two-phase suspension was evaporated to dryness under reduced pressure and recrystallized from ethanol–ether to afford 25 mg, 95% yield: mp 141 °C dec; R_f (E) 0.07. Anal. ($C_{27}H_{26}N_3O_3I$) H; C: calcd, 57.15; found, 49.91; N: calcd, 7.40; found, 6.28.

***N*-Benzoyl-*N*-[(*p*-methoxyphenyl)sulfonyl]-L-arginine (11).** This compound was prepared following the procedure introduced by Nishimura and Fujino.[18] From 5.55 g (19.9 mmol) of *N*-benzoyl-L-arginine and *p*-methoxybenzenesulfonyl chloride (8.24 g, 39.9 mmol) was prepared 5.89 g, 66% yield, of **11** as a hygroscopic solid. For characterization, this compound was converted to its dicyclohexylammonium salt: mp 203–204 °C; R_f (D) 0.58. Anal. ($C_{32}H_{47}N_5O_6S$) C, H, N.

6-[*N*-Benzoyl-*N*-[(*p*-Methoxyphenyl)sulfonyl]-DL-arginylamido]quinoline (12). Into 5 mL of anhydrous dimethylformamide at −20 °C was dissolved 800 mg (1.78 mmol) of **11** and 249 μL (1.78 mmol) of triethylamine. After 5 min, 231 μL (1.78 mmol) of isobutyl chloroformate was added, the resulting suspension was stirred at this temperature for 45 min, whereupon **1** (214 mg, 1.49 mmol) was introduced into the reaction mixture, and the reactants were gradually allowed to reach room temperature. After 24 h, the product was isolated by diluting the organic solvent with water, adjusting the pH to 9 with 5% $NaHCO_3$, and extracting with ethyl acetate. The extracts were dried ($MgSO_4$), and the solvent was removed in vacuo. Crude **12** was purified by preparative scale TLC using multiple elutions in solvent system B to afford 652 mg, 76% yield: mp 136–138

(29) Iden, C., manuscript in preparation.

°C; R_f (C) 0.38. Anal. ($C_{29}H_{30}N_6O_5S$) C, H, N.

6-(*N*-Benzoyl-DL-arginylamido)quinoline (13). To 33 mg (0.06 mmol) of 12 was added 850 μL of methanesulfonic acid containing 15 μL of anisole. After standing at room temperature for 40 min, the product was precipitated by addition of 50 mL of ether, washed several times by decantation, and dried under reduced pressure. Purification was effected by ion-exchange chromatography conducted on Amberlite CG-50 resin (carboxylate form), using a stepwise gradient of ammonium carbonate from 0.1 to 1.0 M. The blue fluorescent fractions were combined and lyophilized to produce 20 mg, 77% yield: mp 230 °C dec; R_f (F) 0.41; positive Sakaguchi test. Anal. ($C_{22}H_{24}N_6O_2 \cdot H_2CO_3$) C, H, N.

1-Methyl-6-(*N*-benzoyl-DL-arginylamido)quinolinium Diiodide (9). A solution consisting of 25 mg (0.05 mmol) of 13, 2 mL of methyl iodide, and 0.5 mL of dimethylformamide was allowed to stand at room temperature in the dark for 48 h. The solvents were removed under reduced pressure and ether was added to solidify the oily residue. Recrystallization from ethanol–ether produced 22 mg, 62% yield: mp 220 °C dec; R_f (E) 0.06; positive Sakaguchi test. Anal. ($C_{23}H_{28}N_6O_2I_2$) C, H, N.

Enzyme Assays. Kinetic constants were determined from initial rates of hydrolysis by the Lineweaver–Burk method. Correlation coefficients for all experiments were 0.998 or higher. Assays using pancreatic elastase were conducted in 67 mM Tris-HCl buffer (pH 8.8) at 37 °C, using a thermostatically controlled cuvette. The specific activity of elastase was determined using orcein-elastin[30] prior to each experiment. Substrates for chymotrypsin were evaluated in 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM $CaCl_2$ at 25 °C. The specific activity of the enzyme was determined with use of the chromogenic inactivator 2-nitro-4-carboxyphenyl diphenylcarbamate.[31] Assays utilizing trypsin were performed in 46 mM Tris-HCl buffer (pH 8.1) containing 11 mM $CaCl_2$ at 25 °C. For the titration of trypsin, *p*-nitrophenyl *p*-guanidinobenzoate was used following the method of Chase and Shaw.[32] The concentrations of enzymes and the ranges at which substrates were employed are listed in Table I. Excitation and emission wavelengths used to monitor the appearance of MAQ^+ were 410 and 550 nm, respectively, and for 6-AQ they were 355 and 550 nm, respectively. All of the substrates prepared in this study were stable to buffer in the absence of enzymes: after they were exposed for 24 h at 25 °C to the assay media alone, no increase in fluorescence due to the appearance of the leaving group was detected.

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Registry No. 1, 580-15-4; 2, 84614-59-5; 3·2HBr, 90605-99-5; 4, 84614-60-8; 5, 90606-00-1; 6, 90606-01-2; 7, 84614-61-9; 8, 90606-02-3; 9, 90606-03-4; 10, 154-92-7; 11, 90606-04-5; 11 dicyclohexylammonium, 90606-06-7; 12, 90693-47-3; 13, 84680-45-5; 15, 90606-05-6; Cbz-Ala, 1142-20-7; Cbz-Ala-Ala, 16012-70-7; Cbz-Phe-6AQ, 80115-53-3; *p*-methoxybenzenesulfonyl chloride, 98-68-0; elastase, 9004-06-2; chymotrypsin, 9004-07-3; trypsin, 9002-07-7.

Supplementary Material Available: The fast atom mass spectra for compounds 5–8 (4 pages). Ordering information is given on any current masthead page.

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Luteinizing Hormone-Releasing Hormone Antagonists Containing Very Hydrophobic Amino Acids[1]

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In a continuation of our studies on the effects of hydrophobic substitutions in analogues of luteinizing hormone-releasing hormone (LH-RH), we have synthesized LH-RH antagonists containing the very hydrophobic amino acid 3-(2-naphthyl)-D-alanine (D-Nal(2)). The D-Nal(2) substitution was found to be effective when incorporated in positions 3 and 6. The most potent analogue containing two D-Nal(2) residues was [N-Ac-Pro^1,D-pF-Phe^2,D-Nal(2)^{3,6}]LH-RH (ED_{50} = 2.2 μg, rat antiovulatory assay, propylene glycol–saline vehicle). This analogue also demonstrates that the N-Ac-Pro^1 substitution is as effective as the more costly N-Ac-Δ^3-Pro^1 modification. Analogues containing D-Nal(2) in combination with the hydrophilic D-Arg residue in position 6 were prepared. Neither N-Ac-Pro at position 1 nor D-Nal(2) at position 3 was effective in combination with D-Arg. N-Ac-D-Nal(2) at position 1 gave a highly potent antagonist ([N-Ac-D-Nal(2)^1,D-pF-Phe^2,D-Trp^3,D-Arg^6]LH-RH; ED_{50} = 2.4 μg) that exhibited a prolonged duration of action (ED_{50} = 9.0 μg, corn oil vehicle, dosing on diestrus II).

The possibility that synthetic analogues of polypeptide hormones might retain their receptor binding ability without appreciable agonistic activity was recognized early in the study of peptide hormone analogues.[2,3] The first competitive inhibitors[4] of luteinizing hormone-releasing hormone (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2,LH-RH) were discovered soon after the disclosure of the structure of the native hormone.[5] Although the initial inhibitors ([Gly^2]LHRH, des-His^2-LH-RH) required high doses in order to be effective[6] (in vitro), they identified position 2 as the crucial site for modification to obtain antagonists. Later substitutions in the des-His^2-LH-RH

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