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New class of sensitive and selective fluorogenic substrates for serine proteinases

Amino acid and dipeptide derivatives of rhodamine

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A series of dipeptide derivatives of Rhodamine, each containing an arginine residue in the P_1 position and one of ten representative benzyloxycarbonyl (Cbz)-blocked amino acids in the P_2 position, has been synthesized, purified and characterized as substrates for serine proteinases. These substrates are easily prepared with high yields. Cleavage of a single amide bond converts the non-fluorescent bisamide substrate into a highly fluorescent monoamide product. Macroscopic kinetic constants for the interaction of these substrates with bovine trypsin, human and dog plasmin, and human thrombin are reported. Certain of these substrates exhibit extremely large specificity constants. For example, the $k_{cat.}/K_m$ for bovine trypsin with bis-(*N*-benzyloxycarbonylglycyl-argininamido)-Rhodamine [(Cbz-Gly-Arg-NH)$_2$-Rhodamine] is 1 670 000 $M^{-1} \cdot s^{-1}$. Certain of these substrates are also highly selective. For example, the most specific substrate for human plasmin, (Cbz-Phe-Arg-NH$_2$)-Rhodamine, is not hydrolysed by human thrombin, and the most specific substrate for human thrombin, (Cbz-Pro-Arg-NH)$_2$-Rhodamine, is one of the least specific substrates for human plasmin. Comparison of the kinetic constants for hydrolysis of the dipeptide substrates with that of the single amino acid derivative, (Cbz-Arg-NH)$_2$-Rhodamine, indicates that selection of the proper amino acid residue in the P_2 position can effect large increases in substrate specificity. This occurs primarily as a result of an increase in $k_{cat.}$ as opposed to a decrease in K_m and, in certain cases, is accompanied by a large increase in selectivity. Because of their high degree of sensitivity and selectivity, these Rhodamine-based dipeptide compounds should be extremely useful substrates for studying serine proteinases.

Recently we reported the use of a Rhodamine derivative as a leaving group in the fluorogenic substrate for serine proteinases (Cbz-Arg-NH)$_2$-Rhodamine (Leytus *et al.*, 1983). Enzymic cleavage of one of the amide bonds in the non-fluorescent bisamide substrate results in a 3500-fold increase in fluorescence intensity by release of the highly fluorescent monoamide product Cbz-Arg-NH-Rhodamine. The molar absorption coefficient of Cbz-Arg-NH-Rhodamine at 492nm is 23 500 $M^{-1} \cdot cm^{-1}$, and the quantum yield is 0.32. A comparison of the sensitivities of (Cbz-Arg-NH)$_2$-Rhodamine and of 7-(*N*-Cbz-L-Arg-NH)-Mec (Zimmerman *et al.*, 1976, 1977) as substrates for bovine trypsin, human and dog plasmin, and human thrombin revealed (Cbz-Arg-NH)$_2$-Rhodamine to be the better substrate by factors ranging from 50- to 300-fold. The greater sensitivity of the Rhodamine-based substrate arises not only from the fluorophoric leaving group being highly detectable, Cbz-Arg-NH-Rhodamine being 4–5-fold more fluorescent than NMec, but also from the reactive-site bonds in (Cbz-Arg-NH)$_2$-Rhodamine being more reactive than that in the coumarin substrate. We suggested that the greater degree of conjugation in the monoamide hydrolysis product relative to that in the bisamide substrate is the origin of the enhanced reactivity of the amide bonds in the substrate.

Abbreviations used: (Cbz-Arg-NH)$_2$-Rhodamine, bis-(*N*-benzyloxycarbonyl-L-argininamido)-Rhodamine; DMF, *NN*-dimethylformamide; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SBu^t, isobutyl thioester; *p*-NA, *p*-nitroanilide; NMec, 7-amino-4-methylcoumarin; <Glu, pyroglutamic acid.

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Because of these properties, we believed (Cbz-Arg-NH)$_2$-Rhodamine could serve as a model for the design and construction of more sophisticated peptide substrates that would offer greater sensitivity and even selectivity.

Here we report the synthesis of ten dipeptide Rhodamine-based substrates designed to measure the amidolytic activity of trypsin-like serine proteinases. Each dipeptide substrate contains an arginine residue in the P_1 position and one of ten representative Cbz-blocked amino acids in the P_2 position. The macroscopic kinetic constants for the interaction of these substrates with bovine trypsin, human and dog plasmin, and human thrombin, were determined to assess the utility of these substrates and to probe the subsite specificity of these enzymes. Certain of these reagents were excellent substrates for these proteinases, exhibiting second-order specificity constants ($k_{cat.}/K_m$ values) 100-fold greater than those for (Cbz-Arg-NH)$_2$-Rhodamine and of the same magnitude as reported for corresponding thioester-based substrates (McRae *et al.*, 1981*a*). In addition, certain of these Rhodamine-based substrates exhibited a high degree of selectivity.

Experimental

Spectrometry

Electronic absorption spectra were measured on a Beckman Acta model cIII spectrophotometer with matched silica cells having 1 cm path-length.

Materials

Cbz-Gln-*p*-nitrophenol was purchased from Vega Biochemicals. All other Cbz-blocked amino acids, as well as 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, were purchased from Sigma Chemical Co. Fluorescein was purchased from the Aldrich Chemical Co. *p*-Nitrophenyl *p*-guanidinobenzoate was purchased from ICN, Cleveland, OH, U.S.A., and dissolved in redistilled DMF. Hepes was purchased from Calbiochem. Rhodamine 110, laser grade, was purchased from Eastman Kodak Co. Fluorescein mono-*p*-guanidinobenzoate was synthesized as described previously (Melhado *et al.*, 1982). (Cbz-Arg-NH)$_2$-Rhodamine and Cbz-Arg-NH-Rhodamine were synthesized as described previously (Leytus *et al.*, 1983). Disposable plastic cuvettes were purchased from Evergreen, Los Angeles, CA, U.S.A. All peptide substrates were stored as dry solids at −20°C or at concentrations of 0.01 M in redistilled DMF at 4°C. In either state, no decomposition was observed over a period of 6 months. Once dissolved in DMF, substrate solutions were incubated at room temperature for 24–48 h until they turned completely colourless. Bovine pancreatic trypsin (three-times-crystallized) was purchased from Worthington. Stock solutions were prepared in 1 mM-HCl and stored at −20°C. Dog and human plasmin were prepared by a slight modification of the procedure of Castellino & Sodetz (1976) as described previously (Leytus *et al.*, 1981; Livingston *et al.*, 1981). Stock solutions were prepared in 10 mM-Hepes/NaOH buffer, pH 7.5, containing 25% (v/v) glycerol, and stored at −20°C. Human thrombin was a gift from Dr. Robert Rosenberg, Harvard Medical School, Boston, MA, U.S.A. Stock solutions of human thrombin were stored in 10 mM-Hepes/NaOH buffer, pH 7.5, containing 0.9 M-NaCl at −20°C. The active-site concentrations of all enzymes were determined with Fluorescein mono-*p*-guanidinobenzoate as described by Melhado *et al.* (1982).

Chromatography

Analytical t.l.c. was performed on Brinkmann silica-gel plates with fluorescent indicator, with butan-2-one/acetone/water (8:1:1, by vol.) as the developing solvent. Detection was under 254 nm and 365 nm light.

Quantum yields

Fluorescence emission spectra were recorded on a Perkin–Elmer MPF-44A spectrofluorimeter equipped with a Universal digital readout (model UDR-1) and connected to a Hewlett–Packard model 7015 *x–y* recorder with time base. A 2 nm bandwidth was used on both the excitation and emission monochromators. The excitation wavelength for Rhodamine and its derivatives in 10 mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol was 460 nm, and the emission was scanned from 470 nm to 670 nm. The same wavelengths were used for a Fluorescein standard solution in 0.1 M-NaOH. The recorded emission spectra were traced with a Hewlett–Packard model 9864 digitizer board interfaced to a Hewlett–Packard model 9825A computer, which then corrected the emission spectra for variations in the response of the photomultiplier tube at different wavelengths. The corrected fluorescence intensities were plotted as a function of wavenumber, and the spectra were integrated. Fluorescence quantum yields were calculated relative to a quantum yield of 0.94 for Fluorescein in 0.1 M-NaOH (Heller *et al.*, 1974).

Complete tryptic hydrolysis

The Rhodamine content of the dipeptide substrates was determined by complete tryptic hydrolysis of the amide bonds followed by measurement of the absorbance increase. A 0.02 ml portion of 12 μM-bovine trypsin was added at zero time and 30 min to a 2.0 ml solution of 10 μM-substrate in 10 mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol, that was prepared from accurately weighed samples.

After 60min, the concentration of Rhodamine released was calculated from A_{492} by using a molar absorption coefficient for Rhodamine in 10mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol, of 66800 $M^{-1} \cdot cm^{-1}$ (Leytus *et al.*, 1983).

Preparation of monoamide products

To 2ml of 0.20mM-bisamide substrate in 10mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol, was added 20μl of 0.12μM-bovine trypsin in 10mM-Hepes buffer, pH7.5, containing 0.02M-$CaCl_2$, and the A_{492} was monitored, as a function of time, against a similar solution without trypsin. When the absorbance reached 0.086, the reaction was stopped by addition of 5μl of 0.50mM-*p*-nitrophenyl *p*-guanidinobenzoate to both the sample and reference cuvettes. Approx. 2% of the bisamide substrate is converted into its corresponding monoamide product as determined by using a molar absorption coefficient for Cbz-Arg-NH-Rhodamine at 492nm in 10mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol, of 23500 $M^{-1} \cdot cm^{-1}$ (Leytus *et al.*, 1983).

Enzyme assays

Enzyme assays were performed at 22°C in 10mM-Hepes buffer, pH7.5, containing 10% (v/v) dimethyl sulphoxide. For bovine trypsin, 0.02M-$CaCl_2$ was also present. Stock solutions of 0.01M-substrate were prepared in DMF and diluted 33–100-fold with buffer before assay. The highest substrate concentration, which contained from 1 to 3% DMF, was then serially diluted with buffer to obtain a range of substrate concentrations. Enzyme stock solutions were diluted from 100- to 100000-fold into buffer immediately before being assayed. For all assays an enzyme concentration was chosen so that less than 5% of the substrate was hydrolysed. Unless otherwise indicated, 0.01ml of enzyme was mixed with 0.04ml of substrate in the bottom of a disposable plastic cuvette. After 5min, 0.95ml of 10mM-Hepes buffer, pH7.5, containing 15% (v/v) ethanol was added, and the fluorescence was immediately recorded. No spontaneous hydrolysis of the substrates was observed during an assay. Fluorescence measurements were made using a Perkin–Elmer 650-40 fluorescence spectrophotometer or a Perkin–Elmer MPF-44A fluorescence spectrophotometer equipped with a Perkin–Elmer Universal digital readout (model UDR-1). The excitation and emission wavelengths were 492 and 523nm respectively, both set with a bandwidth of 4nm. The fluorescence spectrophotometers were standardized with a polymethacrylate block embedded with Rhodamine B to ensure that the relative fluorescence was comparable in different experiments.

Kinetic analysis

The kinetic constants $k_{cat.}$, K_m and $k_{cat.}/K_m$ were determined by measuring the rate of enzyme-catalysed hydrolysis over a range of substrate concentrations, usually from one-fifth to twice the K_m, and then fitting the data points with a computer to the Michaelis–Menten rate equation using the iterative method described by Cleland (1967). Conversion of relative fluorescence units (FU) into molar concentrations (M) of monoamide product formed was accomplished by using a standard curve correlating fluorescence intensities with molar concentrations of Cbz-Arg-NH-Rhodamine in 10mM-Hepes buffer, pH7.5, containing 15% (v/v) ethanol (Leytus *et al.*, 1983).

Preparation of $(Arg\text{-}NH)_2$-Rhodamine

$(Cbz\text{-}Arg\text{-}NH)_2$-Rhodamine (0.4g, 0.40mmol) was deprotected by treatment with 10ml of 4M-HBr in acetic acid for 1h at room temperature. The product was precipitated from the reaction solution with 100ml of diethyl ether and centrifuged at 10000*g* for 20min. This was followed by three cycles of suspension in ethyl ether and centrifugation at 10000*g* for 20min. The product was dried over anhydrous $CaSO_4$ in an evacuated desiccator at room temperature and yielded 0.36g (90%) of an orange powder. The product was judged to be pure by analytical t.l.c., which revealed a single dark spot under u.v. light (Table 1).

Table 1. *T.l.c. and hydrolysis characteristics*

T.l.c. was performed with butan-2-one/acetone/water (8:1:1, by vol.) as eluent with the concentrations of stock solutions 0.1mM in methanol. Complete hydrolysis of the Rhodamine derivatives was performed as described in the Experimental section. The results are expressed as percentages of the values predicted from the weight of the sample and its assigned molecular weight.

Compound	R_F on t.l.c.	Purity by complete trypsin hydrolysis (%)
(Arg-NH)-Rhodamine	0.02	—
$(Cbz\text{-}Arg\text{-}NH)_2$-Rhodamine	0.26	98
$(Cbz\text{-}Ala\text{-}Arg\text{-}NH)_2$-Rhodamine	0.25	103
$(Cbz\text{-}Gln\text{-}Arg\text{-}NH)_2$-Rhodamine	0.12	100
$(Cbz\text{-}\langle Glu\text{-}Arg\text{-}NH)_2$-Rhodamine	0.24	99
$(Cbz\text{-}Gly\text{-}Arg\text{-}NH)_2$-Rhodamine	0.47	105
$(Cbz\text{-}Leu\text{-}Arg\text{-}NH)_2$-Rhodamine	0.23	92
$(Cbz\text{-}Met\text{-}Arg\text{-}NH)_2$-Rhodamine	0.45	104
$(Cbz\text{-}Phe\text{-}Arg\text{-}NH)_2$-Rhodamine	0.49	102
$(Cbz\text{-}Pro\text{-}Arg\text{-}NH)_2$-Rhodamine	0.20	108
$(Cbz\text{-}Trp\text{-}Arg\text{-}NH)_2$-Rhodamine	0.42	102
$(Cbz\text{-}Val\text{-}Arg\text{-}NH)_2$-Rhodamine	0.32	105

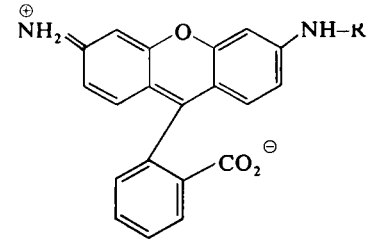
Synthesis of dipeptide derivatives of Rhodamine

The same procedure was used for the synthesis and purification of all the dipeptide Rhodamine substrates, except for that which contained Cbz-Gln in the P_2 position. To 1.22 mmol of Cbz-blocked amino acid in a capped glass vial was added 9 ml of cold dry DMF/pyridine (1:1, v/v) and the contents were stirred at 4°C until solution was complete. To this was added 0.215 g (1.12 mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide. After 5 min of stirring at 4°C, 0.05 g (0.051 mmol) of (Arg-$NH)_2$-Rhodamine, dissolved in 1 ml of DMF/pyridine (1:1, v/v), was added. Stirring was continued at 4°C for 2 h and then at room temperature for 2 days. For the substrate that contained Cbz-Gln in the P_2 position the corresponding activated *p*-nitrophenyl ester (1.22 mmol) was allowed to react with (Arg-$NH)_2$-Rhodamine (0.051 mmol) in DMF/pyridine (1:1, v/v) for 10 days at room temperature. All reaction solutions were then concentrated by precipitation, by using 10 vol. of ethyl acetate to 1 vol. of reaction solution, followed by centrifugation at 10000 ***g*** for 20 min. To the resulting residue, dissolved in 1 ml of DMF, was added 10 ml of 1.2 M-HCl and the solution then centrifuged at 10000 ***g*** for 20 min. The residue was dissolved in 1 ml of DMF, precipitated by the addition of 10 ml of ethyl acetate, and centrifuged at 10000 ***g*** for 20 min. Additional cycles of solution in 1 ml of DMF, precipitation with 10 ml of ethyl acetate and centrifugation at 10000 ***g*** for 20 min were repeated until flocculent, pale orange or pink precipitates were obtained (usually after two to four cycles). The products, after being dried over anhydrous $CaSO_4$ either in an evacuated desiccator at room temperature or an evacuated drying pistol at 78°C, appeared as pale orange or pink powders. Yields ranged from 45 to 85%. The products were judged to be pure by analytical t.l.c., which revealed single dark spots under u.v. light (Table 1).

Results

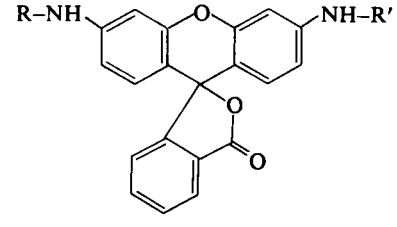
Synthesis

All the dipeptide substrates, except that which contained Cbz-Gln in the P_2 position, were prepared in DMF/pyridine, (1:1, v/v) from (Arg-$NH)_2$-Rhodamine and Cbz-blocked amino acids by condensation with 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride, a water-soluble carbodi-imide. (Arg-$NH)_2$-Rhodamine was obtained by unblocking (Cbz-Arg-$NH)_2$-Rhodamine with 4 M-HBr in acetic acid. For the derivative that contained Cbz-Gln in the P_2 position, the corresponding activated *p*-nitrophenyl ester was allowed to react with (Arg-$NH)_2$-Rhodamine in DMF/pyridine (1:1, v/v). All reactions could be driven to completion by using a 20-fold molar excess of blocked amino acid and carbodi-imide, when present, relative to the concentration of (Arg-$NH)_2$-Rhodamine.



Mono-substituted Rhodamine

Rhodamine	R = H
Cbz-Arg-NH-Rhodamine	R = Cbz-Arg
Cbz-P_2-Arg-NH-Rhodamne	R = Cbz-P_2-Arg



Bis-substituted Rhodamine

(Arg-NH_2)-Rhodamine	R = R' = Arg
(Cbz-Arg-$NH)_2$-Rhodamine	R = R' = Cbz-Arg
(Cbz-P_2-Arg-$NH)_2$-Rhodamine	R = R' = Cbz-P_2-Arg

Purification

Purification of the Rhodamine-based dipeptide substrates, achieved by precipitating the product in reaction mixtures with ethyl acetate and with 1.2 M-HCl, was very effective, since the insoluble product could be separated from excess soluble reactants and the soluble urea side product by

Table 2. *Spectral properties of mono-substituted Rhodamine*

All spectra were measured in 10 mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol. For emission spectra the excitation wavelength was 460 nm and the spectra were corrected as described in the Experimental section: the quantum yield (ϕ) values are given relative to $\phi = 0.94$ for Fluorescein in 0.1 M-NaOH.

Compound	Absorption spectra $\lambda_{max.}$ (nm)	Emission spectra $\lambda_{emission}$ (nm)	ϕ
Cbz-Arg-NH-Rhodamine	467, 490	529	0.32
Cbz-Phe-Arg-NH-Rhodamine	469, 491	529	0.36
Cbz-Pro-Arg-NH-Rhodamine	468, 491	529	0.33

centrifugation. The purity of the products was assessed by t.l.c. on silica gel, which revealed only single spots (Table 1). The purity of the products was also assessed by quantitative analysis of their Rhodamine content, its release being monitored after complete hydrolysis by bovine trypsin as described in the Experimental section. The amount of Rhodamine released was within 8% of that expected, based upon the weight of the sample and the assigned molecular weight of the product.

Spectral properties of monoamide hydrolysis products

During the course of an assay, the non-fluorescent bisamide substrates are enzymically cleaved to yield their corresponding highly fluorescent monoamide products. To assess whether the various monoamide products exhibited the same spectral properties, we obtained absorbance and emission spectra and quantum yields for Cbz-Phe-Arg-NH-Rhodamine and Cbz-Pro-Arg-NH-Rhodamine, prepared as described in the Experimental section, and compared them with those previously reported for Cbz-Arg-NH-Rhodamine (Leytus *et al.*, 1983). The results, summarized in Table 2, suggest that the spectral properties of all Rhodamine monoamide leaving groups are the same, regardless of the composition and length of the peptide chain portion of the molecule. By implication, all Rhodamine monoamide derivatives, or at least those that possess an arginine residue in the P_1 position, should exhibit the same molar fluorescence coefficient. For this reason, the molar fluorescence coefficient originally determined for Cbz-Arg-NH-Rhodamine (Leytus *et al.*, 1983) was also employed for the extended peptide derivatives when converting fluorescence intensities into molar concentrations of monoamide product formed during enzymic hydrolysis.

Kinetic constants

The kinetic constants $k_{cat.}$, K_m and $k_{cat.}/K_m$ for the hydrolysis of $(Arg\text{-}NH)_2$-Rhodamine, $(Cbz\text{-}Arg\text{-}NH)_2$-Rhodamine, and ten Rhodamine-based dipeptide substrates by bovine trypsin, human and dog plasmin, and human thrombin are reported in Table 3. The ten dipeptide substrates each contained an arginine residue in the P_1 position, but different Cbz-blocked amino acids in the P_2 position. With each of the substrates, the kinetics of hydrolysis as a function of substrate concentration conformed to that predicted by the Michaelis–Menten rate equation. An important kinetic parameter is the specificity constant, $k_{cat.}/K_m$, since this constant is probably the most useful parameter for comparing the reactivity of different substrates with the same or different enzymes (Bender & Kézdy, 1965; Knight, 1977; McRae *et al.*, 1981*a*).

Table 3. *Kinetic constants for the hydrolysis of amino acid and dipeptide substrates by bovine trypsin, dog and human plasmin, and human thrombin*

Proteinase ...	Bovine trypsin			Dog plasmin			Human plasmin			Human thrombin		
Substrate	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1}.s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1}.s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1}.s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1}.s^{-1}$)
(Arg-NH)-Rhodamine			454		No hydrolysis			No hydrolysis			No hydrolysis	
$(Cbz\text{-}Arg\text{-}NH)_2$-Rhodamine	23.9	139	172000	0.070	63.4	1100	0.075	62.4	1200	0.072	16.4	4400
$(Cbz\text{-}Ala\text{-}Arg\text{-}NH)_2$-Rhodamine	66.3	42.4	1560000	1.36	116	11700	1.12	138	8120	0.32	21.6	15000
$(Cbz\text{-}Gln\text{-}Arg\text{-}NH)_2$-Rhodamine	44.1	73.7	598000	0.40	42.2	9500	0.80	120	6700	0.15	64.4	2330
$(Cbz\text{-}\langle Glu\text{-}Arg\text{-}NH)_2$-Rhodamine	51.9	110	472000	0.39	73.3	5300	0.27	97.4	2800	1.46	36.6	39900
$(Cbz\text{-}Gly\text{-}Arg\text{-}NH)_2$-Rhodamine	108	64.7	1670000	0.71	66.2	11000	0.40	61.5	6500	0.16	22.8	7000
$(Cbz\text{-}Leu\text{-}Arg\text{-}NH)_2$-Rhodamine	25.0	101	248000	0.28	33.2	8400	0.39	61.8	6300	0.14	23.8	5900
$(Cbz\text{-}Met\text{-}Arg\text{-}NH)_2$-Rhodamine	25.6	100	256000	0.88	124	7100	0.50	91.1	5500	0.053	24.2	2200
$(Cbz\text{-}Phe\text{-}Arg\text{-}NH)_2$-Rhodamine	43.2	57.2	755000	8.79	132	66600	8.92	294	30300		No hydrolysis	
$(Cbz\text{-}Pro\text{-}Arg\text{-}NH)_2$-Rhodamine	62.2	61.6	1010000	1.96	139	14100	0.79	89.7	8800	3.42	9.30	368000
$(Cbz\text{-}Trp\text{-}Arg\text{-}NH)_2$-Rhodamine	6.92	27.2	254000	1.81	121	15000	3.44	128	26900		No hydrolysis	
$(Cbz\text{-}Val\text{-}Arg\text{-}NH)_2$-Rhodamine	21.4	31.3	684000	0.51	28.4	18000	1.14	76.4	14900	0.60	25.8	23000

Discussion

The objectives of the present study were to synthesize a series of dipeptide Rhodamine-based compounds and to assess their properties as substrates for serine proteinases in terms of sensitivity, specificity and selectivity. Rhodamine-based amino acid and dipeptide substrates are extremely sensitive. The sensitivity of a substrate depends on the reactivity of the reactive-site bond and on the detectability of the leaving group as a hydrolysis product. We have shown that the reactive-site bond in (Cbz-Arg-NH)$_2$-Rhodamine is more reactive than that in 7-(Cbz-Arg-NH)-4-Mec and that the hydrolysis product, Cbz-Arg-NH-Rhodamine, is more detectable than NMec (Leytus *et al.*, 1983). Analysis of the spectral properties of Cbz-Phe-Arg-NH-Rhodamine and Cbz-Pro-Arg-NH-Rhodamine, obtained by limited enzymic hydrolysis of the corresponding bisamide substrates, showed them to be identical with those of Cbz-Arg-NH-Rhodamine. These data also allowed us to conclude that neither the length of the peptide chain nor its amino acid composition influences the spectral properties of the hydrolysis products, at least for those Rhodamine-based substrates with an arginine residue in the P_1 position.

The specificity of a substrate for an enzyme can be described by the specificity constant, $k_{cat.}/K_m$, which reflects the efficiency with which the enzyme catalyses the hydrolysis of the substrate. The Rhodamine-based substrates, from the unblocked single-amino-acid substrate (Arg-NH)$_2$-Rhodamine to the blocked single-amino-acid substrate (Cbz-Arg-NH)$_2$-Rhodamine, to the blocked dipeptide substrates, exhibited a wide range of specificity constants. (Arg-NH)$_2$-Rhodamine was not hydrolysed by human or dog plasmin or human thrombin. Addition of Cbz blocking groups to yield (Cbz-Arg-NH)$_2$-Rhodamine resulted in significant hydrolysis by these proteinases. Further extension with certain blocked amino acids in the P_2 position resulted in an even larger increase in the specificity constant. Comparison of the kinetic constants for the best dipeptide substrates with those for (Cbz-Arg-NH)$_2$-Rhodamine indicated that the large increase in $k_{cat.}/K_m$ afforded by extending the single amino acid substrate with an appropriate P_2 residue was primarily the result of a very large increase in $k_{cat.}$ as opposed to a decrease in K_m (Table 3). This large increase in $k_{cat.}$ suggests either that the orientation of the dipeptide substrate in the proteinase's active site allowed for more efficient catalysis compared with the single amino acid substrate, or that the extended chain of the dipeptide substrate in some way destabilized the reactive-site amide bond, making monopeptidyl-Rhodamine a better leaving group than Cbz-Arg-NH-Rhodamine. The latter explanation seems less likely, since it would predict that, in general, all dipeptide substrates should be more reactive than the single amino acid substrate. This was not observed.

The specificity exhibited by many proteinases depends to a large extent upon the interaction of subsite amino acids in the proteinase's active site with extended amino acid residues in the peptide substrate. This can be characterized, with synthetic substrates, by observing variations in the specificity constant upon substituting or altering a single residue in the peptide substrate. Since plasmin and thrombin are trypsin-like serine proteinases and, as such, prefer arginine or lysine in the P_1 position, the specificity constants with (Cbz-Arg-NH)$_2$-Rhodamine were expected to be similar. However, with the substrates in the dipeptide series, each with an arginine residue in the P_1 position and a different Cbz-blocked amino acid in the P_2 position, these two proteinases exhibited distinct preferences for the amino acid in the P_2 position. Whereas human plasmin most preferred phenylalanine in the P_2 position, human thrombin by far preferred proline in the P_2 position. Furthermore, under our assay conditions, human thrombin did not hydrolyse those substrates with phenylalanine or tryptophan in the P_2 position. That (Cbz-Trp-Arg-NH)$_2$-Rhodamine is a poor substrate for human thrombin is consistent with the findings of McRae *et al.* (1981*a*), who reported that Cbz-Trp-Arg-*p*-NA, Cbz-Trp-Arg-NMec and Cbz-Trp-Arg-SBu^t were very poor substrates for bovine thrombin. However, whereas we observed that phenylalanine in the P_2 position also yielded a poor substrate for human thrombin, they found that Cbz-Phe-Arg-SBu^t was a good substrate for bovine thrombin. This difference in specificity could be attributed either to a difference in the subsite requirements of human and bovine thrombin or to a difference in the influence of the leaving groups in these two classes of substrates.

The selectivity of a substrate refers to whether it is efficiently hydrolysed by one enzyme and not by others. With the large range of specificity constants exhibited by the substrates in the dipeptide series, the possibility arose that certain of the dipeptide substrates may be selective. A comparison of the $k_{cat.}/K_m$ values in Table 3 indicates that some of the best substrates for human plasmin were among the worst substrates for human thrombin, and vice versa. The substrates with the two highest specificity constants with human plasmin, (Cbz-Phe-Arg-NH)$_2$-Rhodamine and (Cbz-Trp-Arg-NH)$_2$-Rhodamine, were not hydrolysed by human thrombin. Conversely, the best substrate for human thrombin, (Cbz-Pro-Arg-NH)$_2$-Rhodamine, was one of the worst substrates for human plasmin, the difference in $k_{cat.}/K_m$ values being 40-fold. This high degree of selectivity, observed with two trypsin-like serine proteinases whose substrate specificities might

otherwise have been thought to be similar, was afforded by extending the non-selective substrate (Cbz-Arg-NH)$_2$-Rhodamine by a single amino acid residue.

The principle that the more intrinsically reactive a substrate is the less selectively it will be cleaved by different proteinases can be affirmed by comparing our results with Rhodamine-based substrates with those obtained with peptide thioester substrates (McRae *et al.*, 1981*a*). Thioester bonds are intrinsically more reactive than amide bonds. For example, Cbz-Arg-SBu^t has a high specificity constant for bovine thrombin, 920000 $M^{-1} \cdot s^{-1}$, whereas (Cbz-Arg-NH)$_2$-Rhodamine has a low specificity constant for human thrombin, 4000 $M^{-1} \cdot s^{-1}$. However, the greater reactivity afforded by thioester substrates may be at the expense of selectivity. Addition of another amino acid to Cbz-Arg-SBu^t does not significantly alter its specificity. The specificity constants for Cbz-Phe-Arg-SBu^t and Cbz-Pro-Arg-SBu^t with bovine thrombin are 960000 $M^{-1} \cdot s^{-1}$ and 1500000 $M^{-1} \cdot s^{-1}$ respectively. The addition of another single amino acid to (Cbz-Arg-NH)$_2$-Rhodamine, however, does significantly alter its specificity. (Cbz-Phe-Arg-NH)$_2$-Rhodamine is not hydrolysed by human thrombin, and the specificity constant for (Cbz-Pro-Arg-NH)$_2$-Rhodamine is 368000 $M^{-1} \cdot s^{-1}$. Since different proteinases exhibit a preference for different amino acids in the P_2 position of a substrate, and since occupation of the P_2 position does not enhance the specificity of thioester-based substrates as much as it does the specificity of Rhodamine-based substrates, the potential for selectivity is much greater with the Rhodamine-based substrates.

Recently a thorough study of thioester-based peptide substrates was reported (McRae *et al.*, 1981*a*, *b*) that allows us to compare the properties of this class of substrates with the Rhodamine-based peptide substrates, in terms of sensitivity and utility. The specificity constants for the best Rhodamine-based peptide substrates are of similar magnitude to those of the best thioester-based peptide substrates. For example, the best thioester-based dipeptide substrate for bovine trypsin, Cbz-Phe-Arg-SBu^t, exhibits a $k_{cat.}/K_m$ of 6700000 $M^{-1} \cdot s^{-1}$, whereas the best Rhodamine-based dipeptide, (Cbz-Gly-Arg-NH)$_2$-Rhodamine, exhibits a $k_{cat.}/K_m$ of 1670000 $M^{-1} \cdot s^{-1}$. The best dipeptide thioester-based substrate for bovine thrombin, Cbz-Phe-Arg-SBu^t, exhibits a $k_{cat.}/K_m$ of 1500000 $M^{-1} \cdot s^{-1}$, whereas the best Rhodamine-based dipeptide for human thrombin, (Cbz-Pro-Arg-NH)$_2$-Rhodamine, exhibits a $k_{cat.}/K_m$ of 368000 $M^{-1} \cdot s^{-1}$. In general, fluorophoric leaving groups are more detectable than chromophoric leaving groups. The lowest concentration of a chromophore with a molar absorption coefficient in the range 10000–20000 $M^{-1} \cdot cm^{-1}$ that can be measured with an accuracy of better than 2% is in the micromolar range, whereas the lowest concentration of the monoamide Rhodamine fluorophore that can be measured with a similar degree of accuracy is in the nanomolar range. Thus, in comparing the sensitivities of Rhodamine-based substrates with thioester-based substrates, the small differences in specificity are more than compensated for by the larger differences in detectability, in favour of the Rhodamine-based substrates. Finally, the Rhodamine-based dipeptides should be useful in selective assays of proteinolytic enzymes in complex biological fluids and extracts, e.g. blood plasma, whereas the thioester-based substrates in such assays would be more difficult to use because those plasma proteins with free thiol groups such as serum albumin would interfere with the assay.

In conclusion, Rhodamine-based peptide substrates offer a combination of characteristics which, until now, have not been exhibited by any one class of synthetic substrates for serine proteinases. They are extremely sensitive substrates that exhibit both a high degree of specificity and selectivity. These characteristics should render them useful reagents in studies on proteinolytic enzymes and on the wide variety of physiological processes in which these enzymes are involved.

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