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Rhodamine-based compounds as fluorogenic substrates for serine proteinases

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A new fluorogenic substrate for serine proteinases, bis(*N*-benzyloxycarbonyl-L-argininamido)Rhodamine [(Cbz-Arg-NH)$_2$-Rhodamine], was synthesized, purified and chemically and enzymically characterized. This compound, which employs Rhodamine as a fluorophoric leaving group, is the first in a series of substrates designed to measure the amidase activity of proteinases. Cleavage of one of the amide bonds of (Cbz-Arg-NH)$_2$-Rhodamine by a trypsin-like serine proteinase converts the non-fluorescent bisamide substrate into a highly fluorescent monoamide product. Significant differences in the electronic absorption and fluorescence emission spectra and quantum yields of bis-, mono- and un-substituted Rhodamine are reported. Macroscopic kinetic constants for the interaction of (Cbz-Arg-NH)$_2$-Rhodamine with bovine trypsin, human and dog plasmin and human thrombin were determined. Compared with the corresponding 7-amino-4-methylcoumarin-based analogue, (Cbz-Arg-NH)$_2$-Rhodamine exhibits an increase in sensitivity with these enzymes of 50–300-fold. The physical basis for this increase in sensitivity is discussed.

Synthetic fluorogenic amide substrates have proved to be extremely valuable reagents for the quantitative assay of serine proteinases. Among the primary aromatic amides developed for this purpose (Zimmerman *et al.*, 1976, 1977; Morita *et al.*, 1977; Nieuwenhuizen *et al.*, 1977; Pochron *et al.*, 1978; Bigbee *et al.*, 1978; Pierzchala *et al.*, 1979; Smith *et al.*, 1980; Bissell *et al.*, 1980), coumarin derivatives offer the greatest sensitivity and have been the most widely used.

Two important criteria for selecting a fluorophore for incorporation into a synthetic substrate are the detectability of the fluorophore and the reactivity of the bond undergoing cleavage. Despite the usefulness of coumarin-based substrates, less than optimal conditions for detection of the fluorophore must be used to maximize spectral differences between substrate and product. Furthermore, no structural change occurs within the aminocoumarin moiety on cleavage of the substrate that would be expected to make amides formed from aminocoumarins especially reactive.

Abbreviations used: Cbz, benzyloxycarbonyl; (Cbz-Arg-NH)$_2$-Rhodamine, bis-(*N*-Cbz-L-argininamido)-Rhodamine; Cbz-Arg-NH-Rhodamine, mono-(*N*-Cbz-L-argininamido)Rhodamine; f.d.m.s., field-desorption mass spectrometry; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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Recently we began to study the physical and spectral properties of xanthene dyes and their derivatives to determine their usefulness as substrates for serine proteinases. We synthesized and characterized bis- and mono-substituted derivatives of Fluorescein, Fluorescein di-*p*-guanidinobenzoate and Fluorescein mono-*p*-guanidinobenzoate, and showed them to be excellent active-site titrants for the esterase activity of serine proteinases (Melhado *et al.*, 1982). Fluorescein was chosen as the fluorophoric leaving group because at neutral to basic pH it possesses a large absorption coefficient and quantum yield in the visible region, where the output from a xenon lamp is relatively high and where interference from most biological compounds is low.

Rhodamine is a diamino analogue of Fluorescein that exhibits spectral properties similar to those of Fluorescein. Ioffe & Otten (1965) reported that when the amino groups of Rhodamine are blocked by acetylation this intensely coloured dye is converted into a colourless and presumably non-fluorescent form, implying that the conjugation system of the chromophore is interrupted (Drexhage, 1976). Thus Rhodamine should be a highly reactive fluorophoric leaving group, since loss of acylation is accompanied by a large increase in the degree of conjugation and hence a large increase in stability. For these reasons and because amino acid deriva-

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tives of Rhodamine have not previously been synthesized, we decided to explore the usefulness of amino acid derivatives of Rhodamine as fluorogenic substrates for the amidase activity of serine proteinases.

Experimental

Spectrometry

Electronic absorption spectra were measured on a Beckman Acta model cIII spectrophotometer with matched silica cells having 1cm path length. F.d.m.s. spectra were obtained on a Varian–MAT 731 spectrometer equipped with a Varian–MAT combination electron-impact-field-desorption ion source.

Microanalyses

Microanalyses were performed by Mr. Josef Nemeth and his staff at the University of Illinois.

Materials

N-Cbz-L-arginine hydrochloride, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride and 7-amino-4-methylcoumarin were purchased from Sigma Chemical Co. Fluorescein was purchased from Aldrich Chemical Co. *p*-Nitrophenyl *p*-guanidinobenzoate was purchased from ICN and dissolved in redistilled *NN*-dimethylformamide. Hepes and 7-(*N*-Cbz-L-argininamido)-4-methylcoumarin were purchased from Calbiochem. Rhodamine 110 (laser grade) was purchased from Eastman Kodak Co. Fluorescein mono-*p*-guanidinobenzoate was synthesized and purified as described previously (Melhado *et al.*, 1982). (Cbz-Arg-NH)$_2$-Rhodamine, Cbz-Arg-NH-Rhodamine, Rhodamine, 7-amino-4-methylcoumarin and 7-(*N*-Cbz-L-argininamido)-4-methylcoumarin were stored as dry solids or at concentrations of 10mM in redistilled dimethylformamide at 4°C. In either state, no decomposition was observed in 6 months. Once dissolved in dimethylformamide, solutions of (Cbz-Arg-NH)$_2$-Rhodamine were incubated at room temperature for 24–48h until they turned completely colourless. Thereafter they were stored at 4°C. Bovine pancreatic trypsin (three-times crystallized) was purchased from Worthington Biochemical Corp. Stock solutions were prepared in 1mM-HCl and stored at −20°C. Human and dog plasmin were prepared by a slight modification of the procedure of Castellino & Sodetz (1976) as described previously (Livingston *et al.*, 1981; Leytus *et al.*, 1981). Stock solutions were prepared in 10mM-Hepes/NaOH buffer, pH7.5, containing 25% (v/v) glycerol, and stored at −20°C. Human thrombin was a gift from Dr. Robert Rosenberg, Harvard Medical School. Stock solutions were stored in 10mM-Hepes/NaOH buffer, pH7.5, containing 0.9M-NaCl at −20°C. For each enzyme the concentration of active sites was determined with Fluorescein mono-*p*-guanidinobenzoate as described previously (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 254nm and 365nm light. Preparative t.l.c. was performed on 20cm × 20cm Brinkmann silica-gel plates 2.0mm thick with fluorescent indicator. Detection was under 254nm and 365nm light. The adsorbent used for column chromatography was Silica Woelm 32-63.

Quantum yields

Fluorescence emission spectra were recorded on a Perkin–Elmer MPF-44A spectrofluorometer equipped with a universal digital read-out (model UDR-1) and connected to a Hewlett Packard model 7015 *X–Y* recorder with time base. A 2nm bandwidth was used on both the excitation and emission monochromators. The excitation wavelength for Rhodamine and its derivatives in 10mM-Hepes buffer, pH7.5, containing 15% (v/v) ethanol was 460nm, and the emission spectrum was scanned from 470nm to 670nm. The same wavelengths were used for the Fluorescein standard in 0.1M-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 0.1M-NaOH (Heller *et al.*, 1974).

Complete acid hydrolysis and complete tryptic hydrolysis

The Rhodamine contents of (Cbz-Arg-NH)$_2$-Rhodamine and Cbz-Arg-NH-Rhodamine were determined by complete acid hydrolysis and by complete tryptic hydrolysis of the amide bonds followed by measurement of the fluorescence and absorbance increases respectively. For acid hydrolysis, 5μM solutions of (Cbz-Arg-NH)$_2$-Rhodamine or Cbz-Arg-NH-Rhodamine in 6M-HCl were prepared from accurately weighed samples and were incubated for 2h at 110°C in sealed hydrolysis vials. The fluorescence intensities of 0.01ml samples of these hydrolysates, on dilution into 2.98ml of 10mM-Hepes buffer, pH7.5, containing 15% (v/v) ethanol and 0.01ml of 6M-NaOH, were then measured and compared with a Rhodamine standard curve. The excitation and emission wavelengths were 492nm and 523nm respectively, both

set with a bandwidth of 4nm. For enzymic hydrolysis, 10μM solutions of (Cbz-Arg-NH)$_2$-Rhodamine or Cbz-Arg-NH-Rhodamine were prepared in 10mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol, from accurately weighed samples. To initiate hydrolysis, a 0.02ml portion of 12μM bovine trypsin was added, which was followed 30min later by an additional 0.02ml portion of trypsin. After 1h the concentration of Rhodamine released was calculated from the absorbance at 492nm by using an absorption coefficient for Rhodamine in 10mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol, of 66800 $M^{-1} \cdot cm^{-1}$.

Enzyme assays

Enzyme assays with (Cbz-Arg-NH)$_2$-Rhodamine and 7-(*N*-Cbz-L-argininamido)-4-methylcoumarin were performed at 22°C in 10mM-Hepes buffer, pH 7.5, containing 10% (v/v) dimethyl sulphoxide. For bovine trypsin, 20mM-$CaCl_2$ was also present. Stock solutions of substrates and of enzymes were diluted into the appropriate assay buffer before the assay. For all assays, an enzyme concentration was chosen so that less than 5% of the substrate would be hydrolysed. Unless indicated otherwise, 0.01ml of enzyme solution was mixed with 0.04ml of substrate solution at the bottom of a disposable plastic cuvette (Evergreen) and allowed to react for 5min. Then 0.95ml of 10mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol, was added and the fluorescence immediately recorded. No spontaneous hydrolysis of the substrates was observed during an assay. Fluorescence measurements were made with a Perkin–Elmer MPF-44A fluorescence spectrophotometer equipped with a Perkin–Elmer universal digital readout (model UDR-1). The excitation and emission wavelengths for (Cbz-Arg-NH)$_2$-Rhodamine were 492nm and 523nm respectively, both set with a bandwidth of 4nm. The excitation and emission wavelengths for 7-(*N*-Cbz-L-argininamido)-4-methylcoumarin were 380nm and 460nm respectively (Zimmerman *et al.*, 1976, 1977), both set with a bandwidth of 4nm. The fluorescence spectrophotometer was standardized with a polymethacrylate block in which was embedded 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 0.2 to 2 times the K_m, and then, with a microcomputer, fitting the data points to the Michaelis–Menten rate equation by using the iterative method described by Cleland (1967). Conversion of relative fluorescence units into molar concentrations of Cbz-Arg-NH-Rhodamine or 7-amino-4-methylcoumarin produced was accomplished by using standard curves correlating fluorescence with molar concentrations of either Cbz-Arg-NH-Rhodamine or 7-amino-4-methylcoumarin in 10mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol.

Synthesis of (Cbz-Arg-NH)$_2$-Rhodamine

To 4.0g (11.6mmol) of Cbz-L-arginine hydrochloride in a capped glass vial was added 80ml of cold dry dimethylformamide/pyridine (1:1, v/v) and the contents were stirred at 4°C until solution was complete. To this was added 2.0g (10.4mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide. After 5min of stirring at 4°C, 150mg (0.41mmol) of Rhodamine 110, dissolved in 1.5ml of dimethylformamide/pyridine (1:1, v/v) was added. Stirring was continued for 2h at 4°C and then for 2 days at room temperature. During this time the reaction solution turned from deep orange to colourless. The reaction solution was transferred to a 250ml Nalgene centrifuge bottle and concentrated by precipitation with 150ml of diethyl ether followed by centrifugation at 100000**g** for 20min. The residue was then dissolved in 10ml of dimethylformamide, precipitated by the addition of 200ml of acetone and centrifuged at 10000**g** for 20min. To the resulting gel, dissolved in 10ml of dimethylformamide, was added 100ml of 1.2M-HCl, and the solution was centrifuged at 10000**g** for 20min. The residue was redissolved in 10ml of dimethylformamide, and the precipitation with 1.2M-HCl repeated. The orange–red gel that resulted was next dissolved in 10ml of methanol and precipitated by the addition of 200ml of ethyl acetate. After two additional cycles of solution in methanol and reprecipitation with ethyl acetate, the product was dried in an evacuated desiccator at room temperature to yield 340mg (83%) of a pale pink powder. The solid exhibited no distinct melting point, with gradual evolution of gas above 170°C (evacuated sealed capillary). The product was judged to be pure by analytical t.l.c., which revealed a single dark spot under u.v. light (Table 1). Chemical analysis gave the following: Found: C, 57.89; H, 5.63; N, 13.99; Calc. for $C_{48}H_{50}N_{10}O_9$,2HCl,H_2O: C, 57.53; H, 5.44; N, 13.98%.

Synthesis of Cbz-Arg-NH-Rhodamine

To 40mg (0.12mmol) of Cbz-L-arginine hydrochloride 20mg (0.10mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide and 30mg (0.08mmol) of Rhodamine in a capped glass vial was added 1.5ml of cold dry dimethylformamide/pyridine (1:1, v/v). The solution was stirred for 1h at 4°C and then overnight at room temperature. Purification of Cbz-Arg-NH-Rhodamine was achieved by a modifi-

cation of the flash chromatography technique described by Still *et al.* (1978), with an eluent consisting of butan-2-one/acetone/water (8:1:1, by vol.). A 35 cm × 1.8 cm column was wet-packed with silica gel under 35 kPa (5 lbf/in^2) above atmospheric pressure. The 1.5 ml reaction solution was loaded directly on to the column and forced into the silica-gel bed under 35 kPa above atmospheric pressure. The walls of the column were washed with a small volume of eluent, which was subsequently forced into the gel under pressure, and the column and an auxillary reservoir were filled with eluent. Elution was begun by adjusting the pressure regulator to 35 kPa above atmospheric pressure, which corresponded to a flow rate of 8 ml/min. Fractions of volume 6 ml were collected, and the progress of the separation was monitored by visual examination of the column and by t.l.c. Fast-running greenish-yellow fluorescent fractions, containing unchanged Rhodamine, were discarded. Fractions containing the highly fluorescent tight orange band that closely followed the band of unchanged Rhodamine were pooled and concentrated to dryness under reduced pressure at 45°C. The residue was dissolved in 1 ml of dimethylformamide, transferred to a polypropylene tube and precipitated with 10 ml of 2 M-HCl at 4°C overnight. After centrifugation at 10000 *g* for 20 min, the orange precipitate was dissolved in 1 ml of methanol, precipitated with 10 ml of diethyl ether and centrifuged at 10000 *g* for 20 min. The product was then dried in an evacuated drying pistol at 78°C to yield 7.5 mg (13%) of a red–brown solid. The solid exhibited no distinct melting point, with gradual evolution of gas above 205°C (evacuated sealed capillary). The product was judged to be pure by analytical t.l.c., which revealed a single fluorescent spot under u.v. light (Table 1). Chemical analysis gave the following: Found: C, 57.33; H, 5.41; N, 11.19; Calc. for $C_{34}H_{32}N_6O_6$,2HCl,H_2O: C, 57.38, H, 5.11; N, 11.81%.

Preparation of Cbz-Arg-NH-Rhodamine by trypsin-catalysed hydrolysis of (Cbz-Arg-NH)$_2$-Rhodamine

To 2 ml of 0.2 mM-(Cbz-Arg-NH)$_2$-Rhodamine in 10 mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol, was added 20 μl of 0.12 μM-trypsin in 10 mM-Hepes buffer, pH 7.5, containing 20 mM-$CaCl_2$, and the electronic absorbance at 492 nm was monitored as a function of time against a similar solution that lacked trypsin. When the absorbance reached 0.086, the reaction was stopped by addition of 5 μl of 0.5 mM-*p*-nitrophenyl *p*-guanidinobenzoate to both the sample and reference cuvettes. Approx. 2% of the (Cbz-Arg-NH)$_2$-Rhodamine was converted into Cbz-Arg-NH-Rhodamine, as determined by using a molar absorption coefficient for Cbz-Arg-NH-Rhodamine at 492 nm in 10 mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol, of 23500 M^{-1}·cm^{-1}.

Results

Synthesis and purification

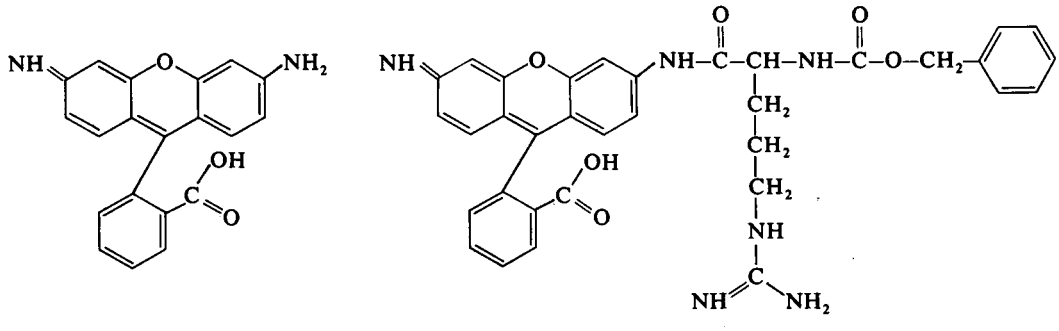
(Cbz-Arg-NH)$_2$-Rhodamine and Cbz-Arg-NH-Rhodamine were prepared in dimethylformamide/pyridine (1:1, v/v) from Rhodamine and Cbz-L-arginine hydrochloride by condensation with 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride, a water-soluble carbodi-imide. Purification of (Cbz-Arg-NH)$_2$-Rhodamine was achieved by precipitating the product with 1.2 M-HCl. On the other hand, purification of Cbz-Arg-NH-Rhodamine could be achieved only by preparative t.l.c. or flash column chromatography. The purity of (Cbz-Arg-NH)$_2$-Rhodamine and Cbz-Arg-NH-Rhodamine was assessed by t.l.c. on silica gel (Table 1). Examination of the plates under 254 nm and 365 nm light revealed no evidence of Cbz-L-arginine hydrochloride or Rhodamine in either product and no evidence of cross-contamination of the bis- and mono-amides.

Assignment of structures

The structures assigned to (Cbz-Arg-NH)$_2$-Rhodamine and Cbz-Arg-NH-Rhodamine on the basis of elemental analysis and method of synthesis were corroborated by f.d.m.s., in which the mass range was scanned from *m/e* 200 to 1000. The peaks with the largest *m/e* values are listed in Table 1, along with their assignments, and correspond to those of the predicted molecular ions (M^+) minus chloride. The assigned structures for (Cbz-Arg-NH)$_2$-Rhodamine and Cbz-Arg-NH-Rhodamine were also supported by analysis of their Rhodamine content on complete hydrolysis by acid and by bovine trypsin as described in the Experimental section. By both methods of hydrolysis, the amount of Rhodamine released was within 2% of that expected from the assigned molecular weights and the weights of the samples (Table 1). These data also indicate the purity of the products. In addition, since the amount of Rhodamine released by complete trypsin hydrolysis is approximately equivalent to that released by complete acid hydrolysis, little or no racemization about the α-carbon atom of arginine occurred during synthesis, as expected.

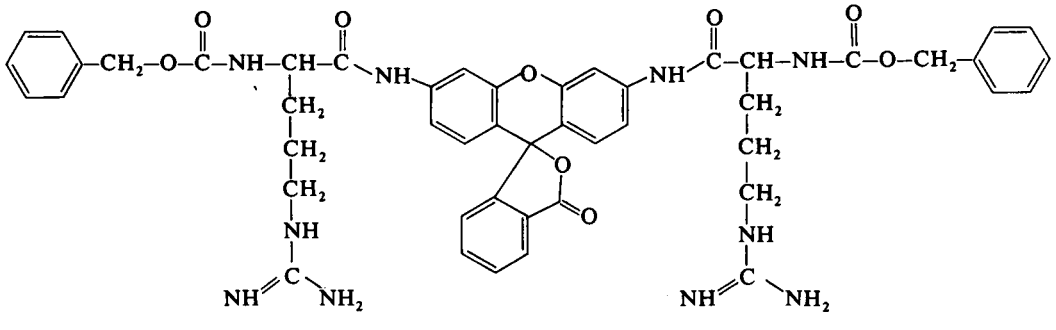
Electronic absorption spectra

The electronic absorption spectra of Rhodamine and Cbz-Arg-NH-Rhodamine are displayed in Fig. 1 and summarized in Table 2, along with their molar absorption coefficients at 492 nm. The occurrence of the longest-wavelength absorption band for Rhodamine at 495 nm rather than 510 nm results from these spectra being recorded in 10 mM-Hepes



Rhodamine

Cbz-Arg-NH-Rhodamine



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

Table 1. *T.l.c., f.d.m.s. and hydrolysis characteristics of Rhodamine, $(Cbz\text{-}Arg\text{-}NH)_2$-Rhodamine and Cbz-Arg-NH-Rhodamine*

T.l.c. was performed with butan-2-one/acetone/water (8:1:1, by vol.) as eluent. 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.	Appearance under light		F.d.m.s.		Purity by complete hydrolysis (%)	
		254 nm	365 nm	*m/e*	(assignment)	6 M-HCl	Trypsin
Rhodamine	0.58*	—	Greenish-yellow			—	—
$(Cbz\text{-}Arg\text{-}NH)_2$-Rhodamine	0.26†	Black	—	948 947 913 911 623	(M^+-Cl) (M^+-HCl) (M^+-2Cl) (M^+-2HCl) (M^+-2Cl)	98	100
Cbz-Arg-NH-Rhodamine	0.46‡	—	Orange	622 621	$(M^+-H-2Cl)$ (M^+-2HCl)	99	98

* Concentration of stock solution 0.05 mg/ml in methanol.
† Concentration of stock solution 1 mg/ml in methanol.
‡ Concentration of stock solution 0.1 mg/ml in methanol.

buffer, pH 7.5, containing 15% (v/v) ethanol, instead of under the standard condition for Rhodamine, namely acidic ethanol (Wehrly, 1979).

The distinctive appearances of the electronic absorption spectra for Rhodamine and Cbz-Arg-NH-Rhodamine and the sensitivity of the mono- and

bis-amides to hydrolysis by bovine trypsin permit electronic absorption spectroscopy to be used to monitor the initial conversion of (Cbz-Arg-NH)$_2$-Rhodamine into Cbz-Arg-NH-Rhodamine, followed by the subsequent conversion of the latter into Rhodamine, as shown in Fig. 2. (Cbz-Arg-NH)$_2$-Rhodamine was incubated with bovine trypsin and, after various time intervals, electronic absorption spectra were recorded. The spectrum of the initial hydrolysis product is identical with that of Cbz-Arg-NH-Rhodamine. The concentration of Cbz-Arg-NH-Rhodamine first increases with time and then begins to decrease, with the concomitant appearance of the spectrum of Rhodamine. On complete hydrolysis, only the spectrum of Rhodamine remains. These data are also consistent with (Cbz-Arg-NH)$_2$-Rhodamine being a bifunctional substrate, with the initial product of hydrolysis of (Cbz-Arg-NH)$_2$-Rhodamine being Cbz-Arg-NH-Rhodamine, and the ultimate product of hydrolysis being Rhodamine.

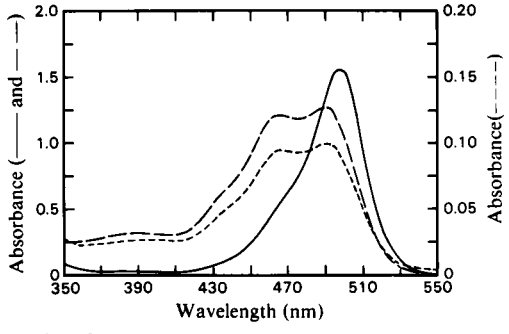


Fig. 1. *Absorption spectra of Rhodamine* (——, *20.6 μM*), *synthetic Cbz-Arg-NH-Rhodamine* (– – –, *38.1 μM*) *and trypsin-produced Cbz-Arg-NH-Rhodamine* (– – – –, *2.95 μM*) *in 10 mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol*

Emission spectra and quantum yields

The corrected fluorescence emission spectra of Rhodamine, (Cbz-Arg-NH)$_2$-Rhodamine and Cbz-Arg-NH-Rhodamine are displayed in Fig. 3 and summarized in Table 2, along with the quantum yields of these compounds. The quantum yield of (Cbz-Arg-NH)$_2$-Rhodamine is about one-tenth that of Rhodamine, whereas the quantum yield of Cbz-Arg-NH-Rhodamine is about one-third that of Rhodamine.

Molar fluorescence coefficients

A molar fluorescence coefficient, whose dimensions are relative fluorescence units per molar concentration of fluorophore, can be used to

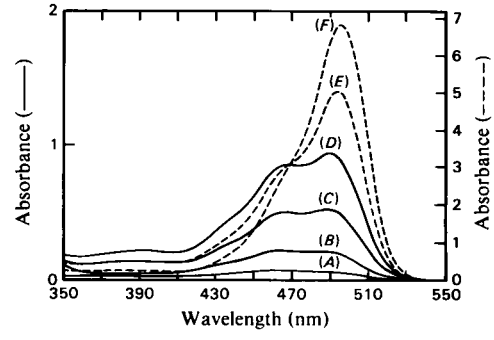


Fig. 2. *Absorption spectra of the hydrolysis of (Cbz-Arg-NH)$_2$-Rhodamine by trypsin as a function of time* To 2 ml of 0.10 mM-(Cbz-Arg-NH)$_2$-Rhodamine in 10 mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol, was added 20 μl of 1.2 μM-trypsin at time 0 and at time 126 min. Absorption spectra were recorded at the following times: *A*, 0 min, *B*, 1 min, *C*, 4 min, *D*, 9 min, *E*, 124 min, and *F*, 154 min. For spectra *E* and *F*, the absorbances were calculated from measurements on diluted solutions.

Table 2. *Physical constants of Rhodamine, (Cbz-Arg-NH)$_2$-Rhodamine and Cbz-Arg-NH-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 ϕ values are given relative to $\phi = 0.94$ for Fluorescein in 0.1 M-NaOH.

		Absorption spectra		Emission spectra	
Compound	Molecular formula	$\lambda_{max.}$ (nm)	ε at 492 nm ($M^{-1} \cdot cm^{-1}$)	$\lambda_{em.}$ (nm)	ϕ
Rhodamine	$C_{20}H_{14}N_2O_3$,HCl	495	66 800	523	0.91
(Cbz-Arg-NH)$_2$-Rhodamine	$C_{48}H_{50}N_{10}O_9$,2HCl,H_2O		29	532	0.09
Cbz-Arg-NH-Rhodamine	$C_{34}H_{32}N_6O_6$,2HCl,H_2O				
(*a*) Synthesized		464, 490	23 500	529	0.29
(*b*) Trypsin-produced		467, 490		529	0.32

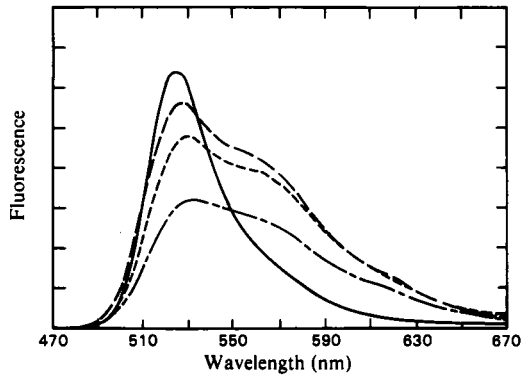


Fig. 3. *Corrected fluorescence emission spectra for Rhodamine* (——, *1.36 μM*), *(Cbz-Arg-NH)$_2$-Rhodamine* (— — —, *0.10 mM*), *synthetic Cbz-Arg-NH-Rhodamine* (— —, *4.78 μM*), *and trypsin-produced Cbz-Arg-NH-Rhodamine* (— — — —, *2.95 μM*) *in 10 mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol*
The spectra are not drawn to scale.

Table 3. *Molar-fluorescence coefficients for Rhodamine, Cbz-Arg-NH-Rhodamine, 7-amino-4-methylcoumarin and (Cbz-Arg-NH)$_2$-Rhodamine at assay wavelengths*
Molar fluorescence coefficients were measured in 10 mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol. Abbreviation: FU/M, relative fluorescence units per molar concentration of solute.

Compound	Molar fluorescence coefficient: Value (FU/M)	Molar fluorescence coefficient: Relative to that of Rhodamine
Rhodamine	1.90×10^{11}*	1
Cbz-Arg-NH-Rhodamine	1.81×10^{10}*	0.095
7-Amino-4-methylcoumarin	4.15×10^{9}†	0.022
(Cbz-Arg-NH)$_2$-Rhodamine	5.20×10^{6}*	2.74×10^{-5}

* $\lambda_{ex.} = 492$ nm, $\lambda_{em} = 523$ nm.
† $\lambda_{ex.} = 380$ nm, $\lambda_{em} = 460$ nm.

compare the relative detectabilities of different fluorophores under enzyme assay conditions. The molar fluorescence coefficients of Rhodamine, Cbz-Arg-NH-Rhodamine, (Cbz-Arg-NH)$_2$-Rhodamine and 7-amino-4-methylcoumarin, obtained from their fluorescence standard curves, are listed in Table 3. The molar fluorescence coefficient for Cbz-Arg-NH-Rhodamine is approx. 4.5-fold greater than that for 7-amino-4-methylcoumarin. This difference is, in part, the result of the absorption coefficient of Cbz-Arg-NH-Rhodamine being approx. 5-fold greater than that of 7-amino-4-methylcoumarin, the quantum yield for Cbz-Arg-NH-Rhodamine being 0.47-fold that of 7-amino-4-methylcoumarin, the efficiency of the excitation optics at 492 nm being 3.1-fold greater than that at 380 nm, and the efficiency of the emission optics at 523 nm being 0.62-fold that at 460 nm. [The molar absorption coefficient at 380 nm for 7-amino-4-methylcoumarin measured in 10 mM-Hepes buffer, pH 7.5, containing 15% (v/v) ethanol, is $4490\ M^{-1} \cdot cm^{-1}$ (S. P. Leytus & W. F. Mangel, unpublished work). The quantum yield of 7-amino-4-methylcoumarin in ethanol is 0.68 (Petrovich & Borisevich, 1963). The relative efficiencies of the excitation optics at 380 nm and 492 nm were determined by using a 15 mg/ml solution of Rhodamine B in ethanol as a 'quantum counter'. The relative efficiencies of the emission optics at 460 nm and 523 nm were determined from emission correction factors.] The high molar fluorescence coefficient of Cbz-Arg-NH-Rhodamine is consistent with the observation that monodecanoyl-Rhodamine is highly fluorescent (Wehrly, 1979), and the low molar fluorescence coefficient of (Cbz-Arg-NH)$_2$-Rhodamine is consistent with the observations that diacetylated Rhodamine is fixed in a colourless state (Ioffe & Otten, 1965) and that disubstituted esters of fluorescein are relatively non-fluorescent (Melhado *et al.*, 1982).

Kinetics of the enzyme-catalysed hydrolysis of (Cbz-Arg-NH)$_2$-Rhodamine and 7-(N-Cbz-L-argininamido)-4-methylcoumarin

The rate of bovine trypsin-catalysed hydrolysis of (Cbz-Arg-NH)$_2$-Rhodamine is linear with time and proportional to the enzyme concentration. Furthermore, the kinetics of the hydrolysis of (Cbz-Arg-NH)$_2$-Rhodamine by bovine trypsin obey the Michaelis–Menten rate equation. The macroscopic kinetic constants for the hydrolysis of (Cbz-Arg-NH)$_2$-Rhodamine by bovine trypsin, human and dog plasmin and human thrombin are listed in Table 4. For comparison, the kinetic constants for the hydrolysis of 7-(*N*-Cbz-L-argininamido)-4-methylcoumarin by these same proteinases are also listed.

Confirmation that the product of limited proteinase-catalysed hydrolysis of (Cbz-Arg-NH)$_2$-Rhodamine is Cbz-Arg-NH-Rhodamine

To confirm that the product of limited hydrolysis of (Cbz-Arg-NH)$_2$-Rhodamine by bovine trypsin is indeed the monoamine Rhodamine derivative, we synthesized Cbz-Arg-NH-Rhodamine and compared some of its spectral properties with those of the product of the enzyme-catalysed hydrolysis of (Cbz-Arg-NH)$_2$-Rhodamine, prepared as described in the Experimental section. Both compounds exhibited the same unique electronic absorbance and emission spectra (Figs. 1 and 3 and Table 2) and the

Table 4. *Kinetic constants for the hydrolysis of (Cbz-Arg-NH)$_2$-Rhodamine and 7-(N-Cbz-L-argininamido)-4-methylcoumarin by bovine trypsin, human and dog plasmin and human thrombin*

Experimental details are as given in the text, although for the 4-methylcoumarin derivative the reaction times were increased to 60 min, except for bovine trypsin.

	(Cbz-Arg-NH)$_2$-Rhodamine			7-(*N*-Cbz-L-argininamido)-4-methylcoumarin		
Proteinase	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Bovine trypsin	23.9	139	172000	1.33	504	2640
				1.3*	250*	5200*
Human plasmin	0.072	62.4	1150	0.022	795	27.7
Dog plasmin	0.070	63.4	1100	0.018	797	22.6
Human thrombin	0.073	16.4	4450	0.024	66.8	359

* Enzyme assays were performed at 24°C in 50 mM-2-{[2-hydroxymethyl)ethyl]amino}ethanesulphonic acid (Tes) buffer, pH 8.0. containing 10 mM-$CaCl_2$ and 1% (v/v) dimethyl sulphoxide (Zimmerman *et al.*, 1977).

same quantum yields (Table 2). By these criteria, the product of limited enzymic hydrolysis of (Cbz-Arg-NH)$_2$-Rhodamine is identical with Cbz-Arg-NH-Rhodamine.

Discussion

Although Rhodamine is one of the most fluorescent compounds known, this xanthene dye has not previously been considered for use as a fluorophoric leaving group in a synthetic substrate for serine proteinases. The Rhodamine-based amino acid derivative prepared here, (Cbz-Arg-NH)$_2$-Rhodamine, exhibits negligible intrinsic fluorescence, its molar fluorescence coefficient being 1/37000th that of Rhodamine (Table 3). (Cbz-Arg-NH)$_2$-Rhodamine is also stable, exhibiting no spontaneous hydrolysis in aqueous solutions during an assay. Most importantly, cleavage of a single amide bond converts the non-fluorescent bisamide substrate into a highly fluorescent monoamide product, a process that is accompanied by a large increase in the degree of conjugation in the Rhodamine moiety.

Previous attempts to obtain a monoamide derivative of Rhodamine, monoacetylRodamine, were unsuccessful (Ioffe & Otten, 1965), and hence, until now, the properties of bis- and mono-amide derivatives of this important xanthene dye were unknown. With the synthesis of (Cbz-Arg-NH)$_2$-Rhodamine and Cbz-Arg-NH-Rhodamine we have begun to investigate the properties of these derivatives and to compare them with the properties of bis- and mono-substituted derivatives of Fluorescein (Melhado *et al.*, 1982). For example, the bis-substituted derivatives of both Rhodamine and Fluorescein possess very small molar fluorescence coefficients that increase on conversion of the bis-substituted derivatives into their corresponding mono-substituted compounds and again on conversion of the mono-substituted compounds into their corresponding unsubstituted dyes.

(Cbz-Arg-NH)$_2$-Rhodamine behaved as a typical substrate with bovine trypsin, human and dog plasmin and human thrombin. Its rate of hydrolysis was linear with time and enzyme concentration and obeyed the Michaelis–Menten rate equation. Although it is a bifunctional substrate, we found that this does not complicate the interpretation of kinetic data, provided that less than 15% of the substrate is hydrolysed during an assay. Under these conditions, the increase in fluorescence is due solely to the production of Cbz-Arg-NH-Rhodamine. The bifunctionality of (Cbz-Arg-NH)$_2$-Rhodamine may even confer certain advantages. For example, the effective concentration of susceptible amide bonds is twice the substrate concentration and would therefore be expected to lower the K_m. Also, the presence of two amino acid residues per Rhodamine moiety may enhance the solubility of (Cbz-Arg-NH)$_2$-Rhodamine.

To assess the potential utility of Rhodamine-based substrates, we compared the effectiveness of (Cbz-Arg-NH)$_2$-Rhodamine and 7-(*N*-Cbz-L-argininamido)-4-methylcoumarin as substrates for bovine trypsin, human and dog plasmin and human thrombin. The effectiveness of a substrate for a serine proteinase can be defined as the product of the second-order rate constant $k_{cat.}/K_m$ and the relative detectability of the fluorophoric leaving group, as signified by the molar fluorescence coefficient. By this criterion (Cbz-Arg-NH)$_2$-Rhodamine is a better substrate than 7-(*N*-Cbz-L-argininamido)-4-methylcoumarin for bovine trypsin by a factor of 280-fold, for human and dog plasmin by a factor of 200-fold and for human thrombin by a factor of 50-fold (Tables 3 and 4).

The differences between the individual $k_{cat.}$ and K_m values for (Cbz-Arg-NH)$_2$-Rhodamine and for 7-(*N*-Cbz-L-argininamido)-4-methylcoumarin are consistent with previous observations that the nature of a chromophoric or fluorophoric leaving group can significantly influence the interaction of a synthetic

substrate with the active site of an enzyme (Chase & Shaw, 1969; Wong & Shaw, 1976; Castillo *et al.*, 1979). The larger $k_{cat.}$ values for $(Cbz\text{-}Arg\text{-}NH)_2$-Rhodamine suggest either that Cbz-Arg-NH-Rhodamine is a better leaving group than 7-amino-4-methylcoumarin, which would render the Rhodamine-based substrate intrinsically more reactive, or that the Rhodamine moiety influences the orientation of the substrate in the enzyme's active site, allowing for more efficient catalysis, or both. The fluorophores in these compounds may also influence the initial binding of substrate to enzyme, because the differences in K_m values between the Rhodamine-based and coumarin-based compounds are too large to result solely from $(Cbz\text{-}Arg\text{-}NH)_2$-Rhodamine being a bifunctional substrate.

In conclusion, Rhodamine offers an attractive alternative to other primary aromatic amines, including 7-amino-4-methylcoumarin, as a fluorophoric leaving group in substrates for serine proteinases. Recently we have begun to synthesize and characterize a series of di- and tri-peptide derivatives of Rhodamine (S. P. Leytus, W. L. Patterson & W. F. Mangel, unpublished work) exhibiting $k_{cat.}/K_m$ ratios with bovine trypsin, human and dog plasmin and human thrombin 10–100-fold larger than those for $(Cbz\text{–}Arg\text{-}NH)_2$-Rhodamine. Certain of these substrates are highly selective. Thus substrates with Rhodamine as the fluorophoric leaving group promise to be very useful in the study of proteolytic enzymes and the wide variety of physiological processes in which they are involved.

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References

Bigbee, W. L., Weintraub, H. B. & Jensen, R. M. (1978) *Anal. Biochem.* **88**, 114–122

Bissell, E. R., Mitchell, A. R. & Smith, R. E. (1980) *J. Org. Chem.* **45**, 2283–2287

Castellino, F. J. & Sodetz, J. M. (1976) *Methods Enzymol.* **45**, 273–286

Castillo, M. J., Nakajima, K., Zimmerman, M. & Powers, J. C. (1979) *Anal. Biochem.* **99**, 53–64

Chase, T., Jr. & Shaw, E. (1969) *Biochemistry* **8**, 2212–2224

Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol.* **29**, 1–32

Drexhage, K. H. (1976) *J. Res. Natl. Bur. Stand. Sect. A* **80**, 421–428

Heller, C. A., Henry, R. A., McLaughlin, B. A. & Bliss, D. E. (1974) *J. Chem. Eng. Data* **19**, 214–219

Ioffe, I. S. & Otten, V. F. (1965) *Zh. Org. Khim.* **1**, 336–339

Leytus, S. P., Peltz, G. A., Liu, H.-Y., Cannon, J. F., Peltz, S. W., Livingston, D. C., Brocklehurst, J. R. & Mangel, W. F. (1981) *Biochemistry* **20**, 4307–4314

Livingston, D. C., Brocklehurst, J. R., Cannon, J. F., Leytus, S. P., Wehrly, J. A., Peltz, S. W., Peltz, G. A. & Mangel, W. F. (1981) *Biochemistry* **20**, 4298–4306

Melhado, L. L., Peltz, S. W., Leytus, S. P. & Mangel, W. F. (1982) *J. Am. Chem. Soc.* **104**, 7299–7306

Morita, T., Kato, H., Iwanaga, S., Takada, K., Kimura, T. & Sakakibara, S. (1977) *J. Biochem. (Tokyo)* **82**, 1495–1498

Nieuwenhuizen, W., Wijngaards, G. & Groeneveld, E. (1977) *Anal. Biochem.* **20**, 143–148

Petrovich, P. I. & Borisevich, N. A. (1963) *Izv. Akad. Nauk. SSSR Ser. Fiz.* **27**, 703–707. Engl. Transl.: *Bull. Acad. Sci. USSR Div. Chem. Sci.* **27**, 701–705

Pierzchala, P. A., Dorn, C. P. & Zimmerman, M. (1979) *Biochem. J.* **183**, 555–559

Pochron, S. P., Mitchell, G. A., Albareda, I., Huseby, R. M. & Gargiulo, R. J. (1978) *Thromb. Res.* **13**, 733–739

Smith, R. E., Bissell, E. R., Mitchell, A. R. & Pearson, K. W. (1980) *Thromb. Res.* **17**, 393–402

Still, W. C., Kahn, M. & Mitra, A. (1978) *J. Org. Chem.* **43**, 2923–2925

Wehrly, J. A. (1979) Ph.D. Thesis, University of Illinois, Urbana

Wong, S.-C. & Shaw, E. (1976) *Arch. Biochem. Biophys.* **176**, 113–118

Zimmerman, M., Yurewicz, E. & Patel, G. (1976) *Anal. Biochem.* **70**, 258–262

Zimmerman, M., Ashe, B., Yurewicz, E. C. & Patel, G. (1977) *Anal. Biochem.* **78**, 47–51