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Synthesis and properties of Cbz-Phe-Arg-CHN_2 (benzyloxycarbonylphenylalanylarginyldiazomethane) as a proteinase inhibitor

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The preparation of peptides terminating in -Arg-CHN_2 has been attempted because of their potential value as proteinase inactivators. We have succeeded in one case, converting Cbz-Phe-ArgOH to the diazomethane without blocking the guanidino group. As expected from previous results with such reagents, the new derivative was extremely effective in inactivating a cysteine proteinase specific for cleaving at arginyl bonds, that is, clostripain. However, in contrast with the inertness of serine proteinases to reagents of this type in the cases examined previously, plasma kallikrein was inactivated by Cbz-Phe-Arg-CHN_2, although with a considerably lower rate constant than with clostripain. Trypsin, however, was not inactivated, but gradually destroyed the reagent, as had been observed previously with chymotrypsin and Cbz-Phe-CHN_2. This has now been re-examined with *p*-nitro-Cbz-Ala-Phe-CHN_2 and shown to involve a cleavage to *p*-nitro-Cbz-Ala-PheOH, probably with liberation of diazomethane.

INTRODUCTION

Peptidyl diazomethanes (R–C=O–CHN_2) are effective inactivators of cysteine proteinases that alkylate the active-centre -SH group by affinity-labelling (Leary *et al.*, 1977). The amino acid side chains in the peptide portion confer affinity to various proteinases, providing a very useful means of obtaining selective inhibitors for individual thiol proteinases (Green & Shaw, 1981). This type of reagent is of additional value, since it has not been found to inactivate other classes of proteinases and, although acting on cysteine proteinases by alkylation of the thiol group, it is inert to simple thiols such as mercaptoethanol. Chymotrypsin was found to destroy Cbz-Phe-CHN_2, without undergoing alkylation, a process which can be monitored by u.v. spectroscopy (Watanabe *et al.*, 1979), but this did not appear to be a general reason for the resistance of serine proteinases to reagents of this type, since in other cases this change is not observed. It has been proposed (Brocklehurst & Malthouse, 1978) that substrate-derived diazomethanes are, in fact, enzyme-activated reagents in the case of cysteine proteinases, owing to the formation of an enzyme adduct, a thiohemiketal, which is an intermediate in the inactivation of cysteine proteinases but is not formed with serine proteinases.

Previous attempts to obtain an arginyl derivative which would be of interest in the study of enzymes specific for this residue have failed because of synthesis difficulties. The usual blocking groups for protecting the guanidino group of arginine require acidic or reductive deblocking conditions that are destructive of the diazomethane group. A protective group for the arginine side chain that is cleavable in the presence of the diazo function has not yet been found. The possibility of protection by protonation of the guanidino group during peptide synthesis has been demonstrated in a number of cases (Anderson, 1953; Wuensch, 1974), and therefore attempts were made to activate peptides containing a *C*-terminal unblocked arginine residue for direct conversion into the diazomethane. Although we failed with a variety of peptides, probably owing to a lack of solubility under the conditions used, we did succeed with Cbz-Phe-ArgOH. By addition of small amounts of acetonitrile to the tetrahydrofuran suspension of the peptide, the solubility could be increased sufficiently to prepare a mixed anhydride for reaction with diazomethane. As described below, the resultant Cbz-Phe-ArgCHN_2 was an effective inactivator of two cysteine proteinases, clostripain and cathepsin B, and the kinetic properties of the inhibitions were determined. We also examined two serine proteinases, trypsin and plasma kallikrein, for a possible susceptibility to Cbz-Phe-ArgCHN_2, although previous results with serine proteinases were negative with peptidyl diazomethanes (Green & Shaw, 1981; Watanabe *et al.*, 1979).

EXPERIMENTAL

Materials

Trypsin [tosylphenylalanylchloromethane ('TPCK')-treated] and chymotrypsin were purchased from Worthington Biochemicals Corp., Freehold, NJ, U.S.A.; plasma kallikrein was from Kabi-Vitrum, Stockholm, Sweden, and clostripain was from Boehringer, Mannheim, Germany. Cathepsin B was prepared from pig liver as described by Evans & Shaw (1983). Bz-Pro-Phe-Arg-Nan acetate was obtained from Kabi-Vitrum and Cbz-Phe-Arg-NHMec, from Bachem, Bubendorf, Switzerland. All other chemicals were from Fluka. Cbz-Phe-ArgOH was synthesized by standard methods (Wuensch *et al.*, 1974).

Abbreviations used: Nan, *p*-nitroanilide; NHMec, amino-4-methyl-7-coumarylamide; DMF, dimethylformamide; DMSO, dimethyl sulphoxide; Cbz, benzyloxycarbonyl; -CHN_2, diazomethane; Bz, benzoyl; OMe, methyl ester; Cit, citrulline.

Table 1. Kinetic constants associated with the inactivation of plasma kallikrein, clostripain and cathepsin B by Cbz-Phe-Arg-CHN_2

Assays were performed and the data analysed as described in the Experimental section to yield the values of the kinetic constants, which are given together with the S.E.M. values obtained in these analyses.

Enzyme	K_i (μM)	$10^2 \times k_i$ (s^{-1})	$10^{-3} \times (k_i/K_i)$ ($M^{-1} \cdot s^{-1}$)
Plasma kallikrein	74±20	1.8±0.4	0.25±0.02
Cathepsin B	0.89±0.26	4.0±0.3	45±3
Clostripain	0.16±0.02	1.4±0.1	86±4

Synthesis

The chemical synthesis of Cbz-Phe-ArgCHN_2 could be accomplished by the usual method for peptidyldiazomethanes: acylation of diazomethane with the mixed carbonic carboxylic acid anhydride of the peptide. According to n.m.r. data, not more than 5% racemization occurred during synthesis. Although DMF is a good solvent for Cbz-Phe-ArgOH, the reaction did not succeed in DMF.

Cbz-Phe-Arg-CHN_2 hydroacetate was synthesized as follows. A suspension of Cbz-Phe-ArgOH (714 mg, 1.57 mmol) in tetrahydrofuran (70 ml) and acetonitrile (7 ml) was cooled to −20 °C. *N*-Methylmorpholine (0.22 ml, 2 mmol) and isobutyl chloroformate (0.26 ml, 2 mmol) were added. After 5 min, ethereal diazomethane (15 ml, 4.5 mmol) was added, and the stirred mixture allowed to warm up to room temperature during 1.5 h. The solution was evaporated under a stream of N_2, and the residue subjected to preparative h.p.l.c. [Whatman Magnum 9; ODS-3; linear gradient elution with a mixture of acetonitrile in 0.1% trifluoroacetic acid/water, from 20 to 100% (v/v) acetonitrile in 5 min]. The main fraction was collected, diluted with water and immediately filtered through a column of 5 g of quaternary aminoethyl-Sephadex A-25 (acetate form). Freeze-drying gave a fluffy, yellowish powder; yield 72 mg (8%). It had retention time on h.p.l.c. (Whatman RAC II ODS-3 column; gradient mixture of 0.1% trifluoroacetic acid/water and acetonitrile; linear gradient from 10% acetonitrile to 100% acetonitrile within 7 min) of 6.47 min (99%). I.r. (KBr): 3350 cm^{-1} (s, broad), 2100 (s), 1660 (s); n.m.r. (p.p.m., 250 MHz, [$^2H_6$]DMSO): 1.2–1.8 [m, 4H, CH_2-β, CH_2-γ(Arg)], 1.67 [s, 3H, CH_3 (acetate)], 2.76, 3.00 [AB part of ABX system, 2H, CH_2-β(Phe)], 3.0 [m, 2H, CH_2-δ(Arg)], 4.1–4.4 (2m, 2H, 2H-α), 4.98 [s, 2H, CH_2 (Z)], 5.86 (s, 1H, CHN_2), 7.3 (m, 10H), 7.5–8.5 [broad, m, 5NH(guanidino)], 7.67 (d, J = 7, 1NH), 8.71, (d, J = 7, 1NH).

Enzyme assays

Plasma kallikrein was assayed at 37 °C in 0.05 M-Tris/HCl buffer, pH 7.8, containing 0.1 M-NaCl and 0.1% poly(ethylene glycol) (M_r 6000). The release of *p*-nitroaniline resulting from the hydrolysis of the substrate Bz-Pro-Phe-Arg-Nan was monitored by measuring the increase in absorbance at 405 nm. Under the assay conditions used, the Michaelis constant of this substrate with plasma kallikrein was determined as described previously (Stone & Hofsteenge, 1985) to be 450±80 μM. Clostripain was assayed at 37 °C in 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.05 M-$CaCl_2$, 0.1% poly(ethylene glycol) (M_r 6000) and 5.0 mM-dithiothreitol. Before assay, clostripain was activated by preincubation in the above buffer solution for 1 h at 37 °C. The release of *p*-nitroaniline from Bz-Pro-Phe-Arg-Nan was monitored as described above. The Michaelis constant for this substrate with clostripain was found to be 248±19 μM. Cathepsin B was assayed at 37 °C in 0.1 M-sodium acetate buffer, pH 5.4, containing 5.0 mM-dithiothreitol. The increase in fluorescence due the hydrolysis of Cbz-Phe-Arg-NHMec was measured with a Perkin–Elmer LS3 fluorescence spectrometer. The concentration of Cbz-Phe-Arg-NHMec in the assays was 20 μM, an order of magnitude lower than the Michaelis constant of this substrate with cathepsin B (Barrett & Kirschke, 1981).

Determination of inactivation kinetic constants

Kinetic constants for the inactivation of plasma kallikrein, clostripain and cathepsin B by Cbz-Phe-Arg-CHN_2 were determined by monitoring the inactivation of the enzymes in the presence of the substrate, as previously described (Stone & Hofsteenge, 1985). Progress-curve data for the inactivation were fitted to eqn. (1):

$$P = \frac{v_i}{k_{app.}}[1 - \exp(-k_{app.}t)] + d \quad (1)$$

where P is the amount of *p*-nitroaniline formed at time t, $k_{app.}$ is the observed inactivation rate constant and d is a displacement term to account for the fact that at $t = 0$ the absorbance or fluorescence is not accurately known. The value of $k_{app.}$ is related to K_i, the dissociation constant of the initial complex formed between the enzyme and inhibitor, and k_i, the rate at which this initial complex forms an inactive enzyme by eqn. (2):

$$k_{app.} = \frac{k_i[I]}{[I] + K_i[1 + ([S]/K_m)]} \quad (2)$$

where [S] is the concentration of substrate in the assay and K_m is the Michaelis constant of the substrate. Progress-curve data were obtained for at least five concentrations of the inhibitor, and these data were fitted to eqn. (1) by non-linear regression. The resulting values of $k_{app.}$ were fitted to eqn. (2) by weighted linear regression to obtain values for k_i and $K_i[1 + ([S]/K_m)]$. The value of K_i could then be calculated using the known concentration of substrate together with the previously determined value of the Michaelis constant. For cathepsin B, the concentration of substrate was much less than the value of K_m, such that $K_i[1 + ([S]/K_m)]$ was assumed to equal K_i.

Reaction of Cbz-Phe-Arg-CHN_2 with trypsin

Cbz-Phe-Arg-CHN_2 (1 mg, 1.8 μmol) dissolved in DMSO (0.2 ml) was added to 0.1 M-Tris buffer, pH 7.4, containing 0.03 M-$CaCl_2$ (1.2 ml). A solution of trypsin (0.2 mg) in 10 mM-HCl (0.08 ml) was added at room temperature. The reaction was monitored by h.p.l.c. (conditions described above). The diazoketone disappeared within 4 h. The only detectable product co-chromatographed with Cbz-Phe-ArgOH.

Reaction of *p*-nitro-Cbz-Ala-Phe-CHN_2 with chymotrypsin

p-Nitro-Cbz-Ala-Phe-CHN_2 [20 mg, 45 μmol, pre-

pared by standard procedures (Green & Shaw, 1981)] dissolved in methanol (50 ml) was added to 0.05 M-Tris buffer containing 0.5 mM-$CaCl_2$ (100 ml). Chymotrypsin (20 mg) dissolved in 1 mM-HCl (1 ml) was added, and the mixture was stirred at room temperature overnight. With h.p.l.c. (Whatman RAC II ODS-3 column operated with a gradient of aq. 0.1% trifluoroacetic acid and acetonitrile with a linear gradient from 40% acetonitrile to 100% acetonitrile in 7 min) a single product could be detected (retention time 2.88 min) that co-chromatographed with synthetic *p*-nitro-Cbz-Ala-PheOH. The solution was heated to boiling, cooled and filtered through Celite. The filtrate was extracted with ethyl acetate (5 × 2 ml), the combined organic phases dried with anhydrous $MgSO_4$ and concentrated. The residue in dichloromethane (3 ml) was treated with ethereal diazomethane (3 ml, 1 mmol) for 1 h. The solution was evaporated with a stream of N_2 and the residue chromatographed on a preparative silica plate with ethyl acetate/hexane (3:2, v/v). Isolation of the main band gave a yellowish oil (16.1 mg, 83%) which was compared with a synthetic sample of *p*-nitro-Cbz-Ala-Phe-OMe_3. The m.s., i.r. and n.m.r. spectra were identical. Crystallization of the oil from ethyl acetate/hexane gave almost colourless needles (7.5 mg; 39%), which had m.p. 150 °C (synthetic sample: 147–149 °C).

RESULTS AND DISCUSSION

Clostripain and cathepsin B are both cysteine proteinases. The former has a strict proteolytic specificity, cleaving at arginyl carboxy groups (Porter *et al.*, 1971). Its marked susceptibility to Cbz-Phe-$ArgCHN_2$ (Table 1) is in keeping with this property, and is seen to involve both high affinity and reactivity. This proteinase was previously found to be insensitive to Cbz-Phe-Ala-CHN_2 (Green & Shaw, 1981); no effect was seen with 0.25 mM reagent after several hours of exposure, a difference of about 10^9 in reactivity. This is a great contrast with the situation with cathepsin B. Although conveniently measured with arginyl substrates, cathepsin B has no preference for this bond in proteins, but seems to prefer hydrophobic amino acid side chains in the P_2 and P_3 positions (Barrett & Kirschke, 1981). The kinetic properties of its inactivation by Cbz-Phe-$ArgCHN_2$ (Table 1) can be compared with those of Cbz-Phe-Cit-CHN_2 (Table 3 of Shaw *et al.*, 1983); affinities are comparable, with $K_i = 0.89$ and 0.49 μM respectively, but k_{2nd} is 45 as against 6.7 $M^{-1} \cdot s^{-1}$ for the uncharged inhibitor.

It was noted previously (Green & Shaw, 1981) that chymotrypsin was not inactivated by Cbz-PheCHN_2, but instead treated it as a substrate in an unknown manner. This has now been investigated with the use of *p*-nitro-Cbz-Ala-PheCHN_2, whose easily detectable *p*-nitrobenzyl group permits a more thorough analysis of the reaction. The formation of a single product was observed, namely the parent peptide, *p*-nitro-Cbz-Ala-PheOH, indicating that the diazoketone was cleaved by the enzyme into the free acid and diazomethane. A similar reaction took place when Cbz-Phe-$ArgCHN_2$ was incubated with trypsin. The single detectable product had the same retention time on h.p.l.c. as Cbz-Phe-ArgOH. It is therefore probable that, in this case, the diazoketone is also cleaved into the parent peptide and diazomethane. No hydroxymethyl ketone was detected. Diazoketones have been reported to be cleaved enzymically to yield free acids, for example in the case of the action of glutaminase on the glutamine analogue 6-diazo-5-oxonorleucine (Hartman & McGrath, 1973). A hydroxymethyl ketone was observed to form when asparaginase acts on 5-diazo-4-oxo-norvaline (Jackson & Handschumacher, 1970). In the latter reaction, the inhibitor–enzyme complex followed either of two pathways. One of these was hydrolysis, as noted, but occasionally also a covalent bond formed, with inactivation of the enzyme, a pathway favoured by increasing concentrations of DMSO (Lachman & Handschumacher, 1976).

The inactivation of plasma kallikrein was unexpected, since previous studies had shown serine proteinases to be indifferent to diazomethanes. The post-proline-cleaving enzyme from macrophages was shown to be readily inactivated by Cbz-Ala-Ala-Pro-CHN_2 (Green & Shaw, 1983). This is regarded as a serine proteinase, owing to its di-isopropyl fluorophosphate-sensitivity (Walter *et al.*, 1980), although it is also susceptible to *N*-ethylmaleimide. We regard the classification as controversial. On the other hand, the inactivation of plasma kallikrein may represent the initial observation of the modification of a serine proteinase by this class of reagents. It should be noted, however, that the affinity is very poor relative to that shown by the trypsin-like cysteine proteinase clostripain. The site of reaction remains to be determined. It is of interest that, in the case of the reaction of *Escherichia coli* asparaginase with 5-diazo-4-oxo-L-norvaline, evidence has been obtained for a modification of a serine side chain (Peterson *et al.*, 1977).

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