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PREPARATION AND PROPERTIES OF SOME CITRULLINE *p*-NITROANILIDE DERIVATIVES FOR POSSIBLE USE AS PROTEASE SUBSTRATES†

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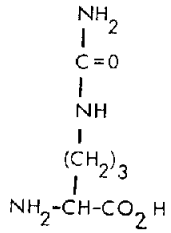
Abstract—A number of citrulline *p*-nitroanilides have been synthesised as potential substrates for proteolytic enzymes. N$^{\alpha}$-Benzyloxycarbonyl-L-citrulline *p*-nitroanilide, a key starting material, was prepared by the phosphoazo method. During this reaction, depending on the conditions, lactam formation and decarbamoylation took place. It is probable that decarbamoylation took place subsequent to the lactamisation step. The derivatives prepared included some protected tripeptide nitroanilides, benzyloxycarbonylglycyl-L-prolyl-L-citrulline *p*-nitroanilide, benzyloxycarbonyl-D-phenylalanyl-L-prolyl-L-citrulline *p*-nitroanilide, benzyloxycarbonylglycyl-L-phenylalanyl-L-citrulline *p*-nitroanilide, methyloxycarbonylglycyl-L-phenylalanyl-L-citrulline *p*-nitroanilide and a protected tetrapeptide, benzyloxycarbonylglycyl glycyl-L-phenylalanyl-L-citrulline *p*-nitroanilide.

Preliminary results have indicated that citrulline *p*-nitroanilides are far more susceptible to hydrolysis by plant thiol enzymes such as papain, ficin and bromelain than by mammalian serine proteases.

Chromogenic derivatives (particularly *p*-nitroanilides) of amino acids and peptides are finding considerable use as substrates for the routine estimation of proteolytic and peptidolytic enzymes of clinical importance.[1-3] Assays based on such synthetic substrates possess many advantages over the previously-used methods involving the evaluation of biological properties.

It is a feature of many of the synthetic substrates applicable to proteases in blood – and a consequence of the specificities of the enzymes concerned – that either arginyl- or lysyl- bonds are cleaved.[1,2] We wished to extend the possibilities of further selectivity by examining the effect of substituting arginine (or lysine) residues by citrulline **1**, an analogue of arginine possessing an ureido-group in its side-chain in place of the guanido-group. The effect of the substitution is that although the bulk of



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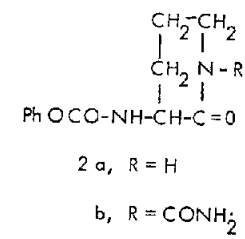
the side chain is similar to that of arginine, the citrulline carries no positive charge.

The *p*-nitroanilide group is the most common chromogen used in such substrates, despite the fact that amino-acid *p*-nitroanilides are not easily synthesised.

In general it appears that the only successful methods for the preparation of protected arginine *p*-nitroanilides are those proceeding through the activation of the *p*-nitroaniline, such as the isocyanate method,[4,5] the phosphoazo method[6,8] and the use of phosphorus pentoxide.[9,10]

The same appears to be the case for the protected citrulline derivatives. We were unable to prepare benzyloxycarbonyl-L-citrulline *p*-nitroanilide (Z–Cit–pNP), which is the key starting material for all the derivatives reported here, using the mixed anhydride method[11] or the dicyclohexylcarbodiimide method,[10] with or without the addition of 1-hydroxybenzotriazole.

Eventually, this compound was prepared using the phosphoazo method but not without difficulty. In accordance with the method described by Kasafirek *et al.*,[7] the "phosphorus trichloride-*p*-nitroaniline" intermediate was prepared in pyridine at −20°. N$^{\alpha}$-Benzyloxylcarbonyl-L-citrulline was added and the mixture refluxed for 3 hr. The only products isolated were N, N′-bis(*p*-nitrophenyl)urea[4,12,13] (16%) whose structure was confirmed by ^{1}H NMR and IR spectroscopy and by elemental analysis, and 3-(benzyloxycarbonylamido) piperidin-2-one (**2a**, the lactam of N$^{\alpha}$-benzyloxycarbonyl-L-ornithine) in 17% yield; the ^{1}H and ^{13}C NMR spectra and elemental analysis confirmed the structure of this compound.



2 a, R = H
b, R = CONH$_2$

When, instead of refluxing for 3 hr, the reaction was carried out for 3 days at room temperature, the desired *p*-nitroanilide (**3**; R=Z, see Table 1) was isolated in 28% yield. In addition a 30% yield of a compound identified as N^1-carbamoyl -3- (benzyloxycarbonylamido)piperidin -2-one (**2b**; the lactam of N$^{\alpha}$-benzyloxycarbonyl-L-citrulline) was obtained. The structure of **2b** was established by elemental analysis, ^{1}H NMR studies (including ^{1}H-^{2}H exchange and spin decoupling) and mass spectrometry (M$^+$ = 291).

Thus, at the lower temperature, lactamisation to **2b** occurred along with the production of the *p*-nitroanilide (**3**; R=Z), although the yield of the desired produce was reduced because of the side reaction. At the higher temperature, decarbamoylation probably took place

†Some of this material was presented at the National Meeting of the Association of Clinical Biochemists July, 1980 at Birmingham, England.

Table 1. Derivatives of citrulline *p*-nitroanilide (R-Cit-*p*NA) (3)

R	m.p. (°C)	R_f	$[\alpha]_D^{20}$ (C=1, DMSO) (°)	Found (%) C	H	N	S	Formula	Required (%) C	H	N	S
Z	219–220	0.62	+21.5	56.0	5.1	16.3		$C_{20}H_{23}N_5O_6$	55.9	5.4	16.3	
H[a]		0.11	+20.8									
$CH_3C_6H_4SO_2$	214–215	0.52	−39.4	50.9	5.4	15.9	7.0	$C_{19}H_{23}N_5O_6S$	50.8	5.1	15.6	7.1
C_6H_5CO	237–240	0.33	+92.6	57.3	5.5	17.2		$C_{19}H_{21}N_5O_5$	57.1	5.3	17.5	
Z-Gly	189–191	0.55	−28.0	54.2	5.7	17.6		$C_{22}H_{26}N_6O_7$	54.3	5.4	17.3	
Z-Phe	197–200	0.68	+9.0	60.2	5.4	14.8		$C_{29}H_{32}N_6O_7$	60.4	5.6	14.6	
Z-Pro	203–205	0.34	−61.5	57.3	6.0	15.7		$C_{25}H_{30}N_6O_7$	57.0	5.7	16.0	
Z-Gly-Pro	213–215	0.38	−53.0	55.1	6.2	16.3		$C_{27}H_{33}N_7O_8$	55.6	5.7	16.8	
Z-D-Phe-Pro	100–110	0.35	−54.3	59.9	6.0	14.4		$C_{34}H_{39}N_7O_8 \cdot \frac{1}{2}H_2O$	59.8	5.9	14.4	
CH_3CO	237–239	0.39	+24.3	50.0	5.6	20.5		$C_{14}H_{20}N_5O_5$	49.8	5.9	20.7	
H.Phe[a]		0.41	−7.0									
Z-Gly-Gly-Phe	178–205	0.63	+5.8	56.3	6.0	15.8		$C_{33}H_{38}N_8O_9 \cdot H_2O$	55.9	5.7	15.8	
Z-Gly-Phe	167–170	0.72	+3.8	58.8	5.4	15.4		$C_{31}H_{35}N_7O_8$	58.8	5.5	15.5	
CH_3CCO-Gly-Phe	204–208	0.58	+5.0	51.9	5.2	16.7		$C_{25}H_{31}N_7O_8 \cdot H_2O$	52.2	5.8	17.0	

[a] Hydrobromides, hygroscopic.

subsequent to cyclisation, since we have shown that the carbamoyl derivative **2b**, isolated from the reaction at room temperature, gave the lactam **2a** when refluxed with *p*-nitroaniline in pyridine. N,N'-Bis(*p*-nitrophenyl)urea was also produced in this reaction. It is interesting to note that this same substituted urea could be formed directly from *p*-nitroaniline and urea by heating in acetic acid–water.[4]

Lactam formation has been reported with N^{α}-benzyloxycarbonyl-G-nitroarginine on treatment with dicyclohexylcarbodiimide,[14] and when N^{α}-benzyloxycarbonyl-G-nitroarginine *p*-nitrophenyl ester (an oil) was allowed to stand.[15] In those cases the lactam of benzyloxycarbonylornithine **2a** was reported as a product or by-product[14] of subsequent reactions. The *p*-nitroanilide of N^{α}-tosyl-L-arginine was prepared (at room temperature) by Kasafirek *et al.*[6] by the PCl_3 method, although the yield was low and no by-product was reported.

This is the first example, so far as we are aware, of lactamisation involving a citrulline derivative.

A consequence of this side reaction was to reduce the obtainable yield of *p*-nitroanilide. However, the phosphorus pentoxide method[9,10] gave even lower yields.

Benzyloxycarbonyl-L-citrulline *p*-nitroanilide (**3**; R=Z) was deprotected in the standard way using hydrogen bromide in acetic acid[16] and from the resulting product (**3**; R=H) were prepared the acetyl (**3**; R=CH_3CO), tosyl (**3**; R=$CH_3C_6H_4SO_2$) and benzoyl (**3**; R=C_6H_5CO) derivatives. Three dipeptide derivatives, benzyloxycarbonyl-L-phenylalanyl-L-citrulline *p*-nitroanilide (**3**; R=Z-Phe), benzyloxycarbonyl-L-prolyl-L-citrulline *p*-nitroanilide (**3**; R=Z-Pro) and benzyloxycarbonylglycyl-L-citrulline *p*-nitroanilide (**3**; R=Z-Gly) were also prepared from the citrulline *p*-nitroanilide, in each case by the use of the *p*-nitrophenyl ester of the corresponding benzyloxycarbonylamino-acid.

Treatment of the protected dipeptide derivative benzyloxycarbonyl-L-prolyl-L-citrulline *p*-nitroanilide (**3**; R=Z-Pro) with hydrogen bromide in acetic acid afforded L-prolyl-L-citrulline *p*-nitroaniline hydrobromide. From this were prepared two tripeptide derivatives, **3**(R=Z-D-Phe-Pro) and **3**(R=Z-Gly-Pro), again by the use of the *p*-nitrophenyl esters of the corresponding benzyloxycarbonylamino-acids.

In a similar manner, deprotection of **3**(R=Z-Phe) gave **3**(R=H-Phe) which allowed the preparation of the protected tetrapeptide derivative (**3**; R=Z-Gly-Gly-Phe) by the use of benzyloxycarbonylglycylglycine *p*-nitrophenyl ester.

The protected tripeptide derivative Z-Gly-Phe-Cit-*p*NA was prepared by coupling H-Cit-*p*NA with benzyloxycarbonylglycylphenylalanine using the dicyclohexylcarbodiimide method. 1-Hydroxybenzotriazole was added in order to suppress racemisation.

Finally, methyloxycarbonylglycyl-L-phenylalanyl-L-citrulline *p*-nitroanilide was prepared by the deprotection and subsequent treatment with methyloxycarbonyl chloride of Z-Gly-Phe-Cit-*p*NA.

Analytical data are given in Table 1 and in the Experimental section.

As a result of the synthetic work a number of derivatives of citrulline *p*-nitroanilide were available for testing as substrates of a range of proteolytic enzymes.

Preliminary examination with enzymes showed that Z-Cit-*p*NA, Tos-Cit-*p*NA, Z-Gly-Cit-*p*NA, Z-Pro-Cit-*p*NA and Z-Gly-Pro-Cit-*p*NA were not detectably hydrolysed by any of the serine proteases thrombin, trypsin, pancreatic kallikrein, urokinase and bovine plasmin. However papain did hydrolyse Z-Cit-*p*NA, Z-

Table 2. Action of enzymes on citrulline derivatives (relative rates of hydrolysis at 25°, pH 7.0; ratios of change in O.D. at 400 nm[a] in 5 min) (± 10)

Substrate (6.36×10^{-4} M)	Papain	Ficin	Bromelain	Trypsin	Thrombin
Bz-D,L-Arg-pNA[b]	500	150	61	385	-
Z-Gly-Pro-Arg-pNA[c]	-	-	-	-	690
Z-Gly-Pro-Cit-pNA	360	59	416	0	0
Z-Cit pNA	172	112	200	0	0
Tos-Cit-pNA	0	0	33	0	-
H-Cit-pNA.HBr	0	0	168	0	-
Z-Gly-Cit-pNA	0	16	132	0	-
Z-Pro-Cit-pNA	100	35	42	0	-
H.Pro-Cit.pNA.HBr	0	0	102	0	-

[a] 100 = 0.100 O.D. units = 1×10^{-5} M p-nitroaniline

[b] Hydrochloride $2 \times 6.36 \times 10^{-4}$ M

[c] Hydrochloride 1.4×10^{-4} M

Pro–Cit–*p*NA and Z–Gly–Pro–Cit–*p*NA but not Tos–Cit–*p*NA or Z–Gly–Cit–*p*NA.

In view of the susceptibility of some of the citrulline derivatives to the plant thiol protease papin, but not to the mammalian serine proteases, two other plant thiol proteases, ficin and thrombin, were also tested. The results of the preliminary enzyme studies are shown in Table 2 and confirm that certain citrulline *p*-nitroanilides may be valuable substrates for the determination of plant thiol enzymes. We hope to publish more detailed results of enzyme studies elsewhere.

EXPERIMENTAL

Tlc was carried out using DC-Plastikfolien Kieselgel 60 F_{254} (Merck) with acetone as eluant. Components were detected by using iodine vapour or by UV absorption. M.ps are uncorrected and are given in the Table 1, along with R_f values, optical rotations (obtained with a Perkin–Elmer 41 Polarimeter) and elemental analyses where appropriate. Amino-acid analyses were obtained on a Locarte automatic amino-acid analyser. Peptides were hydrolysed in sealed tubes in 6M HCl at 110° for 24 hr. Under these conditions some citrulline was converted to ornithine.

Attempted synthesis of N$^{\alpha}$*-benzyloxycarbonyl-*L*-citrulline* p*-nitroanilide by the phosphoazo method*

(a) A soln of *p*-nitroaniline (1.4 g; 0.010 mole) in dry pyridine (20 ml), in a flask equipped with a $CaCl_2$ tube, was cooled to −(20–30)° by using an acetone-solid CO_2 bath. PCl_3 (0.45 cm^3; 0.0051 mole) in dry pyridine (4 ml) was added and the mixture was kept for 30 min at approximately −20° and for a further 30 min at room temp. N$^{\alpha}$-Benzyloxycarbonyl-L-citrulline (3.09 g; 0.010 mole)[16] was added and the mixture was refluxed for 3 hr. The soln was evaporated to dryness and water (80 cm^3) was added. The solid product was filtered off, washed twice with water (10 mL portions), and dissolved in hot EtOH (60 ml) to which glacial AcOH (0.5 ml) had been added. Some of the solid (0.118 g) did not dissolve and was filtered off and washed 3 times with EtOH. This yellow solid which sublimed above 327° was identified by NMR and IR as N, N'-bis(*p*-nitrophenyl) urea. More of this product separated from the EtOH soln (0.358 g). Total yield 0.476 g (16%). A small amount was recrystallized twice from dimethylformamide for analysis. R_f 0.74 (yellow); trace at R_f 0.37 (iodine positive) (Found: C, 51.8; H, 3.0; N, 18.2. Calc. for $C_{13}H_{10}N_4O_5$: C, 51.7; H, 3.3; N, 18.5%). NMR: (d^6-Dimethyl sulphoxide at 70°): δ 7.70 (doublet; 4 protons: part A of an AB system, $J_{AB} \simeq 9$ Hz), δ 8.18 (doublet; 4 protons: part B of an AB system, $J_{BA} \simeq 9$ Hz), δ 9.52 (singlet; 2 protons: NH).

The remainder of the solution and washings were evaporated to dryness and redissolved in hot EtOH–MeOH (70: 30; 80 ml). After standing for a few days, pale yellow crystals m.p. 172–174° appeared. These were filtered off and washed with EtOH. On further concentration, more crystals were obtained, total yield: 0.421 g (17%). This produce was identified by ^{1}H and ^{13}C NMR as 3-(benzyloxycarbonylamido)-piperidin-2-one. A small amount was recrystallized from EtOH for analysis to give colourless crystals m.p. 173–174° (Lit. 171–173°,[14] 174–175°[15]). $[\alpha]_D^{20}$ 0 to 0.4° (cl, DMSO) R_f($CHCl_3$) 0.44 (iodine positive). (Found: C, 63.1; H, 6.6; N, 11.5. Calc. for $C_{13}H_{16}N_2O_3$: C, 62.9; H, 6.5; N, 11.3%). ^{1}H NMR: (d^6-Dimethyl sulphoxide): δ 1.56–2.10 (broad multiplet; 4 protons: $-CH_2-CH_2-CH_2-NH-$), δ 3.11 (multiplet; 2 protons $-CH_2-CH_2-NH-$), δ 3.9 (multiplet; 1 proton: $-NH-CH-CO-$), δ 5.03 (singlet; 2 protons: $Ph-CH_2-O-$), δ 7.18–7.55 (multiplet; 7 protons: $Ph-CH_2-$ and NH).

D_2O *exchange and spin decoupling.* The changes observed in the NMR spectrum after leaving the sample with D_2O overnight are shown below together with the results from spin decoupling in the normal and D_2O-exchange spectra:

D_2O-exchange spectrum.

proton(s)	δ	Normal NMR	D_2O exchange
$-Ch_2^b-CH_2^a-NH$	3.11	multiplet	triplet $J_{ab} \simeq 6$ Hz
O O ‖ ‖ -C-NH-CH-C- and O ‖ -C-NH-CH_2- Ph-CH_2-	7.18–7.55	multiplet (7 protons)	singlet (5 protons)

Spin decoupling.

proton(s)	δ	Normal NMR	When irradiation at around δ 1.72	When irradiation at around δ 7.42
$-CH_2^b-CH_2^a-NH^c-$	3.11	multiplet	doublet, $J_{ac} \simeq 3$ Hz	triplet, $J_{ab} = 5$ Hz
$-NH^f-CH^e(CH_2^g)-CO-NH$	3.9	multiplet	doublet, $J_{ef} \simeq 9$ Hz	"broad" triplet

Also when irradiating at either around δ 3.11 or 3.5 or 3.9 the broad multiplet at δ 1.56–2.10 simplifies.

Spin decoupling on the D_2O exchange product.

Proton(s)	δ	D_2O exchange	When irradiating at around δ 1.84
$-CH_2^b-CH_2^a-NH^c-$	3.11	triplet, $J_{ab} \simeq$ Hz	singlet
$-NH^f-CH^e(CH_2^g)-CO-NH$	3.9	signal overlaps with H_2O signal	singlet at 3.88

Mass Spectrum: Top mass (M^+) = 248

(b) A solution of *p*-nitroaniline (9.8 g; 0.070 mole) in dry pyridine (150 ml), in a flask equipped with a $CaCl_2$ tube, was cooled to -(20–30)° by using an acetone-solid CO_2 bath. PCl_3 (3.15 ml; 0.036 mole) was added and the mixture was kept for 30 min at -20° and for a further 30 min at room temp.

N^α-Benzyloxycarbonyl-L-citrulline (21.63 g; 0.070 mole) was added and the mixture was left stirring for 3 days at room temp. (15–18°). It was then evaporated to dryness at 50–55° and warm water (~ 300ml) was added. A thick yellow oil was precipitated, washed twice with water (50 ml each time) and crystallized from EtOH (250 cm^3). The product obtained was recrystallized from EtOH (350 ml) to give N^α-*benzyloxycarbonyl*-L-*citrulline* p-*nitroanilide* (8.34 g., 28%) m.p. 219–220° $[\alpha]_D^{20}$ + 21.5 (*c* = 1, DMSO) R_f 0.62 (iodine and UV positive) (Found: C, 56.0; H, 5.1; N, 16.3. $C_{20}H_{23}N_5O_6$ requires C, 55.9; H, 5.4; N, 16.3%). NMR (d^6-Dimethyl sulphoxide): δ 1.3–1.85 (broad singlet; 4 protons: $-\underline{CH_2}-\underline{CH_2}-CH_2-NH-$), δ 3.02 (multiplet; 2 protons: $-CH_2-\underline{CH_2}-NH-$), δ 4.26 (multiplet; 1 proton: $-NH-\underline{CH}-CO-$), δ 5.04 (singlet; 2 protons: $Ph-CH_2-O-$), δ 5.44 (singlet; 2 protons: $-CO-\underline{NH_2}$), δ 6.01 (triplet; 1 proton: $-CH_2^b-\underline{NH}^a-CO-NH_2$, $J_{ab} \simeq 6$ Hz), δ 7.34 (singlet; 5 protons: $\underline{Ph}-CH_2-$), δ 7.65 (doublet; 1 proton: $-O-CO-\underline{NH}^f-CH^d-$ $J_{fd} \simeq 8$ Hz), δ 7.86 (doublet; 2 protons: part A of an AB system, $J_{AB} \simeq 9$ Hz), δ 8.22 (doublet; 2 protons: part B of an AB system, $J_{BA} \simeq 9$ Hz), δ 10.65 (singlet; 1 proton: $-CO-\underline{NH}-Ar$).

D_2O exchange. The changes observed in the NMR spectrum after adding D_2O are:

Proton(s)	δ	Normal NMR	D_2O exchange
$-CH-\underline{NH_2}$	5.44	singlet	small residue
$-CO-\underline{NH}-Ar$	10.65	singlet	small residue

Spin Decoupling on the D_2O exchange product

Proton	δ	D_2O exchange	When irradiating at around δ 4.26
$-O-CO-NH-\underline{CH}-$	7.65	doublet	singlet

On standing, crystals were deposited from the ethanol filtrates. These were filtered off, and identified by NMR as N^1-*carbamoyl*-3-*benzyloxycarbonylamidopiperidin*-2-*one*. Total yield; 6.0 g (30%) m.p. 116–117°C. This compound was recrystallized from EtOAc/Et_2O, m.p. 132–133°. $[\alpha]_D^{20}$, 4.2° (cl, DMSO). R_f 0.64 (iodine and UV positive). (Found: C, 57.5; H, 6.1; N, 14.6. $C_{14}H_{17}N_3O_4$ requires C, 57.7; H, 5.8; N, 14.4%). NMR (d^6-Dimethyl sulphoxide): δ 1.55–2.25 (multiplet; 4 protons: $-\underline{CH_2}-\underline{CH_2}CH_2-N-$), δ 3.52 (multiplet; 1 proton: $-CH_2-CH_2-N-$, the axial proton), δ 3.9 (multiplet; 1 proton: $-CH_2-\overline{CH_2}-N-$, the equatorial proton), δ 4.28 (multiplet; 1 proton: $-NH-\underline{CH}-$), δ 5.04 (singlet; 2 protons: $Ph-\underline{CH_2}-$, δ 7.36 (singlet; 7 protons: $\underline{Ph}-CH_2-$ and 2 NH protons) δ 8.36 (broad singlet; 1 proton NH).

D_2O *exchange.* The changes observed in the NMR spectrum after shaking with D_2O are: The N–H signal at δ 8.36 disappeared and while the relative area of the singlet at σ 7.36 was decreased from 7 to 5 because the 2 NH protons under this peak were exchanged.

Spin decoupling.

Proton	δ	Normal NMR	When irradiating at around δ 1.7–2.2
$-CH_2-C(H^{equatorial})(H^{axial})-N-$	3.52	multiplet	doublet $J_{ax.eq} \simeq 13$ Hz
$-CH_2-C(H^{equatorial})(H^{axial})-N$	3.9	multiplet	doublet $J_{eq.ax} \simeq 13$ Hz
$-NH^f-CH^d-CH_2-$	4.28	multiplet	doublet $J_{fd} \simeq 8$ Hz

Spin decoupling on the D_2O exchange product. The proton at δ 4.28 appears as a singlet when irradiating around δ 1.7–2.2. *Mass spectrum*: Top mass (M^+) = 291.

The reaction between N^1-carbamoyl-3-benzyloxycarbonyl-amidopiperidine-2-one and p-nitroaniline. A soln of N^1-carbamoyl-3-benzyloxycarbonylamidopiperidine-2-one (0.250 g; 0.00086 mole) and *p*-nitroaniline (0.124 g; 0.00090 mole) in dry pyridine (5 ml) was refluxed for 3½ hr with exclusion of moisture and then evaporated to dryness. The resulting oil was dissolved in hot ethanol and a yellow solid precipitated, which was shown to be N, N'-bis-(*p*-nitrophenyl)urea, sublimes above ~320°). Yield: 0.037 g (14%).

From the ethanol another compound crystallised out after cooling m.p. 175–176°, which was 3-(benzyloxycarbonylamido)-piperidin-2-one, yield: 0.028 g (13%).

These compounds were identified by IR and tlc comparison with authentic samples.

L-*Citrulline p-nitroanilide hydrobromide.* To a suspension of N^α-benzyloxycarbonyl-L-citrulline *p*-nitroanilide (1.52 g; 0.00234 mole) in glacial AcOH (5 ml), a soln of HBr in AcOH (5 ml; 40% v/v) was added. After stirring for 1½ hr at room temp. ether (180 ml) was added. The semi-solid HBr salt was precipitated and washed twice with ether (total: 80 ml) by decantation and then was dried *in vacuo* over NaOH. The yield of this hygroscopic product was almost quantitative and it was pure enough to be used for subsequent reactions. NMR (D_2O) δ 1.50–2.10 (broad multiplet; 4 protons: $-\underline{CH_2}-\underline{CH_2}-CH_2-NH-$), δ 3.22 (triplet; 2

protons: $-CH_2^c-CH_2^b-NH-$, $J_{bc} \simeq 6$ Hz), δ 4.32 (triplet, 1 proton: $-NH-CH^d-CH_2^c-$ $J_{dc} \simeq 5$ Hz), δ 7.77 (doublet; 2 protons: part A of an AB system, $J_{AB} \simeq 9$ Hz), δ 8.28 (doublet; 2 protons: part B of an AB system, $J_{BA} \simeq 9$ Hz).

N$^{\alpha}$-*Tosyl*-L-*citrulline*-p-*nitroanilide.* To a soln of L-citrulline *p*-nitroanilide hydrobromide (0.253 g; 0.00067 mole) in redistilled DMF (1.5 ml), triethylamine (0.58 ml; 0.00420 mole) and tosyl chloride (0.134 g; 0.00070 mole) were added with stirring and cooling. After 5 min cooling, stirring was continued for a further 15 min at room temp. Then water (14 ml) was added and an oil was precipitated, which crystallized from absolute EtOH (2 ml)to give crystals of N$^{\alpha}$-tosyl-L-*citrulline* p-*nitroanilide*, 0.047 g, m.p. 209–210°.

From the aqueous dimethylformamide soln more crystals came out after standing. Total yield: 0.256 g; 85%. NMR (d^6-dimethyl sulphoxide). δ 1.1–1.8 (multiplet; 4 protons: $-CH_2CH_2-CH_2-NH-$), 2.18 (singlet; 3 protons: CH_3-), δ 2.9 (multiplet; 2 protons; $-CH_2-CH_2-NH-$), δ 3.9 (multiplet; 1 proton: $-NH^f-CH^d-CO-$, $J_{fd} \simeq 6$ Hz), δ 7.17 (doublet; 2 protons: aromatic $J \simeq 9$ Hz), δ 7.62 (two doublets one on top of the other; 4 protons: aromatic), δ 8.13 (doublet; 2 protons: aromatic, $J \simeq 9$ Hz), δ 10.47 (singlet, 1 proton: –CO–NH–Ar).

N$^{\alpha}$-*Benzoyl*-L-*Citrulline* p-*nitroanilide.* This was prepared similarly using benzoyl chloride. The *benzoyl derivative* (3; R = C_6H_5CO) was crystallized from ethanol, yield 43%. NMR (d^6-dimethyl sulphoxide): δ 1.30–2.05 (broad multiplet; 4 protons: $-CH_2-CH_2-CH_2-NH-$), δ 3.10 (multiplet; 2 protons: $-CH_2-CH_2-NH-$), δ 4.65 (multiplet; 1 proton: –NH–CH–CO–), δ 5.46 (singlet; 2 protons: $-CONH_2$), δ 6.07 (triplet; 1 proton: $-CH_2^b-NH^a-CONH_2$, $J_{ab} \simeq 6$ Hz), δ 7.50 (multiplet; 3 protons: the 2 meta H's + the para H of the Ph group), δ 7.90 (multiplet; 4 protons: 2 H's the A part of the AB system of the p-nitroanilide + the 2 ortho H's of the Ph group), δ 8.24 (doublet; 2 protons; the B part of AB system of the p-nitroanilide, $J_{BA} \simeq 9$ Hz), δ 8.72 (doublet; 1 proton: $-NH^f-CH^d-$, $J_{fd} \simeq 7$ Hz), δ 10.78 (singlet; 1 proton: –CO–NH–Ar).

N$^{\alpha}$-*Acetyl*-L-*citrulline* p-*nitroaniline* was also prepared as above using acetic anhydride. The *acetyl derivative* (3; R = CH_3CO) crystallized from EtOH, yield 37%. NMR (d^6-dimethyl sulphoxide): δ 1.30–1.82 (broad multiplet; 4 protons: $-CH_2-CH_2-CH_2-NH-$), δ 1.91 (singlet; 3 protons: CH_3CO-), δ 3.01 (multiplet; 2 protons $-CH_2-CH_2-NH-$), δ 4.40 (multiplet; 1 proton: –NH–CH–CO–), δ 5.43 (singlet; 2 protons: $-CONH_2$), δ 5.99 (triplet; 1 proton: $-CH_2^b-NH^{\alpha}-CONH_2$, $J_{ab} \simeq 6$ Hz), δ 7.86 (doublet; 2 protons: part A of the AB system, $J_{AB} \simeq 9$ Hz), δ 8.22 (doublet; 3 protons: 2 H's part B of the AB system, $J_{BA} \simeq 9$ Hz + 1H, $Ch_3CONH-CH-$) δ 10.66 (singlet; 1 proton: –CO–NH–Ar).

D_2O *exchange.* The D_2O exchange NMR spectrum confirms that there is an amide proton at δ 8.22, the same position as the part B (doublet) of the AB system.

Benzyloxycarbonylglycyl-L-*citrulline* p-*nitroanilide* (3; R=Z–Gly). To a soln of L-citrulline *p*-nitroanilide hydrobromide (0.405 g; 0.00108 mole) in dimethylformamide (2.5 ml), triethylamine (0.5 ml; 0.364 g; 0.00360 mole) and benzyloxycarbonylglycine *p*-nitrophenyl ester[18] (0.446 g; 0.00135 mole) were added. After the mixture was left stirring at room temp. for 18 hr, with exclusion of moisture, water (20 ml) was added and a yellow oil precipitated. This was crystallized from warm abs EtOH (3–4 ml) and the pale yellow crystals were washed three times with EtOH (total amount: 12 ml) and twice with water (9 ml each time). After drying *in vacuo* over P_2O_5, 0.200 g of *benzyloxycarbonylglycyl*-L-*citrulline* *p*-nitroanilide, m.p. 181–183° was obtained. From the washings more product crystallized out (0.074 g), m.p. 189–191°. The total yield was: 0.274 g (52%). NMR (d^6-dimethyl sulphoxide). δ 1.1–1.95 (broad multiplet; 4 protons: $-CH_2-CH_2-CH_2-NH-$), δ 3.0 (multiplet; 2 protons: $-CH_2-CH_2-NH-$), δ 3.73 (doublet; 2 protons: $-NH^b-CH_2^g-CO-$, $J_{gb} \simeq 6$ Hz), δ 4.48 (broad singlet; 1 proton: –NH–CH–CO), δ 5.05 (singlet; 2 protons: $Ph-CH_2-$), δ 5.42 (singlet; 2 protons: $-CO-NH_2$), δ 6.0 (triplet; 1 proton: $-CH_2^g-NH^b-CO-NH_2$ $J_{ab} \simeq 6$ Hz), δ 7.35 (singlet; 6 protons: 5 aromatic, $Ph-CH_2-$ and 1 NH, $-OCO-NH-CH_2-$) δ 7.88 (doublet; 2 protons: part A of an AB system, $J_{AB} \simeq 9$ Hz), δ 8.25 (doublet overlapping with a peak at 8.3; 3 protons: part B of an AB system and $-CH_2-CO-NH-CH-$), δ 10.65 (singlet, 1 proton: –CO–NH–Ar).

Benzyloxycarbonyl-L-*prolyl*-L-*citrulline* p-*nitroanilide* (3; R = Z-Pro) was prepared in same manner as immediately above, except that benzyloxycarbonyl-L-proline *p*-nitrophenyl ester[19] was used. This p-*nitroanilide* was crystallized from ethanol, yield 79%. NMR (d^6-Dimethylsulphoxide). δ 1.0–2.3 (multiplet, 8 protons: 4 from the citrulline side chain and 4 from the proline ring), δ 2.96 (multiplet; 2 protons: $-CH_2-CH_2-NH-$), δ 3.50 (multiplet; 2 protons, $-N-CH_2-CH_2-$), δ 4.35 (multiplet; 2 protons: the CH from proline and the CH from citrulline residues) δ 5.02 and 5.08 (2 singlets; 2 protons: $PhCH_2O-$) 5.40 (singlet: 2 protons: $-CONH_2$), δ 5.95 (multiplet; 1 proton: $-NHCONH_2$), δ 7.27 (singlet; 5 protons: $Ph-CH_2-O-$), δ 7.84 (doublet; 2 protons: part A of an AB system, $J_{AB} \simeq 9$ Hz), δ 8.20 (doublet; 2 protons: part B of an AB system, $J_{BA} \simeq 9$ Hz), δ 10.65 (singlet; 1 proton: –CH–CONH–CH–), δ 10.84 (singlet; 1 proton: –CONH–Ar).

Benzyloxycarbonyl-L-*phenylalanyl*-L-*citrulline* p-*nitroanilide* (3; R = Z–Phe) was prepared in the same way using benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester.[20] The *protected dipeptide* p-*nitroanilide* crystallised from ethanol, yield 61%. NMR (d^6-dimethyl sulphoxide): δ 1.30–1.90 (broad multiplet; 4 protons: $-CH_2-CH_2-CH_2-NH-$), δ 2.60–3.25 (comfilex multiplet pattern; 4 protons; $Ph-CH_2-CH-$ and $-CH_2-CH_2-NH-$), δ 4.40 (broad multiplet; 2 protons: the 2 α-CH's of phenylalanine and citrulline residues), δ 4.94 (singlet; 2 protons: $Ph-CH_2-O-$), δ 5.42 (singlet; 2 protons: $-CO-NH_2$), δ 5.98 (broad triplet; 1 proton: $-CH_2^b-NH^a-CO-NH_2$, $J_{ab} \simeq 6$ Hz), δ 7.28 (singlet; 11 protons: the 2 Ph's plus a doublet at δ 7.40 due to $PhCH_2OCO-NH-$), δ 7.85 (doublet; 2 protons: part A of an AB system, $J_{AB} \simeq 9$ Hz), δ 8.22 (doublet; 3 protons: part B of an AB system, $J_{BA} \simeq 9$ Hz, together with a doublet at δ 8.30 due to $-CH-CO-NH-CH-$), δ 10.67 (singlet; 1 proton: –CO–NHAr).

L-*Proline*-L-*citrulline* p-*nitroanilide hydrobromide* (3; R = H-Pro). To a suspension of benzyloxycarbonyl-L-prolyl-L-citrulline *p*-nitroanilide (0.58 g; 0.0011 mole) in glacial acetic acid (2.3 ml), a soln of HBr in AcOH (2.7 ml; 40% v/v) was added. After stirring for 1½ hr at room temp. ether (80 ml) was added. The semi-solid HBr salt was precipitated, triturated and washed twice with ether by decantation and then dried overnight *in vacuo* over sodium hydroxide. The yield of this product was quantitative and it was pure enough to be used for the next step.

Benzyloxycarbonylglycyl-L-*prolyl*-L-*citrulline* p-*nitroanilide* (3; R=Z–Gly-Pro). To a soln of L-proline-L-citrulline *p*-nitroanilide hydrobromide (0.30 g; 0.00063 mole) in redistilled dimethylformamide (1.5 ml), triethylamine (0.3 ml; 0.128 g; 0.00216 mole) and benzyloxycarbonylglycine *p*-nitrophenyl ester (0.25 g; 0.00075 mole) were added. The mixture was left standing for 48 hr at room temp. and then water (11 ml) was added and the product was precipitated as a semisolid which was washed twice with water (4 ml portions) and solidified on trituration in dichloromethane-ether. The yield was 2.2 g (60%), m.p. 213–215°. Amino acid analysis: The molar ratio of Gly: Pro: (Cit + Orn) was found to be 0.98:1.03:0.98. NMR (d^6-Dimethyl sulphoxide): δ 1.3–2.3 (multiplet; 8 protons: 4 from the citrulline side chain and 4 from the proline ring), δ 3.00 (multiplet; 2 protons: $N-CH_2-CH_2-$) δ 3.76 (doublet; 2 protons: $-CONH^h-CH_2^g-CO-$, $J_{gh} \simeq 6$ Hz), δ 4.32 (multiplet; 2 protons: $Ph-CH_2-O-$), δ 7.31 (singlet; 5 protons: $PhCH_2O-$), δ 7.92 (doublet; 2 protons: part A from an AB system, $J_{AB} \simeq 9$ Hz), δ 8.18 (doublet; 3 protons: part B from an AB system, $J_{BA} \simeq 9$ Hz, plus the NH:–CO–NH–CH_2–CO–), δ 10.60 (singlet; 1 proton: –CH–CONH–CH–), δ 11.10 (singlet; 1 proton: CONH–Ar).

Benzyloxycarbonyl - D - phenylalanyl - L - prolyl - L - citrulline p - *nitroanilide* (3; R = Z-D-Phe-Pro) To a solution of L-proline-L-citrulline *p*-nitroanilide (0.40 g; 0.00084 mole) in redistilled dimethylformamide (2 ml), triethylamine (0.5 ml; 0.364 g; 0.0036 mole) and benzyloxycarbonyl-D-phenylalanine-*p*-nitrophenyl ester (purchased from Sigma) (0.40 g; 0.00095 mole) were added. The mixture was left standing for 5 days at room temp. with exclusion of moisture and then water (20 ml) was added and an oil was precipitated. The *protected tripeptide* p-*nitroanilide* was precipitated from CH_2Cl_2 with ether as a solid (0.234 g; yield = 41.3%) m.p. 100–110°, which was slightly hygroscopic. NMR: (d^6-Dimethyl sulphoxide). The most significant peaks are at: δ 7.20 and δ 7.28 (2 singlets; 10 protons: the 2 Ph-groups), δ

7.50–8.30 (AB system from *p*-nitroanilide together with one NH; 5 protons).

L-Phenylalanyl-L-citrulline p-nitroanilide hydrobromide (3; R = H-Phe). Benzyloxycarbonyl - L - phenylalanyl - L - citrulline *p* - nitroanilide (0.518 g; 0.00090 mole) was dissolved in a mixture of hydrogen bromide in acetic acid (40% v/v, 2.5 ml) and glacial AcOH (2 ml). After standing for 1 hr and 40 min, ether (80 ml) was added and the ppt. was triturated and washed twice with ether (50 ml portions) by decantation and the white crystals of the product were dried overnight *in vacuo* over potassium hydroxide. The yield was almost quantitative. NMR: (d^6-dimethyl sulphoxide + D_2O): δ 1.32–1.90 (broad multiplet; 4 protons: CH_2–CH_2–CH_2–NH–), δ 3.08 (multiplet; 2 protons: –CH_2–CH_2–NH–), ~ δ 4.20 (multiplet; 1 proton: –NH–CH–CO, overlaps with the H_2O peak, but it can be seen clearly in the D^6–DMSO NMR spectrum), δ 4.50 (multiplet; 1 proton: NH_3^+–CH(CH_2Ph)–), δ 7.26 (singlet; 5 protons: Ph–CH_2–), δ 7.84 (doublet; 2 protons: part A of an AB system, $J_{AB} \simeq 9$ Hz), δ 8.25 (doublet; 2 protons: part B of an AB system, $J_{BA} \simeq 9$ Hz).

Benzyloxycarbonyl-glycyl-glycyl-L-phenylalanyl-L-citrulline p-nitroanilide (3; R = Z-Gly-Gly-Phe). To a soln of L-phenylalanyl-L-citrulline *p*-nitroanilide hydrobromide (0.235 g; 0.00045 mole), N-ethylmorpholine (0.059 ml); 0.00045 mole) and 1-hydroxybenzotriazole (0.061 g; 0.00045 mole) in redistilled dimethylformamide (1 ml), benzyloxycarbonylglycylglycine *p*-nitrophenyl ester (0.174 g; 0.00045 mole) was added and the reaction mixture was left standing at room temp. for 30 min. Then ether was added and the precipitate was washed successively with 2N Na_2CO_3, water, $KHSO_4$ aq and water. After it was dried *in vacuo* over P_2O_5, it was crystallized from EtOH-pet. ether 60–80° to give *benzyloxycarbonylglycylglycyl - L - phenylalanyl - L - citrulline p-nitroanilide* (0.037 g; yield = 12%); m.p. 178–205°. Amino acid analysis: The molar ratio of Gly: Phe: (Cit + Orn) was found to be 2.04: 1.00: 0.93. NMR: (d^6-dimethyl sulphoxide). The most significant peaks are at: δ 5.00 (singlet; 2 protons: Ph–CH_2–O–), δ 5.42 (singlet; 2 protons: –CO–NH_2), δ 5.96 (broad triplet; 1 proton: –NH–$CONH_2$), δ 7.10–7.48 (2 singlets; 12 protons: the 2 Ph groups +2 NH's), δ 7.75–8.40 (a broad pattern including an AB quartet; 6 protons: from an AB system +2 NH's), δ 10.58 (singlet; 1 proton: –CO–NHAr).

Benzyloxycarbonyl - glycyl - L - phenylalanyl - L - citrulline p - nitroanilide (3; R = Z-Gly-Phe). Benzyloxycarbonyl-L-citrulline *p*-nitroanilide (0.558 g; 0.00130 mole) was deprotected as described and the product, L-citrulline-*p*-nitroanilide hydrobromide, was dissolved in dimethylformamide (5 ml). To this soln, N-methylmorpholine (0.14 ml; 0.013 g; 0.00130 mole), 1-hydroxybenzotriazole (0.270 g; 0.00200 mole), and benzyloxycarbonyl-glycyl-L-phenylalanine (0.390 g; 0.00109 mole) were added, followed by dicyclohexylcarbodiimide (0.277 g; 0.00110 mole) at −5°. The mixture was left stirring for 45 min at −5° and 20 hr at room temp with exclusion of moisture. Then glacial AcOH (2 drops) was added and after 10 min the N, N'-dicyclohexylurea was filtered off and washed with dimethylformamide (1 ml). Water (60 ml) was added to the filtrate and the ppt. was filtered off, after standing for 2 hr in the freezer, and washed twice with water (5 ml portions).

The solid was crystallized from EtOH-$EtOAc_2$ to give *benzyloxycarbonyl-glycyl-L-phenylalanyl-L-citrulline p-nitroanilide* (66%). Amino acid analysis: The molar ratio of Gly: Phe: (Cit + Orn) was found to be 0.99: 0.94: 1.06. NMR: (d^6-dimethyl sulphoxide): δ 1.30–1.96 (multiplet; 4 protons: –CH_2–CH_2–CH_2–NH–), δ 2.70–3.90 (complex multiplet pattern including a peak due to H_2O and the 3CH_2's: –CH_2–NH–$CONH_2$, –CH(CH_2Ph)– and –NH–CH_2–CO–), δ 4.58 (broad multiplet; 2 protons: the two –CH's of the phenylalanine and citrulline residues), δ 5.02 (singlet; 2 protons: Ph–CH_2–O–), δ 5.48 (singlet; 2 protons: –CO–NH_2), δ 6.02 (broad triplet; 1 proton: –CH_2^b–NH^a–$CONH_2$, $J_{ab} \simeq$ 6 Hz), δ 7.22 and 7.36 (2 singlets; 11 protons: the 2 Ph's + $PhCH_2OCO$–NH–), δ 7.80–8.50 (a multiplet pattern containing an AB system; 6 protons: 4 of the AB system and the 2 NH's. NH–CH_2– and –CH–CO–NH–CH–), δ 10.60 (singlet; 1 proton: –CO–NH–Ar).

Glycyl-L-phenylalanyl-L-citrulline p-nitroanilide hydrobromide (3; R = H-Gly-Phe). To a suspension of benzyloxycarbonyl-glycyl-L-phenylalanyl-L-citrulline *p*-nitroanilide (0.200 g; 0.000316 mole) in glacial AcOH (0.5 ml), hydrogen bromide in acetic acid (0.7 ml); 45% v/v) was added. After the mixture was left standing for 1½ hr, ether (~15 ml) was added. The colourless ppt. was separated by decantation, washed twice with ether and dried *in vacuo* for 24 hr with KOH.

Methoxycarbonylglycyl-L-phenylalanyl-L-citrulline p-nitroanilide (3; R = CH_3OCO–Gly–Phe). to a soln of glycyl-L-phenylalanyl-L-citrulline *p*-nitroanilide hydrobromide (0.147 g; 0.000253 mole) at 5°, triethylamine (0.070 ml; 0.000505 mole) and methoxycarbonyl chloride (0.024 ml; 0.00030 mole) were added with stirring. After 45 min at −5°, stirring was continued at room temp for a further 2 hr. The mixture was cooled to 10° and water (10 ml) was then added. After 20 min in the freezer, the semisolid ppt. was separated by decantation, washed with water and dissolved in hot EtOH. Ether was added and the soln was concentrated. Yellow crystals of *methoxycarbonylglycyl-L-phenylalanyl-L-citrulline p-nitroanilide* were filtered off and washed with ethyl acetate and ether, yield: 0.078 g (56%), m.p. 204–208°.

Enzyme studies

The citrulline substrate solns were prepared as follows. Substrate (10 mg) was dissolved in DMSO (1.0 ml) and sufficient acetonitrile added so that the substrate concentration in the assays was 6.36×10^{-4} (Benzoyl-D,L-arginine *p*-nitroanilide and Z–Gly–Pro–Arg–*p*NA solns were prepared in a similar way but at different concentrations. (Table 2). To the substrate soln (0.40 ml) at 25°, was added phosphate buffer (0.05 M, pH 7.0; 1.5 ml) at the same temp. followed by enzyme solns in the same buffer. The rate of change in O.D. at 400 nm was measured continuously for 5 min. Initial velocities were constant with respect to time. The enzymes, papain, ficin and bromelain were made up in phosphate buffer containing EDTA (0.001 M) and cysteine hydrochloride (0.005 M). Bromelain and ficin solutions were centrifuged before us. A period of 20 min was allowed for activation of the thiol enzymes before assay. The results are shown in Table 2.

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