Confirmation of the Site of Nitrosation in Tryptophan Derivatives by α-Chymotrypsin

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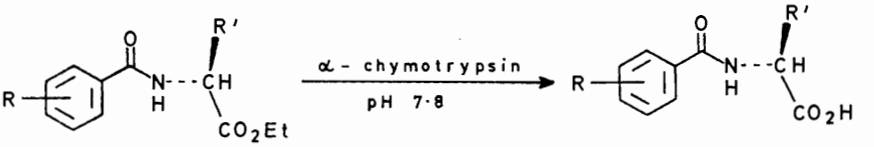
N-Aroyl derivatives of aromatic hydrophobic L-amino-acid esters (1)—(7) are hydrolysed by α-chymotrypsin to the corresponding L-amino-acids (8)—(14). When the amidic nitrogen atom is incorporated into a heterocyclic ring, *e.g.* in the benzotriazinones (15a and b), substrate properties are lost. The *N*-nitroso-derivatives of *N*-acetyl-L-tryptophan methyl and ethyl esters are smoothly hydrolysed by α-chymotrypsin; their nitroso-groups are therefore attached to the indolic rather than the amidic nitrogen atom.

THE esters of *N*-acyl and *N*-aroyl derivatives of the hydrophobic aromatic amino-acids L-phenylalanine, L-tyrosine, and L-tryptophan can serve as substrates for the proteolytic enzyme α-chymotrypsin. *N*-Benzoyl-L-phenylalanine ethyl ester (1a), for example, is hydrolysed by this enzyme to the corresponding carboxylic acid (9a).[1]

In an earlier paper [2] the chemistry of aroyl-L-phenylalanine ethyl esters (2a)—(7a) and the related L-tyrosine esters (2b)—(7b) was described. Hydrolysis of the phenylalanine ester (2a) in 8N-sulphuric acid gave *o*-nitrobenzoic acid, whereas hydrolysis of the related tyrosine ester (2b) in alkali afforded the amino-acid (9b) which was substantially racemised. We now report the hydrolysis of these and other derivatives of hydrophobic

The 3-substituted 1,2,3-benzotriazin-4(3*H*)-ones (15a and b), prepared by diazotisation of the anthraniloyl amino-acid esters (4a and b),[2] were originally required for examination as photoaffinity probes [3] for the hydrogen-bonding site of α-chymotrypsin, but proved of no interest because of their photostability. In the present work they were found to be neither substrates nor inhibitors of α-chymotrypsin: they remained unchanged following treatment with enzyme at pH 7.8, and subsequent addition of the known substrate (2a) led to smooth hydrolysis of the latter to the corresponding carboxylic acid (9a). This unreactivity can be attributed to the lack of an amidic N–H bond. This feature is essential for productive binding at the hydrogen-bonding locus



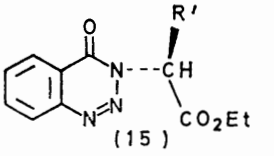
(1)	R = H	(8)
(2)	R = *o*-NO_2	(9)
(3)	R = *p*-NO_2	(10)
(4)	R = *o*-NH_2	(11)
(5)	R = *p*-NH_2	(12)
(6)	R = *o*-N_3	(13)
(7)	R = *p*-N_3	(14)

SCHEME 1

L-amino-acids by α-chymotrypsin, and the use of this enzyme to confirm the site of nitrosation of some *N*-acetyl-L-tryptophan derivatives.

The L-phenylalanine and L-tyrosine series (2)—(7) (a and b) were available from earlier work,[2] and the *N*-aroyl-L-tryptophan derivatives (2c)—(5c) were prepared by adaptation of the same syntheses. All these esters proved to be substrates for α-chymotrypsin at pH 7.8 (Scheme 1) and the hydrolysis, as shown by pH-stat titration, was nearly quantitative in all cases. Yields of the acids in preparative-scale syntheses ranged from moderate to good (Table 1), and in some cases specific rotations were determined to confirm that the products were optically active.

on the enzyme which orients the ester group at the hydrolytic site.[4]



(15)

a; R′ = benzyl
b; R′ = *p*-hydroxybenzyl
c; R′ = indol-3-ylmethyl

In contrast to the cyclisations mentioned above, diazotisation of *N*-anthraniloyl-L-tryptophan ethyl ester (4c) was not straightforward: a dark oil was obtained and none

[1] S. Kaufmann and H. Neurath, *Arch. Biochem.*, 1949, **21**, 437.
[2] A. C. Mair and M. F. G. Stevens, *J. Chem. Soc.* (*C*), 1971, 2317.
[3] J. R. Knowles, *Accounts Chem. Res.*, 1972, **5**, 155.
[4] T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, 1969, **46**, 337.

of the expected benzotriazinone (15c), which would be formed by intramolecular coupling of the diazonium ion, was isolated. In the tryptophan derivative (4c) alternative sites for attack by the electrophilic nitrosating species are available, notably the indole fragment and possibly also the amidic group. In addition, indole derivatives serve as substrates for intermolecular coupling reactions with diazonium salts.[5] Because of similar extensive formation of by-products we were unable to prepare the azides (6c) and (7c) by the diazotisation–azidation route.

Bonnett and Holleyhead [6] have recently examined the nitrosation of the DL-form of *N*-acetyltryptophan methyl ester (16a) and the corresponding DL-acid (18a). The ester gave a yellow *N*-nitroso-derivative which was formulated as the N(1)-isomer (17a) (in the DL-form) rather than the nitroso-amide isomer (19a). The spectroscopic evidence supporting structure (17a) was somewhat contradictory. The mass spectrum of the nitroso-derivative (like that of 3-methylindole) showed a base peak at *m/e* 130 attributed to the indol-3-ylmethyl cation (or a rearrangement product), and is more consistent with the nitrosoamide formula (19a); on the other hand the electronic absorption spectrum indicated the nitroso-group to be attached to the indole chromophore.

We considered it feasible to identify the site of nitrosation in *N*-acetyltryptophan esters by exploiting the specificity of α-chymotrypsin. *N*-Acetyl-L-tryptophan methyl and ethyl esters (16a and b) are substrates for α-chymotrypsin,[7] and are hydrolysed to *N*-acetyl-L-tryptophan (18). The *N*(1)-nitroso-L-tryptophan esters (17a and b), having amidic N–H groups, should be substrates for the enzyme, whereas the nitroso-amide isomers (19a and b), lacking this crucial feature, should not (Scheme 2). Yellow *N*-nitroso-derivatives of the L-tryptophan esters (16a and b) were obtained with spectro-

TABLE 1

N-Aroyl-L-amino-acids prepared by hydrolysis of *N*-aroyl-L-amino-acid esters by α-chymotrypsin

Starting ester	Product acid	Yield (%)	M.p. (°C)	Formula	Found (%) (Required) C	H	N	Physical characteristics of product*
(3a)	(10a)	68	140—142	$C_{16}H_{14}N_2O_5$	60.9 (61.1)	4.4 (4.5)	8.7 (8.9)	$\nu_{max.}$ 3 350 (NH), 3 300—2 300 (bonded OH), 1 730 (CO_2H), 1 650 (CONH), 1 525 and 1 355 cm^{-1} (NO_2): $[\alpha]_D^{25}$ −60.0° (*c* 0.9)
(4a)	(11a) †	85	142—143	$C_{16}H_{16}N_2O_3$	67.8 (67.6)	5.7 (5.6)	9.7 (9.9)	$\nu_{max.}$ 3 375 and 3 280 (NH), 3 080—2 340 (bonded OH) 1 710 (CO_2H), 1 630 cm^{-1} (CONH): $[\alpha]_D^{25}$ +44.0° (*c* 1)
(5a)	(12a) †	63	210—212	$C_{16}H_{16}N_2O_3$	67.5 (67.6)	5.4 (5.6)	9.6 (9.9)	$\nu_{max.}$ 3 300 (NH), 3 150—2 500 (bonded OH), 1 710 (CO_2H), 1 635 cm^{-1} (CONH): $[\alpha]_D^{25}$ −12.3° (*c* 0.8)
(6a)	(13a)	42	39—40 (decomp.)	$C_{16}H_{14}N_4O_3$	62.2 (61.9)	4.8 (4.5)	17.9 (18.1)	$\nu_{max.}$ 3 400 (NH), 3 200—2 500 (bonded OH), 2 130 and 2 110 (N_3), 1 710 (CO_2H), 1 680 cm^{-1} (CONH): $[\alpha]_D^{25}$ +13.3° (*c* 1.2)
(7a)	(14a)	73	89—91 (decomp.)	$C_{16}H_{14}N_4O_3$	61.6 (61.9)	4.3 (4.5)	17.7 (18.1)	$\nu_{max.}$ 3 320 (NH), 3 300—2 300 (bonded OH), 2 120 and 2 085 (N_3), 1 730 (CO_2H), 1 640 cm^{-1} (CONH)
(2b)	(9b)	40	224—225	$C_{16}H_{14}N_2O_6$	58.0 (58.2)	4.4 (4.2)	8.8 (8.5)	$\nu_{max.}$ 3 280 (NH), 3 100—2 300 (bonded OH), 1 720 (CO_2H), 1 640 (CONH), 1 530 and 1 345 cm^{-1} (NO_2)
(3b)	(10b)	64	270 (decomp.)	$C_{16}H_{14}N_2O_6$	58.5 (58.2)	4.0 (4.2)	8.2 (8.5)	$\nu_{max.}$ 3 420 (NH), 3 250—2 300 (bonded OH), 1 695 (CO_2H), 1 610 (CONH), 1 533 and 1 353 cm^{-1} (NO_2)
(4b)	(11b) †	54	246—247	$C_{16}H_{16}N_2O_4$	63.8 (64.0)	5.6 (5.3)	9.0 (9.3)	$\nu_{max.}$ 3 460 (NH), 3 200—2 300 (bonded OH), 1 710 (CO_2H), 1 660 cm^{-1} (CONH)
(5b)	(12b) †	60	185—186	$C_{16}H_{16}N_2O_4$	64.2 (64.0)	5.4 (5.3)	9.1 (9.3)	$\nu_{max.}$ 3 300 (NH), 3 100—2 300 (bonded OH), 1 700 (CO_2H), 1 660 cm^{-1} (CONH)
(6b)	(13b)	30	178—179 (decomp.)	$C_{16}H_{14}N_4O_4$	58.7 (58.9)	4.2 (4.3)	17.0 (17.2)	$\nu_{max.}$ 3 240 (NH), 3 100—2 500 (bonded OH), 2 135 and 2 090 (N_3), 1 720 (CO_2H), 1 640 cm^{-1} (CONH)
(7b)	(14b)	82	176—177	$C_{16}H_{14}N_4O_4$	59.2 (58.9)	4.7 (4.3)	17.6 (17.2)	$\nu_{max.}$ 3 300 (NH), 3 100—2 300 (bonded OH), 2 120 and 2 080 (N_3), 1 730 (CO_2H), 1 630 cm^{-1} (CONH)
(2c)	(9c)	75	207—208	$C_{18}H_{15}N_3O_5$	61.0 (61.2)	4.0 (4.2)	11.7 (11.9)	$\nu_{max.}$ 3 410 (NH), 3 120—2 400 (bonded OH), 1 725 (CO_2H), 1 630 (CONH), 1 525 and 1 345 cm^{-1} (NO_2)
(3c)	(10c)	35	120—122	$C_{18}H_{15}N_3O_5$	60.9 (61.2)	3.9 (4.2)	11.8 (11.9)	$\nu_{max.}$ 3 400 (NH), 3 100—2 530 (bonded OH), 1 735 (CO_2H), 1 645 (CONH), 1 520 and 1 340 cm^{-1} (NO_2)
(4c)	(11c) †	37	241—242	$C_{18}H_{17}N_3O_3$	66.6 (66.9)	5.1 (5.3)	12.9 (13.0)	$\nu_{max.}$ 3 460 (NH), 3 200—2 380 (bonded OH)
(5c)	(12c) †	40	213—214	$C_{18}H_{17}N_3O_3$	67.3 (66.9)	5.6 (5.3)	13.3 (13.0)	$\nu_{max.}$ 3 410 and 3 300 (NH), 3 100—2 500 (bonded OH), 1 710 (CO_2H), 1 630 cm^{-1} (CONH)

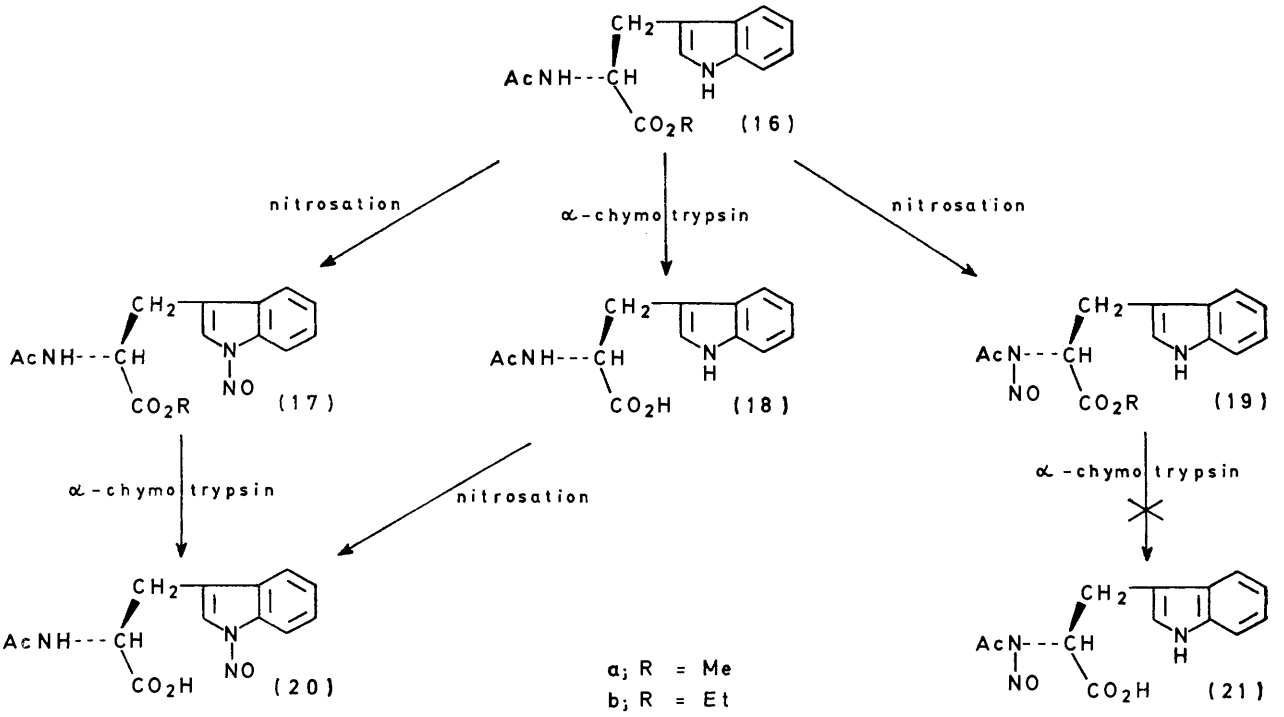
* I.r. spectra were recorded for KBr discs on a Perkin-Elmer 257 instrument (slow scan). Specific rotations were determined on a Perkin-Elmer 141 polarimeter in 95% ethanol. † pH Adjusted to 3 before extraction of the acids into chloroform.

[5] M. F. G. Stevens, *J.C.S. Perkin I*, 1975, 1555.
[6] R. Bonnett and R. Holleyhead, *J.C.S. Perkin I*, 1974, 962.
[7] B. Zerner, R. P. M. Bond, and M. L. Bender, *J. Amer. Chem. Soc.*, 1964, **86**, 3674.

scopic characteristics in accord with those reported for the nitroso-derivative of *N*-acetyl-DL-tryptophan methyl ester.[6] Comparison of the solid phase and solution spectra of the nitroso-derivatives and their precursors was informative (Table 2). In all cases both N–H and C=O (amide) stretching absorptions were shifted to higher frequency in solution, whereas the C=O (ester) band moved to lower frequency. Solvent-induced shifts of N–H and C=O absorptions to higher frequency are characteristic of secondary amides where both groups participate in intermolecular hydrogen bonding in the solid phase.[8] The nitroso-group absorption on the other hand was little influenced by phase change.

Both nitroso-derivatives were smoothly hydrolysed by α-chymotrypsin. In both cases the only product was an unstable nitroso-carboxylic acid, identical with the product obtained by nitrosation of *N*-acetyl-L-tryptophan (18).

These two new pieces of evidence prove that the nitroso-esters have an intact AcNH group, and that nitrosation involves the indolic NH group, as originally proposed.[6] Additional confirmation of the unreactivity of the amidic NH in *N*-acetyl-L-amino-acid esters towards nitrosation was provided when *N*-acetyl-L-phenylalanine methyl ester and *N*-acetyl-L-tyrosine ethyl ester were unchanged following treatment with nitrous acid under conditions which nitrosated the indolic NH of the corresponding tryptophan analogues.



SCHEME 2

TABLE 2

I.r. data ($\nu_{max.}$/cm^{-1}) of *N*-acetyl-L-tryptophan derivatives *

Compound	N–H	C=O (ester)	C=O (amide)	Other
(16a) (KBr)	3 290br	1 749	1 640	
(16a) ($CHCl_3$)	3 470 and 3 420	1 735	1 670	
(16b) (KBr)	3 290br	1 744	1 640	
(16b) ($CHCl_3$)	3 470 and 3 420	1 735	1 670	
(17a) (KBr)	3 312	1 760	1 647	1 438 (N=O)
(17a) ($CHCl_3$)	3 415	1 740	1 672	1 435 (N=O)
(17b) (KBr)	3 320	1 754	1 648	1 440 (N=O)
(17b) ($CHCl_3$)	3 420	1 734	1 670	1 437 (N=O)
(18) (KBr)	3 415 and 3 360		1 620	3 100—2 300 (bonded OH), 1 708 (CO_2H)
(20) (KBr)	3 300br		1 605	3 100—2 400 (bonded OH), 1 705 (CO_2H) 1 442 (N=O)

* Recorded on a Perkin-Elmer 157G spectrometer (slow scan).

EXPERIMENTAL

Enzymic hydrolyses were conducted with an automatic titrator (TTT2) and autoburette (ABU 12) obtained from Radiometer, Copenhagen. Salt-free, lyophilised α-chymotrypsin from bovine pancreas was purchased from Koch–

[8] L. J. Bellamy, 'The Infra-red Spectra of Complex Molecules,' Methuen, London, 1964, p. 213.

Light. ^{1}H N.m.r. spectra were recorded with a Varian HA-100D instrument (tetramethylsilane as internal standard) and mass spectra with an A.E.I.–G.E.C. MS 902 instrument (70 eV; source temperature 100—150°).

N-o-*Nitrobenzoyl*-L-*tryptophan Ethyl Ester* (2c).—This *ester* (84%) was prepared from L-tryptophan ethyl ester hydrochloride and *o*-nitrobenzoyl chloride (1.1 mol. equiv.) in an emulsion of chloroform and aqueous N-sodium carbonate.[2] It crystallised from aqueous ethanol with m.p. 148—149° (Found: C, 63.3; H, 5.0; N, 10.8. $C_{20}H_{19}N_3O_5$ requires C, 63.0; H, 5.0; N, 11.0%), $\nu_{max.}$ (KBr) 3 300(NH), 1 730 (CO_2Et), 1 658 (CONH), and 1 525 and 1 352 cm^{-1} (NO_2). N-p-*Nitrobenzoyl*-L-*tryptophan ethyl ester* (3c), similarly prepared (80%), had m.p. 131—132° (Found: C, 63.3; H, 5.0; N, 10.9%), $\nu_{max.}$ (KBr) 3 280 (NH), 1 745 (CO_2Et), 1 645 (CONH), and 1 518 and 1 342 cm^{-1} (NO_2).

N-o-*Aminobenzoyl*-L-*tryptophan Ethyl Ester* (4c).—Catalytic hydrogenation of the corresponding *N*-*o*-nitrobenzoyl derivative in ethanol over 10% palladium–charcoal afforded the *aminobenzoyltryptophan ester* (80%), m.p. 106—107° (Found: C, 68.6; H, 6.1; N, 11.6. $C_{20}H_{21}N_3O_3$ requires C, 68.4; H, 6.0; N, 11.9%), $\nu_{max.}$ (KBr) 3 430 and 3 340 (NH), 1 740 (CO_2Et), and 1 621 cm^{-1} (CONH). N-p-*Aminobenzoyl*-L-*tryptophan ethyl ester* (5c), similarly prepared (82%), had m.p. 186—187° (Found: C, 68.1; H, 6.0; N, 11.7%), $\nu_{max.}$ (KBr) 3 422 and 3 338 (NH), 1 738 (CO_2Et), and 1 626 cm^{-1} (CONH).

Enzymic Hydrolysis of N-o-*Nitrobenzoyl*-L-*phenylalanine Ethyl Ester* (2a).—The substrate (1.0 g)[2] was finely dispersed in water (60 ml) at 25 °C, and the pH set at 7.8 with the instrument operating in the pH-stat mode. α-Chymotrypsin (70 mg) in water (10 ml) was added in one portion. The liberated acid required 29.5 ml of 0.1N-sodium hydroxide in 3.5 h to maintain the pH at 7.8 (100% hydrolysis). The aqueous layer was acidified to pH 1 with 2N-hydrochloric acid, and the product extracted into chloroform. Care was required to avoid formation of an emulsion. The dried (Na_2SO_4), evaporated, chloroform layer afforded *N*-*o*-nitrobenzoyl-L-phenylalanine (9a), which crystallised from benzene–light petroleum as white needles (66%), m.p. 154—155° (Found: C, 61.2; H, 4.6; N, 8.8. Calc. for $C_{16}H_{14}N_2O_5$: C, 61.1; H, 4.5; N, 8.9%), $\nu_{max.}$ (KBr) 3 380 (NH), 3 180—2 350 (bonded OH), 1 735 (CO_2H), 1 625 (CONH), and 1 530 and 1 350 cm^{-1} (NO_2), $[\alpha]_D^{25}$ −20.0° (*c* 1 in EtOH) (lit.,[2] $[\alpha]_D^{25}$ −18.75°).

Other *N*-aroyl-L-amino-acids prepared by the same general procedure are listed in Table 1.

The hydrolysis could be greatly accelerated by dissolving the substrate in 50% aqueous dimethyl sulphoxide. Reactions were complete within 15 min. However, isolation of the L-amino-acids was more difficult owing to the formation of stable emulsions.

Attempted Hydrolysis of the Triazinone (15a).—The triazinone (1.0 g) [2] suspended in water (100 ml) at 25 °C and pH 7.8 was treated with α-chymotrypsin (70 mg). The mixture was stirred for 2 h but no hydrolysis occurred. A suspension of *N*-*o*-nitrobenzoyl-L-phenylalanine ethyl ester (1.0 g) in water (100 ml) was added; the reaction consumed 28.7 ml of 0.1N-sodium hydroxide (*i.e.* 97% hydrolysis of substrate) in 3.5 h. The mixture was adjusted to pH 10 and extracted with chloroform. The dried (Na_2SO_4), evaporated chloroform layer afforded unchanged triazinone (0.9 g), m.p. 61—63° (lit.,[2] 60—62°) (from aqueous ethanol) (identical i.r. spectrum).

The aqueous layer, acidified to pH 1, was extracted with chloroform and the material obtained crystallised as before. The product, *N*-*o*-nitrobenzoyl-L-phenylalanine (56%) was identical (i.r.) with the aforementioned sample, and had m.p. 154—155°, $[\alpha]_D^{25}$ −19.3° (*c* 1.3 in EtOH).

N-*Acetyl*-N(1)-*nitroso*-L-*tryptophan Methyl Ester* (17a).—*N*-Acetyl-L-tryptophan methyl ester (0.56 g) (prepared by esterification of *N*-acetyl-L-tryptophan with thionyl chloride in anhydrous methanol) and sodium nitrite (0.32 g) were shaken at 0 °C in ether (10 ml), water (2 ml), and acetic acid (0.4 ml) for 4 h. The yellow nitrosotryptophan methyl ester (60%) crystallised from ether–ethyl acetate as needles, m.p. 139—140° (lit.,[6] 145—149° for the DL form); *m/e* 289 (M^+, <1%), 130 (indol-3-yl-CH_2^+, 63%), 43 (CH_3–C≡O$^+$, 44%), and 30 (NO^+, 100%); δ ($CDCl_3$) 8.1 (1 H, m), 7.6—7.3 (4 H, m), 6.45br (s, NH), 5.0 (m, asym. C–H), 3.7 (s, O–CH_3), 3.3 (m, –CH_2·CH<), and 1.98 (s, Ac); $\lambda_{max.}$ (EtOH) 266, 273, and 334 nm.

N-*Acetyl*-N(1)-*nitroso*-L-*tryptophan Ethyl Ester* (17b).—Similarly prepared, from *N*-acetyl-L-tryptophan ethyl ester and nitrite, the *nitroso-derivative* (60%) crystallised from ether–ethyl acetate as yellow needles, m.p. 134—135 (Found: C, 59.6; H, 5.4; N, 13.7. $C_{15}H_{17}N_3O_4$ requires C, 59.4; H, 5.6; N, 13.9%); *m/e* 303 (M^+, <1%), 130 (100%), and 30 (22%); δ ($CDCl_3$) 8.1 (1 H, m), 7.4 (4 H, m), 6.25br (NH), 4.9 (m, asym. CH), 4.07 (q, CH_2·CH_3), 3.15 (d, –CH_2·CH<), 1.90 (s, Ac), and 1.15 (t, CH_2·CH_3); $\lambda_{max.}$ (EtOH) 266, 274, and 335 nm.

Attempted nitrosation of *N*-acetyl-L-phenylalanine methyl ester or *N*-acetyl-L-tyrosine ethyl ester under the same conditions led to the quantitative recovery of the starting ester.

N-*Acetyl*-N(1)-*nitroso*-L-*tryptophan*(20).—*N*-Acetyl-L-tryptophan (2.64 g) and sodium nitrite (1.62 g) in water (100 ml) were protected from light and shaken at 5—10 °C for 2 h. The yellow solution was acidified with 10N-hydrochloric acid and the nitroso-derivative immediately extracted into ethyl acetate. The concentrated solution deposited yellow plates of the nitrosotryptophan (0.5 g), m.p. 125—127° (decomp) (lit.,[6] 137—139° for the DL-form): $\lambda_{max.}$ (EtOH) 265, 273infl., and 333 nm. A further 0.6 g of product was obtained by diluting the mother liquor with light petroleum.

Enzymic Hydrolysis of N-*Acetyl*-N(1)-*nitroso*-L-*tryptophan Methyl Ester* (17a).—Finely powdered ester (17a) (116 mg) was suspended in water (10 ml) at 25 °C and hydrolysed by α-chymotrypsin (7 mg) at pH 7.8 in a light-protected flask. In 3.5 h, 19.8 ml of 0.02N-sodium hydroxide was required to maintain the pH at 7.8 (*i.e.* to 99% hydrolysis). The clear pale yellow hydrolysate was first extracted with ether (2 × 10 ml) to remove unchanged ester, then acidified to pH 1 and rapidly re-extracted with ether (3 × 20 ml). The combined, dried (Na_2SO_4) extracts were evaporated to dryness. The yellow crystalline residue (90 mg) was identical (t.l.c.; u.v. and i.r. spectra) with the sample of *N*-acetyl-*N*(1)-nitroso-L-tryptophan prepared by nitrosation of *N*-acetyl-L-tryptophan.

Hydrolysis of *N*-acetyl-*N*(1)-nitroso-L-tryptophan ethyl ester (17b) with α-chymotrypsin likewise afforded *N*-acetyl-*N*(1)-nitroso-L-tryptophan (82%).

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