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Pyridyl Esters of Peptides As Synthetic Substrates of Pepsin*

Goverdhan P. Sachdev and Joseph S. Fruton†

ABSTRACT: The synthesis of a series of new pepsin substrates of the type A-Phe-Phe-B is described, in which B is a pyridylalkyloxy group, A is benzyloxycarbonyl (Z), Z-Gly, or Z-Gly-Gly, and the Phe-Phe linkage is the only pepsin-sensitive bond. In their cationic form, these compounds are moderately soluble in aqueous solution at pH 2–4, and some of the compounds (*e.g.*, with A = Z-Gly-Gly) are among the most sensitive synthetic substrates hitherto found for pepsin. The determination of the kinetic parameters for their enzymic hydrolysis has shown that changes in the structure of the A and B groups of A-Phe-Phe-B may have very large effects on the value of k_{cat} (*ca.* 500-fold change) with only small accompanying changes in the value of K_M. These kinetic data emphasize the importance, for the kinetic specificity of pepsin, of interactions between the A or B groups of the substrate with enzymic loci that are relatively distant from the site of catalytic action, and are consistent with the possibility that such secondary interactions may alter the conformation of catalytically important groups in the enzyme so as to alter greatly the efficiency of catalysis.

Recent work in this laboratory has been directed toward the delineation of the specificity of pepsin action on the X–Y bond of synthetic peptide substrates of the general type AX–YB. The study of the effect of varying the nature of the amino acid residue X or Y (the other is always L-phenylalanyl) has shown that when A = Z-His[1] and B = OMe or OEt, pepsin exhibits preference for the cleavage of X–Y units in which X = Phe and Y = Trp, Phe, or Tyr (Inouye and Fruton, 1967; Hollands *et al.*, 1969; Trout and Fruton, 1969). In the present study, the effect of altering the nature of the groups A and B in substrates of the type A-Phe-Phe-B has been examined; in these substrates, the Phe–Phe bond is the only one cleaved by the enzyme, and the initial rate of cleavage was determined by an automatic ninhydrin method. As in earlier studies, cationic substrates were employed, since they confer moderate solubility in the pH range 1–5 on peptide derivatives blocked at both the amino- and carboxyl-terminal residues. The present series of substrates contains a cationic group in the B portion of A-Phe-Phe-B, in the form of a pyridiniumalkyloxy group, and the substrates are therefore esters of pyridine alcohols. The 4-picolyl esters of peptides have recently been used with marked success in a repetitive method of peptide synthesis, introduced by Young and his colleagues (Garner *et al.*, 1968; Garner and Young, 1969). These investigators prepared the esters by the reaction of a benzyloxycarbonylamino acid with 4-picolyl chloride in the presence of an organic base, with dimethyl-

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† To whom requests for reprints should be addressed.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 2485 (1966), are: $TyrI_2$, 3,5-diiodo-L-tyrosyl; $Phe(NO_2)$, *p*-nitro-L-phenylalanyl; OM3P, 3-pyridylmethoxy; OM4P, 4-pyridylmethoxy; OP3P, 3-(3-pyridyl)propyl-1-oxy; OP4P, 3-(4-pyridyl)propyl-1-oxy; In, isonicotinoyl; Osu, *N*-oxysuccinimido. Unless otherwise noted, the abbreviated designation of amino acid residues denotes the L form.

formamide as the solvent. In the present work, the esterification was performed by the reaction of the acid with one of several pyridine alcohols [3-pyridylcarbinol, 4-pyridylcarbinol, 3-(3-pyridyl)propan-1-ol, and 3-(4-pyridyl)propan-1-ol] in the presence of dicyclohexylcarbodiimide at 0°, with methylene chloride or ethyl acetate as the solvent. Epand and Wilson (1963) prepared Bz-Gly-OM4P by prolonged heating of hippuric acid with 4-pyridylcarbinol in the presence of H_2SO_4, with toluene as the solvent.

Experimental Section

Chromatography. Examination of the peptides prepared in this work and of the cleavage products released by pepsin was performed with silica gel G as the supporting phase (Eastman Chromagram sheets 6061). The following solvent systems were used: (A) benzene–ethyl acetate (1:1, v/v), (B) ethyl acetate–methanol (95:5, v/v), (C) ethyl acetate, and (D) 1-butanol–acetic acid–water (4:1:1, v/v).

Z-Phe-OM3P. Z-Phe (1.5 g, 5 mmoles) and 3-pyridylcarbinol[2] (0.56 g, 5 mmoles) were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (1.1 g, 5 mmoles), with CH_2Cl_2 (20 ml) as the solvent, to yield 1.9 g (96%) of the product. After recrystallization from ethyl acetate–hexane, it melted at 93–94°, $[\alpha]_D^{23}$ +10.0° (*c* 1, $CHCl_3$). Chromatography (solvent A) gave a single spot of R_F 0.43 (iodine). *Anal.* Calcd for $C_{23}H_{22}N_2O_4$ (390.5): N, 7.2. Found: N, 7.0.

Z-Gly-OM3P. Z-Gly (1.1 g, 5 mmoles) and 3-pyridylcarbinol (0.56 g, 5 mmoles) were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (1.1 g, 5 mmoles), with ethyl acetate (25 ml) as the solvent, to yield 1.4 g (93%) of the product. After recrystallization from ethyl acetate-hexane, it melted at 107–108°. Chromatography (solvent B) gave a single spot of R_F 0.71 (iodine). *Anal.* Calcd for $C_{16}H_{16}N_2O_4$ (300.3): N, 9.3. Found: N, 9.2.

Z-Phe-Phe-OM3P. Z-Phe-OM3P (1.0 g, 2.5 mmoles) was treated with a saturated solution of HBr in glacial acetic acid (5 ml) for 30 min at room temperature, and ether (100 ml) was then added. After 18 hr at 0°, the solid hydrobromide was collected by filtration, washed twice with 25-ml portions of ether, and dissolved in water (5 ml). The aqueous solution was chilled in an ice bath, CH_2Cl_2 (50 ml) was added, and the mixture was shaken with a 50% saturated aqueous K_2CO_3 solution (8 ml). The aqueous layer was extracted twice with 20-ml portions of CH_2Cl_2, and the combined CH_2Cl_2 extract was dried over anhydrous $MgSO_4$. Evaporation of the solvent under reduced pressure gave an oily Phe-OM3P (0.62 g) which was coupled in the usual manner with Z-Phe (0.8 g, 2.6 mmoles) in the presence of dicyclohexylcarbodiimide (0.65 g, 2.9 mmoles), with CH_2Cl_2 (20 ml) as the solvent, to yield 1.2 g (88%) of the product. After recrystallization from ethanol, it melted at 154–155°, $[\alpha]_D^{23}$ +7.6° (*c* 1, $CHCl_3$). Chromatography (solvent B) gave a single spot of R_F 0.88 (iodine). *Anal.* Calcd for $C_{32}H_{31}N_3O_5$ (537.6): C, 71.5; H, 5.8; N, 7.8. Found: C, 71.4; H, 6.1; N, 7.7.

Z-Phe-Gly-OM3P. Z-Phe (1.35 g, 4.5 mmoles) was coupled in the usual manner with Gly-OM3P (derived from 1.5 g (5 mmoles) of Z-Gly-OM3P in the manner described above for Phe-OM3P, except that the treatment with HBr was restricted to 10 min) in the presence of dicyclohexylcarbodiimide (1.1 g, 5 mmoles), with CH_2Cl_2 (25 ml) as the solvent. After recrystallization of the product from ethyl acetate–hexane, the yield was 1.1 g (50%), mp 106–107°. Chromatography (solvent B) gave a single spot of R_F 0.57 (iodine). *Anal.* Calcd for $C_{25}H_{25}N_3O_5$ (447.5): N, 9.4. Found: N, 9.3.

When this compound was subjected to the action of pepsin (1 mM substrate, 0.68 μM enzyme, pH 2.0, 37°, 20 min), there was no detectable release of ninhydrin-reactive material.

Z-Gly-Phe-Phe-OM3P. Z-Gly (0.32 g, 1.5 mmoles) and Phe-Phe-OM3P (derived from 0.81 g (1.5 mmoles) of Z-Phe-Phe-OM3P in the manner described for Phe-OM3P) were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (0.35 g, 1.6 mmoles), with tetrahydrofuran (15 ml) as the solvent, to yield 0.65 g (74%) of the product. After recrystallization from ethyl acetate, it melted at 141–142°, $[\alpha]_D^{23}$ −7.7° (*c* 1, $CHCl_3$). Chromatography (solvent C) gave a single spot of R_F 0.50 (iodine). *Anal.* Calcd for $C_{34}H_{34}N_4O_6$ (594.6): C, 68.7; H, 5.8; N, 9.4. Found: C, 68.7; H, 5.8; N, 9.3. The same product was obtained in 67% yield by the coupling of Z-Gly-Phe with Phe-OM3P in the presence of dicyclohexylcarbodiimide, with tetrahydrofuran as the solvent.

Chromatographic examination (solvent D) of a peptic hydrolysate of this compound (1.25 mM substrate, 0.14 μM pepsin, pH 2.0, 37°, 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.26 (identical with that of Phe-OM3P). There was no ninhydrin-positive component of R_F 0.53 (the value for Phe-Phe-OM3P, prepared by acidolysis of Z-Phe-Phe-OM3P).

Z-Phe-Phe-Gly-OM3P. Z-Phe (0.72 g, 2.2 mmoles) and Phe-Gly-OMP (derived from 1.0 g (2.2 mmoles) of Z-Phe-Gly-OM3P in the manner described for Phe-OM3P) were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (0.55 g, 2.5 mmoles), with tetrahydrofuran (20 ml) as the solvent, to yield 1.2 g (93%) of the product. After recrystallization from ethyl acetate, it melted at 180–181°, $[\alpha]_D^{23}$ −29.9° (*c* 1, $CHCl_3$). Chromatography (solvent C) gave a single spot of R_F 0.45 (iodine). *Anal.* Calcd for $C_{34}H_{34}N_4O_6$ (594.6): C, 68.7; H, 5.8; N, 9.4. Found: C, 68.7; H, 5.8; N, 9.4. The same product was obtained in 98% yield by the coupling of Z-Phe-Phe with Gly-OM3P in the presence of dicyclohexylcarbodiimide, with tetrahydrofuran as the solvent.

Chromatographic examination (solvent D) of a peptic hydrolysate of this compound (1.25 mM substrate, 0.35 μM pepsin, pH 2.0, 37°, 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.29 (identical with that for Phe-Gly-OM3P, prepared by acidolysis of Z-Phe-Gly-OM3P). There was no ninhydrin-positive component of R_F 0.37 (the value for Gly-OM3P, prepared by acidolysis of Z-Gly-OM3P).

Z-Gly-Gly-Phe-Phe-OM3P. Z-Gly-Gly (0.5 g, 2 mmoles) and Phe-Phe-OM3P (derived from 1.0 g (1.8 mmoles) of Z-Phe-Phe-OM3P in the manner described above for Phe-OM3P) were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (0.45 g, 2.1 mmoles), with tetrahydrofuran (200 ml) as the solvent. A portion of the product separated with the dicyclohexylurea and was extracted with dioxane: total yield 0.75 g (64%), mp 179–180°, $[\alpha]_D^{23}$ −3.2°

[2] The pyridine alcohols were supplied by Fluka A. G., Buchs, Switzerland (U. S. distributor, Columbia Organic Chemicals Co., Columbia, S. C. 29205).

(*c* 0.5, $CHCl_3$). Chromatography (solvent B) gave a single spot of R_F 0.70 (iodine). *Anal.* Calcd for $C_{36}H_{37}N_5O_7$ (651.7): C, 66.3; H, 5.7; N, 10.7. Found: C, 65.9; H, 5.8; N, 10.7.

Chromatographic examination (solvent D) of a peptic hydrolysate of the compound (1.25 mM substrate, 0.16 μM pepsin, pH 2.0, 37°, 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.26 (identical with that for Phe-OM3P). There was no ninhydrin-positive component of R_F 0.53 (the value for Phe-Phe-OM3P).

Z-Gly-Phe-Phe-Gly-OM3P. Z-Gly-Phe (0.6 g, 1.7 mmoles) and Phe-Gly-OM3P (derived from 0.77 g (1.7 mmoles) of Z-Phe-Gly-OM3P in the manner described for Phe-OM3P) were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (0.38 g, 1.8 mmoles), with tetrahydrofuran (15 ml) as the solvent, to yield 0.77 g (70%) of the product: mp 166–167°, $[\alpha]_D^{23}$ −25.8° (*c* 1, $CHCl_3$). Chromatography (solvent B) gave a single spot of R_F 0.72 (iodine). *Anal.* Calcd for $C_{36}H_{37}N_5O_7$ (651.7): C, 66.3; H, 5.7; N, 10.7. Found: C, 66.1; H, 6.2; N, 10.6.

Chromatographic examination (solvent D) of a peptic hydrolysate of this compound (1.25 mM substrate, 0.35 μM pepsin, pH 2.0, 37°, 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.29 (identical with that for Phe-Gly-OM3P). There was no ninhydrin-positive component of R_F 0.21 (the value for Phe-Phe-Gly-OM3P).

Z-Phe-OM4P. This compound was prepared from Z-Phe and 4-pyridylcarbinol in the manner described above for Z-Phe-OM3P: yield 92%, mp 87–88°, $[\alpha]_D^{23}$ +11.7° (*c* 1, $CHCl_3$) and −31.5° (*c* 1, dimethylformamide); lit. (Garner *et al.*, 1968) mp 87.5–89.5°, $[\alpha]_D^{20}$ −33° (*c* 1, dimethylformamide). Chromatography (solvent B) gave a single spot of R_F 0.72 (iodine). *Anal.* Calcd for $C_{23}H_{22}N_2O_4$ (390.5): N, 7.2. Found: N, 7.3.

Z-Phe-Phe-OM4P. Z-Phe (1.3 g, 4.3 mmoles) and Phe-OM4P (derived from 4.4 mmoles of Z-Phe-OM4P in the usual manner) were coupled in the presence of dicyclohexylcarbodiimide (1.0 g, 4.7 mmoles). After recrystallization from ethyl acetate–hexane, the product melted at 126–127°: yield 1.5 g (59%), $[\alpha]_D^{23}$ +11.7° (*c* 1, $CHCl_3$). Chromatography (solvent B) gave a single spot of R_F 0.66 (iodine). *Anal.* Calcd for $C_{32}H_{31}N_3O_5$ (537.6): C, 71.5; H, 5.8; N, 7.8. Found: C, 71.3; H, 5.8; N, 7.9. The same product was obtained in 54% yield by coupling Phe-OM4P with Z-Phe-Osu (Anderson *et al.*, 1964) with dimethoxyethane as the solvent.

Z-Phe-Phe-OP3P. Z-Phe (2.0 g, 6.6 mmoles) and 3-(3-pyridyl)propan-1-ol (0.93 g, 6.6 mmoles) were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (1.6 g, 6.6 mmoles) with CH_2Cl_2 (40 ml) as the solvent to yield Z-Phe-OP3P as an oily residue that could not be crystallized. The product was converted into Phe-OP3P which was coupled in the usual manner with Z-Phe (2.0 g, 6.6 mmoles) with tetrahydrofuran as the solvent (40 ml) to yield 2.4 g (65%) of the dipeptide derivative. After recrystallization from ethyl acetate–hexane, it melted at 86–87°, $[\alpha]_D^{23}$ +17.1° (*c* 1, $CHCl_3$). Chromatography (solvent B) gave a single spot of R_F 0.65 (iodine). *Anal.* Calcd for $C_{34}H_{35}N_3O_5$ (565.7): C, 72.2; H, 6.2; N, 7.4. Found: C, 72.0; H, 6.3; N, 7.3.

Z-Gly-Phe-Phe-OP3P. Z-Gly (0.36 g, 1.7 mmoles) and Phe-Phe-OP3P (obtained from 0.89 g of Z-Phe-Phe-OP3P) were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (0.36 g, 1.7 mmoles) with tetrahydrofuran (40 ml) as the solvent to yield 0.71 g (68%) of the product. After recrystallization from ethyl acetate–hexane, it melted at 126–127°, $[\alpha]_D^{23}$ +2.1° (*c* 1, $CHCl_3$). Chromatography (solvent B) gave a single spot of R_F 0.66 (iodine). *Anal.* Calcd for $C_{36}H_{38}N_4O_6$ (622.7): C, 69.6; H, 6.2; N, 9.0. Found: C, 70.0; H, 6.2; N, 9.2.

Chromatographic examination (solvent D) of a peptic hydrolysate of the compound (1.2 mM substrate, 0.27 μM pepsin, pH 2.0, 37°, 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.29 (identical with that of Phe-OP3P). There was no ninhydrin-positive component of R_F 0.55 (the value for Phe-Phe-OP3P, prepared by acidolysis of Z-Phe-Phe-OP4P).

Z-Gly-Gly-Phe-Phe-OP3P. Z-Gly-Gly (0.43 g, 1.7 mmoles) and Phe-Phe-OP3P (derived from 0.89 g (2.9 mmoles) of Z-Phe-Phe-OP3P in the manner described for Phe-OM3P) were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (0.36 g, 1.7 mmoles), with tetrahydrofuran (75 ml) as the solvent, to yield 0.82 g (71%) of the product. After recrystallization from ethyl acetate–hexane, it melted at 129–130°, $[\alpha]_D^{23}$ +19.1° (*c* 1, $CHCl_3$). Chromatography (solvent B) gave a single spot of R_F 0.28 (iodine). *Anal.* Calcd for $C_{38}H_{41}N_5O_7$ (679.8): C, 67.1; H, 6.1; N, 10.3. Found: C, 67.1; H, 6.3; N, 10.4.

Chromatographic examination (solvent D) of a peptic hydrolysate of the compound (1.25 mM substrate, 0.27 μM pepsin, pH 2.0; 37°; 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.29 (identical with that for Phe-OP3P). There were no ninhydrin-positive components of R_F 0.55 (the value for Phe-Phe-OP3P) or of R_F 0.25 (the value for Gly-Phe-Phe-OP3P).

Z-Phe-Phe-OP4P. Z-Phe (1.5 g, 5 mmoles) and 3-(4-pyridyl)-propan-1-ol (0.69 g, 5 mmoles) were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (1.1 g, 5 mmoles), with CH_2Cl_2 (15 ml) as the solvent, to yield Z-Phe-OP4P as an oil that could not be crystallized. The product was converted into Phe-OP4P (in the manner described for Phe-OM3P), which was coupled in the usual manner with Z-Phe (1.5 g, 5 mmoles) in the presence of dicyclohexylcarbodiimide (1.1 g, 5 mmoles), with CH_2Cl_2 (15 ml) as the solvent, to yield 2.1 g (75%) of the dipeptide derivative. After recrystallization from ethyl acetate, it melted at 120–121°, $[\alpha]_D^{23}$ +19.1° (*c* 1, $CHCl_3$). Chromatography (solvent B) gave a single spot of R_F 0.83 (iodine). *Anal.* Calcd for $C_{34}H_{35}N_3O_5$ (565.7): C, 72.2; H, 6.2; N, 7.4. Found: C, 72.0; H, 6.5; N, 7.2.

Z-Phe(NO_2)-Phe-OP4P. Z-Phe(NO_2) (1.9 g, 5.5 mmoles) (Inouye and Fruton, 1967) was coupled with Phe-OP4P in the manner described above for Z-Phe-Phe-OP4P. After recrystallization from ethyl acetate–hexane, it melted at 111–112°, yield 1.9 g (58%). Chromatography (solvent B) gave a single spot of R_F 0.84 (iodine). *Anal.* Calcd for $C_{34}H_{34}N_4O_7$ (610.7): C, 66.8; H, 5.6; N, 9.2. Found: C, 66.5; H, 5.9; N, 9.2.

Z-Gly-Phe-OP4P. Z-Gly (0.5 g, 2.4 mmoles) and Phe-OP4P (derived from 1.0 g of the Z compound) were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (0.5 g, 2.4 mmoles). After recrystallization from ethyl acetate–hexane, the product melted at 71–72°, $[\alpha]_D^{24}$ +26.0° (*c* 1, $CHCl_3$), yield 0.92 g (80%). Chromatography (solvent B) gave a single spot of R_F 0.55 (iodine). *Anal.* Calcd for $C_{27}H_{29}N_3O_5$ (475.6): N, 8.8. Found: N, 9.0.

When this compound was subjected to the action of pepsin (1.25 mM substrate, 0.68 μM enzyme, pH 2.0; 37°; 20 min),

there was no detectable release of ninhydrin-reactive material.

Z-Gly-Phe-Phe-OP4P. Z-Gly (0.35 g, 1.7 mmoles) and Phe-Phe-OP4P (derived from 1.0 g (1.7 mmoles) of Z-Phe-Phe-OP4P in the manner described for Phe-OM3P) were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (0.35 g, 1.7 mmoles), with CH_2Cl_2 (35 ml) as the solvent, to yield 0.85 g (80%) of the product. After recrystallization from ethyl acetate–petroleum ether (bp 30–60°), it melted at 126–127°, $[\alpha]_D^{23}$ +3.2° (*c* 1, $CHCl_3$). Chromatography (solvent B) gave a single spot of R_F 0.55 (iodine). *Anal.* Calcd for $C_{36}H_{38}N_4O_6$ (622.7): C, 69.6; H, 6.2; N, 9.0. Found: C, 69.4; H, 6.2; N, 8.7.

Chromatographic examination (solvent D) of a peptic hydrolysate of the compound (1.25 mM substrate, 0.27 μM pepsin, pH 2.0, 37°, 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.19 (identical with that for Phe-OP4P). There was no ninhydrin-positive component of R_F 0.48 (the value for Phe-Phe-OP4P, prepared by acidolysis of Z-Phe-Phe-OP4P).

Z-Phe-Phe-Gly-OP4P. Z-Gly-OP4P was prepared in a manner analogous to that described above for Z-Gly-OM3P, but could not be crystallized. The oil was converted into Gly-OP4P by treatment with HBr–acetic acid, and conversion of the hydrobromide into the free ester, in the manner described above for Gly-OM3P. Gly-OP4P (1.5 g, 7.7 mmoles) was coupled with Z-Phe (2.3 g, 7.7 mmoles) in the presence of dicyclohexylcarbodiimide (1.6 g, 7.7 mmoles), with CH_2Cl_2 (20 ml) as the solvent, to yield Z-Phe-Gly-OP4P (3.0 g, 80%) which could not be crystallized. This product (1.5 g, 3.2 mmoles) was converted into Phe-Gly-OP4P (in the manner described for Phe-OM3P) which was coupled with Z-Phe (1.0 g, 3.3 mmoles) in the presence of dicyclohexylcarbodiimide (0.7 g, 3.3 mmoles), with tetrahydrofuran (30 ml) as the solvent, to yield 1.8 g (90%) of the tripeptide derivative. After recrystallization from ethyl acetate–petroleum ether, it melted at 105–106°, $[\alpha]_D^{23}$ −36.5° (*c* 1, $CHCl_3$). Chromatography (solvent A) gave a single spot of R_F 0.57 (iodine). *Anal.* Calcd for $C_{36}H_{38}N_4O_6$ (622.7): C, 69.6; H, 6.2; N, 9.0. Found: C, 70.0; H, 6.1; N, 9.0.

Chromatographic examination (solvent D) of a peptic hydrolysate of the compound (1.25 mM substrate, 0.27 μM pepsin, pH 2.0, 37°, 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.14 (identical with that for Phe-Gly-OP4P). There was no ninhydrin-positive component of R_F 0.33 (the value for Gly-OP4P).

Z-Gly-Gly-Phe-Phe-OP4P. Z-Gly-Gly (0.45 g, 1.8 mmoles) and Phe-Phe-OP4P (derived from 1.0 g (1.8 mmoles) of Z-Phe-Phe-OP4P in the manner described for Phe-OM3P) were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (0.4 g, 1.8 mmoles), with tetrahydrofuran (40 ml) as the solvent, to yield 1.9 g (85%) of the product. After recrystallization from tetrahydrofuran–hexane, it melted at 156–157°, $[\alpha]_D^{23}$ +9.7° (*c* 1, $CHCl_3$). Chromatography (solvent B) gave a single spot of R_F 0.32 (iodine). *Anal.* Calcd for $C_{38}H_{41}N_5O_7$ (679.8): C, 67.1; H, 6.1; N, 10.3. Found: C, 66.9; H, 6.1; N, 10.0.

Chromatographic examination (solvent D) of a peptic hydrolysate of the compound (0.75 mM substrate, 0.27 μM pepsin, pH 2.0, 37°, 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.19 (identical with that for Phe-OP4P). There were no ninhydrin-positive components of R_F 0.48 (the value for Phe-Phe-OP4P) or of R_F 0.23 (the value for Gly-Phe-Phe-OP4P).

Gly-Gly-Phe-Phe-OP4P. The above compound (0.5 g, 0.73 mmole) was treated with HBr in glacial acetic acid (3 ml) in the usual manner, and the resulting tetrapeptide hydrobromide was converted into the free base in the manner described above for Phe-OM3P. The product was recrystallized from chloroform–petroleum ether: yield 0.18 g, mp 111–112°. Chromatography (solvent D) gave a single spot of R_F 0.21 (ninhydrin). *Anal.* Calcd for $C_{30}H_{35}N_5O_5$ (545.65): C, 66.0; H, 6.5; N, 12.8. Found: C, 65.8; H, 6.7; N, 12.7.

Z-Gly-Gly-Phe(NO_2)-Phe-OP4P. Z-Gly-Gly (0.6 g, 1.6 mmoles) and Phe(NO_2)-Phe-OP4P (derived from 1.0 g of the Z compound) were coupled in the manner described above for Z-Gly-Gly-Phe-Phe-OP4P. After recrystallization from ethyl acetate–petroleum ether, the product melted at 154–155°, yield 0.94 g (72%). Chromatography (solvent B) gave a single spot of R_F 0.34 (iodine). *Anal.* Calcd for $C_{38}H_{41}N_6O_9$ (724.8): C, 63.0; H, 5.6; N, 11.6. Found: C, 62.8; H, 5.9; N, 11.4.

Z-Gly-Phe-Phe-Gly-OP4P. Z-Gly-Phe (0.84 g, 2.5 mmoles) and Phe-Gly-OP4P (derived from 1.2 g of the oily Z-Phe-Gly-OP4P in the manner described for Phe-OM3P) were coupled in the usual manner in the presence of dicyclohexylcarbodiimide (0.52 g, 2.5 mmoles), with tetrahydrofuran (40 ml) as the solvent, to yield 1.35 g (80%) of the product: mp 167–168°, $[\alpha]_D^{23}$ −28.7° (*c* 1, $CHCl_3$). Chromatography (solvent B) gave a single spot of R_F 0.45 (iodine). *Anal.* Calcd for $C_{38}H_{41}N_5O_7$ (679.8); C, 67.1; H, 6.1; N, 10.3. Found: C, 67.2; H, 6.1; N, 10.1.

Chromatographic examination (solvent D) of a peptic hydrolysate of the compound (0.2 mM substrate, 0.27 μM pepsin, pH 2.0, 37°, 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.14 (identical with that for Phe-Gly-OP4P, prepared by acidolysis of Z-Phe-Gly-OP4P). There were no ninhydrin-positive components of R_F 0.10 (the value for Phe-Phe-Gly-OP4P) or of R_F 0.33 (the value for Gly-OP4P).

Z-Gly-Gly-Gly-Phe-OP4P. Z-Gly-Gly-Gly (1.1 g, 3.3 mM) and Phe-OP4P (1.0 g, 3.4 mM) (derived from Z-Phe-OP4P (1.5 g) in the manner described for Phe-OM3P) were coupled in the presence of dicyclohexylcarbodiimide (0.65 g, 3.5 mM) with dimethylformamide (40 ml) as the solvent to yield 1.2 g (61%) of the product. After recrystallization from ethyl acetate–hexane, it melted at 124–125°, $[\alpha]_D^{24}$ −10.3° (*c* 1, dimethylformamide). Chromatography (solvent B) gave a single spot of R_F 0.55. *Anal.* Calcd for $C_{31}H_{35}N_5O_7$ (589.7): C, 63.1; H, 6.0; N, 11.9. Found: C, 62.8; H, 6.2; N, 12.0.

When this compound was subjected to the action of pepsin (1 mM substrate, 0.68 μM enzyme, pH 2.0, 37°, 20 min), there was no detectable release of ninhydrin-reactive material.

Isonicotinic Acid N-Hydroxysuccinimido Ester. Isonicotinic acid (1.23 g, 10 mM) and *N*-hydroxysuccinimide (1.15 g, 10 mM) were coupled in the presence of dicyclohexylcarbodiimide (2.1 g, 10 mM), with dimethylformamide (55 ml) as the solvent. Crystallization from ethyl acetate–hexane yielded 1.4 g (64%) of the product, mp 139–140°. After recrystallization from ethanol, it melted at 141–142°. Chromatography (ethyl acetate–methyl ethyl ketone–toluene–formic acid, 5:3:1:1, v/v) gave a single spot of R_F 0.73 (iodine). *Anal.* Calcd for $C_{10}H_8N_2O_4$ (220.2): N, 12.7. Found: N, 12.8.

In-Phe-Phe-OP4P. Phe-Phe-OP4P (0.65 g, 1.5 mmoles),

(obtained from Z-Phe-Phe-OP4P (1.0 g) in the manner described for Z-Phe-OM3P) and In-Osu (0.33 g, 1.5 mM) were coupled at room temperature for 45 min, with dimethoxyethane (20 ml) as the solvent. Water (100 ml) was added, and the resulting crystalline product was dissolved in CH_2Cl_2 (25 ml) and washed successively with $NaHCO_3$ (5%) and water. After being dried over $MgSO_4$, the CH_2Cl_2 solution was evaporated *in vacuo*. The product crystallized slowly from ethyl acetate–hexane: 0.75 g (95.2%), mp 112–113°, $[\alpha]_D^{24}$ −43.1° (*c* 1, dimethylformamide). Chromatography (solvent B) gave a single spot of R_F 0.35 (iodine). *Anal.* Calcd for $C_{32}H_{32}N_4O_4$ (536.6): C, 71.6; H, 6.0; N, 10.4. Found: C, 71.6; H, 6.2; N, 10.6.

Chromatographic examination (solvent D) of a peptic hydrolysate of the compound (1.25 mM substrate, 0.27 μM pepsin, pH 2.0, 37°, 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.19 (identical with that for Phe-OP4P, prepared by acidolysis of Z-Phe-OP4P).

In-Gly-Phe-Phe-OP4P. Gly-Phe-Phe-OP4P (0.8 g, 1.6 mM) (derived from Z-Gly-Phe-Phe-OP4P (1.0 g)) and In-Osu (0.36 g, 1.6 mM) were coupled at room temperature for 75 min. Water (125 ml) was added, and the reaction mixture was treated as described for In-Phe-Phe-OP4P to give 0.56 g (60%) of the product. After recrystallization from ethyl-acetate–hexane, it melted at 154–155°, $[\alpha]_D^{24}$ −25.6° (*c* 1, dimethylformamide). Chromatography (solvent B) showed a single spot of R_F 0.17 (iodine). *Anal.* Calcd for $C_{34}H_{35}N_5O_5$ (593.7): C, 68.8; H, 5.9; N, 11.8. Found: C, 68.6; H, 6.1; N, 11.85.

Chromatographic examination (solvent D) of a peptic hydrolysate of the compound (1.25 mM substrate, 0.27 μM pepsin, pH 2.0, 37°, 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.19 (identical with that for Phe-OP4P prepared by acidolysis of Z-Phe-OP4P).

Z-Trp-Phe-OM3P. Phe-OM3P (0.62 g, 2.4 mM) (obtained from Z-Phe-OM3P (1.0 g) as described earlier) was coupled in the usual manner with Z-Trp (0.73 g, 2.4 mM) in the presence of dicyclohexylcarbodiimide (0.5 g, 2.4 mM), with CH_2Cl_2 (40 ml) as the solvent, to yield 1.1 g (81%) of the product. After recrystallization from ethyl acetate, it melted at 140–141°, $[\alpha]_D^{23}$ +5.2° (*c* 1, $CHCl_3$). Chromatography (solvent B) gave a single spot of R_F 0.69 (iodine). *Anal.* Calcd for $C_{34}H_{32}N_4O_5$ (576.6): C, 70.8; H, 5.6; N, 9.7. Found: C, 70.4; H, 5.3; N, 10.1.

Chromatographic examination (solvent D) of a peptic hydrolysate of the compound (1.25 mM substrate, 0.27 μM pepsin, pH 2.0, 37°, 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.26 (identical with that for Phe-OM3P, prepared by acidolysis of Z-Phe-OM3P).

Z-Trp-Phe-OP4P. Phe-OP4P (1.4 g, 4.7 mM) (obtained from Z-Phe-OP4P (2.0 g) as described above) was coupled in the usual manner with Z-Trp (1.6 g, 4.7 mM) in the presence of dicyclohexylcarbodiimide (1.0 g, 4.7 mmoles) with CH_2Cl_2 (50 ml) as the solvent, to yield 2.0 g (74%) of the product. After recrystallization from ethyl acetate–hexane, it melted at 163–164°, $[\alpha]_D^{23}$ +11.3° (*c* 1, $CHCl_3$). Chromatography (solvent B) gave a single spot of R_F 0.66 (iodine). *Anal.* Calcd for $C_{36}H_{36}N_4O_5$ (604.7): C, 71.2; H, 6.0; N, 9.3. Found: C, 70.8; H, 6.2; N, 9.4.

Chromatographic examination (solvent D) of a peptic hydrolysate of the compound (1.25 mM substrate, 0.27 μM pepsin, pH 2.0, 37°, 24 hr) showed the presence of a single ninhydrin-positive component of R_F 0.19 (identical with that for Phe-OP4P prepared by acidolysis of Z-Phe-OP4P).

Enzyme Studies. The two pepsin preparations (Worthington Biochemical Corp. lots 708 and 693-7) employed in this work had the same activity toward Z-His-Phe-Phe-OEt at pH 4.0 and 37°, and gave the same k_{cat} and K_M values for this substrate (within the precision of the data) as those given by a pepsin preparation made from pepsinogen in the manner described by Rajagopalan *et al.* (1966). Enzyme solutions were prepared freshly before each set of kinetic runs. The initial rate of cleavage (5–10%) of each substrate was determined at 37.0 ± 0.1° by means of the automatic ninhydrin method described previously (Hollands and Fruton, 1968) over the range of substrate concentration, (S), permitted by the solubility at the pH of the HCl–NaCl or sodium formate buffer system (0.04–0.08 M) used. Satisfactory Michaelis–Menten kinetics were found, and the precision (95% confidence limits) of the kinetic parameters, k_{cat} and K_M, was estimated by computer analysis (Hanson *et al.*, 1967). The enzyme concentration, (E), was determined spectrophotometrically at 278 mμ, with the assumption that pepsin has a molar absorptivity of 50,900 and a molecular weight of 34,200. To bring some of the substrates into aqueous solution, it was necessary to dissolve them in formic acid, and to dilute the solution with buffer to obtain the stock solution of substrate used for the kinetic runs.

Results

Synthesis of Pyridylalkyl Esters of Peptides. Young and his colleagues (Garner *et al.*, 1968) prepared the 4-picolyl esters of several N-protected amino acids by reaction of the acid with 4-picolyl chloride at 100°, in the presence of an organic base (triethylamine or tetramethylguanidine), with dimethylformamide as the solvent. The general procedure used in the present work, involving condensation in the presence of dicyclohexylcarbodiimide, appears to be equally satisfactory for the preparation of Z-Phe-OM4P. As noted by Garner *et al.* (1968), the pyridylalkyl group of Z-peptide esters is relatively stable to treatment with HBr–acetic acid. We confirm this observation, and have used this reagent for removal of the benzyloxycarbonyl group in the synthesis of the peptide substrates examined in this work. Upon prolonged incubation with pepsin, the substrates were completely cleaved at the Phe–Phe bond, indicating the absence of significant racemization of either Phe residue in the recrystallized products. Thus, duplicate experiments with Z-Gly-Phe-Phe-Gly-OP4P (prepared from Z-Gly-Phe and Phe-Gly-OP4P) gave values of 103 and 105% for the extent of cleavage of the Phe–Phe bond of this substrate. It had been shown previously (Inouye and Fruton, 1967) that the peptide bond of Phe–D-Phe and D-Phe–Phe units are resistant to pepsin action.

Kinetics of Pepsin Action on Pyridyl Esters. In Table I are collected the values for k_{cat} and K_M determined in the present study. All the substrates are of the type A-Phe-Phe-B, the locus of enzymic attack being the Phe–Phe bond. All the members of the series having amino-terminal benzyloxycarbonyl groups are moderately soluble in water at pH 2, but some of them are not sufficiently soluble at pH 4 for kinetic studies over a sufficiently large range of concentration. For this reason, most of the recorded data were obtained at pH 2; in those instances in which kinetic measurements

TABLE I: Kinetics of Pepsin Action on Synthetic Substrates.

Substrate	S^a (mM)	E (μM)	pH	k_{cat} (sec^{-1})	K_M (mM)	k_{cat}/K_M (sec^{-1} mM^{-1})
Z-Phe-Phe-OM3P	0.1–1.0 (10)	0.49	2.0	0.14 ± 0.01	0.81 ± 0.11	0.17
Z-Phe-Phe-OM4P	0.1–1.0 (12)	0.68	2.0	0.17 ± 0.01	0.85 ± 0.11	0.20
Z-Phe-Phe-OP3P	0.1–0.8 (12)	0.30	2.0	0.50 ± 0.03	0.56 ± 0.07	0.89
Z-Phe-Phe-OP4P	0.1–1.0 (11)	0.45	2.0	0.49 ± 0.06	0.71 ± 0.14	0.69
	0.08–0.8 (11)	0.125	3.0	0.74 ± 0.03	0.21 ± 0.03	3.5
Z-Phe(NO_2)-Phe-OP4P	0.1–0.85 (12)	0.26	2.0	0.69 ± 0.07	0.5 ± 0.1	1.4
	0.02–0.2 (12)	0.12	3.0	1.2 ± 0.2	0.13 ± 0.04	9.7
In-Phe-Phe-OP4P	0.2–2.6 (10)	1.7	2.0	1.7 ± 0.3	8.7 ± 1.2	0.19
	0.2–1.6 (11)	0.35	4.0	1.5 ± 0.1	0.32 ± 0.06	4.8
Z-Gly-Phe-Phe-OM3P	0.1–1.0 (10)	0.49	2.0	0.7 ± 0.1	1.6 ± 0.5	0.44
Z-Gly-Phe-Phe-OP3P	0.1–1.0 (11)	0.06	2.0	2.3 ± 0.3	0.9 ± 0.1	2.5
Z-Gly-Phe-Phe-OP4P	0.1–1.0 (11)	0.10	2.0	2.2 ± 0.1	1.1 ± 0.1	2.0
	0.08–1.0 (10)	0.03	3.5	3.1 ± 0.1	0.36 ± 0.04	8.7
In-Gly-Phe-Phe-OP4P	0.1–1.0 (9)	0.05	2.0	2.3 ± 0.5	4.1 ± 1.0	0.55
	0.2–1.0 (9)	0.035	4.0	1.75 ± 0.2	0.6 ± 0.1	3.0
Z-Gly-Gly-Phe-Phe-OM3P	0.1–1.0 (9)	0.03	2.0	10.0 ± 1.9	1.8 ± 0.3	5.5
Z-Gly-Gly-Phe-Phe-OP3P	0.08–0.6 (10)	0.0035	2.0	64.9 ± 3.9	1.16 ± 0.17	55.5
Z-Gly-Gly-Phe-Phe-OP4P	0.1–0.8 (10)	0.0043	2.0	56.5 ± 5.4	0.8 ± 0.2	70.6
	0.03–0.4 (11)	0.0023	3.5	71.8 ± 3.6	0.42 ± 0.08	171
Z-Gly-Gly-Phe(NO_2)-Phe-OP4P	0.2–1.2 (12)	0.02	2.0	8.1 ± 0.7	1.1 ± 0.1	7.4
Gly-Gly-Phe-Phe-OP4P	0.16–1.0 (12)	0.20	2.0	1.5 ± 0.4	1.4 ± 0.4	1.1
	0.08–0.8 (10)	0.087	3.5	3.8 ± 0.6	1.3 ± 0.3	2.9
Z-Gly-Phe-Phe-Gly-OM3P	0.05–0.5 (10)	0.47	2.0	0.54 ± 0.07	0.9 ± 0.1	0.6
Z-Gly-Phe-Phe-Gly-OP4P	0.1–1.0 (10)	0.16	2.0	0.7 ± 0.1	0.7 ± 0.1	0.1
Z-Phe-Phe-Gly-OM3P	0.1–1.0 (10)	0.87	2.0	0.18 ± 0.02	0.8 ± 0.1	0.22
Z-Phe-Phe-Gly-OP4P	0.1–1.0 (12)	0.35	2.0	0.15 ± 0.02	0.45 ± 0.1	0.33
Z-Trp-Phe-OM3P	0.1–1.0 (10)	2.2	2.0	0.027 ± 0.003	0.64 ± 0.1	0.042
Z-Trp-Phe-OP4P	0.1–0.8 (9)	1.2	2.0	0.098 ± 0.008	0.55 ± 0.1	0.18

[a] The numbers in parentheses denotes the number of runs.

were conducted at a higher pH value (Z-Phe-Phe-OP4P, Z-Gly-Phe-Phe-OP4P, and Z-Gly-Gly-Phe-Phe-OP4P), the increase in pH was accompanied by a markedly higher value of k_{cat}/K_M, largely in consequence of large decrease in the value of K_M.

The first point to be noted in Table I is that the k_{cat} and K_M values for the peptic cleavage of Z-Phe-Phe-OM3P and Z-Phe-Phe-OM4P are the same, within the precision of the data, indicating that the difference in the position of the pyridinium nitrogen does not affect the catalytic action of pepsin under the conditions of this study. Where comparisons can be made for other pairs of substrates that differ only in the location of the pyridinium nitrogen (*e.g.*, Z-Phe-Phe-OP3P and Z-Phe-Phe-OP4P, Z-Gly-Phe-Phe-OP3P and Z-Gly-Phe-Phe-OP4P, and Z-Gly-Gly-Phe-Phe-OP3P and Z-Gly-Gly-Phe-Phe-OP4P), only small differences are seen in the values of k_{cat} and K_M at a given pH.

It is especially noteworthy that an increase in the hydrophobic character of the B group of A-Phe-Phe-B by the addition of two methylene groups to the picolyl group causes a significant enhancement in the value of k_{cat}/K_M; this increase is largely a reflection of a rise in k_{cat}, the K_M values for comparable A-Phe-Phe-OM3P (or -OM4P) and A-Phe-Phe-OP3P (or -OP4P) substrates being essentially the same at a given pH.

The most striking result reported in Table I relates to the effect of changing the A group from Z to Z-Gly-Gly. It will be noted that when A is changed from Z to Z-Gly, a moderate increase in k_{cat}/K_M is observed; this effect is principally due to a fourfold increase in k_{cat}, with a slight increase in K_M. When the A group is Z-Gly-Gly, however, the value of K_M is further increased, but that of k_{cat} is about 100 times greater than for the value for the comparable Z-Phe-Phe-B. The k_{cat} of 72 sec^{-1} at pH 3.5 and 37° for Z-Gly-Gly-Phe-Phe-OP4P is highest hitherto observed for a synthetic peptide substrate of pepsin.

For comparison, it may be noted that the most sensitive known substrate of the series Z-His-X-Y-OR, namely, Z-His-Phe-Trp-OEt, is cleaved with a k_{cat} = 0.6 sec^{-1} and K_M = 0.2 mM at pH 3.5 (Hollands *et al.*, 1969). The most sensitive known substrate of the series Ac-X-Y, namely, Ac-Phe-TyrI_2, has been found to be cleaved at pH 2 with a k_{cat} = 0.2 sec^{-1} and K_M = 0.08 mM (Jackson *et al.*, 1965); at higher pH values the value of k_{cat}/K_M for this substrate drops sharply because of a large increase in K_M. It may be concluded, therefore, that the pyridylalkyl esters of suitable A-Phe-Phe compounds

represent the first members of a useful new series of synthetic substrates of pepsin.

During the course of these studies, two members of the type A-Phe-Phe-OP4P, in which A = isonicotinoyl (In) or In-Gly, were prepared to examine the effect of the presence of cationic groups on both sides of the sensitive Phe–Phe bond.[3] These compounds were much more soluble in the pH range 1–5 than the corresponding benzyloxycarbonyl derivatives, and it was noteworthy that while the value of k_{cat} was the same at pH 2 and 4, the value of K_M was markedly lower at the higher pH. It was also of interest to note that the k_{cat} values for comparable compounds differing in the A group (Z-Gly or In-Gly) were nearly the same.

To test the effect of removal of the benzyloxycarbonyl group of the sensitive substrate Z-Gly-Gly-Phe-Phe-OP4P, the corresponding unblocked tetrapeptide ester was tested as pepsin substrate. It will be noted in Table I that this compound is cleaved much more slowly than the N-protected substrate, but it should be added that the observed k_{cat} is about ten times greater than that found for Gly-Gly-Phe-Phe-OEt, the K_M values for the two substrates being nearly the same (Hollands *et al.*, 1969). This comparison provides further evidence of the considerable effect of the pyridylalkyl group on the catalytic efficiency of pepsin.

The introduction of a glycyl residue between the Phe–Phe unit and the pyridylalkyl group appears to cause in some cases a significant decrease in k_{cat} (for example, compare the values for Z-Gly-Phe-Phe-Gly-OP4P and Z-Gly-Phe-Phe-OP4P). When one of the two phenylalanyl residues of the sensitive Phe–Phe unit of a substrate was replaced by a glycyl residue (as in Z-Phe-Gly-OM3P, Z-Gly-Phe-OP4P, or Z-Gly-Gly-Gly-Phe-OP4P), there was no measurable hydrolysis under conditions where the corresponding Phe–Phe compound was hydrolyzed rapidly.

To examine whether the side-chain specificity with respect to the X–Y unit of a pepsin substrate AX–YB, examined earlier for the series where A = Z-His and B = OMe, also applies to compounds of the series where A = Z and B = OP4P, the compounds Z-Trp-Phe-OM3P and Z-Trp-Phe-OP4P were prepared and tested. It will be noted from Table I that the ratio of k_{cat}/K_M values for the pairs of substrates differing in X = Phe or Trp is about 4. A similar ratio was found for the k_{cat}/K_M values at pH 2.5 for the pair of substrates Z-His-X-Phe-OMe, where X = Phe or Trp (Hollands *et al.*, 1969). It is of interest, however, that for the pair of substrates A-Phe(NO_2)-Phe-OP4P, where A = Z-Gly-Gly or Z, the ratio of k_{cat}/K_M values is about 5, in contrast to the ratio of about 40 for the comparable pair of substrates in the Phe–Phe series. A similarly lower ratio of k_{cat}/K_M values has been found in this laboratory for the pair of substrates A-Phe-(NO_2)-Phe-OMe, where A = Z-Gly-His or Z-His (K. Medzihradszky, unpublished data), than for the comparable Phe–Phe pair (Hollands *et al.*, 1969). It would appear therefore that the effect of the A group on the sensitivity of the X–Y bond may depend in part upon the nature of the X residue of a pepsin substrate.

[3] D. G. Doherty and J. James (abstract in *Federation Proc. 27*, 784 (1968)) have prepared picolinoyl, nicotinoyl, and isonicotinoyl derivatives of Phe-Phe-OEt and of related peptides, and have examined the kinetics of their cleavage by pepsin. A full report on this work is in preparation (Dr. D. G. Doherty, personal communication).

Discussion

The data presented in Table I show that the introduction of the cationic and hydrophobic pyridylalkyl group into the B portion of a pepsin substrate A-Phe-Phe-B markedly increases the susceptibility of the Phe–Phe bond to enzymic attack. It may be expected that further structural modifications of peptide pyridyl esters may yield extremely sensitive synthetic substrates for studies on the kinetics and mechanism of pepsin action.

Earlier work in this laboratory had provided evidence for the view that, in the hydrolysis of peptide substrates by pepsin, K_M approximates the dissociation constant of the enzyme–substrate complex, indicating that the release of the first reaction product occurs after the rate-limiting step in the over-all process (Inouye and Fruton, 1967, 1968). The kinetically determined value of K_M may therefore be assumed to be a measure of binding specificity. Studies on the kinetic parameters in the peptic cleavage of substrates of the type Z-His-X-Y-OMe have shown that there is no correlation between apparently tighter binding of a substrate (as measured by a lower K_M value) and the susceptibility of the X–Y bond (Hollands *et al.*, 1969; Trout and Fruton, 1969). The data in Table I offer additional evidence in agreement with this conclusion. It is of special interest that for the series A-Phe-Phe-B, where B is a pyridylalkyloxy group, the value of K_M is markedly decreased on raising the pH, suggesting the possibility that the pyridinium group may form an ion pair with one of the enzymic carboxylate groups that appear as the pH is raised from 2 to 4. This lower K_M value is not accompanied, however, by a comparable change in k_{cat}.

The most significant effects on catalytic efficiency toward the substrates of Table I are those caused by the nature and location of hydrophobic groups in the A and B portions of A-Phe-Phe-B in relation to the sensitive Phe–Phe peptide bond. The significant increase in k_{cat} in going from picolyl esters to the pyridylpropyl esters is noteworthy; a similar effect had been noted previously in the Z-His-Phe-Phe-B series when the B group was changed from OMe to OEt (Inouye and Fruton, 1967). The largest effect observed in the present studies was that caused by moving the benzyloxycarbonyl group of Z-Phe-Phe-OP4P away from the Phe–Phe unit by the introduction of two glycyl residues; this structural change leads to a higher K_M value, indicating poorer binding, and a 100-fold increase in k_{cat}. This result suggests that the pepsin molecule may have a locus of relatively strong affinity for a hydrophobic phenyl group when it is separated from the sensitive Phe–Phe unit by about two α-amino acid residues. A similar effect was noted previously for the pair of substrates A-Phe-Phe-OEt, where a change from A = Z-His to Z-Gly-His caused a considerable increase in k_{cat} (Inouye and Fruton, 1967; Hollands *et al.*, 1969). These results raise the possibility that secondary hydrophobic interactions at some distance from the site of catalytic action may promote better positioning of the catalytic groups of the enzyme in relation to the sensitive peptide bond. It must be added, however, that in those cases where k_{cat} and K_M are both increased to a similar extent, and Michaelis–Menten kinetics are obeyed, the possibility of significant nonproductive binding of the more resistant substrate must be considered (Hollands *et al.*, 1969). This does not appear to apply to the pair of substrates A-Phe-Phe-OP4P, where A = Z or Z-Gly-Gly, and it seems likely that

the changes in the catalytic efficiency of pepsin for such pairs of substrates may arise from the effect of secondary hydrophobic interactions on the three-dimensional structure of the catalytic site of the enzyme (Hollands *et al.*, 1969). The presence of secondary binding sites in pepsin has been demonstrated through equilibrium binding measurements, using substrate analogs (Humphreys and Fruton, 1968), and their hydrophobic character is indicated by recent studies in our laboratory on the effect of temperature, ionic strength, and buffer species on the binding of such substrate analogs by pepsin (E. V. Raju, R. E. Humphreys, and J. S. Fruton, paper in preparation).

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Added in Proof

Since the submission of this paper, it has been found that Z-Ala-Ala-Phe-Phe-OP4P is hydrolyzed by pepsin at pH 3.5 and 37° with k_{cat} = 260 sec^{-1} and K_M = 0.04 mM (G. P. Sachdev and J. S. Fruton, unpublished data).

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