Effect of Piperidine on Benzylaspartyl Peptides in Solution and in the Solid Phase

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Basic conditions (*e.g.* piperidinolysis), used for the removal of the N^{α}-9-fluorenylmethyloxycarbonyl group, lead to significant cleavage of side-chain benzyl esters of peptides containing benzylaspartyl residues both in solution and solid-phase syntheses; the intermediate imide derivative is slowly transformed to a mixture of piperidides.

Recently a synthesis of thymosin-α_1 on *p*-nitrobenzhydrylamine resin was reported,[1] using benzyl esters for protection of side-chain carboxy groups. The temporary N^{α}-9-fluorenylmethoxycarbonyl[2,3] (Fmoc) protecting group was removed by piperidinolysis. During the purification of the crude thymosin-α_1, fractions corresponding to minor peaks in the elution profile obtained from DEAE-Sephadex A-25 column chromatography were shown to contain materials which could not be completely digested with leucine-aminopeptidase, suggesting that undesired reactions may have occurred during the synthesis.

Knowing the general base-sensitivity of β-esters of aspartyl peptides[4-7] we have examined the possibility that the repeti-

tive piperidinolytic removal of the Fmoc group might have affected the benzylaspartyl residues. Boc† protected model compounds were used in order to minimize the extent of the potential transformations and to simplify the evaluation of results.

For this purpose Boc-Asp(OBzl)-Phe-NH_2 (**1**), m.p. 141—142 °C, R_f^1 [solvent system ethyl acetate–(pyridine–acetic acid–water, 20:6:11), 9:1] 0.61—0.66, R_f^2 ($CHCl_3$–MeOH, 9:1) 0.57—0.64, $[\alpha]_D^{25}$ − 19.7° (*c* 0.98, EtOH),‡ was prepared from Boc-Asp(OBzl)-OSu[8] and H-Phe-NH_2·HBr[9] in solution. Boc-Asp(OBzl)-Phe-resin (**2**) was synthesized by coupling the preformed symmetrical anhydride of Boc-Phe-OH to a 4-aminomethyl-3-nitrobenzyl resin,[10] followed by removal of the Boc group with 50% (v/v) trifluoroacetic acid–CH_2Cl_2, neutralization, and coupling of Boc-Asp(OBzl)-OH in the same way. (**1**) and (**2**) were treated for a long time with 55% (v/v) piperidine–dimethylformamide.

In solution the transformation of (**1**) to Boc-Asu-Phe-NH_2 (**3**) then to a mixture of α- and β-piperidides (**4**) was followed by t.l.c. Aliquot samples were taken at intervals, the solvent was removed at room temperature *in vacuo*, and the residue dried over P_2O_5 for 24 h, then dissolved in $CDCl_3$ for ^1H n.m.r. and i.r. studies. The intermediate (**3**) was identified by spectroscopic comparison with an authentic sample, m.p. 77—81 °C, R_f^1 0.63—0.68, R_f^2 0.52—0.60, $[\alpha]_D^{25}$ −91.9° (*c* 0.99, EtOH),‡ prepared both from (**1**) and from Boc-Asp(Phe-NH_2)-OBzl (**5**),‡§[12] m.p. 165—168 °C, R_f^1 0.58—0.63, R_f^2 0.51—0.59, $[\alpha]_D^{25}$ −3.7° (*c* 1.01, dimethylformamide). (**5**) was synthesized from Boc-Asp(OPfp)-OBzl, m.p. 75—76 °C, R_f^1 0.80, $[\alpha]_D^{25}$ −12.9° (*c* 1.01, ethyl acetate), prepared in the usual way,[11] and from H-Phe-NH_2·HBr.[9]

The ^1H n.m.r. spectra (60 MHz) obtained from the series of samples showed that (**1**) disappeared in the first hour, being transformed mainly to (**3**), then further to (**4**). The semi-quantitative estimation of this conversion was based on the relative intensities of the CH_2 resonances for the benzyl alcohol (δ 4.60, s) and the CH_2 resonances for the β-benzyl ester (δ 5.01, s): 40, 75, 90, and 100% conversion after 10, 20, 30, and 60 min, respectively. A similar change in the relative intensity of the aromatic protons was observed at δ 7.06 (s, benzyl alcohol aromatic resonance) and at δ 7.13 (s, β-benzyl ester aromatic resonance) in favour of the former. At the same time the intensity of the Phe aromatic resonance at δ 7.23 remained unchanged. In the spectrum of the 1 h sample the appearance of the broad peak at δ 1.5 [β-, β′-, and γ-CH_2 in the piperidides (**4**)] showed the detectable start of the incorporation of piperidine into (**3**). This reaction was complete within 24 h, and the ^1H n.m.r. spectrum of the 24 h sample was identical with that of another isolated sample containing no (**1**) and (**3**). The transiently formed (**3**) has a characteristic i.r. band at 1775 cm^{-1}, so the formation of (**3**) and its conversion into (**4**) can be followed by i.r. spectroscopy, too.

Boc-Asp(OBzl)-Phe-NH_2
(**1**)

Boc-Asp(OBzl)-Phe-resin
(**2**)

Boc-Asu-Phe-NH_2
(**3**)

Boc-Asp(X)-Phe-NH_2 + Boc-Asp(Phe-NH_2)-X
(**4**), X = piperidide

Boc-Asp(Phe-NH_2)-OBzl
(**5**)

Ac-Gly-Asp(*O*-[$^{14}C^{\alpha}$]-Bzl)-Phe-Val-Gly-Ala-resin
(**6**)

In the next series of experiments (**2**) was treated with 55% piperidine–dimethylformamide. Aliquot samples were removed at intervals, and the anchored peptide amides were photolytically cleaved from the resin in an anhydrous deoxygenated methanolic suspension by irradiation at 350 nm for 12 h at 24—25 °C.[10] The suspension was filtered, and the resin was washed with methanol and acetone. The combined filtrates were evaporated *in vacuo* at 25 °C, then the residues were dried for 36 h over P_2O_5, and dissolved in $CDCl_3$ for ^1H n.m.r. studies (200 MHz).

The disappearance of the β-benzyl ester of the aspartyl residue was estimated on the basis of the change of the relative intensities of the CH_2 resonances for the β-benzyl ester (δ 5.09, s) and the Boc Bu^t group (δ 1.37, s). 2.0, 4.5, 8.0, 16, 22, and 42% of the β-benzyl groups were piperidinolytically cleaved within 10, 20, and 30 min, 1, 2, and 24 h, respectively. The products obtained from the photolysis of (**2**), before and after treatment with piperidine, were submitted to amino acid analyses after acid hydrolyses; the ratio of Asp to Phe was as expected.

The complex ^1H n.m.r. spectra preclude the use of this technique for monitoring the piperidinolysis of a longer peptide derivative. Therefore Boc-Asp(*O*-[$^{14}C^{\alpha}$]-Bzl)-OH {m.p. 102—103 °C, $[\alpha]_D^{25}$ −21.3° (*c* 2.0, dimethylformamide), specific radioactivity 5.41 × 10^6 d.p.m./mmol, prepared from H-Asp(*O*-[$^{14}C^{\alpha}$]-Bzl)-OH with Boc_2O in the usual way. ^{14}H-Asp(*O*-[$^{14}C^{\alpha}$]-Bzl)-OH was synthesized using benzyl alcohol enriched with [$^{14}C^{\alpha}$]-benzyl alcohol (New England Nuclear)} was incorporated into a model peptide. The loss of benzyl alcohol could be then followed by radioactivity counting. Ac-Gly-Asp(*O*-[$^{14}C^{\alpha}$]-Bzl)-Phe-Val-Gly-Ala-resin (**6**) (amino acid analysis after acid hydrolysis: Gly 2.00, Ala 0.98, Val 1.03, Phe 1.01, Asp 0.97; 5.56 × 10^5 d.p.m./g) was synthesized from aminomethyl-co(polystyrene–1% divinylbenzene)[15] by the usual solid-phase procedure, using preformed symmetrical anhydrides of Boc amino acids, except for the last coupling which was performed using the symmetrical anhydride of Ac-Gly-OH.

In these series of experiments (**6**) was treated successively 8 times with 20 and 55% piperidine–dimethylformamide for 10 and 20 min, respectively, at room temperature. After each treatment the resin was filtered off, and washed (×3) with

† Boc = t-butoxycarbonyl, Bzl = benzyl, Asu = L-aminosuccinyl, OSu = succinimidoxy, OPfp = pentafluorophenoxy.

‡ Spectroscopic data: (**1**), ν(KBr) 1726 (ester), 1688 (urethane), 1640 (br., amide), 1173 (COC), 750, 740, and 700 cm^{-1} (aromatic); δ($CDCl_3$ + CD_3SOCD_3) 1.4 (s, 9H, Bu^t), 2.8 (d, 2H, Phe-CH_2), 3.1 (d. 2H, Asp-CH_2), 4.2—4.9 (m, 2H, 2 × CH), 5.05 (s, 2H, Bzl-CH_2), 6.13 (d, 1H, NH), 6.3 and 6.9 (br. d, 2H, $CONH_2$), 7.20 (s, 5H, Bzl-ArH), 7.28 (s, 5H, Phe-ArH), and 7.48 (d, 1H, NH); (**3**), ν(KBr) 1775 (imide), 1750—1640 (imide + urethane + amide), 1390 and 1160 (COC), 1600, 755, and 703 cm^{-1} (aromatic); δ($CDCl_3$) 1.3 (s, 9H, Bu^t), 2.7 (m, 2H, Phe-CH_2), 3.4—3.7 (m, 3H, Asp-CH_2 + CH), 4.8 (m, 1H, CH), 5.7 and 7.0 (br., 2H, NH_2), 6.0 (d, 1H, NH), 7.13 (br. s, 5H, ArH); (**5**), ν(KBr) 1750 (ester), 1687 (urethane), 1638 (amide), 1165 (COC), 752, 739, and 698 cm^{-1} (aromatic); δ($CDCl_3$) 1.33 (s, 9H, Bu^t), 2.7 and 2.9 (m, 4H, Asp- + Phe-CH_2), 4.55 (br., 2H, 2 × CH), 5.1 (br. s, 2H, Bzl-CH_2), 5.8 (br., 2H, NH_2), 6.15 (br., 1H, NH), 7.16 and 7.24 (2 × s, 10H, ArH).

§ A 0.31 M solution of (**1**) in dimethylformamide was treated with 5 equiv. of triethylamine. Conversion of (**1**) into (**3**) was complete in 7 days. Under the same conditions (**5**) was transformed to (**3**) within 6 h. This significant difference in rates of reaction is in accordance with differences reported earlier for the reverse ring opening reactions.[13]

dimethylformamide. An exactly measured portion of the combined filtrates was counted for radioactivity. The counts were corrected for background, quench, and sample dilution. The loss of benzyl alcohol ranged from 1.1 to 3.5%/cycle and 2.8 to 6.7%/cycle by treatment with 20 and 55% piperidine–dimethylformamide, respectively.

From these results the following conclusions can be drawn. (i) The removal of the Fmoc group by piperidinolysis in solution and solid-phase synthesis of peptides containing benzylaspartyl residues is accompanied by the partial cleavage of benzyl esters. Under the generally accepted conditions this combination of protective groups is not appropriate for solution and solid-phase syntheses of peptides. (ii) The rate of benzyl ester cleavage by piperidinolysis is significantly slower under conditions of solid-phase peptide synthesis than in solution. (iii) In basic media the reactivity of the α-benzyl ester is significantly higher than that of the β-benzyl ester. (iv) The piperidinolysis of benzyl esters of aspartyl peptides leads to the expected cyclic intermediates which are transformed to mixtures of α- and β-piperidides, the former probably being favoured.

We thank Dr. B. Hegedüs for interpretation of the i.r. spectra, Mrs. Zs. Muck for skilful technical assistance, and Dr. G. Sitta for the synthesis of H-Asp(*O*-[$^{14}C^{\alpha}$]-Bzl)-OH.

Received, 31st January 1983; Com. 138

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