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Ultrasound in Peptide Synthesis. 4[1]: Rapid Cleavage of Polymer-Bound Protected Peptides by Alkali and Alkanolamines

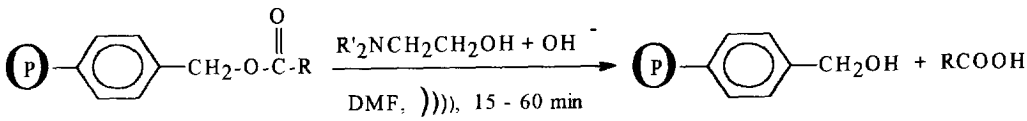
M. V. Anuradha and B. Ravindranath*

Vittal Mallya Scientific Research Foundation
P. O. Box 406, K. R. Road, Bangalore 560004, India

Abstract: Hydrolysis of polystyrene-bound protected amino acids and peptides has been achieved using dimethylaminoethanol or triethanolamine, dimethylformamide and aqueous sodium hydroxide and the reaction is accelerated by ultrasound. Rapid cleavage and excellent yields of the products in high purity, without racemization, are the main advantages of the method.

Cleavage of the peptides from polymeric resins is an important step in the solid-phase peptide synthesis sequence. In the more traditional Merrifield protocol, involving benzyl ester linkage and t-butyloxycarbonylamino acids[2], the preferred method is the use of anhydrous hydrofluoric acid, requiring special (Teflon) equipment and condensation of gaseous hydrogen fluoride at low temperatures[3]. Almost all the protective groups are also cleaved in the process to yield the free (or unprotected) peptide, which is not always advantageous, particularly for segmented peptide synthesis of large peptides[4,5]. Other acidolysis reagents[6] often yield mixtures of products due to partial hydrolysis of some peptide bonds and protective groups. Nucleophilic cleavage to give esters[7], amides[8] and hydrazides[9] is practiced, when these derivatives are desired, but are not favoured when the free terminal carboxylic acid is required. In an ingenious use of transesterification reaction, dimethylaminoethanol is employed to yield the corresponding esters that are readily hydrolysed to the carboxylic acids by aqueous sodium bicarbonate or even by water[10]. However, the reaction is extremely slow, requiring about 72 h in favourable cases. Formation of peptide dimethylamides and partial decomposition of dimethylformamide are some of the other drawbacks of the method. Even in the subsequent finding of the thallous ethoxide catalysis[11], the reactions are slow, needing 2-24 h under dry conditions, to be followed by a hydrolysis step (12-24 h) and cumbersome recovery procedure for the peptide, involving evaporation of the dimethylformamide and dimethyaminoethanol under vacuum.

We now report our finding that triethanolamine or dimethylaminoethanol and aqueous sodium carbonate or sodium hydroxide rapidly cleaved benzyl esters and alkyl esters of nonaromatic acids and that the transesterification and hydrolysis reactions could be combined into one step. Dimethylformamide was used as a cosolvent and the reaction was accelerated several fold by ultrasound. Also being reported in this paper is a convenient solid-phase extraction procedure for recovery of the protected peptides from the reaction mixture.



(R' = CH_3 or CH_2CH_2OH)

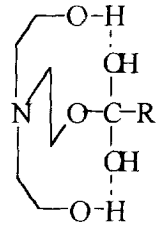
In comparative study using benzyl acetate, benzyl benzoate, methyl benzoate and phenethyl acetate (with anisole as internal standard for GC-MS study to monitor the hydrolysis of the esters), very little reaction (0-21%) occurred when the esters were shaken for 50 h with homogeneous mixtures of (1) dimethylformamide and 0.5 M aqueous sodium carbonate (10:1); (2) dimethylformamide and dimethylaminoethanol (1:1); (3) dimethylformamide, dimethylaminoethanol and aqueous sodium carbonate (5:5:1); and (4) dimethylformamide and triethanolamine (1:1), but when a mixture of dimethylformamide, triethanolamine and aq. sodium carbonate (5:5:1) was used, the esters were hydrolysed to the extent of over 40% (except for methyl benzoate, which was cleaved to the extent of only 18%; Table 1). Similar results were obtained when the mixtures were sonicated for 30 min.

Table 1. Hydrolysis of esters using aq. sodium carbonate and alkanolamines*

Reagent	Benzyl acetate	Benzyl benzoate	Phenethyl acetate	Methyl benzoate
A	0	6	4	0
B	9	16	8	2
C	21	20	11	0
A + B	12	14	20	7
A + C	43	41	43	18

*Reaction conditions: Ester (50 mg) in dimethylformamide (1 mL) shaken with the appropriate reagent for 50 h. Reagents : (A) aq. sodium carbonate (0.5 molar; 0.1 mL). (B) dimethylaminoethanol (1 mL). (C) triethanolamine (1 mL).

Transesterification is the likely mechanism in the case of triethanolamine also as triethanolamine esters could be detected in the product by GC-MS, when the reactions were conducted in the absence of water and alkali. The superior efficacy of this reagent over dimethylaminoethanol could be due to the possible stabilization of the transition state by hydrogen-bonding as in **1**.



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When the sodium carbonate was substituted by equivalent concentration of sodium hydroxide, complete hydrolysis of the esters occurred in 10 min under ultrasound conditions; again, hydrolysis of methyl benzoate was relatively sluggish with only 16% hydrolysis with alkali and 32% with alkali and alkanolamine (Table 2)[12]. Interestingly, there was no difference in the rates of reaction between the two alkanolamines in this case, possibly due to the stronger nucleophilicity of the hydroxide ion.

Table 2. Hydrolysis of esters using aq. sodium hydroxide and alkanolamines*

Reagent	Benzyl acetate	Benzyl benzoate	Phenethyl acetate	Methyl benzoate
A	44	64	42	16
A + B	96	99	91	32
A + C	97	98	92	32

*Reaction conditions: Ester (50 mg) in dimethylformamide (1 mL), sonicated with the appropriate reagent for 10 min. Reagents: (A) aq. sodium hydroxide (1 molar; 0.1 mL). (B) dimethylaminoethanol (1 mL). (C) triethanolamine (1 mL).

The reaction under sonication conditions was readily applicable to the cleavage of protected amino acids and peptides linked to polystyrene resins through benzylic ester bond. Results with representative examples are given in Table 3. As expected, t-butyloxycarbonylamino and benzyloxycarbonylamino groups as well as nitro and benzyl ether groups were stable to this reagent. The cleaved amino acid derivatives were identified by comparison (m.p., TLC, HPLC and $[\alpha]_D$) with standard samples. Stability of the peptide bond to the hydrolysis procedure was confirmed by preparation of Boc-L-Leu-L-PheOMe by both solid-phase and classical solution phase methods using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole in dichloromethane and direct comparison of the products by IR, ^{1}HNMR and $[\alpha]_D$. HPLC comparison with the diastereomeric dipeptides obtained using Boc-L-LeuOH and DL-PheOMe showed that there was no detectable racemization during cleavage from the resin under the present conditions.

Use of the method to cleave a synthetic dodecapeptide, **2**[13], showed the method yields over 96% pure product, as determined by HPLC. For recovery of the peptide, the reaction mixture was diluted 10-fold with water, acidified to pH 4 with 1N HCl and the mixture passed through one or more "Sep-Pak" cartridges, packed with octadecylsilica adsorbent; alternatively, a column packed with a porous nonionic polymer like "Amberlite XAD-7" (based on cross-linked polyacrylamide) could be used. After washing the bed with water, the protected peptide was eluted with methanol. The peptide was then deprotected by hydrogenolysis and acidolysis to yield the homogeneous free peptide, as shown by HPLC.

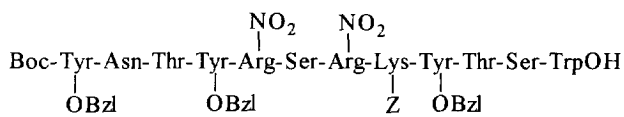

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Table 3. Cleavage of polymer-bound protected amino acids and peptides using alkanolamine and alkali (for conditions and analytical methods, see **Experimental**).

Protected amino acid	Reaction time (min)	Yield[a] %	M.p. °C (Lit)[c]	$[\alpha]_D$[b] Observed	$[\alpha]_D$[b] Expected[c]
Boc-TrpOH	25	97	140 (138)	-16.7	-16.7
Boc-AsnOH	30	90	180 (183)	- 7.9	- 7.6
Boc-PheOH	30	94	90 (87)	+17.5	+17.9
Boc-MetOH	60	95	45 (45-47)	-12.2	+13.0
Boc-LysOH(Z)	30	97	118 (117)	- 6.8	- 6.9
Boc-TyrOH[d]	60	96	225 (228)	+15.5	+15.3
Boc-HisOH[d]	60	94	182 (185)	+ 6.0	+ 6.0
Boc-SerOH(OBzl)	90	96	62 (61)	- 1.8	- 1.4
Boc-AspOH[d]	60	95	165 (168)	- 7.9	- 7.7
Boc-ArgOH(NO_2)	26	100	182 (185)	+ 5.4	+ 5.4
Boc-IleOH	30	95	68 (66-69)	-12.5	+12.7
Boc-ProOH	60	82	136 (135)	-41.5	-41.8
Boc-Leu-PheOH	30	94	152 (155)	- 8.6	- 8.3

[a] yield of the isolated product.
[b] c = 1 in ethanol.
[c] based on the values of the starting N-Boc amino acid or standard compounds.
[d] as dicyclohexylamine salt.

EXPERIMENTAL

N-Boc-amino acids and other side-chain protected amino acid derivatives, as well as Amberlite XAD-7, were purchased from Sigma Chemical Co., U.S.A. Solvents and reagents were dried and distilled before use. GC-MS was carried out on a Hewlett-Packard 5970A quadrupole instrument. HP-Ultra-2 capillary column (0.2 X 250 mm) was used with helium as the carrier gas at a flow rate of 1 mL/min.; a temperature programme of 60-10-250°C was used for the analysis. HPLC was carried out on a Waters instrument which consisted of 510 reciprocating pump, U6K injector and 484 variable wavelength detector set at 210 nm; column used was Novapak C18 (3.9 X 150 mm); mobile phase was acetonitrile:water (3:2), containing 0.1% trifluoroacetic acid at 1.0 mL/min. Optical rotations were measured using JASCO DIP-370 digital polarimeter. ^{1}HNMR spectra were recorded on a Bruker 270 MHz instrument in $CDCl_3$ using TMS as internal standard. "Sep-Pak" (C18) cartridges, packed with octadecylsilyl silica (200mg), were procured from Waters Associates, U.S.A. Ultrasonic bath (Model 8890), with tank capacity of 1.91 L, equipped with digital timer and temperature control and sonication frequency of 47 kHz (input power 80 W), was procured from Cole-Parmer, U.S.A.

Model hydrolysis experiments. Anisole (internal standard, 0.1 g) and 0.1 g of each of benzyl acetate, benzyl benzoate, methyl benzoate and phenethyl acetate were taken in dimethylformamide (1 mL). To these test mixtures were added, individually, the reagents as given under Tables 1 and 2. One set of the samples were shaken for 50 h and another set sonicated for 10 or 30 min. The samples were then diluted with water (10 mL) and extracted with ether and the ether extract analysed on GC-MS. Disappearance of the ester was measured by comparison with anisole peak area in the test and control experiments. Each of the results in Tables 1 and 2 are the mean of three experiments.

Standardized cleavage procedure for protected amino acids and peptides. Polymer-bound N-Boc amino acids were obtained as described[1]. A stock mixture of dimethylformamide (10 mL), triethanolamine (10 mL) and aqueous sodium hydroxide (1 M; 2 mL) was prepared. The polymer (0.1 g) was suspended in the stock mixture (1 mL) and sonicated for 20-60 min. The mixture was filtered and resin washed with water (2 mL). The filtrate was titrated against standard oxalic acid using phenolphthalein as indicator. The stock mixture (1 mL) was similarly titrated and the difference gave the quantity of acid liberated from the resin. The reaction time for complete cleavage of each amino acid was thus determined (Table 3). The progress of the reaction could also be monitored by HPLC of the acidified reaction mixture. To recover the product, the reaction mixture was diluted with water (10 mL), acidified (pH 4) and extracted with ethyl acetate. The ethyl acetate extract was dried (Na_2SO_4), evaporated and the residue recrystallized from ethyl acetate and hexane.

Synthesis of N-Boc-Leu-PheOMe. The standard compound (N-Boc-L-Leu-L-PheOMe) was prepared by the solution phase method, using N-Boc-L-LeuOH and L-PheOMe.HCl, according to the procedure described in the literature[14]; m.p 86°C; $[\alpha]_D$ -27.2° (MeOH, c = 1). N-Boc-L-Leu-DL-PheOMe was similarly prepared starting from N-Boc-L-LeuOH and DL-PheOMe.HCl.

Polymer-bound N-Boc-L-phenylalanine (0.5 g; prepared as described[1]) was stirred with 40% trifluoroacetic acid in dichloromethane (5 mL) for 30 min. The reagents were filtered and the resin washed with 5% diisopropylethylamine in dichloromethane (3 X 5 mL), followed by dichloromethane (3 X 5 mL). The resin was then stirred with N-Boc-L-LeuOH (0.12 g) and 1-hydroxybenzotriazole (0.0625 g) in 0.25 M dicyclohexylcarbodiimide in dichloromethane (4 mL) for 2 h; the condensation reaction was repeated to ensure completion.

The above polymer-bound peptide (0.5 g) was suspended in a mixture of dimethylformamide (5 mL), triethanolamine (5 mL) and 1 M aqueous sodium hydroxide (1 mL) and sonicated for 0.5 h. The resin was then filtered off and washed with water. The filtrate was diluted 10-fold with water, acidified to pH 4 with 1 N hydrochloric acid and extracted with ethyl acetate (3 X 10 mL). The ethyl acetate extract was dried (Na_2SO_4) and evaporated to yield N-Boc-L-Leu-L-PheOH, which was recrystallised from ethyl acetate-hexane; yield 94%.

The above peptide (200 mg) in ether (5 mL) was treated with ethereal diazomethane at 5-10°C till the yellow colour persisted. The solvent was evaporated and the residue recrystallised from ethyl acetate-hexane to yield N-Boc-L-Leu-L-PheOMe as a white crystalline solid (170 mg); m.p. 87°C, $[\alpha]_D$ -27.5° (MeOH, c = 1). ^{1}HNMR ($CDCl_3$): δ 0.91 (6H, *d*, J = 2.7 Hz), 1.44 (9H, *s*), 1.64 (5H, *br*), 3.12 (1H, *m*), 3.71 (3H, *s*), 4.08 (1H, *br*), 4.83 (1H, *m*), 6.51 (1H, *d*, J = 7 Hz), 7.10 (2H, *d*, J = 6Hz), 7.27 (2H, *d*, J = 6Hz). IR superimposable over that of N-Boc-L-Leu-L-Phe-OMe prepared by the solution phase method described above. HPLC showed a single peak (t_R = 3.45 min), while the dipeptide from Boc-L-LeuOH and DL-PhOMe showed two peaks of comparable intensity at t_R values of 3.45 and 3.62 min.

Synthesis and cleavage of protected dodecapeptide (**2**). N-Boc-Tryptophan (0.235 g; 0.77 mmol) was coupled to Merrifield resin (1 g; 0.7 mmol) as described[1]. Removal of the N-Boc protective group with 40% trifluoroacetic acid in dichloromethane and coupling with successive protected amino acids using 1-hydroxybenzotriazole and dicyclohexylcarbodiimide was carried out using standard protocols[15]. The resin-bound protected peptide (200 mg) was cleaved as described above (60 min). The filtrate from the resin was diluted 10-fold with water, acidified with 1N HCl to pH 4 and passed through a series of five "Sep-Pak C18" cartridges. The cartridges were washed with water and then eluted with methanol (5 X 3 mL) to recover the peptide, **2** (82 mg); m.p. 260°C; $[\alpha]_D$ -132° (MeOH, c = 0.1,). HPLC: t_R = 8.30 min. (96.25%). More conveniently, a column of 5 g of Amberlite XAD-7 could be used with similar results (86 mg).

The above protected peptide (60 mg) in methanol-acetic acid (1:1; 10 mL) was hydrogenated at 40 psi in a Parr apparatus over 10% Pd-C (25 mg) for 6 h. The catalyst was filtered off and the filtrate evaporated under reduced pressure. The residue was treated with 40% trifluoroacetic acid in dichloromethane (5 mL) for 30 min and evaporated to dryness. The residue was triturated with ether to yield the free peptide, as a buff coloured solid. HPLC: single peak at t_R = 2.95 min.

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