AN EFFICIENT MILD ACIDOLYTIC DEPROTECTION PROCEDURE FOR Boc/Bzl-BASED SOLID PHASE PEPTIDE SYNTHESIS

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Using the mild acidolytic deprotecting reagents 0·1M MSA or 0·1M TMSOTf in TFA in combination with PMB, which simultaneously accelerates the cleavage and acts as irreversible scavenger, the rate of cleavage of a model dipeptide from the appropriate N^{α}-Fmoc-dipeptidyl resin (polymer-bound benzyl ester, *p*-MBHA and BHA resin, respectively) was studied. The new deprotecting method was successfully applied to the synthesis of three N^{α}-halogenoacetyl modified octapeptides (*I*, *II*) and a 16-residue peptide (*III*). In all cases, the crude products were of the same quality as peptides obtained via the HF/10% anisole technique.

The combination of N^{α}-Boc* groups with various benzyl-type side chain protecting groups is still the most popular strategy for both classical solution and solid phase peptide synthesis[1]. However, the main disadvantage of this strategy is the need for deblocking reagents of strongly acidic character such as HF or TFMSA, which can promote a variety of side reactions and loss of peptide products (for a review, see ref.[2]). Therefore, milder acidolytic deprotection conditions are desirable for the further use and development of this so-called Boc/Bzl protection strategy. Milder acids such as TFA are not of sufficient acidity to remove the common benzyl-based protecting groups[3] even in the presence of thioanisole, which accelerates the deprotection process and acts as a cation scavenger at the same time. Recently, Yoshino et al.[4] described a similar effect for several methylbenzenes. These investigators reported that such additives had the ability to accelerate the cleavage rate to an extent based on the number of methyl groups in the benzene ring. PMB was the most effective scavenger among those tested. For example, complete deblocking of Tyr(Bzl) could

* Symbols and abbreviations are used according to the rules of the IUPAC IUB Commission on Biochemical Nomenclature (Eur. J. Biochem. *138*, 9 (1984)). Additional abbreviations; BHA benzhydrylamine; Br-Ac bromoacetyl; Cl-Ac chloroacetyl; DMF N,N-dimethylformamide: HF hydrogen fluoride; MSA methanesulfonic acid; *p*-MBHA *p*-methylbenzhydrylamine; PMB pentamethylbenzene; RP-HPLC reverse phase high performance liquid chromatography; TFA trifluoroacetic acid; TMSOTf trimethylsilyl trifluoromethanesulfonate; Ad 1-adamantyl; Cl_2Bzl 2,6-dichlorobenzyl; 2-ClZ 2-chlorobenzyloxycarbonyl; MBzl 4-methoxybenzyl; MeBzl 4-methylbenzyl.

be achieved in TFA with 10 equivalents of PMB at room temperature in less than two hours.

Here we describe the application of PMB to the deprotection of the commonly used benzyl-type protecting groups as well as various anchors used in solid phase peptide synthesis[5,6].

As a model for our investigation we chose first the cleavage of Fmoc-Leu-Pro-OH from the corresponding polymer-bound benzyl ester. In order to determine the cleavage rate, samples of deblocked peptide resins were withdrawn at various time intervals, the residual loading being determined by measurement of the characteristic ultraviolet absorption of 9-fluorenylmethylpiperidine, which is formed upon removal of the N^{α}-Fmoc group[7] by means of piperidine. We found that detachment of the model dipeptide from the resin via TFA/PMB according to Yoshino et al. was too slow and incomplete even after 24 hours showing that this reagent is not useful for practical peptide syntheses. However, addition of the strong acid MSA at a concentration of 0·1 mol l^{-1} strongly accelerates the cleavage reaction. Moreover, the rate of cleavage increased with increasing amounts of PMB as shown in Fig. 1.

PMB shows nearly the same accelerating effect as the well known additive thioanisole (Table I), but in contrast to the latter, PMB acts as an irreversible scavenger and shows no alkylating activity. With increasing acid concentration, however, the promoting effect disappears and with 0·5M MSA in TFA neither PMB nor thioanisole showed an influence on the cleavage rate. The results for thioanisole are contrary to those described by Yajima et al.[8] for deprotection with 1M MSA or TFMSA in TFA.

TABLE I

Cleavage of Fmoc-Leu-Pro-OH from Fmoc-Leu-Pro-OCH_2-C_6H_4-resin (0·82 mmol peptide/g resin (1% DVB, Bachem)) at 22°C after 4 h

Acid	Scavenger			
	PMB	%	thioanisole	%
TFA	10 eq.	20·7	10 eq.	39·0
0·1M MSA/TFA	—	69·5	—	69·5
0·1M MSA/TFA	10 eq.	81·7	10 eq.	85·4
0·1M MSA/TFA	30 eq.	91·5	30 eq.	89·6
0·5M MSA/TFA	—	92·2	—	92·2
0·5M MSA/TFA	10 eq.	90·2	10 eq.	91·7

We also studied the use of TMSOTf (refs[9,10]) in place of MSA. With 0·1M TMSOTf in TFA the cleavage is much faster than described above with detachment from the polymer support reaching about 90% after only one hour. Remarkably, the reaction is so fast that the PMB concentration has practically no effect. On the other hand, at a lower TMSOTf concentration (0·02 mol l^{-1}) we could again observe the promoting effect of PMB as shown in Fig. 2. With TFMSA in place of TMSOTf nearly identical results were obtained.

Interestingly, we also observed a remarkable temperature dependence. At 0°C both MSA- and TMSOTf-containing reagents react too slow with only 9% and 13% cleavage, respectively, occurring after one hour. The deprotection reaction proceeds much faster at room temperature (59% and 88%, respectively) and can be further enhanced at 40°C to the extent of 90% in the case of MSA. Furthermore, we have studied the release of Fmoc-Leu-Phe-OH from this resin as a typical example for some amino acids with slower detachment rate[11]. In such a special case both methods are useful, but require a 24 hours treatment for reaching a cleavage of 80%.

In conclusion, high concentrations of strong acids are unnecessary in the cleavage of benzyl-linked peptides from polymeric supports. At room temperature these "low MSA/PMB" and "low TMSOTf/PMB" reagents are equivalent to more acidic media lacking the hydrocarbon additive.

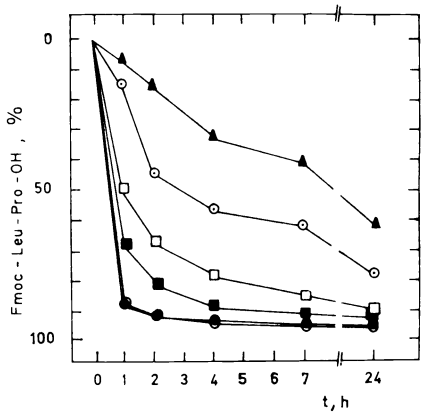

FIG. 1

Effect of PMB and MSA on the release of Fmoc-Leu-Pro-OH from Merrifield-resin in TFA at 22°C, ▲ 10 eq. PMB; ○ no additive/0·1M MSA; ● 10 eq. PMB/0·1M MSA; ◒ 30 eq. PMB/0·1M MSA; ⊙ 10 eq. PMB/0·1M MSA at 0°C; □ 10 eq. PMB/0·1M MSA at 40°C

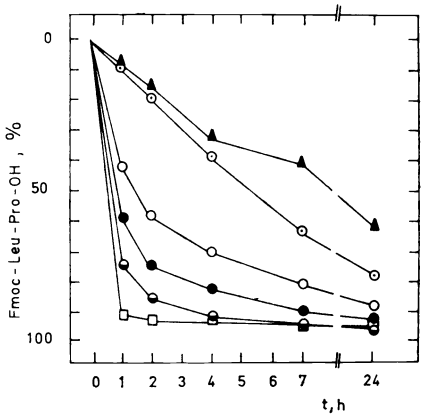

FIG. 2

Effect of PMB and TMSOTf on the release of Fmoc-Leu-Pro-OH from Merrifield-resin in TFA at 22°C. ▲ 10 eq. PMB; □ no additive/0·02M TMSOTf; ■ 10 eq. PMB/0·02M TMSOTf; ○ no additive/0·1M TMSOTf; ● 10 eq. PMB/0·1M TMSOTf; ⊙ 10 eq. PMB/0·1M TMSOTf at 0°C

Using a similar model system we also investigated the cleavage of Fmoc-Leu-Pro--NH_2 from a *p*-MBHA resin. Generally, the TMSOTf reagent was found to be most efficient. The results, which are similar to those described above, are shown in Fig. 3 and summarized here: (i) incomplete cleavage with the TFA/PMB reagent[4]; (ii) pronounced enhancement after TMSOTf addition, e.g. from 0% to 67% after one hour; (iii) further enhancement with increasing amounts of PMB, e.g. from 67% to 88% and (iv) very slow reaction at 0°C due to establishment of an equilibrium.

Finally, cleavage of the model dipeptide from the more stable BHA-resin after four hours (90%), using our standard "low TMSOTf/PMB" reagent is considerable and comparable to that for the *p*-MBHA-resin after two hours. The release of the peptide from this resin types is depending on the nature of the C-terminal amino acid. In contrast to Pro and many of the others amino acids[12], the release of the model compound Fmoc-Leu-Phe-NH_2 from the BHA resin was very low in 7 hours and comparable with a 1M TMSOTf-thioanisole/TFA deprotection (9% and 11%, respectively). For such critical amino acids (Phe, Ile, Val) more acid labile resin types are the only alternative and the MBHA resin will be investigated in the next time. With these exceptions, the results offer the possibility of using our reagent for less expansive resin of this type.

For the final deprotection step, in addition to cleavage of the anchor we should consider the possibility of cleaving all other protecting groups at the same time. Therefore we examined the time for complete removal of the various benzyl-type protecting groups commonly used in the Boc/Bzl strategy. All such groups were cleaved in less than 90 min with the "low MSA/PMB" reagent or in 15 min with the "low TMSOTf/PMB" method according to the order Lys(Z) > Glu(OBzl) > > Thr(Bzl) = Tyr(Bzl) = Ser(Bzl). The TMS triflate technique furthermore deblocked the more stable derivatives, like Arg(Mts), Lys(2-ClZ), Tyr(Cl_2Bzl), Cys-

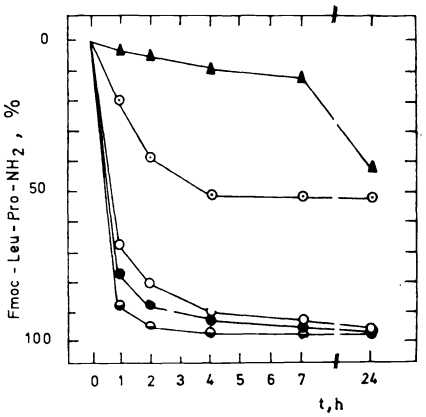

FIG. 3

Effect of PMB and TMSOTf on the release of Fmoc-Leu-Pro-NH_2 from *p*-MBHA-resin in TFA at 22°C. ▲ 10 eq. PMB; ○ no additive/0·1M TMSOTf; ● 10 eq. PMB/0·1M TMSOTf; ◒ 30 eq. PMB/0·1M TMSOTf; ⊙ 10 eq. PMB/0·1M TMSOTf at 0°C

(MBzl) in less than 1 hour and even Cys(MeBzl) in 2 hours. An incomplete removal was observed in the case of Asp(OChx), Glu(OChx) and Cys(Ad), whereas other groups were stable for four hours (i.e., Arg(Tos), Arg(NO_2), Met(O), Trp(For)). The latter should be employed for the synthesis of Trp-containing peptides, because the cleavage of peptides without indol protection is accompanied by the well known formation of some side products[11].

An interesting feature of this mild acidolytic reagent is the absence of succinimide formation[11]. After the cleavage of the sensitive model compound Boc-Asp(OBzl)--Gly-OBzl, the isolated product was free of the corresponding aminosuccinimide--peptide even after a 18 hours treatment.

In practice, for a peptide bound to a Merrifield-resin with benzyl protecting groups only, we recommend the milder „low MSA/PMB" method. Generally, for the cleavage from different resins and in combination with more stable protecting groups commonly used in the Boc/Bzl strategy for longer peptides, the "low TMSOTf/PMB" method is judged to be the more effective reagent.

In order to demonstrate the reliability of our deprotecting method in practical solid phase peptide syntheses, we have prepared two model peptides (*I*, *II*) predicted to be antigenic determinants of the growth-related 25-kDa protein of Ehrlich ascites tumor (EAT p25)[13]. After modification at the N-terminus by the chloroacetyl[14] or the more reactive bromoacetyl[15,16] linker so as to provide for selective coupling to carrier proteins carrying thiol residues, the peptides were detached from the resin using our new methods.

Naturally, PMB-containing reagents can also be used in solution phase syntheses as demonstrated for the VP_1 sequence 144–159 of the foot-and-mouth disease virus[17] (*III*). After purification by preparative RP-HPLC the structures of all peptides were confirmed by amino acid analysis and fast atom bombardment mass spectra (FAB-MS).

Our results indicate that these mild deprotecting procedures using 0·1M MSA or or 0·1M TMSOTf in TFA in combination with PMB have some attractive features. Unlike HF, our reagents do not require special apparatus for its handling and in contrast to the more acidic 1M sulfonic acid–thioanisole/TFA methods there is no risk of succinimide formation during the deprotection.

EXPERIMENTAL

The purity and the stability of amino acid derivatives and peptides was checked by thin layer chromatography (TLC) on Merck silica gel G and cellulose plates in the solvent system: 1-butanol--pyridine–acetic acid–water (4 : 1 : 1 : 2) and 1-butanol–acetic acid–water (4 : 1 : 1). UV, ninhydrin and the modified Reindel-Hoppe procedure[16] were used for detection of compounds on TLC plates. Samples for amino acid analysis were hydrolyzed with 6M HCl in the gas phase for 24 h and the resulting amino acid mixtures were analyzed by precolumn derivatization with the dabsylation technique[17]. To determine the residual loading of the deblocked peptide resins,

samples were hydrolyzed in evacuated sealed tubes with a mixture of propionic acid — 12M HCl (1 : 1) at 130°C for 16 h (ref.[18]) and the fragments analyzed on an amino acid analyzer AAA 881 (Mikrotechna, Praha, Czechoslovakia). Fast atom bombardment mass spectra were obtained on a ZAB-EQ spectrometer (VG Analytical Ltd., Manchester) with xenon at 8 kV as the bombarding gas. Analytical RP-HPLC was carried out on a 75 × 4·6 mm Vydac (5 μm) column, using a 0—100% gradient of CH_3CN in 0·05% TFA-water; flow 1 ml/min; detection: 220 nm. Final purification of the peptides was achieved by preparative RP-HPLC on a 1·6 × 50 cm RP C-18 (13 μm) column, using a 10—40% gradient of MeOH in 0·1% TFA–water; flow 5 ml/min; detection: 220 nm.

Measurement of Cleavage Rates for Model Dipeptides

Model dipeptide resins were prepared by solid phase synthesis[11,19] using *p*-MBHA copoly(styrene–1% divinylbenzene) (Fluka, 1·0—1·2 mmol NH_2/g), BHA copoly(styrene–2% divinylbenzene) (UR-95 from G.D.R., 0·85 mmol NH_2/g), and chloromethylated copoly(styrene–1% divinylbenzene) (Bachem, U.S.A., 1·2 mmol Cl/g) resins. The loading at the beginning of the test and the residual loadings were determined by measurement of the characteristic ultraviolet absorption of the dibenzofulvene–piperidine adduct after N^{α}-Fmoc group cleavage from resin samples with 20% piperidine in DMF (ref.[7]).

General procedure: Fmoc-Leu-Pro-*p*-MBHA resin (33·3 mg, 0·025 mmol), Fmoc-Leu-Pro--BHA resin (30·5 mg, 0·025 mmol) or Fmoc-Leu-Pro-O-CH_2-C_6H_4-resin (30·5 mg, 0·025 mmol) was stirred with 0·1M TMSOTf in TFA (0·5 ml) in the presence of PMB (37·0 mg, 0·25 mmol). Samples of deblocked peptide resins were withdrawn at various periods of time (1, 2, 4, 7 and 24 h), washed, dried in vacuo over phosphorus pentoxide for 2 h and the residual loading determined.

General Methods for Cleavage of Peptides from the Resin

Method A: Cleavage of peptides (0·05 mmol) from the resin and removal of protecting groups were effected by stirring with 0·1M TMSOTf in TFA (1·0 ml) in the presence of PMB (0·50 mmol, 10 eq. protecting group) for 4 h at room temperature. Dry ether was added and the resulting unprotected peptides, together with the resin, were filtered or centrifuged, washed with ether (to remove excess PMB) and the free peptides extracted successively with portions of acetic acid, 0·1M acetic acid and water. The solvent was evaporated in vacuo at 30°C and the residue was desalted twice by gel chromatography on a Sephadex G-10 column using 0·1M acetic acid as eluent. After lyophilization of the desired fractions (monitored by UV absorption measurement at 220 nm) the crude peptides were purified to homogeneity by preparative RP-HPLC.

Method B: Cleavage of the peptides (0·05 mmol) from the resin and removal of protecting groups was accomplished with 0·1M MSA in TFA (1·0 ml) containing PMB (1·50 mmol, 30 eq./ /protecting group) for 4 h at room temperature. Purification followed that of method *A*.

Method C: For comparison the peptide derivatives were cleaved from the carrier by treatment of the peptide-resin with liquid HF containing 10% anisole at 0°C for 1 h. The HF was evaporated at 0°C (bath) during 30 min, the residue was triturated three times with dry ether, the further purification being the same as described in method *A*.

Cl-Ac-Gly-Pro-Leu-Pro-Lys-Ala-Val-Thr-NH_2 (*I*)

Starting with BHA resin (content of amino group 0·85 mmol/g), the title peptide was prepared by stepwise coupling of the appropriate Boc protected amino acids. N-Chloroacetylation of the

TABLE II

Analytical data for N-halogenoacetyl-modified peptides (*I*, *II*)

Peptide	Amino acid analysis				R_F	R_t min	Purity[a], %			$(M + H)^+$, m/z
	Gly Lys	Pro Val	Leu Thr	Ala			method *A*	method *B*	method *C*	Calculated/Found
I	1·10 0·91	1·93 0·96	0·86 0·86	1·00	0·47	12·6	75·8	70·7	72·5	856·4 857
IIa	2·02 1·42	2·31 —	1·01 —	2·00	0·48	10·8	51·4	53·6	72·7	786·3 787
IIb	1·99 1·04	2·09 —	1·02 —	2·00	0·45	10·8	36·3	49·8	38·7	830·8 832

[a] Relative area of the main peak in the crude product measured at 220 nm.

N-terminus of the protected peptide was performed with a 3-fold excess of chloroacetic anhydride in CH_2Cl_2. Fifty milligram samples of the resulting Cl-Ac-Gly-Pro-Leu-Pro-Lys(Z)-Ala-Val--Thr(Bzl)-BHA resin were cleaved with 0·1M TMSOTf in TFA (0·7 ml) containing PMB (189·0 mg, 1·275 mmol) as described for method *A* and after purification by preparative RP-HPLC the product was found to contain 97·2% of peptide *I*. Amino acid analysis of samples obtained after cleavage from the support indicated the following residual loadings of the deblocked peptide resins: 19·9% (method *A*); 25·0% (method *B*); 7·3% (method *C*). Analytical data are shown in Table II.

Cl-Ac-Gly-Pro-Ala-Pro-Lys-Leu-Gly-Ala-OH (*IIa*)

Starting with Boc-Ala-O-CH_2-C_6H_4-resin (0·35 mmol/g of Boc-Ala) the title peptide was prepared by stepwise coupling of the appropriate Boc protected amino acid. N-Chloroacetylation was performed as previously reported for peptide *I*.

A sample of 50·0 mg of the protected Cl-Ac-Gly-Pro-Ala-Pro-Lys(Z)-Leu-Gly-Ala-O-CH_2--C_6H_4-resin was cleaved with 0·1M MSA in TFA (0·7 ml) containing PMB (156·0 mg, 1·05 mmol) as described for method *B* and after purification by preparative RP-HPLC the product analysed for 99·5% of peptide *IIa*. Residual loading of the deblocked peptide resins: 6·7% (method *B*); 2·3% (method *C*). Analytical data are shown in Table II.

Br-Ac-Gly-Pro-Ala-Pro-Lys-Leu-Gly-Ala-OH (*IIb*)

The title peptide was prepared as previously given for peptide *II*. N-Bromoacetylation of the N-terminus of the protected peptide was performed with a 3-fold excess of bromoacetic anhydride in CH_2Cl_2. A 50·0 mg sample of the protected peptide resin was cleaved with 0·1M MSA in TFA (0·7 ml) containing PMB (156·0 mg, 1·05 mmol) as described for method *B* and after purification by preparative RP-HPLC the product was shown to contain 84·0% of peptide *IIb*. Analytical data are shown in Table II.

H-Leu-Arg-Gly-Asp-Leu-Gln-Val-Leu-Ala-Gln-Lys-Val-Ala-Arg--Thr-Leu-OH VP_1 (144 – 159) (*III*)

The title peptide was synthesized in solution by a [3 + (7 + 6)]-segment condensation from conventionally prepared segments. A 17·0 mg sample of the protected VP_1 (144 – 159)-sequence (0·007 mmol, Boc-Leu-Arg(Mts)-Gly-Asp(OBzl)- Leu-Gln-Val-Leu-Ala-Gln-Lys(Z)-Val-Ala-Arg-(Mts)-Thr-Leu-OBzl) was deprotected with 0·1M TMSOTf in TFA (1·0 ml) containing PMB (103·8 mg, 0·7 mmol) as described for method *A*. Peptide *III* obtained in this way was homogeneous according to analytical RP-HPLC (R_t 16·3 min; 65·2% method *A*, 60·8% method *C*). TLC R_F 0·40 1-butanol–pyridine–acetic acid–water (4 : 1 : 1 : 2). Amino acid analysis: Gly 1·04, Leu 4·12, Arg 1·01, Asp 1·03, Glu 1·98, Val 1·96, Lys 0·97, Thr 0·93, Ala 2·00, FAB MS (*m/z*): calculated: 1 781·2; found: 1 781·6 $(M + H)^+$.

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