Journal of Peptide Science
J. Peptide Sci. **6**: 264–270 (2000)

The Deprotection of Lys(Mtt) Revisited

LINE BOUREL*, OLIVIER CARION, HÉLÈNE GRAS-MASSE and OLEG MELNYK

UMR 8525 CNRS-Université de Lille 2, Institut de Biologie et Institut Pasteur de Lille, 1 rue du Pr Calmette, BP 447, 59021 Lille, France

Received 20 October 1999
Accepted 24 October 1999

Abstract: The selective deprotection of Lys(Mtt)-containing peptidyl resins was successfully monitored by RP-HPLC using very short linear gradients. RP-HPLC analyses of the acidic filtrates also revealed the partial cleavage of the Trt groups and of the peptide–resin bond. The absorbance of the Mtt carbocation at 470 nm is only twice that of the Trt cation. Thus, the UV monitoring at 470 nm seems to be inappropriate, especially at the end of the deprotection, when the Mtt and the Trt levels are comparable. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Mtt; deprotection; RP-HPLC; monitoring; Wang resin; Rink amide resin

INTRODUCTION

The synthesis of branched peptides or the modification of peptides with fluorescent labels [1], fatty acids, or particular functional groups often requires the derivatization of an ε-amino group. The selective unmasking of the Lys side chain during the solid phase synthesis of the peptide necessitates the use of an orthogonal protection [2]. If we consider the 9-fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl (*t*Bu) strategy, this orthogonal protection can be achieved by introducing Lys(Mtt) in the sequence, since the 4-methyltrityl (Mtt) group can be cleaved using mild acidic conditions such as trifluoroacetic acid (TFA)/dichloromethane (DCM) (1:99, v/v) [3]. The ease of removal of the Mtt group depends strongly upon the sequence, thus requiring a simple, rapid and reliable detection method for the Mtt carbocation liberated in solution. We report in this paper that for Trt-containing peptidyl resins the yellow color released during the acidic washings or the absorption at 470 nm, two criteria often used for the monitoring of the deprotection, were found misleading, owing to a simultaneous liberation into solution of some Trt carbocations. Alternately, the reverse phase high performance liquid chromatography (RP-HPLC) analysis of the acidic solution appeared to be more appropriate since it was possible to separate Trt and Mtt alcohols. In addition, Wang [4] and Rink amide [5] peptidyl resins were found to be partially cleaved in TFA/DCM (1:99). Thus, exposure of the peptidyl resin to TFA must be maintained as short as possible, reinforcing the need for a reliable detection method for the Mtt carbocation.

Abbreviations: APCI, atmospheric pressure chemical ionization; BHT, 2,6-di-*tert*-butyl-4-methylphenol; DCM, dichloromethane; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOBt, *N*-hydroxybenzotriazole; LCMS, liquid chromatography mass spectrometry; Mtt, 4-methyltrityl; Rink amide resin, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin; RP-HPLC, reverse phase high performance liquid chromatography; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Wang resin, *p*-benzyloxybenzyl alcohol resin.

* Correspondence to: UMR 8525 CNRS-Université de Lille 2, Institut de Biologie et Institut Pasteur de Lille, 1 rue du Pr Calmette, BP 447, 59021 Lille, France; e-mail: line.bourel@pasteur-lille.fr

MATERIALS AND METHODS

Peptide Synthesis

Peptidyl resins **1**, **3**, **5**–**17** and **2**, **4** were obtained starting from Rink amide resin [5] (0.58 mmol/g, France Biochem, Meudon, France) and Fmoc-L-Asn(Trt)-Wang resin [4] (0.44 mmol/g, France Biochem, Meudon, France) respectively, using

standard Fmoc/*tert*-butyl strategy [6] and HBTU/HOBt activation in an Applied Biosystem 431A peptide synthesizer (Foster City, USA). Fmoc-protected amino acids were purchased from France Biochem, Meudon, France. Side-chain protections were as follows: Arg(Pmc), Asn(Trt), Asp(O*t*Bu), Gln(Trt), Glu(O*t*Bu), His(Trt), Lys(Boc), Lys(Mtt), Ser(*t*Bu), Thr(*t*Bu), Tyr(*t*Bu), Trp(Boc).

Typical Mtt Removal Procedure and RP-HPLC Monitoring

Peptidyl resin (0.25 mmol) was treated with 15 ml of TFA/DCM (1:99, v/v). The beads were stirred for 2 min, filtered and immediately resuspended in the same volume of TFA/DCM (1:99). The procedure was repeated until Mtt-OH disappeared from the filtrates. The filtrates were analysed by RP-HPLC as follows: aliquots (200 μl) were concentrated *in vacuo*, redissolved in 1 ml of a water/tetrahydrofuran (THF) (1:1) mixture containing 62.5 ppm of 2,6-di-*tert*-butyl-4-methylphenol (BHT) used as an internal standard, and injected (30 μl) on a C18 TSKgel column (super-ODS, Tosohaas), 50°C, 2 ml/min; eluant A: TFA 0.05% in water, eluant B: TFA 0.05% in water/acetonitrile (1:4 v/v), linear gradient 0–100% B in 10 min, then 100% B for 1 min, detection at 215 nm.

Liquid chromatography mass spectrometry (LCMS) analyses (see Figure 1) were performed on a LCMS Micromass Platform using a C18 TSKgel column (super-ODS), 60°C, 2.75 ml/min; eluant A: TFA 0.05% in water, eluant B: TFA 0.0425% in water/acetonitrile (1:4), linear gradient 0–100% B in 3 min, then 100% B for 1 min, sum of the absorbances between 200 and 400 nm.

Cleavage of the Peptidyl Resins

An aliquot of the washing solutions was hydrolysed in evacuated sealed tubes with 6 N HCl/phenol (10:1, v/v) at 110°C for 24 h. The amount of cleaved peptide was determined by quantitative amino acid analysis on a Beckman amino acid analyser model 7300 using ninhydrin detection.

RESULTS AND DISCUSSION

Exposure of Lys(Mtt) to mild acidic conditions, such as 1% TFA in DCM, generates the stable Mtt carbocation, whose yellow color is used for the monitoring of the deprotection. However, during the Mtt removal of peptidyl resins **5**–**17**, the yellow color did not disappear even after prolonged acidic treatments. We hypothesized that a partial cleavage of the Trt groups, or of the peptide–resin link could be responsible for this residual absorbance. Thus, the deprotection of Lys(Mtt) was carefully studied using a RP-HPLC monitoring protocol which allowed to distinguish precisely the different products of the acidolysis.

For all the deprotection experiments, the concentration of TFA in DCM was maintained constant (1%, v/v). When necessary, the number of equivalents of TFA per Mtt group was varied by changing the volume of the TFA/DCM solution. After each acidic treatment, an accurately measured aliquot of the filtrate was concentrated *in vacuo* and redissolved in THF/H_2O (1:1). THF was stabilized with BHT, which served as an internal standard. The monitoring was performed using a high speed and high resolution TSKgel C18 RP-HPLC column. A 3 min gradient permitted the separation of Trt-OH and Mtt-OH without any difficulty. The duration of analysis (5 min, including the re-equilibration of the column) was short enough to adjust the number of washings to the degree of deprotection difficulty.

Deprotection of Model Peptidyl Resins 1–4

Peptidyl resins **1**–**4** served as models to correlate the absence of Mtt-OH in the RP-HPLC profiles with the completion of the Mtt removal. Fmoc-Lys(Mtt)-Asn(Trt)-Rink amide resin **1** was washed several times with 1% TFA in DCM (18 equivalents of TFA each time per Mtt group, see entry 1 of Table 1 and Figure 1). As expected, a yellow color appeared immediately, decreased until the 7th acidic treatment yet remained up to the 20th cycle of deprotection. The RP-HPLC trace corresponding to the 10th washing (Figure 1(d)) showed the presence of trace amounts of Mtt-OH, the absence of Trt-OH but the persistence of a large peak which corresponded to Fmoc-Lys-Asn(Trt)-NH_2 by APCI-MS. Deprotection and cleavage of the peptidyl resin in concentrated TFA assured the complete removal of the Mtt group (Figure 1(e)).

For peptidyl resin **1**, the residual absorption at 470 nm observed following complete removal of the Mtt group could be attributed to the presence of Fmoc-Lys-Asn(Trt)-NH_2 in solution. Indeed, the visible absorption of the TFA/DCM mixtures was very similar to those given by Fmoc-L-Asn(Trt)-OH when dissolved in the same solvent (data not shown). For peptidyl resin **1**, Trt-OH could not be detected in the filtrates. However, it will be seen later that for

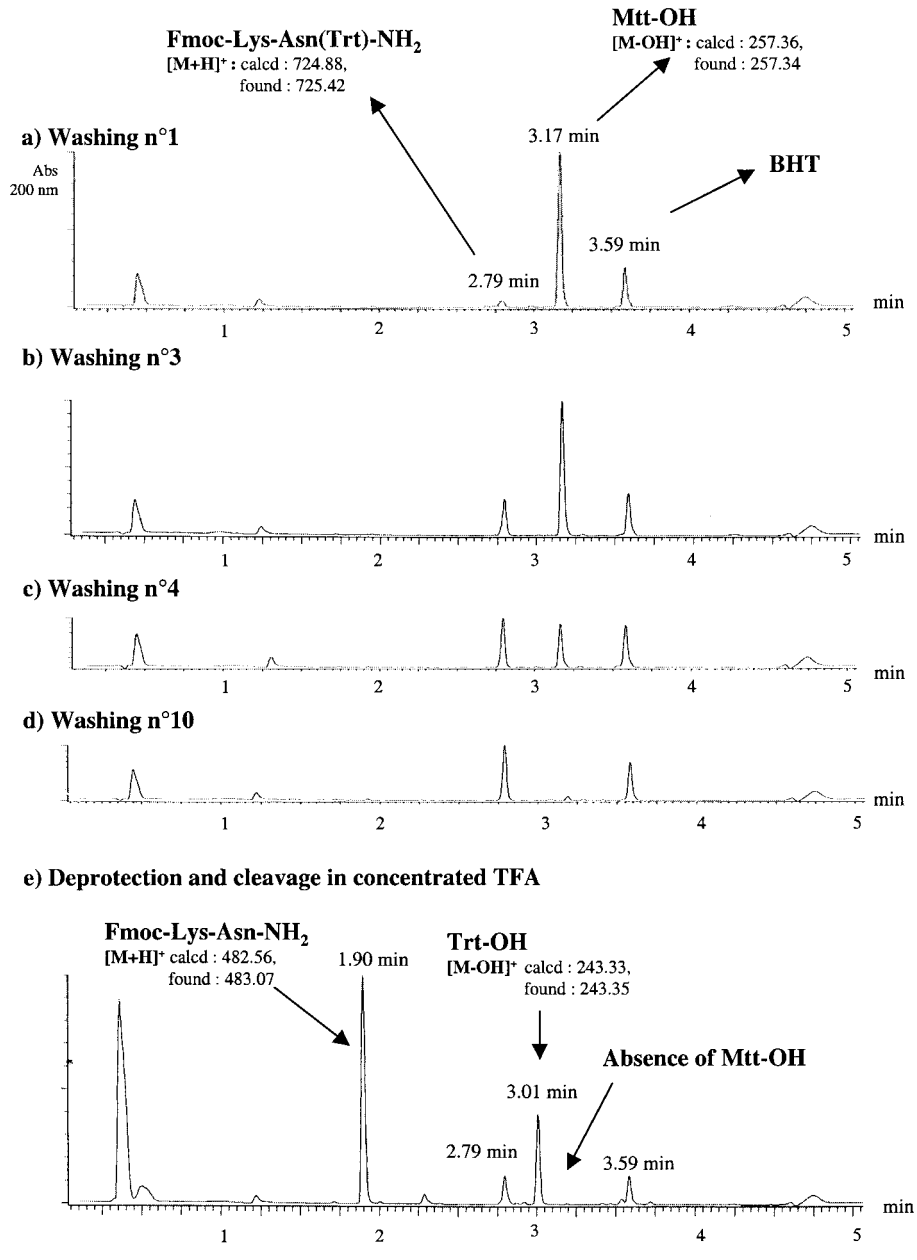

Figure 1 Deprotection of peptidyl resin **1**. LCMS analyses.

peptidyl resins containing a large number of Trt-protected side chains, Trt-OH was present in significant amounts in the RP-HPLC profiles. The partial cleavage of the protected peptidyl resin was also observed with the Wang linker (resin **2**).

The same analysis was repeated with peptidyl resins **3–4**. For resin **3**, the Mtt group disappeared after seven washings, whereas the yellow color persisted up to the 14th acidic treatment. The 3rd washing yielded a new peak, which was attributed to the protected peptide Ac-Y(*t*Bu)KN(Trt)-NH_2. Amino acid analysis of an aliquot following total acid hydrolysis confirmed the presence of the peptide in the washing mixtures and allowed its quantitation. The cleavage was found to be as high as 1.8% of the initial loading per washing. The completion of the deprotection was checked by acetylation of the peptidyl resin following the disappearance of Mtt-OH from the RP-HPLC profiles. Cleavage and deprotection of the peptide in concentrated TFA led to the crude tripeptide Ac-YK(Ac)N-NH_2, which was found to be free of the non-acetylated analog. Thus,

Table 1 Deprotection of Peptidyl Resins **1–17**

Entry	Peptidyl resin[a]	PS resin	Nb of Asn(Trt) and/or Gln(Trt)	TFA 1% in DCM for 0.25 mmol (ml per cycle)	Nb of cycles[e]
1	Fmoc-**K**(**Mtt**)N-resin	RA[b]	1	35	7
2	Fmoc-**K**(**Mtt**)N-resin	W[c]	1	35	7
3	Ac-Y**K**(**Mtt**)N-resin	RA	1	35	7
4	Ac-Y**K**(**Mtt**)N-resin	W	1	35	7
5	Fmoc-EKGGLEGIYY SARRHRILDMYLE**K**(**Mtt**)-resin	RA	0	15	9
6	Fmoc-DWQDYTSGPG IRYPKTFGWLWKLV**K**(**Mtt**)-resin	RA	1	15	9
7	Fmoc-SKWDDPWGEV LAWKFDPTLAY-TYEA**K**(**Mtt**)-resin	RA	0	15	9
8	Fmoc-SVRPKVPLRA MTYKLAIDMSH-FIKEK**K**(**Mtt**)-resin	RA	0	15	9
9	Fmoc-YTYEAYARYP EELEASQACQRKR-LEEG**K**(**Mtt**)-resin	RA	2	15	9
				30	2
10	Fmoc-KFGAEVVPGF QALSEGCTPYDINQML-NCVG DKE**K**(**Mtt**)-resin	RA	3	15	9
				30	2
11	Fmoc-QIQWMYRQQM PIPVGNIYRRWIQL-GLQKCV RMYNPTN**K**(**Mtt**)-resin	RA	9	15	9
				30	2
12	Fmoc-DELFNELLNS VDVNGEVKE-NILEESQ**K**(**Mtt**)-resin	RA	5	15	15
				20	8
13	Ac-LEESQVNDDI FNSLVKSVQQEQQHNV**K**(**Mtt**)-resin	RA	8	15	12
				20	12
14	Fmoc-LLSNEEPKE NIIDNLLNNI**K**(**Mtt**)-resin	RA	5	20	25
15	Fmoc-**K**(**Mtt**)VESVAPSVE ESVAPSVEESVAEN-VEESV-resin	RA	1	15	12
16	Ac-**K**(**Mtt**)GKVLTSFTN KELQAYAKG**K**(**Mtt**)-resin	RA	2	30[d]	15
17	Ac-**K**(**Mtt**)GSRDDDMET KRQNENG**K**(**Mtt**)-resin	RA	3	30[d]	12

[a] Protecting groups of functional side chains were omitted for clarity. Amino acids were protected as described in the 'Materials and Methods'.
[b] RA, Rink amide linker.
[c] W, Wang linker.
[d] The volume corresponds to 8 equivalents of TFA per Mtt group. The peptidyl resin was washed with DCM between each acidic treatment.
[e] Number of cycles required to obtain the complete desappearance of MttOH during RP-HPLC monitoring of the acidic washings.

as for peptidyl resin **1**, the disappearance of Mtt-OH in the RP-HPLC trace of the washing mixtures could be correlated with a complete deprotection of the ε-amino group.

Application to Larger Sequences

The previous model experiments set the stage for the deprotection of the peptidyl resins **5**–**17**, which present the Lys(Mtt) residue in different environments. Lys(Mtt) is situated at the *C*-terminus for peptidyl resins **5**–**14** yet at the *N*-terminus for peptidyl resin **15**. Resins **16**–**17** present two Lys(Mtt) residues at both *N*- and *C*-termini. In addition, two resins contained a large number of Trt-protected residues, i.e. peptidyl resins **11** and **13**.

As previously described, the model experiments were performed using 18 equivalents of TFA per washing and per Mtt group. For the Rink amide peptidyl resin **3**, the amount of cleaved peptide (about 2% of the initial loading per washing) was found to be high. Therefore, the number of TFA equivalents, and thus the volume of the TFA/DCM (1:99) solution was lowered. Typically, the deprotection was initiated with 8 equivalents of TFA per Mtt group (for 0.25 mmol of peptidyl resin, 15 ml of TFA/DCM solution per cycle). Depending upon the difficulty of the deprotection, the volume of the acidic solution was increased (see entries 9–13, Table 1). For resins **13**–**17**, the amount of cleaved peptide was determined as described above for peptidyl resin **3** (Table 2), and was found to represent 0.10–0.20% of the initial loading per washing. For peptidyl resin **14**, which was found to be particularly difficult to deprotect, about 4% of the product was lost during the overall process. For the other examples, the loss was slightly lower (2–3%).

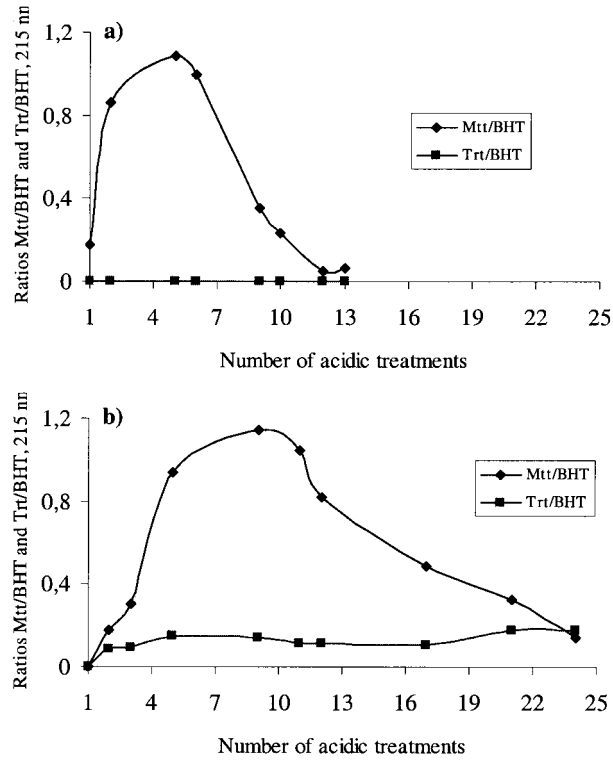

Figure 2 Determination of the Mtt removal for peptidyl resins **15** (a) and **13** (b). BHT is used as an internal reference for the RP-HPLC analyses.

Peptidyl resin **15**, containing only one Trt protected amino acid, behaved like model resins **1** and **3**, i.e. Trt-OH was not detected in the RP-HPLC

Table 2 Determination of the Amount of Cleaved Peptide during Acidic Treatments

Peptidyl resins	Amount of cleaved peptide (% of the initial loading)[a]				
Washing step	13	14	15	16	17
5	0.03	0.20	0.25	0.28	0.15
10	0.04	0.12	0.37	0.26	0.27
12	nd	nd		nd	0.30
14	nd	nd		0.26	
16	0.07	nd			
17	nd	0.16			
23	0.11	0.17			

[a] Determined by quantitative amino acid analysis following total acid hydrolysis of an aliquot of the washing solution.
nd: not determined.

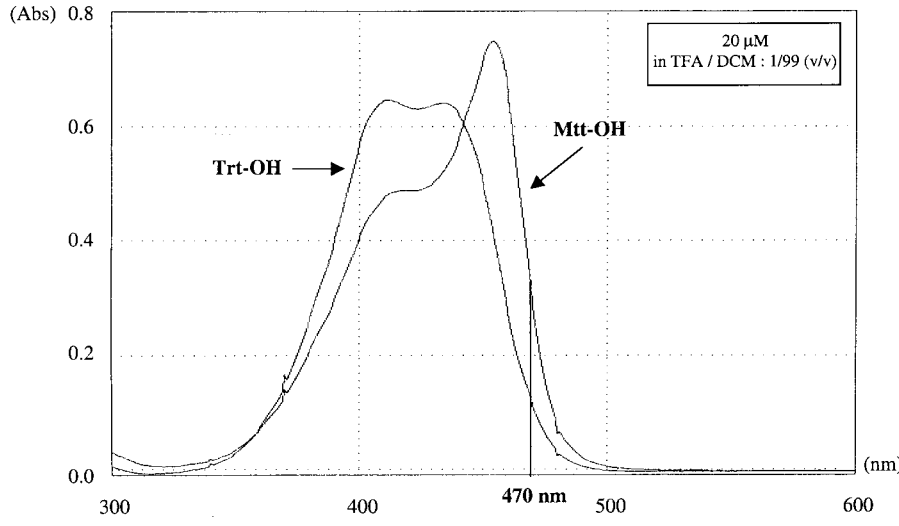

Figure 3 Spectrophotometric analysis of Mtt-OH and Trt-OH in TFA/DCM (1:99, v/v).

traces (Figure 2(a)). Alternately, the level of Trt group released during the deprotection of peptidyl resin **13** was found to be high (Figure 2(b)), and could be related to the large number of Trt-protected amino acids in the sequence. In this case, the partial removal of the Trt group was particularly troublesome at the end of the deprotection, where the Trt and Mtt levels were comparable. Indeed, the monitoring of the end of the deprotection could not be performed spectrophotometrically, since the absorption at 470 nm of Mtt-OH in TFA/CH_2Cl_2 (1:99) is only twice that of Trt-OH (Figure 3). Peptidyl resins **11** and **14**, containing nine and five Trt groups respectively, gave similar results.

Finally, the ease of removal of the Mtt moiety was found to be highly dependent on the sequence (Table 1). Peptidyl resins **5**–**11**, assembled on a *C*-terminal Lys(Mtt) residue, were easily deprotected, while resins **12**–**14** were found to be more difficult cases.

CONCLUSION

In conclusion, deprotection of Lys(Mtt) on the solid phase was found to cleave partially the Trt group. The level of Trt release was particularly high for peptidyl resins containing a large number of Trt-protected side chains, and perturbed the spectrophotometric monitoring of the deprotection. Alternately, RP-HPLC permitted the rigorous monitoring of the deprotection, thanks to the ability to separate Trt-OH and Mtt-OH using very short (3 min) linear gradients. Finally, Wang and Rink amide peptidyl resins were found to be partially cleaved in TFA/DCM (1:99). The RP-HPLC monitoring described here minimizes the exposure of the peptidyl resin to the acidic dichloromethane solution and consequently the loss of product during this deprotection step.

Acknowledgements

We gratefully acknowledge financial support from Institut Pasteur de Lille, Université de Lille 2, ANRS and CNRS. We thank also Dr Steve Brooks for proofreading the manuscript and Christophe Landry for performing LCMS analyses.

REFERENCES

1. Thiam K, Loing E, Verwaerde C, Auriault C, Gras-Masse H. Influence of lipid modification on the conformation and the conformation and the ability to induce MHC class II expression on murine and human cells. *J. Med. Chem.* 1999; **42**(18): 3732–3736.
2. Lloyd-Williams P, Albericio F, Giralt E. *Chemical Approaches to the Synthesis of Peptides and Proteins*. CRC Press: Boca Raton, New York, 1997; 12.
3. Aletras A, Barlos K, Gatos D, Koutsogianni S, Mamos P. Preparation of the very acid-sensitive Fmoc-Lys(Mtt)-OH. *Int. J. Peptide Protein Res.* 1995; **45**: 488–496.
4. Wang SS. *p*-Alkoxybenzyl alcohol resin and *p*-alkoxybenzyloxycarbonylhydrazide resin for solid phase synthesis of protected peptide fragments. *J. Am. Chem. Soc.* 1973; **95**(4): 1328–1333.

5. Rink H. Solid-phase synthesis of protected peptide fragments using a trialkoxy-diphenyl-methylester resin. *Tetrahedron Lett*. 1987; **28**: 3787–3790.

6. Fields GB, Noble RL. Solid phase peptide synthesis utilizing 9-fluorenyl methoxycarbonyl amino acids. *Int. J. Peptide Protein Res*. 1990; **35**: 161–214.

