Int. J. Peptide Protein Res. **37**, 1991, 513–520

2-Chlorotrityl chloride resin

Studies on anchoring of Fmoc-amino acids and peptide cleavage

KLEOMENIS BARLOS, OLGA CHATZI, DIMITRIOS GATOS and GEORGE STAVROPOULOS

Department of Chemistry, University of Patras, Patras, Greece

Received 26 July, accepted for publication 21 November 1990

The esterification of 2-chlorotrityl chloride resin with Fmoc-amino acids in the presence of DIEA is studied under various conditions. High esterification yields are obtained using 0.6 equiv. Fmoc-amino acid/mmol resin in DCM or DCE, in 25 min, at room temperature. The reaction proceeds without by product formation even in the case of Fmoc-Asn and Fmoc-Gln. The quantitative and easy cleavage of amino acids and peptides from 2-chlorotrityl resin, by using AcOH/TFE/DCM mixtures, is accomplished within 15–60 min at room temperature, while t-butyl type protecting groups remain unaffected. Under these exceptionally mild conditions 2-chlorotrityl cations generated during the cleavage of amino acids and peptides from resin do not attack the nucleophilic side chains of Trp, Met, and Tyr.

Key words: 2-chlorotrityl chloride resin; esterification; Fmoc-amino acids; peptide cleavage

In recent years base labile Fmoc-amino acids **2** protected at their side chains with acid sensitive groups of the t-butyl type have found rapidly increasing application in solid phase peptide synthesis by using 4-alkoxybenzyl alcohol resins. Their esterification catalyzed by DMAP and utilizing DCC is accompanied by a significant degree of racemization and dipeptide formation and several methods have been applied to overcome these serious problems. The peptide ester bond to these resins can be cleaved by using at least 50% TFA in DCM (1). Resins of the triphenylmethyl (trityl, Trt) (2, 3), diphenylmethyl (4–6), and dialkoxybenzyl type (7–13) have been proved to be much more sensitive to acids. By their application not only free peptides, but also peptide fragments, partially protected at their side chains with groups of the t-butyl type can be obtained.

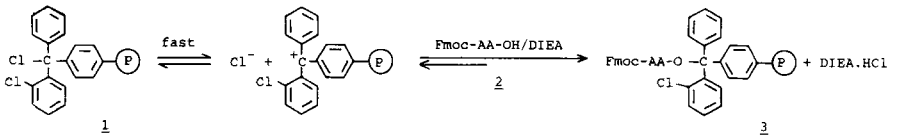
Initial studies (2) concerning the applicability of the Trt-resins showed that the best combination of properties required for the solid phase peptide synthesis (SPPS) is revealed from the 2-chlorotrityl chloride resin (**1**). We have therefore investigated thoroughly the properties of this resin in order to elucidate its application in SPPS.

Abbreviations used are in accordance with rules of IUPAC-IUB Commission on Biochemical Nomenclature in *European J. Biochem.* (1984) **138**, 9–37; *J. Biol. Chem.* (1989) **264**, 663–673. Other abbreviations: AA, amino acid; Acm, acetamidomethyl; AcOH, acetic acid; BOP, benzotriazolyl *N*-oxytrisdimethylaminophosphonium hexafluorophosphate; Bu^t, *tert.*-butyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCE, 1,2-dichloroethane; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DMAC, *N,N*-dimethylacetamide; DMAP, *p*-dimethylaminopyridine; DMF, *N,N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; Mbh, dimethoxybenzhydryl; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TDO, 2,5-diphenyl-2,3-dihydro-3-oxo-4-hydroxythiophene dioxide; TFA, trifluoroacetic acid; TFE, trifluoroethanol; THF, tetrahydrofuran; TLC, thin-layer chromatography; Tmob; 2,4,6-trimethoxybenzyl; Trt, triphenylmethyl; Xan, 9-xanthenyl.

RESULTS AND DISCUSSION

Anchoring of Fmoc-amino acids to 2-chlorotrityl resin (1)

It is known from the chemistry of tritylchloride in solution that its reaction with nucleophiles proceeds via an exceptionally fast heterolytic cleavage and formation of the very stable trityl cation. This is followed by a comparatively slow reaction between the formed cation and the nucleophile. It seems reasonable that the reaction of the polymeric 2-chlorotrityl chloride



SCHEME 1

(**1**) with Fmoc-amino acids **2** follows a similar path (Scheme 1).

In order to examine the properties of **1** during its esterification with Fmoc-AA-OH we first investigated the effect of various solvents. Our results for the sterically hindered Fmoc-Ile-OH are summarized in Table 1. Thus, a high loading of the resin corresponding to an almost quantitative esterification of Fmoc-Ile-OH is obtained in DCM (99%) or DCE (98%), while in THF, DMF, and DMAC the esterification yield is somewhat lower (85–89%). Dioxane has been found to be less suitable for the amino acid anchoring to the resin.

The above experiments have been carried out using a 3.2-fold molar excess of DIEA over Fmoc-Ile-OH. Further investigation of the amount of DIEA needed to obtain maximum esterification showed that the resin loading remains stable during decreasing DIEA to an 1:1 molar ratio to Fmoc-Ile-OH (Table 1). Nevertheless we continued to use excess of DIEA in order to prevent possible hydrolysis of resin-Cl bond and as an acceptor of HCl formed during the destruction process of the unreacted chloride which follows the esterification.

The degree of racemization occurring with the applied procedure was determined by preparing the dipeptides L-Leu-L-Phe-OH and L-Leu-D-Phe-OH and was found by HPLC-analysis to be less than 0.05%. This is not surprising because the applied procedure for esterification of **1** avoids electrophilic activation of the carboxyl group. For the attachment of Fmoc-amino acids to the alkoxybenzyl and dialkoxybenzyl alcohol resins, which have routinely been used in SPPS, strong electrophilic activation of the acid (1) or troublesome prior transformation of the resin to the corresponding benzyl halide has to be realized (14, 15).

In all procedures described in literature, which avoided the addition of DMAP, the esterification of **2** on the resins of the alkoxybenzyl alcohol or alkoxybenzyl halide type is slow (> 10 h) with the exception of applying TDO esters of **2** (16, 17). Even in the latter case, which normally leads to a sufficient loading within 1–2 h at room temperature, the esterification of a sterically hindered amino acid, such as Fmoc-Ile-OH, proceeds rather slowly, requiring 10 h at room temperature to obtain a good loading, although the amino acid is applied in excess.

The esterification of Fmoc-Ile-OH with resin **1** proceeds very rapidly (Fig. 1). Thus, in 2 min at room temperature a loading of 0.60 mmol Ile/g resin is obtained, corresponding to a 66% reaction of Fmoc-Ile-OH. It was found that in 10 min an almost quantitative esterification was achieved (96%). According to our knowledge such fast esterification has not been observed for any other resin, regardless of the procedure used. In addition an almost linear correlation between loading of the resin and the used mmol of Fmoc-Ile-OH in the reaction is obtained (Fig. 2). This correlation constitutes an advantage and provides the possibility to calculate the required load of the amino acid from the beginning.

TABLE 1

Resin loading by Fmoc-Ile-OH in various solvents[a]

Solvent	Resin substitution on H-Ile-OH [mmol/g]	% Esterification of Fmoc-Ile-OH
DCM	0.90	99
DCE	0.89	98
THF	0.81	89
DMF	0.79	87
DMAC	0.77	85
Dioxane[b]	0.48	53

[a]Conditions are: 1 mmol Fmoc-Ile-OH; 1 g resin **1** (1.6 mmol Cl^-/g). By using several substitutions of active chloride, like 1.18, 1.41, and 1.60 mmol Cl^-/g resin **1**, the esterification, carried out in DCE, was always > 93%; 3.2 mmol DIEA. The use of various amounts between 1.0 and 3.2 mmol DIEA, in DCE, gave > 94% esterification of Fmoc-Ile-OH on the resin; 15 mL solvent; 25 min at room temperature. [b]Prolongation of the reaction for 2 h increased the yield to 72%.

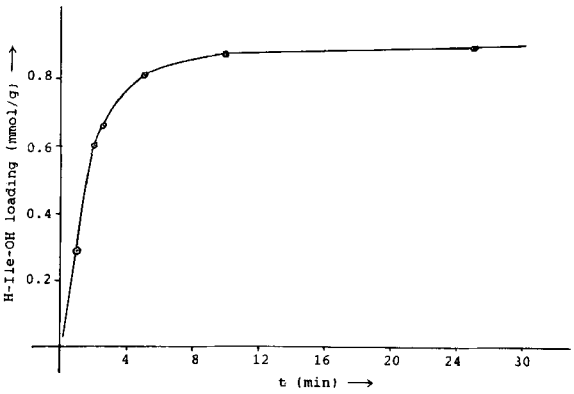


FIGURE 1

Time dependence of H-Ile-OH loading to resin **1**.

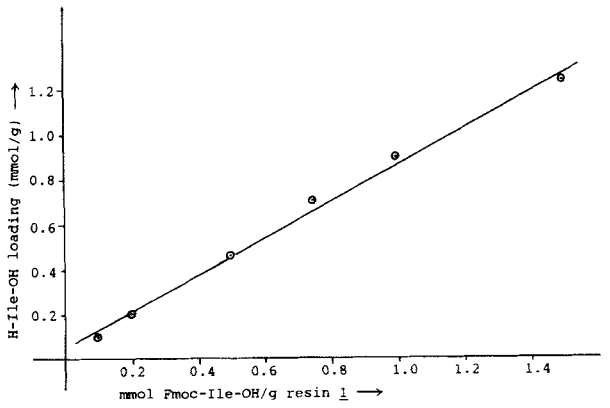


FIGURE 2
Correlation between the used mmol Fmoc-Ile-OH and the succeeded loading to resin **1**.

High esterification yields of Fmoc-Ile-OH are also achieved using **1** with various chloride substitution (Table 1). In all cases examined in DCE the yield was higher than 93%. An efficient esterification has also been achieved with most amino acids applied for anchoring (Table 2). The exception, Fmoc-Cys(Trt)-OH reacting with the 2-chlorotrityl chloride resin in an analogous high yield, can not be explained by the steric hindrance due to the bulky side chain Trt-group, because similar amino acid derivatives, such as Fmoc-Ser(Trt)-OH (18) and Fmoc-Thr(Trt)-OH (19), were esterified onto the resin with much higher yield. Similarly, lower yields have been obtained with Fmoc-Asp(Bu^t)-OH and Fmoc-Trp-OH.

Electrophilic activation of Asn and Gln derivatives by DCC or other methods results in nitrile formation. Side reaction during activation of Asn and Gln derivatives can be minimized by blocking their side chain carboxamide by base stable groups Mbh, Tmob, Trt,

TABLE 2
Resin loading by Fmoc-AA-OH[a]

No.	AA	Fmoc-AA-OH [mmol]	Resin substitution on H-AA-OH[mmol/g]	% Esterification of Fmoc-AA-OH
1	Ala	0.9	0.70	82
2	Arg(Pmc)	1.0	0.68	96
3	Arg(Mtr)	0.8	0.53	85
4	Asn	0.3	0.23	78
5	Asn	1.0	0.74	81
6	Asp(Bu^t)	0.5	0.25	54
7	Asp(Bu^t)	1.0	0.56	64
8	Cys(Bu^t)	0.9	0.57	71
9	Cys(Trt)	0.5	0.24	56
10	Cys(Trt)	1.0	0.46	61
11	Gln	0.7	0.51	78
12	Glu(Bu^t)	0.4	0.26	68
13	Glu(Bu^t)	1.0	0.67	78
14	Gly	0.7	0.52	76
15	Gly	1.0	0.79	82
16	His(Trt)	0.6	0.45	92
17	D-His(Trt)	0.6	0.46	94
18	Ile	1.0	0.89	98
19	Leu	1.0	0.90	99
20	Met	0.6	0.53	95
21	Phe	1.0	0.83	93
22	Pro	0.8	0.65	87
23	Ser(Bu^t)	0.8	0.56	77
24	Ser(Trt)	0.6	0.45	90
25	Thr(Bu^t)	0.7	0.56	88
26	Thr(Trt)	0.5	0.41	95
27	Trp	0.5	0.24	51
28	Trp	0.8	0.35	50
29	Tyr(Bu^t)	0.6	0.49	91
30	Tyr(Trt)	0.4	0.30	86
31	Val	0.9	0.84	100

[a]Conditions are: 1 g resin **1** (1.6 mmol Cl^-/g); 2.5 mmol DIEA; 15 mL DCE; 25 min at room temperature.

and Xan. Their acidic cleavage requires the use of concentrated TFA solutions and prolonged treatment and therefore the advantage of applying acid sensitive resins is decreased (1). To overcome this problem side chain attachment of Fmoc-Asp-OBu^t and Fmoc-Glu-OBu^t to acid sensitive resin-amines is recommended (20, 21). In contrast, it is obvious that by applying resin **1** the side chains of Gln and Asn can be left unprotected. As expected, no nitrile formation or any other byproduct is observed by TLC and HPLC after their esterification with **1** and their subsequent splitting from resin with acetic acid.

During the esterification reaction of both Fmoc-Arg(Mtr)-OH and Fmoc-Arg(Pmc)-OH the resin is colored red-violet. We did not find any explanation for this observation. The HPLC analysis showed for both derivatives, after their cleavage from resin, that no side reactions occurred during the esterification process, while the pale-yellow color of resin recurred after the removal of the Fmoc-group.

It is important to note for all cases we have tested (Table 2) that a good loading does not require more than 0.6 equiv. Fmoc-amino acid (1 mmol/g resin) over the active chloride of the resin. In contrast, according to the literature, it is generally assumed that for an acceptable loading of a resin of the benzyl alcohol type at least 1.5 equiv. Fmoc-amino acid over the free hydroxyl functions is necessary.

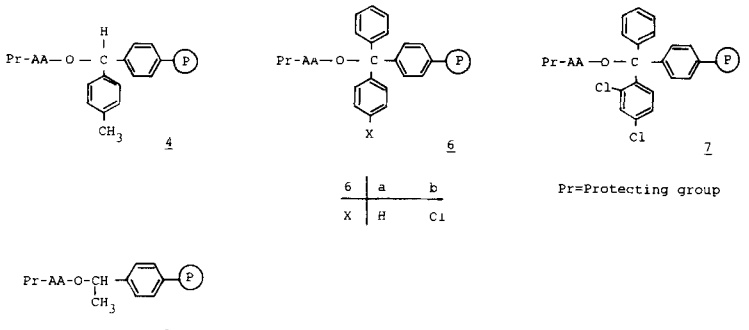
After the attachment to the resin of the first Fmoc-amino acid, the excess of active chloride remaining unreacted has to be destroyed. This can be rapidly succeeded (in 10 min) by addition of methanol, TFE, acetic acid or water and excess of DIEA in the reactor, when the 2-chlorotrityl chloride group is transformed to the corresponding methyl ether, trifluoroethyl ether, 2-chlorotrityl acetate or 2-chlorotrityl carbinol.

*Cleavage of amino acids and peptides from 2-chlorotrityl resin (**1**)*

The most important property of an acid labile resin, suitable for the preparation of free or protected peptides at their side chains with groups of the benzyl as well as the t-butyl type, is the correct balanced stability of the peptide-resin ester bond. Thus, the stability of the ester bond against acid has to be low enough to ensure quantitative splitting of the peptide from the resin with intact remaining side chain protecting groups of the t-butyl type and the N^{im}-Trt group of histidine. For this selectivity to succeed, the use of strong acids like TFA has to be avoided even at low concentrations. On the other hand the same ester bond has to have sufficient stability during the coupling, washing, and N^{α}-deprotection steps of the synthesis. This stability area is limited, as we have found by recent studies on the properties of several acid sensitive resins (6). For example, the ester bond of the 4-methyl benzhydryl esters (**4**) and of the α-methylbenzyl esters (**5**) has been proved to be quite sensitive against 1% TFA, but this can not be split off fast enough with weaker acids to ensure selective splitting in the presence of t-butyl and N^{im}-Trt protecting groups. On the other hand the ester bond on the trityl and 4-chlorotrityl resins (**6**) has been proved to be sensitive against HOBt or treatment with alcohols. Thus, loss of peptide chains during the synthetic procedure has been observed to a significant degree even during the preparation of small peptides (2). The same has also been observed in the case of trialkoxybenzhydryl resin (22). To surmount this problem, base has to be added during the coupling step, but in this case it is extremely difficult to avoid racemization of activated Cys and His derivatives. For this reason the resins **6a** and **6b** (Scheme 2) have not been considered suitable for the solid phase peptide synthesis (2).

Correctly balanced stability for the properties of the ester bond, as they described above, has been observed in our studies for 2-chlorotrityl (**3**) and 2,4-dichlorotrityl (**7**) resins. Their acid sensitivity has been found to be equivalent and therefore all further investigations have been performed with resin **3**.

Our studies showed that **3** is absolutely stable during both activation procedures with DCC/HOBt and BOP/DIEA. For example, no loss of peptide chains was observed during the synthesis of the human chorionic gonadotropin fragment 109-145 consisting of 37 amino acid residues. This peptide was synthesized by chain elongation using Fmoc-AA-OH preactivated with DCC and excess HOBt. The ester bond is also stable during washings of the resin with the usual applied solvents like DMF, DMAC, DCM,



SCHEME 2

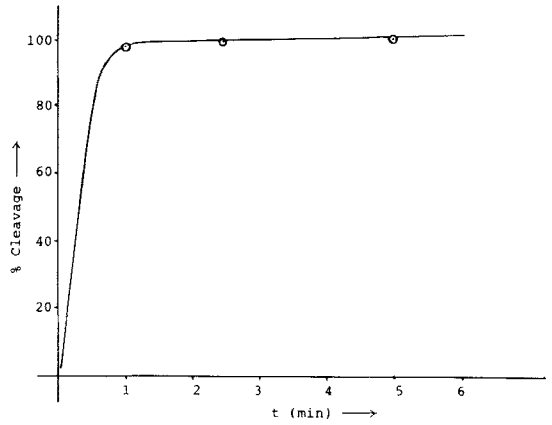


FIGURE 3
Time dependence of Fmoc-Ala-OH cleavage from resin **1**.

DCE, 2-propanol, and methanol. In our synthetic studies we routinely used alternative washings with DMF and 2-propanol. Several weak acids like acetic acid, formic acid, mono-, di-, and trichloroacetic acid, as well as low concentrations of strong acids like TFA and HCl have been applied for quantitative splitting of the peptide from 2-chlorotrityl resin. The best results for cleavage rates, yields, and selectivity in the presence of protecting groups of the t-butyl and Trt-type have been obtained with the application of AcOH/TFE/DCM (solvent A). We found by TLC analysis that only the very acid sensitive side chain Trt-group used for the protection of the N^{im}-function of His and the hydroxyl functions of Ser and Thr are very slightly affected. In contrast, when Trt, t-butyl, or Acm groups are used for the protection of the thiol function of Cys and Mtr or Pmc for the protection of quanidine function of Arg, they remain completely unaffected during splitting of the amino acids and peptides from the resin.

It must be noted that addition of TFE in the used solvent is a very effective accelerator for splitting of the peptide from 2-chlorotrityl resin. Mixtures containing MeOH (solvent B) instead of TFE are far less effective than solvent A. It is interesting that total absence of alcohols or moisture from the solvent leads to an uncompleted splitting from the resin even after 24 h treatment with AcOH/DCM (1:9). An impressive demonstration of the great difference between solvent A and B constitutes the splitting of Fmoc-Ala-OH from 2-chlorotrityl resin. It has been found that a 97.9% removal of Fmoc-Ala-OH is achieved with solvent A in 1 min at room temperature, while the use of solvent B leads to a quantitative removal in 30 min at the same temperature (Fig. 3).

As a peptide chain is elongated the splitting rate

TABLE 3
Conditions for peptide cleavage from 2-chlorotrityl resin[a]

No.	Peptide [fragment]	Position and side protecting group	AcOH/TFE/DCM [composition]	Time [min]
1	Leu[5]-enkephalin	1 = Bu[t]	1:1:8	15
2	Met[5]-enkephalin	1 = Bu[t]	1:1:8	15
3	N^{α}-Fmoc-ACTH[1-10]	1-3,5 = Bu[t]; 6 = Trt; 8 = Pmc	1:1:8	30
4	ACTH [1-24]	2,5 = Bu[t]; 1,3,6 = Trt; 8 = Pmc	1:2:7	30
5	ACTH [15-27]	15,16,26 = Boc; 17,18 = Mtr, 23 = Bu[t]	1:2:7	30
6	Angiotensin II	1,4 = Bu[t]; 2 = Pmc; 6 = Trt	1:1:8	30
7	Tetragastrin[b]	—	1:1:8	30
8	Leu[15]-gastrin I[c]	6-10,12 = Bu[t]	1:1:8	60
9	Peptide T[d]	2-5,7,8 = Trt	1:1:8	60
10	Chorionic gonadotropin [109-145]	109,111,112,117-121,127, 130,132,138,140 = Bu[t]; 110 = SBu[t]; 114, 113 = Pmc	1:2:7	60
11	N^{γ}-Fmoc-human calcitonin [1-10]	1,7 = Trt; 5,6 = Bu[t]	1:2:7	30
12	Magainin [1-10]	4,7,8 = Trt	1:2:7	30
13	N^{α}-Fmoc-human prothymosin [76-86][e]	76-80,82,85 = Bu[t] 83 = Trt	1:2:7 and 2:2:6	120
14	Human prothymosin [59-109]	60-67, 69-74, 76-80, 82 83,85, 91-97, 99,100, 105-109 = Bu[t]; 87,101,102, 104 = Boc; 88 = Pmc	1:2:7	60

[a]Conditions: 15 mL solvent A/g peptide- resin ester; room temperature. [b]Anchoring to the resin from Asp side chain. [c]Anchoring to the resin from Asp[16] side chain. [d]The O-Trt protecting group of Tyr is simultaneously partially removed. [e]The cleavage was quantitative in all other cases by treating the peptides with the appropriate solvent for the indicated time.

from the resin becomes somewhat slower. For example, in the case of the protected peptide fragments of human prothymosin 59-109 and chorionic gonadotropin 109-145 the splitting from the resin was quantitatively completed within 1 h at room temperature by using solvent A (with the composition 1:2:7 by vol.). When Pro is the *C*-terminal amino acid, the observed splitting rates are slower than usual. In this case solvent B fails to split > 80% peptide after 1 h treatment at room temperature and therefore solvent A has to be used for quantitative splitting. Also the particular peptide structure seems to be important. Thus, when small peptides are *C*-terminated with the dipeptide Asn-Gly or Gln-Gly and solvent A is applied, the splitting rate is somewhat slower and takes 1 h to be completed at room temperature. The splitting rate is increased and > 80% of the peptides is removed in 20 min at room temperature after the addition of 4–5 amino acid residues on the peptide chain.

We have encountered as yet only one case among the peptides being split from 2-chlorotrityl resin (Table 3) where the cleavage was not quantitative after 1 h treatment with solvent A. This was the prothymosin fragment 76-86, where treatment with solvent A (1:2:7) led to a 40% peptide detachment from the resin. A further 50% of the peptide was obtained when the treatment was repeated for another hour using solvent A with the composition 2:2:6 by vol.*

Cleavage of Trp-containing peptides from TFA sensitive resins of the alkoxybenzyl type results in irreversible alkylation of the indole ring by the alkoxybenzyl cations, generated during the cleavage. This side reaction can be suppressed by the addition of scavengers to the TFA mixtures during cleavage or side chain deprotection (1). An analogous side reaction is not expected in the case of trityl resins as they generate, during cleavage, cations with very weak electrophilic character due to the mesomeric effect of three aromatic rings. In addition the extreme steric hindrance of trityl cations impedes the electrophilic substitution of aromatic compounds and reduces considerably the reaction rate. According to our findings, when Fmoc-Trp is loaded on 2-chlorotrityl resin, the splitting, after the Fmoc group has been removed, is complete by applying solvent A or 5% TFA in DCM for 1 min at room temperature and is confirmed by a negative Kaiser test. Similar results are obtained for peptides incorporating Trp in their chain (Table 3).

Cleavage of peptides from resin **1** utilizing 50% TFA in DCM for simultaneous removal of t-butyl type protecting groups results in time-dependent alkylation of the indole ring of Trp, observed by a progressive increase in coloring of the resin beads after the Kaiser test. To avoid this side reaction the peptide can be quantitatively cleaved from the resin in 1 min and separated from the solid support by filtration. Subsequently, deprotection of the Trp-containing peptide from t-butyl groups is carried out without the danger of Trp alkylation by the resin. In contrast to Trp, Met and Tyr can be quantitatively cleaved from resin **1** independently of the concentration of TFA used.

*The synthesis of peptides contained in Table 3 will be published separately.

EXPERIMENTAL PROCEDURES

2-Chlorotrityl chloride resin, which can be prepared in an analogous manner to that used for the preparation of unsubstituted polymeric trityl chloride (23), was obtained from Biohellas S.A. Fmoc-amino acids and their derivatives were purchased either from Nova-Biochem or Biohellas. Solvents, AcOH, TFA, and TFE were of analytical grade, purchased from Fluka and used without further purification. The purity of Fmoc-amino acids obtained after splitting the 2-chlorotrityl resin esters was checked by TLC and HPLC. TLC was performed on precoated silica gel 60 F_{254} (Merck) aluminium plates employing the following solvents systems: toluene/acetic acid (9:1) and (8:2), chloroform/methanol/acetic acid (88:10:2) and (8:1:1). All HPLC runs were performed on a Waters 501 apparatus with Automated Gradient Controller, equipped with Spectro-Monitor UV detector using pre-packed analytical Hibar column [LiChrosorb RP 18(5 μm), 250 × 4mm] and a gradient solvent system of 0.1% TFA in water/0.1% TFA in acetonitrile.

Determination of active chloride on resin 1

This procedure is suitable for determination of the alkyl-chloride (active chloride) on resin **1** while the aryl-chloride remains unaffected.

Dry 2-chlorotrityl chloride resin (100 mg) was swelled for 5 min in dioxane (1 mL) and an excess of 0.01 N NaOH (30 mL) was added dropwise and slowly by stirring. The mixture was dilute with water to a total volume 100 mL, the indicator (methyl red) was added and the unreacted NaOH was titrated back with 0.01 N HCl.

Esterification of Fmoc-L-isoleucine with resin 1

2-Chlorotrityl chloride resin (1 g) was swelled in 10 mL of solvent from 2–3 min. Then a freshly prepared solution of Fmoc-Ile-OH and DIEA, in 5 mL of the used solvent, was added and the mixture was stirred for the appropriate time. The reaction was terminated by addition 20 mL of a mixture methanol/DIEA (9:1) and immediate filtration. Subsequently the Fmoc-Ile-resin ester was washed (3 × DMF, 2 × 2-propanol, 2 × DMF, 2 × 2-propanol, methanol and 2 × ether) and dried *in vacuo* for 24 h at room temperature. The kind of solvent, mmol amount of Fmoc-Ile-OH and DIEA, chloride loading on resin, and reaction time are shown in Table 1 and Figs. 1 and 2.

Esterification of Fmoc-AA-OH with resin 1. General procedure

2-Chlorotrityl chloride resin (1 g, 1.6 mmol Cl^-/g) and the appropriate quantity of the Fmoc-amino acid in DCE (10 mL) were stirred for 5 min at room temperature. Then DIEA (0.44 mL, 2.5 mmol) in DCE (5 mL) was added dropwise in a period of 5 min and the mixture was stirred for other 20 min. Subsequently methanol (1 mL) was added and stirring was continued for other 10 min. The resin was filtered, washed (3 × DCE, 2 × DMF, 2 × 2-propanol, 2 × DMF, 2 × 2-propanol, methanol and 2 × ether) and dried *in vacuo* for 24 h.

Determination of substitution of amino acid-resin esters

The substitution of N^{α}-deprotected amino acid-resin esters was quantitatively determined by using the ninhydrin procedure (24) and measuring the absorbance at 570 nm (ε values of literature have been generally used except for H-Ile-OH, which has been determined to be 16.543, according to the measurement conditions). The mean of three measurements with difference < 5% each other are presented in Tables 1 and 2.

The esterification yields were calculated by comparison of the experimental determination for each amino acid with the theoretical values. The theoretical loading (L) of any one amino acid on the resin corresponding to 100% esterification was calculated by the following formula:

$$L(\text{mmol/g}) = \frac{x}{1 + x(w-36.5)\cdot 10^{-3}}$$

where: x = mmol amino acid used for esterification per 1 g resin **1**
w = molecular weight of the amino acid

This formula does not take into account the small difference in weight between the substituted chloride by methoxide, when the latter is formed on the resin during the destruction of the excess chloride.

Cleavage of amino acids and peptides from resin 1

The amino acid or peptide-resin ester was suspended in the appropriate mixture of AcOH/TFE/DCM (15 mL/g ester) and stirred at room temperature. The resin was separated by filtration and washed four times with the used splitting mixture. The progress of the cleavage was checked by taking small aliquots of the resin ester, treatment with 25% piperidine in DMF for removal of the Fmoc-group followed by quantitative determination of existing amino groups on the resin by the ninhydrin procedure (see above). The cleavage was assumed complete after a negative Kaiser test. The obtained results are summarized in Table 3.

Determination of the racemization degree

The dipeptides L-Leu-L-Phe-OH and L-Leu-D-Phe-OH were prepared according to the usual manner. Their crude samples were chromatographed using isocratic elution with 0.1% TFA in H_2O/CH_3CN (67/33, v/v) at a flow rate of 1 mL/min. The detection was performed at 218 nm and the retention time for L-Leu-L-Phe-OH was 6.4 min and for L-Leu-D-Phe-OH 9.3 min. The proportion of the diastereomers was found to be less than 0.05%.

ACKNOWLEDGMENT

We thank the Ministry of Industry, Research and Technology and the "Chemical and Biopharmaceutical Laboratories of Patras, S.A." for financial support.

REFERENCES

1. Fields, G.B. & Noble, R.L. (1990) *Int. J. Peptide Protein Res.* **35**, 161–214 (for a recent review)
2. Barlos, K., Gatos, D., Kallitsis, K., Papaphotiu, G., Sotiriu, P., Wenqing, Y. & Schäfer, W. (1989) *Tetrahedron Lett.* **30**, 3943–3946
3. Barlos, K., Gatos, D., Kapolos, S., Papaphotiu, G., Schäfer, W. & Wenqing, Y. (1989) *Tetrahedron Lett.* **30**, 3947–3950
4. Rink, H. (1987) *Tetrahedron Lett.* **28**, 3787–3790
5. Barlos, K., Gatos, D., Kallitsis, J., Papaioannou, D., Sotiriou, P. & Schäfer, W. (1987) *Liebigs Ann. Chem.*, 1031–1035
6. Barlos, K., Gatos, D., Hondrelis, J., Matsoukas, J., Moore, G.J., Schäfer, W. & Sotiriou, P. (1989) *Liebigs Ann. Chem.*, 951–955
7. Sheppard, R.C. & Williams, B.J. (1982) *Int. J. Peptide Protein Res.* **20**, 451–454
8. Atherton, E., Brown, E., Priestley, G., Sheppard, R.C. & Williams B.J. (1981) in *Peptides: Synthesis, Structure, Function* (Rich, D.H. & Gross, E., eds.), pp. 163–175, Pierce Chemical Company, Rockford, IL
9. Sheppard, R.C. & Williams, B.J. (1982) *J. Chem. Soc. Chem. Commun.*, 587–589
10. Atherton, E., Dryland, A., Sheppard, R.C. & Wade, J.D. (1983) in *Peptides: Structure and Function* (Hruby, V.J. & Rich, D.H., eds.), pp. 45–54, Pierce Chemical Company, Rockford, IL
11. Mergler, M., Tanner, R., Gosteli, J. & Grogg, P. (1988) *Tetrahedron Lett.* **29**, 4005–4008
12. Mergler, M., Nyfeler, R., Tanner, R., Gosteli, J. & Grogg, P. (1988) *Tetrahedron Lett.* **29**, 4009–4012
13. Mergler, M., Nyfeler, R., Gosteli, J. & Grogg, P. (1988) in *Peptides: Chemistry and Biology* (Marshall, G.R., ed.), pp. 259–260, Escom, Leiden
14. Colombo, R., Atherton, E., Sheppard, R.C. & Woolley, V. (1983) *Int. J. Peptide Protein Res.* **21**, 118–126
15. Mergler, M., Gosteli, J., Nyfeler, R., Tanner, R. & Grogg, P. (1989) in *Peptides 1988* (Jung, G. & Bayer, E., eds.), pp. 133–135, Walter de Gruyter and Company, Berlin
16. Kirstgen, R., Sheppard, R.C. & Steglich, W. (1987) *J. Chem. Soc. Chem. Commun.*, 1870–1871
17. Kirstgen, R. & Steglich, W. (1989) in *Peptides 1988* (Jung, G. & Bayer, E., eds.), pp. 148–150, Walter de Gruyter and Company, Berlin

18. Barlos, K., Mamos, P., Papaioannou, D., Patrianakou, S., Sanida, C. & Schäfer, W. (1987) *Liebigs Ann. Chem.*, 1025–1030
19. Barlos, K., Gatos, D., Koutsogianni, S., Schäfer, W., Stavropoulos, G. & Wenqing, Y. (1990) *Tetrahedron Lett.*, in press
20. Albericio, F., van Abel, R. & Barany, G. (1990) *Int. J. Peptide Protein Res.* **35**, 284–286
21. Breipohl, G., Knolle, J. & Stüber, W. (1990) *Int. J. Peptide Protein Res.* **35**, 281–283
22. Nokihara, K., Hellstern, H. & Höfle, G. (1990) in *Peptides: Chemistry, Structure and Biology* (Rivier, J.E. & Marshall, G.R., ed.), pp. 1046–1048, Escom, Leiden
23. Fréchet, J.M.J. & Nuyens, L.J. (1976) *Can. J. Chem.* **54**, 926–934
24. Sarin, V.K., Kent, S.B.H., Tam, J.P. & Merrifield, R.B. (1981) *Anal. Biochem.* **117**, 147–157

Address:

Dr. *Kleomenis Barlos*
Department of Chemistry
University of Patras
Patras 26010
Greece