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Solid-Phase Peptide Synthesis using N^α-Trityl-Amino Acids.

Beatriz G. de la Torre,¹ Miguel A. Marcos,¹ Ramon Eritja¹ and Fernando Albericio.²

¹Department of Molecular Genetics. Instituto de Biología Molecular de Barcelona. C.S.I.C.,
Jordi Girona 18-26, E-08034 Barcelona, Spain.

²Department of Organic Chemistry. University of Barcelona, Martí i Franquès 1-11, 5
planta, E-08028 Barcelona, Spain.

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Abbreviations: AcOH: acetic acid, Ar: aromatic, Boc: t-butyloxycarbonyl, Bzl: benzyl, DCM: dichloromethane, DIEA: ethyldiisopropylamine, DIPC: diisopropylcarbodiimide, DMF: dimethylformamide, Et₃N: triethylamine, Et₂O: ethyl ether, Fmoc: fluorenylmethyloxycarbonyl, HATU: *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridino-1-ylmethylene-*N*-methylmethanaminium hexafluorophosphate *N*-oxide], HOBT: hydroxybenzotriazole, MeOH: methanol, NPE: 2-(*o*-nitrophenyl)ethyl, PAL: 5-(4-aminomethyl-3,5-dimethoxyphenoxy)-valeric acid, PEG-PS: polyethyleneglycol-polystyrene, pip: piperidine, tBu: tert-butyl, TCA: trichloroacetic acid, Tfa: trifluoroacetyl, TFA: trifluoroacetic acid, TFE: trifluoroethanol, Trt: trityl.

ABSTRACT

The preparation of N^α-trityl-amino acids is described. Several derivatives of trifunctional amino acids carrying acid- and base-labile side-chain protecting groups and the trityl group at the N^α position are prepared for first time. The incorporation of N^α-trityl-amino acids into peptide sequences using solid-phase protocols was achieved. The use of the trityl group for the protection of the α-amino group in conjunction with base-labile side-chain protecting groups constitutes a new method for the assembly of peptides in mild conditions.

INTRODUCTION

The solid-phase peptide synthesis (SPPS) methodology has evolved from Boc/Bzl to Fmoc/tBu strategy.¹ The main advantage of the latter is that the final release of the peptide from the solid support with concomitant removal of the side-chain protecting groups does not require the addition of strong acids like, anhydrous HF. Furthermore, Fmoc- and tBu- based protecting groups are orthogonal, which allows removal in any order and in the presence of each other, and facilitates the synthesis of complex peptides. Unfortunately, these mild conditions are not suitable for the preparation of sensitive biomolecules such as glyco- or phosphopeptides or oligonucleotide-peptide conjugates.

A milder strategy devoid of a base treatment uses Trityl (Trt) and tBu for the protection of α-amino group and side-chain functions respectively. The Trt group was introduced for peptide synthesis in solution² and can be removed in diluted acidic solutions (TFA-DCM (1:99), diluted AcOH or HOBt in TFE).^{3, 4} Although N^α-Trt-amino acid residues are resistant to racemization,⁵ their use in SPPS has not been described perhaps owing to their low reactivity.⁶

In the present communication, we describe the preparation of N^α-Trt-amino acids and several small peptides using N^α-Trt-amino acids and various strategies and supports. The results show that N^α-Trt-amino acids are useful derivatives for the preparation of peptides in solid-phase in mild conditions compatible with sensitive biomolecules.

Experimental part

Materials and methods

Amino acids and amino acid methyl ester hydrochlorides were purchased from Bachem (Switzerland) and NovaBiochem (Switzerland). Solvents including HPLC grade solvents were from SDS and E. Merck. Reagents were from Aldrich and Fluka and they were used without further purification. Analytical TLC was run on aluminium sheets coated with silica gel 60 F₂₅₄ from Merck. Silica gel column chromatography was performed with Chromatogel 60 A C.C. (40-60 microns, 230-400 mesh, SDS). NMR spectra were recorded on a Varian 300 MHz spectrometer.

General procedure for the preparation of N-trityl-amino acids using amino acid methyl esters (method A).

The N-Trt derivatives of Gly, Leu, Ile, Tyr(tBu) and Asp(OtBu) were prepared by a modification of the protocol described by Zervas.² To a stirred solution of amino acid methyl ester hydrochloride (5 mmol) in 20 ml of DMF was added 1.4 ml (10 mmol) of triethylamine and the solution was cooled with ice. 1.36 g of trityl chloride (5 mmol) dissolved in 7 ml of DCM was added and the mixture was stirred vigorously for 2.5 h at room temperature. Precipitated salts were then removed by filtration and ethanol (10 ml) was added to the solution, which was concentrated to dryness. The residue was treated with water and ethyl acetate. The organic phase was washed in water (2 x 30 ml), dried and concentrated to dryness yielding the desired N-Trt-amino acid methyl ester as white powders, which were used directly without further purification.

N-Trt-Gly-OMe, yield 1.84 g (99%). N-Trt-Leu-OMe, yield 2.12 g (93%). N-Trt-Val-OMe, yield 1.97 g (95 %). N-Trt-Ile-OMe, yield 2.0 g (90 %). N-Trt-Asp-(OtBu)-OMe, yield 2.1 g (94 %). N-Trt-Tyr(tBu)-OMe, yield 2.1 g (95 %).

General method for the hydrolysis of the methyl esters.

N-Trt-amino acid methyl ester (1.6 mmol) was dissolved in dioxane (25 ml/g of amino acid derivative), cooled with ice and then 1 N LiOH in MeOH/ water (1:1) (50 ml/ g of amino acid derivative) was added. The ice bath was removed and the mixture was left to stir (see each derivative for stirring time and temperature). The solution was neutralized with 2M KHSO₄ and the product was extracted with ethyl acetate. The combined organic layers were dried over Na₂SO₄, filtered and evaporated to dryness to give the target compounds.

Trt-Gly-OH. The hydrolysis was run at R.T. for 3 h. Yield 40%. $^1\text{H-NMR}$ (Cl_3CD) δ : 7.4-7.2 (m, 15H), 5.4 (broad s), 3.20 (s, 2H); $^{13}\text{C-NMR}$ (Cl_3CD) δ : 174.0 (COOH), 144.1, 128.5, 128.2 and 127.0 (Ar, trityl), 71.5 (Cq, trityl), 45.7 ($\alpha\text{-CH}_2$). Anal. Calcd for $\text{C}_{21}\text{H}_{18}\text{NO}_2$: C, 79.47; H, 6.03; N, 4.41. Found C, 79.33; H, 6.07; N, 4.39.

Trt-Leu-OH: The hydrolysis was run at 50°C for 5 h. Yield 98%. $[\alpha]_{\text{D}}^{22} = +48^\circ$ ($c=1$, MeOH); $^1\text{H-NMR}$ (Cl_3CD) δ : 7.42 (m, 6H), 7.20 (m, 9H), 3.32 (d, 1H), 1.82 (m, 1H), 1.41 (m, 1H), 1.26 (m, 1H), 0.98 (d, 3H), 0.87 (t, 3H); $^{13}\text{C-NMR}$ (Cl_3CD) δ : 178.0 (COOH), 145.7, 128.8, 127.8 and 126.6 (Ar, trityl), 71.4 (Cq, trityl), 59.3 ($\alpha\text{-CH}$), 40.7 ($\beta\text{-CH}$), 26.6 ($\gamma\text{-CH}_2$), 14.8 ($\gamma\text{-CH}_3$), 12.1 ($\delta\text{-CH}_3$). Anal. Calcd for $\text{C}_{25}\text{H}_{27}\text{NO}_2$: C, 80.40; H, 7.29; N, 3.76. Found C, 80.02; H, 7.32; N, 3.83.

Trt-Ile-OH. The hydrolysis was run at 50°C for 16 h. Yield 36%. $[\alpha]_{\text{D}}^{22} = +51^\circ$ ($c=1$, MeOH); $^1\text{H-NMR}$ (Cl_3CD) δ : 7.42 (m, 6H), 7.20 (m, 9H), 3.27 (t, 1H), 1.68 (m, 1H), 1.49 (m, 2H), 0.87 (dd, 6H); $^{13}\text{C-NMR}$ (Cl_3CD) δ : 180.3 (COOH), 145.7, 128.8, 127.0 and 126.5 (Ar, trityl), 71.2 (Cq, trityl), 55.3 ($\alpha\text{-CH}$), 45.0 ($\beta\text{-CH}$), 24.6 ($\gamma\text{-CH}$), 23.5 ($\delta\text{-CH}_3$), 21.9 ($\delta\text{-CH}_3$). Anal. Calcd for $\text{C}_{25}\text{H}_{27}\text{NO}_2$: C, 80.40; H, 7.29; N, 3.76. Found C, 80.90; H, 7.38; N, 3.73.

Trt-Asp(OtBu)-OH: The hydrolysis was run at 50°C for one day. Yield 76%. $[\alpha]_{\text{D}}^{22} = -6.5^\circ$ ($c=1$, MeOH); $^1\text{H-NMR}$ (Cl_3CD) δ : 7.43 (m, 6H), 7.25 (m, 9H), 3.45 (m, 1H), 2.4 (m, 2H), 1.34 (s, 9H); $^{13}\text{C-NMR}$ (Cl_3CD) δ : 172 (COOH and COOR), 145.6, 128.6, 127.9 and 126.7 (Ar, trityl), 71.5 (Cq, trityl), 67.0 (Cq, tBu), 54.3 ($\alpha\text{-CH}$), 37.0 ($\beta\text{-CH}_2$), 27.9 (CH_3 tBu); Anal. Calcd for $\text{C}_{27}\text{H}_{29}\text{NO}_4$: C, 75.15; H, 6.77; N, 3.25. Found C, 75.01; H, 6.56; N, 3.10.

N-Trt-Tyr(tBu)-OH: The hydrolysis was run at 50°C for one day. Yield 82%. $[\alpha]_{\text{D}}^{22} = +38^\circ$ ($c=1$, MeOH); $^1\text{H-NMR}$ (Cl_3CD) δ : 7.5-6.9 (m, 19H), 3.5 (m, 1H), 2.8 (m, 2H), 1.34 (s, 9H); $^{13}\text{C-NMR}$ (Cl_3CD) δ : 175.0 (COOH), 154.0, 145.8, 132.2, 130.1, 128.7, 127.8, 126.3 and 123.9 (Ar, Trt and Tyr), 78.3 (Cq, tBu), 70.9 (Cq, Trt), 58.3 ($\alpha\text{-CH}$), 41.7 ($\beta\text{-CH}_2$), 28.8 (CH_3 tBu), Anal. Calcd for $\text{C}_{32}\text{H}_{33}\text{NO}_3$: C, 80.14; H, 6.94; N, 2.92. Found C, 80.30; H, 7.07; N, 2.88.

Trt-Val-OH: After 6 days at 50°C , the hydrolysis of the ester was not complete and the reaction mixture was discarded.

General procedure for the preparation of N^α-trityl amino acids using amino acid trimethylsilyl esters (method B).

The N-Trt derivatives of Gly, Ala, Leu, Val, Phe, Lys(Tfa), Asn(Trt) and Gln(Trt) were prepared as described by Barlos et al.⁷ To a stirred suspension of amino acid (10 mmol) in 18 ml of CHCl₃-acetonitrile (5:1) was added 1.27 ml of trimethylsilyl chloride (10 mmol) at room temperature. The mixture was heated under reflux for 2 h and then allowed to cool to room temperature. 2.79 ml of Et₃N (20 mmol) and 2.79 g of trityl chloride (10 mmol) dissolved in 10 ml of CHCl₃ were then added. The resulting mixture was stirred for 1 h at room temperature. Thereafter, 2 ml of methanol (50 mmol) was added. For Gly, Ala, Leu, Val and Phe: the mixture was concentrated to dryness and the residue was treated with a mixture of 50 ml of Et₂O and 50 ml of a 5% precooled solution of citric acid. The organic phase was washed with 1 N NaOH (2 x 20 ml) and water (2 x 20 ml). The combined aqueous layers were washed with Et₂O, cooled with ice and neutralized with acetic acid. The product was extracted with Et₂O and dried. Evaporation of the solvent gave the desired products. Trt derivatives of Gly, Ala, Leu, Val, and Phe are described in ref. 7, except for NMR data, which is shown below. NMR data for Trt-Gly-OH and Trt-Leu-OH are described above.

Trt-Ala-OH: ¹H-NMR (Cl₃CD) δ: 7.42 (m, 6H), 7.20 (m, 9H), 3.32 (m, 1H), 1.09 (m, 3H); ¹³C-NMR (Cl₃CD) δ: 179.3 (COOH), 145.1, 128.7, 128.0 and 126.8 (Ar, Trt), 71.7 (Cq, Trt), 52.8 (α-CH), 20.6 (β-CH₃).

Trt-Val-OH: ¹H-NMR (Cl₃CD) δ: 7.42 (m, 6H), 7.20 (m, 9H), 3.17 (t, 1H), 2.00 (m, 1H), 0.97 (dd, 6H); ¹³C-NMR (Cl₃CD) δ: 178.6 (COOH), 145.9, 128.9, 127.7 and 126.5 (Ar, Trt), 71.2 (Cq, Trt), 61.3 (α-CH), 33.5 (β-CH), 19.7 and 17.8 (γ-CH₃).

Trt-Phe-OH: ¹H-NMR (Cl₃CD) δ: 7.4-7.1 (m, 20H), 3.57 (t, 1H), 2.88-2.68 (m, 2H); ¹³C-NMR (Cl₃CD) δ: 178.5 (COOH), 145.4, 136.8, 129.9, 128.6, 128.4, 127.9 126.9 and 126.6 (Ar, Trt and Phe), 71.2 (Cq, Trt), 58.0 (α-CH), 40.7 (β-CH₂).

Trt-Lys(Tfa) triethylammonium salt. Synthesis started with 2.5 mmol. After the first Et₂O extraction, the product was purified by silica gel chromatography. The column was packed with 1% triethylamine in DCM and eluted with a 0-5% MeOH gradient in DCM. Yield 0.2 g (0.34 mmol, 14%). TLC (CHCl₃/MeOH 9:1) R_f 0.49. ¹H-NMR (Cl₃CD) δ: 7.5-7.1 (m, 15H), 3.23 (m, 3H), 2.88 (q, CH₂, Et₃NH) 1.46 (m, 4H), 1.25 (m, 2H), 1.14 (t, CH₃, Et₃NH);

^{13}C -NMR (Cl_3CD) δ : 179.0 (COOH), 157.2 (COCF_3 , $J_{\text{C-F}}$ 36Hz), 115.9 (CF_3 , $J_{\text{C-F}}$ 288Hz), 146.5, 128.9, 127.6 and 126.2 (Ar, Trt), 71.4 (Cq, Trt), 56.8 (α -CH), 44.7 (CH_2 , Et_3NH), 39.6 (ϵ - CH_2), 34.4 (β - CH_2), 28.4 (δ - CH_2), 21.9 (γ - CH_2), 8.4 (CH_3 , Et_3NH); Anal. Calcd for $\text{C}_{33}\text{H}_{42}\text{N}_3\text{O}_3\text{F}_3$: C, 67.67; H, 7.23; N, 7.17. Found 67.63; H, 7.11; N, 6.68.

Trt-Asn(Trt)-OH. Synthesis started with 2.5 mmol of Asn(Trt). After the first Et_2O extraction, the product was purified by silica gel chromatography eluted with a 0-5% MeOH gradient in DCM. Yield 0.15 g (0.25 mmol, 10%). TLC ($\text{CHCl}_3/\text{MeOH}$ 9:1) Rf 0.40. ^1H -NMR (Cl_3CD) δ : 7.5-7.1 (m, 30H), 3.61 (m, 1H), 2.33 (m, 1H), 1.15 (m, 1H); ^{13}C -NMR (Cl_3CD) δ : 174.0 (COOH), 171.2 (CONH), 144.4, 144.0, 128.6, 128.5, 128.0, 127.9, 127.4 and 127.1 (Ar, Trt), 71.9 (Cq, Trt), 70.9 (Cq, Trt), 54.4 (α -CH), 37.0 (β - CH_2); Anal. Calcd for $\text{C}_{42}\text{H}_{36}\text{N}_2\text{O}_3$: C, 81.79; H, 5.88; N, 4.54. Found C, 80.91; H, 5.65; N, 4.45.

Trt-Gln(Trt)-OH. Synthesis started with 2.5 mmol of Gln(Trt). After the first Et_2O extraction, the product was purified by silica gel chromatography eluted with a 0-5% MeOH gradient in DCM. Yield 0.35 g (0.55 mmol, 22%). TLC ($\text{CHCl}_3/\text{MeOH}$ 9:1) Rf 0.50. ^1H -NMR (Cl_3CD) δ : 7.5-7.1 (m, 30H), 3.65 (m, 1H), 2.61 (m, 1H), 2.38 (m, 1H), 1.07 (m, 2H); ^{13}C -NMR (Cl_3CD) δ : 174.8 (COOH), 174.0 (CONH), 144.2, 141.5, 128.7, 128.5, 128.5, 127.8 and 126.9 (Ar, Trt), 73.4 (Cq, Trt), 70.9 (Cq, Trt), 57.6 (α -CH), 32.6 (β - CH_2), 26.3 (γ - CH_2); Anal. Calcd for $\text{C}_{43}\text{H}_{38}\text{N}_2\text{O}_3$: C, 81.88; H, 6.07; N, 4.44. Found C, 80.97; H, 6.07; N, 4.17.

Solid-phase peptide synthesis.

The following peptide sequences were assembled: 1) H-Leu-Ala-Gly-Val-OH, 2) H-Leu-Ala-Gly-Val-NH₂, 3) H-Tyr-Gly-Gly-Phe-Leu-OH, 4) H-Tyr-Gly-Gly-Phe-Leu-NH₂ and 5) H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-NH₂.

Sequences 1 and 3 was assembled on PEG-PS carrying the 2-(o-nitrophenyl)ethyl (NPE) linkage. Sequences 2, 4 and 6 were assembled on PEG-PS carrying the 5-(4-aminomethyl-3,5-dimethoxyphenoxy)-valeric acid (PAL) linker (Table 2).

The following synthesis cycle was used: 1) detritylation: 3% TCA in DCM (2 x 1 min), 2) neutralization: 5% DIEA in DCM (2 x 1 min, 1 x 5 min), 3) washings: DCM (3x) and DMF (3x), 4) Coupling: either DIPC/ HOBt protocol: N $^\alpha$ -Trt-amino acid (5 eq), HOBt (5 eq) and

DIPC (5 eq) in DMF, 1 h or HATU/ DIEA protocol: N^α-Trt-amino acid (2 eq), DIEA (4 eq) and HATU (2 eq) in DMF, 10 min and 5) washings: DMF (3x), and DCM (3x).

After the assembly of the desired sequences supports carrying NPE linkages were treated with 20% piperidine in DMF for 2 h and the support was removed by filtration. The resulting solutions were concentrated to dryness and products were purified by HPLC. Supports carrying the sequence 3, which contains Tyr(tBu) and the NPE linkage, were treated with 50% TFA in DCM for 30 min before piperidine treatment to remove tBu ether. Peptide amides prepared on supports carrying the PAL linker were cleaved and deprotected by treatment with 70% TFA in DCM for 2-3 h. The support was then filtered out and washed with 70% TFA in DCM, DCM and water. The combined filtrates were concentrated to dryness and purified by HPLC.

HPLC purification was performed using C18 columns. Solvent A: 0.045% TFA in water, solvent B: 0.035% TFA in acetonitrile. Gradient A: 30 min linear gradient from 10% to 100% B. Gradient B: 30 min linear gradient from 5% to 50% B. Monitored at 220 nm. In all cases except sequence 5, a major peak was collected and analyzed by mass spectrometry. Sequence 1) H-Leu-Ala-Gly-Val-OH, retention time 15.6 min (gradient A), MS (expected for C₁₆H₃₀N₄O₅ 358.4 amu) found 360.2 (M+H), 380.6 (M+Na); sequence 2) H-Leu-Ala-Gly-Val-NH₂, retention time 14.5 min (gradient A), MS (expected for C₁₆H₃₁N₅O₄ 358.4 amu) found 359.5 (M+H); sequence 3) H-Tyr-Gly-Gly-Phe-Leu-OH, retention time 15.4 min (gradient B), MS (expected for C₂₈H₃₇N₅O₇ 555.6 amu) found 554.0 (M-H) and sequence 4) H-Tyr-Gly-Gly-Phe-Leu-NH₂ retention time 23.31 min (gradient B), MS (expected for C₂₈H₃₈N₆O₆ 554.6 amu) found 552.8 (M-H).

Results

Preparation of N^α-Trt-amino acids.

Several methods for the preparation of N^α-Trt-amino acids have been described: 1) Tritylation of amino acid methyl esters and subsequent hydrolysis with NaOH in dioxane/water mixtures,² 2) direct tritylation of amino acids using a mixture of trityl chloride/diethylamine/isopropanol/water^{2,8} and 3) one-pot synthesis of N-Trt-amino acids using trimethylsilyl esters.⁷ The latter method may be more suitable since starts with free

amino acids and, according to the literature, yields are better than those of the first two methods.

Therefore, the N^α-Trt derivatives of Gly, Ala, Leu, Val, Phe, Lys(Tfa), Asn(Trt) and Gln(Trt) were prepared by the one-pot method using trimethylsilyl esters following Barlos et al.⁷ However, the yields were lower than described (39-64%, Table 1). Moreover, when we applied this method to the trifunctional amino acids Lys(Tfa), Asn(Trt) and Gln(Trt) (which have not been described elsewhere), products resulting from the work-up were not homogenous, an extra step of purification using silica gel chromatography was needed and the yields very even lower (10-22%, Table 1).

Table 1. N^α-Trt-amino acids prepared in this study.

N ^α -Trt-amino acid	Method ^a	Yield (%)	[α] _D ²² deg ^c
Gly	A	40	
Gly	B	43	
Ala	B	55	
Leu	A	98	+48
Leu	B	37	
Ile	A	36	+51
Val	A	Not isolated ^b	
Val	B	39	
Phe	B	64	
Lys(Tfa)	B	14	
Asn(Trt)	B	10	
Gln(Trt)	B	22	
Tyr(tBu)	A	82	+38
Asp(OtBu)	A	76	-6.5

^aA: from methyl ester derivative, B: from trimethylsilyl ester derivative

^bnot isolated owing to incomplete hydrolysis

^c(c=1, MeOH)

Thus the use of amino acid methyl esters as starting derivatives was investigated. These derivatives have been described for the preparation of N^α-Trt amino acids² but they were not used owing to the low rates of hydrolysis of methyl esters observed in sterically hindered N^α-Trt amino acid methyl esters.

The N^α-Trt derivatives of Gly, Leu, Ile, Val, Tyr(tBu) and Asp(OtBu) were prepared using amino acid methyl esters. Reaction of amino acid methyl ester hydrochlorides with trityl chloride in DMF in the presence of triethylamine⁹ gave the desired N^α-Trt-amino acid methyl esters in excellent yields (90-99%). Saponification of the Trt-Gly-OMe was assayed in various conditions: 1) KOH in methanol/dioxane/water,¹ 2) LiOH in methanol/dioxane/water⁹ and 3) iodotrimethylsilane¹⁰. The best results were obtained with LiOH and the methyl ester was completely removed in 3 h at room temperature. The rest of N^α-Trt-amino acid (Leu, Ile, Val, Tyr(tBu) and Asp(OtBu)) methyl esters were treated with LiOH but hydrolysis was slower due to steric hindrance² and lower solubility in the aqueous saponification solution. Heating the mixture to 50 °C improved the hydrolysis by increasing reactivity and solubility of the methyl ester. In these conditions, all N^α-Trt-amino acid methyl esters except Val were completely hydrolyzed in 5-24 h. N^α-Trt-amino acid free acids were isolated in good yields (40-98%, Table 1) with a simple work-up. The N^α-Trt derivative of Val was not prepared by this method owing to incomplete saponification.

Solid-phase peptide synthesis.

To our knowledge, there is one single report on the use of N^α-Trt-amino acids in solid-phase peptide synthesis. In this report, N^α-Trt-amino acids were added in the third position of a peptide sequence assembled with Fmoc-amino acid to avoid diketopiperazine formation.¹¹ The use of Trt-amino acids in peptide synthesis is limited owing to low yields in the preparation of Trt-amino acids and their failure to couple with other amino acids in acceptable yields.⁷

Table 2. Peptide sequences assembled using N^α-Trt-amino acids.

Sequence	Linker	Coupling	Deprotection
H-LAGV-OH	NPE	DIPC/HOBt	20% pip/ DMF
H-LAGV-NH ₂	PAL	HATU/DIEA	TFA
H-YGGFL-OH	NPE	DIPC/HOBt	1) TFA, 2) 20% pip/DMF
H-YGGFL-OH	NPE	HATU/DIEA	1) TFA, 2) 20% pip/DMF
H-YGGFL-NH ₂	PAL	DIPC/HOBt	TFA
H-YGGFL-NH ₂	PAL	HATU/DIEA	TFA
H-VQAAIDYING-NH ₂	PAL	HATU	TFA

Therefore, the coupling efficiency of Trt-amino acids was analyzed on test peptide sequences. The test sequences were the Merrifield tetrapeptide, Leu-enkephaline and the well-known difficult sequence from the acyl carrier protein ACP(65-74) (Table 2). Peptide sequences were assembled on polyethyleneglycol-polystyrene (PEG-PS) supports. We used the 2-(*o*-nitrophenyl)ethyl¹² (NPE) linker for the preparation of peptide free carboxylic acids and the 5-(4-aminomethyl-3,5-dimethoxyphenoxy)-valeric¹³ (PAL) linker for the preparation of peptide amides (Scheme 1).

Scheme 1. Chemical structure of NPE and PAL linkers

The synthesis cycle is described in the experimental part. The Trt group is removed with diluted (0.1-1%) solutions of TFA in DCM, as reported elsewhere.¹¹ The 3% TCA solution in DCM used to remove the dimethoxytrityl (DMT) group in oligonucleotide synthesis also remove efficiently the Trt group on solid-phase supports. Therefore, we used 3% TCA solution in DCM for the removal of the Trt group. After detritylation, we included a neutralization step with 5% DIEA in DCM. Two coupling protocols were assayed: a) diisopropylcarbodiimide (DIPC)-HOBt activation using 5 equivalents of Trt-amino acid and b) HATU/ DIEA activation using 2 equivalents of Trt-amino acid.

After the assembly of the sequences, peptides were deprotected and released from the solid support. The NPE ester linkage was cleaved with 20% piperidine solution in DMF and the PAL amide linkage was cleaved with 70% TFA in DCM. In addition to the 20% piperidine solution, we tested the treatment of the support with concentrated ammonia and NaOH in water/dioxane for the cleavage of the NPE linker. Cleavage was inefficient with NaOH and the treatment with concentrated ammonia gave a mixture of two products, the free carboxylic acid and the amide, in agreement with literature data (data not shown).¹⁴ In contrast treatment of NPE-peptide supports with 20% piperidine solution resulted in high yields and formation of the piperidine amide was not observed.

In all cases except for the ACP(65-74) peptide, we detected a major peak that was isolated and characterized by mass spectrometry (Figures 1 and 2). We found slightly better HPLC profiles when using HATU as coupling agent than when using DIPC (Figure 2). In any case, HATU is preferred because it requires only two equivalents of Trt-amino acid. The synthesis of the sequence ACP(65-74) gave several peaks, indicating incomplete coupling reactions. The failure of this synthesis confirms that Trt-amino acids are less reactive than the corresponding Boc- or Fmoc-amino acids but the reactivity is still high enough to produce short peptides that may carry sensitive biomolecules.

Figure 1. Reversed-phase HPLC chromatogram of A) H-Leu-Ala-Gly-Val-NH₂ and B) H-Tyr-Gly-Gly-Phe-Leu-NH₂ after cleavage from PAL solid supports. See methods for HPLC conditions.

Discussion

The use of Trt group for the protection of the temporary amino group during solid-phase peptide synthesis has been explored. As described elsewhere, the preparation of Trt-amino acids is not as straightforward as urethane protecting groups like Boc- or Fmoc-amino acids and coupling of Trt-amino acids may be less efficient. However, Trt-amino acids are an interesting alternative to the synthesis of peptides carrying molecules sensitive to acids (such as oligonucleotide-peptide conjugates and glycopeptides) because the removal of the Trt group can be performed in diluted acid solutions. We showed that Trt-amino acids can be combined with base labile supports, generating peptides in conditions compatible with oligonucleotides and glycopeptides. Moreover, tBu group can be used for side chain protection and acid labile supports in conjunction with Trt-amino acids. This strategy yields peptides with a single TFA treatment like the Fmoc-strategy but without the need to use bases for the removal of the amino protecting group. Overall, Trt-amino acids offer great potential for the synthesis of peptides in very mild conditions.

Figure 2. Reversed-phase HPLC chromatogram of H-Tyr-Gly-Gly-Phe-Leu-OH after cleavage from NPE solid support. A) crude from preparation using HATU/DIEA as coupling activators. B) crude from preparation using DIPC/HOBt. See methods for HPLC conditions.

Here we show that the problems faced in the preparation of Trt-amino acids can be solved by using amino acid esters rather than free amino acids. Although methyl esters have been used for several amino acids, saponification of Trt-amino acids methyl esters are still too harsh and cannot be used as a general method for all amino acids. We believe that the preparation of Trt-amino acids will improve by selecting a more labile group for the carboxyl function that should be removed in the presence of the trityl group.

We also address the problem of the activation of Trt-amino acids for solid-phase peptide synthesis. We found that DIPC and HATU can be used for the assembly of peptides, although HATU is preferred. Although Trt-amino acids are less efficient in coupling reactions than Boc- or Fmoc-amino acids, they can be used to produce short peptides. Therefore, they may increase the scope of peptide molecules accessible to chemical synthesis.

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