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Use of *N*-Tritylamino Acids and PyAOP[1] for the Suppression of Diketopiperazine Formation in Fmoc/^{t}Bu Solid-Phase Peptide Synthesis Using Alkoxybenzyl Ester Anchoring Linkages

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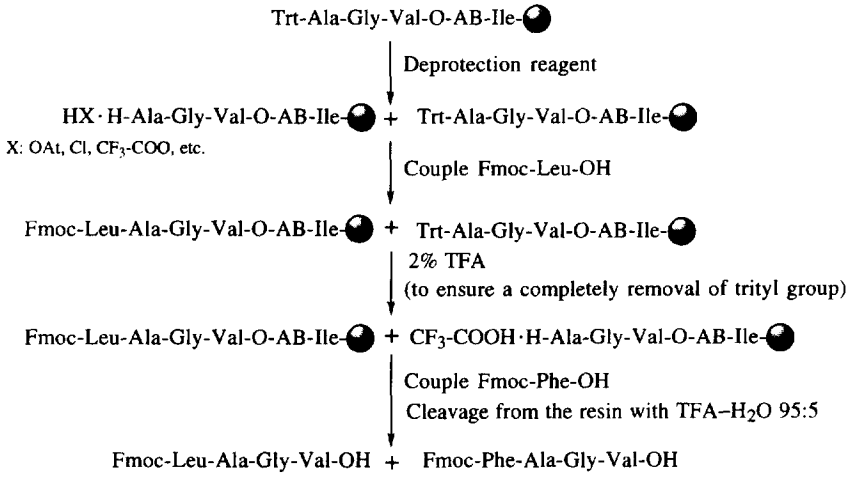
In order to suppress diketopiperazine formation in the solid-phase coupling of the third amino acid to dipeptides in Fmoc/^{t}Bu peptide synthesis using alkoxybenzyl ester resins, the Trt group was chosen for α-amino protection of the second amino acid since it can be removed by mild acid treatment. Subsequently, the coupling may be carried out without a prior neutralisation step using PyAOP as coupling reagent. This methodology also avoids diketopiperazine formation during the preparation of protected peptides with the HMPB handle since this is stable to the extremely mild acid treatment used to remove the Trt group. Copyright © 1996 Elsevier Science Ltd

The formation of diketopiperazines (DKP) is an undesired side reaction in solid-phase peptide synthesis,[2] and not only produces a decrease in the overall yield of final peptide, but also leads to the formation of hydroxyl sites[3] on the polymer which can give rise to other side reactions. This intramolecular aminolysis has been established to be either acid[2a] or base catalysed[2b-c,4] and it is strongly dependent on the nature and the sequence of the amino acids. Otherwise, DKP formation rates differ considerably for different peptide-resin linkages.[4,5]

Regarding the Boc/Bzl strategy, Gisin *et al* [2a] have described that DKP formation was repressed by adding the carbodiimide reagent prior to the carboxyl component, whereas Suzuki *et al* [6] have suppressed this side reaction by using 4N HCl in dioxane for Boc-deprotection, followed by addition, without prior neutralisation of the resin, of the *N*-methyl morpholine salt of the third amino acid along with DCC. Our group[7] has described the use of BOP reagent for the suppression of DKP formation when the coupling is carried out without a prior neutralisation step. It would be valuable to have available a general methodology for the classical alkoxybenzyl based linkages [Wang type resin (AB resin)[8] for free peptides, and HMPB resin[9] for protected peptides] in Fmoc/^{t}Bu strategy, in order to overcome this side reaction. So far, the only way to reduce DKP formation is using bulky resins such as the 2-chlorotrityl resin[10a] and *tert*-butyl based resin[10b] or to avoid the dipeptide stage in the synthesis incorporating the second and the third amino acid in form of a protected dipeptide.[11]

It is well known that the trityl (Trt) group, as an α-amino protecting group in peptide synthesis, can be selectively removed by mild acid treatment in excellent yields and in the presence of other acid-sensitive protecting groups.[12] Furthermore, it offers excellent resistance to racemisation even in the case of strongly activated chiral amino acid derivatives.[13] Thus, the Trt group could be used in combination with a Fmoc strategy in order to avoid DKP formation in a similar manner to that which we described earlier for a Boc strategy.[7]

Optimization of trityl group removal. First of all in this study, we have tried to define the mildest conditions required to remove the trityl group quantitatively . We have chosen Trt-Ala-Gly-Val-AB-Ile-MBHA[14] as a model to check the extention of deprotection by using a deprotection-coupling protocol shown in the Scheme 1.[15] After treatment of our model peptide with a given deprotection reagent, two possible resin-bound species can be obtained: unchanged Trt-Ala-Gly-Val and Ala-Gly-Val with a liberated N^{α}-amino group. These two possibilities were distinguished by a sequence involving coupling of Fmoc-Leu-OH, treatment with 2% TFA to ensure a complete removal of the remaining trityl groups, coupling of Fmoc-Phe-OH, and evaluation by HPLC and amino acid analysis (Table 1) of the two peptides obtained after that the peptide-resin was treated with TFA–H_2O (95:5).



Scheme 1

Bodanzsky *et al* [16] have described the removal in solution of the trityl group with several weak acids and the coupling of the amino salt without the addition of a tertiary base, in order to prevent side reactions. Initially, we tried to find a weak acid, other than a carboxylic acid, which could provide the acidity needed for quantitative acidolytic removal of the trityl group on the solid-phase at a practical rate, without catalysing DKP formation.[2,4,5] For this reason several acids were tested as acidolytic reagents (entries 1 and 2, Table 1). Since the deprotection rates were not practical in these cases, and taking into account that recently, Kaiser Sr. *et al* [17] have described the use of chlorotrimethylsilane–phenol in DCM as a mild removal reagent for the Boc protecting group, we have tried to find a similar cocktail reagent that was suitable for removing the trityl group without bringing about loss of peptide by acidolytic cleavage of peptide from the resin (entries 3 and 4, Table 1). We have found that 0.1M HOAt and 0.12M Me_3SiCl in TFE (entry 3.2) and 0.02M Me_3SiCl in TFE (entry 4.3) quantitatively removed the trityl group, and they were also completely suitable when 3-(4-hydroxymethylphenoxy) propionic acid (AB)[8b] is used to link the peptide to the resin. Unfortunately, we have detected that these conditions lead to partial loss of peptide when the HMPB[9] handle is used to link the peptide to the resin. Since our objective was to achieve a general methodology for all alkoxybenzyl ester anchoring linkages used in Fmoc/^{t}Bu strategy, we have finally decided to investigate the minimum amount of TFA needed for quantitative removal of the trityl group using the above protocol[15] evaluating this as 0.2% TFA and 1% H_2O in DCM (entry 5.2).

Table 1

Entry	Deprotection condition	Amino Acid ratio[a] Leu	Phe	Stability[b] of peptide-resin linkage (AB resin)
1.1	0.1 M HOAt in TFE[c]	56	44	+
1.2	0.1 M HOAt in TFE–H_2O (8:2)	45	55	+
1.3	0.1 M HOAt in TFE–Et_3SiH (95:5)	18	82	+
1.4	0.2 M HOAt in TFE–DMF (9:1)	16	84	+
2.1	0.1 M HOBt in TFE	11	89	+
2.2	0.5 M HOBt in TFE	18	84	+
2.3	0.5 M Tetrazole in TFE	45	55	+
2.4	0.5 M HOSu in TFE	19	81	+
3.1	0.1 M HOAt and 0.10 M Me_3SiCl in TFE	93	7	+
3.2	0.1 M HOAt and 0.12 M Me_3SiCl in TFE	100	0	+
3.3	0.1 M HOAt and 0.15 M Me_3SiCl in TFE	100	0	- (6%)
4.1	0.10 M Me_3SiCl in TFE	100	0	- (22%)
4.2	0.05 M Me_3SiCl in TFE	100	0	- (8%)
4.3	0.02 M Me_3SiCl in TFE	100	0	+
4.4	0.01 M Me_3SiCl in TFE	87	13	+
5.1	0.1% TFA and 1% H_2O in DCM	81	19	+
5.2	0.2% TFA and 1% H_2O in DCM	100	0	+

a) Determined by amino acid analysis. b) +: stable; -: not stable (% loss of peptide). c) Solution saturated.

Once we had determined the removal conditions of the trityl group, and in view of our previous experience of the problem of DKP formation in the synthesis of the dipeptide D-Val-Pro,[2a,7] we chose Fmoc-Lys-D-Val-Pro-OH as a model to test our methodology. Trt-D-Val-Pro-AB-Ile-MBHA and Trt-D-Val-Pro-HMPB-Ile-MBHA were synthesized by a standard coupling protocol.[14] The incorporation of the third amino was carried out by removing the trityl groups of the dipeptides with 0.2 % TFA and 1% H_2O in DCM[18] and then (see Figure 1) without prior neutralisation, coupling with Fmoc-Lys(Boc)-OH (5 fold excess relative to the amino function), using PyAOP[19,20] reagent (5 fold excess) with DIEA (10 fold excess) in DMF (complete coupling within 60 minutes). The formation of the DKP should compete with the formation of the new peptide bond leading to the tripeptide resin. This PyAOP-mediated coupling leads to the suppression of DKP formation when the AB linker was used, and to 5% of loss of peptide when HMPB was used (in this case the acidolytic removal of the trityl group probably leads to partial loss of peptide).[21,22]

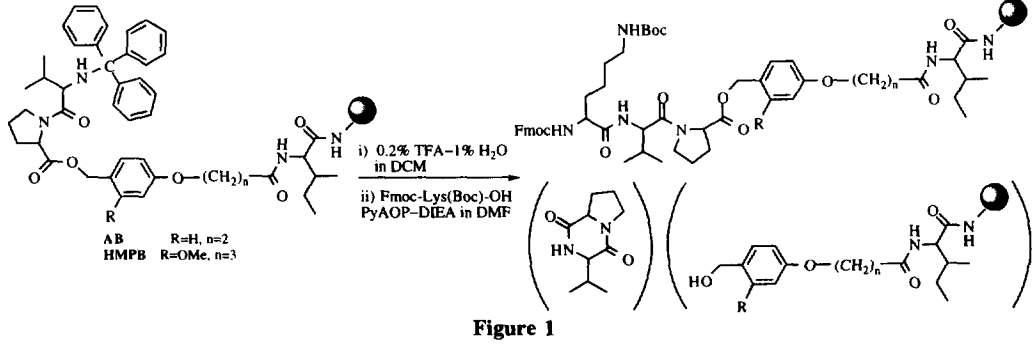


Figure 1

These results demonstrate the usefulness of *N*-tritylamino acids together with the use of PyAOP reagent in the solid-phase coupling of the third amino acid to dipeptides in Fmoc/^{t}Bu strategy using

alkoxybenzyl ester resins, in order to suppress the diketopiperazine formation in cases where this side reaction is troublesome. This methodology allows protected peptides to be prepared with the HMPP handle, because this is practically stable to the mild acid treatment used to remove the Trt group. Furthermore, several conditions using non carboxylic acids have been described for the removal of Trt groups on solid-phase.

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References and Notes

1. Abbreviations used in this article: AB, 3-(4-hydroxymethylphenoxy) propionic acid; Boc, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yloxy-tris-(dimethylamino)phosphonium hexafluorophosphate; Bzl, benzyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DIPCDI, *N,N'*-diisopropylcarbodiimide; DKP, diketopiperazine; DMAP, *N,N*-dimethylaminopyridine; DMF, *N,N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HMPB, 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid; HPLC, high performance liquid chromatography; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; MBHA, *p*-methylbenzhydrylamine; Nbb, α-[3-nitrobenzamido]benzylpolystyrene); PyAOP, 7-azabenzotriazol-1-yl-oxytris(pyrrolidino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; ^{t}Bu, *tert*-butyl; TFE, 2,2,2-trifluoroethanol; Trt, triphenylmethyl (trityl).
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14. Fmoc-Ile-OH was attached directly to a *p*-methylbenzhydrylamine resin by a standard coupling protocol and served as an internal standard for estimating the stability of the peptide-resin linkage. 3-(4-Hydroxymethylphenoxy) propionic acid was coupled to the *N*-group of Ile by the same coupling method. Fmoc-Val-OH was incorporated using DIPCDI in the presence of DMAP (0.3 fold excess relative to the amino function), and Fmoc-Gly-OH and Trt-Ala-OH (Barlos, K.; Papaioannou, D.; Theodoropoulos, D. *J. Org. Chem.* **1982**, 47, 1324-1326) using a standard coupling protocol. A standard protocol in Fmoc/^{t}Bu solid-phase peptide synthesis was as follows: The resin was swollen by washing with DMF (3x1 min) and removed the Fmoc group with piperidine–DMF (2:8) (2x1 min, 1x10 min), washing with DMF (5x1 min). Fmoc-AA-OH and HOAt (3 fold excess relative to the amino function in both cases) was added as a solution in DMF followed by DIPCDI. The mixture was left 60 min and then filtered, washing with DMF (5x1 min).
15. The Trt-Ala-Gly-Val-O-AB-resin (0.05 g, 0.57 mmols/g resin) was swollen by washing with DMF (3x1 min) and deprotected with the appropriate reagent (2 mL, 2x1 min, 1x20 min), washing with DMF (3x1 min), followed by neutralisation with DIEA–DCM (1:19) (3x1 min) and washing again with DMF (3x1 min). Fmoc-Leu-OH and HOAt (3 fold excess relative to the amino function in both cases) was added as a solution in DMF followed by DIPCDI. The mixture was left 60 min and then filtered, washing with DMF (5x1 min), DCM (3x1 min). Subsequently, the resin was deprotected with TFA–H_2O–DCM (2:1:97) (5x1 min), washing with DCM (3x1 min), followed by neutralization with DIEA–DCM (1:19) (3x1 min) and washing again with DCM (3x1 min), DMF (3x1 min). Fmoc-Phe-OH and HOAt (3 fold excess relative to the amino function in both cases) was added as a solution in DMF followed by DIPCDI. The mixture was left 60 min and then filtered, washing with DMF (5x1 min), DCM (3x1 min). In both cases, qualitative ninhydrin test was used to determine completion of coupling.
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21. Yields were calculated by amino acid analysis of the resins before and after the incorporation of the Fmoc-Lys(Boc)-OH.
22. Synthesis of the same tripeptides using standard Fmoc chemistry [deprotection with piperidine–DMF (2:8) (2x1 min, 1x10 min)] gave rise 89% (AB-resin) and 67% (HMPB-resin) of DKP formation.

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