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A Dde resin based strategy for inverse solid-phase synthesis of amino terminated peptides, peptide mimetics and protected peptide intermediates

AMAN RAI and WILLIAM G. GUTHEIL*
Department of Pharmaceutical Sciences, University of Missouri-Kansas City, 5005 Rockhill Road, Kansas City, Missouri 64110-2265, USA

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Abstract: This report describes a Dde resin based attachment strategy for inverse solid-phase peptide synthesis (ISPPS). This attachment strategy can be used for the synthesis of amino terminated peptides with side chains and the carboxyl terminus either protected or deprotected. Amino acid *t*-butyl esters were attached through their free amino group to the Dde resin. The *t*-butyl carboxyl protecting group was removed by 50% TFA, and inverse peptide synthesis cycles performed using an HATU/TMP based coupling method. Protected peptides were cleaved from the resin with dilute hydrazine. Side chain protecting groups could then be removed by treatment with TFMSA/TFA. The potential of this approach was demonstrated by the synthesis of several short protected and unprotected peptides in good yield and with low epimerization. Its potential for peptide mimetic synthesis was demonstrated by the synthesis of two peptide trifluoromethylketones.

Keywords: inverse solid-phase peptide synthesis; Dde resin; peptide mimetic; peptide trifluoromethylketone; amino acid *t*-butyl esters; protected peptide fragment

INTRODUCTION

Solid-phase peptide synthesis (SPPS) has become a routine tool for the synthesis of peptides and small proteins since its introduction in 1963 [1]. SPPS in the *C*-to-*N* direction is well established (reviewed in [2]). Strategies for *N*-to-*C* (inverse) SPPS (ISPPS) are much less well developed [3–8]. A previous report demonstrated the use of amino acid *t*-butyl esters for ISPPS [9]. Amino acid *t*-butyl esters are easily prepared, stable and a substantial selection is commercially available. This earlier strategy used dicarboxylic acid linkers on either PAM or MBHA resin, with the linker remaining in the final product. Other approaches to ISPPS have solved the attachment problem in several ways, including the use of a TFA cleavable trityl-amine attachment [5,8], a TFA cleavable urethane attachment [6], and with photocleavable linkers [7].

Compatible attachment strategies which can flexibly provide a variety of products are necessary to exploit the full potential of ISPPS. Dde (1-(4,4-dimethyl-2,6-dioxocyclohexylidene) ethyl) is an amine protecting group that can be used orthogonally with *t*-butyl protecting groups [10]. Dde protection has been reported as a side chain protecting group in the solid-phase synthesis of branched [10] and cyclic [11] peptides. A Dde based resin has been used for the solid-phase synthesis of polyamine analogues of philanthotoxins [12]. Since cleavage from a Dde based resin can be accomplished using dilute hydrazine, the use of a Dde attachment strategy for ISPPS appeared likely to allow for the synthesis of both side chain and *C*-terminally protected peptides, without a linker in the product. Such a strategy would provide several conversion pathways for ISPPS (Scheme 1).

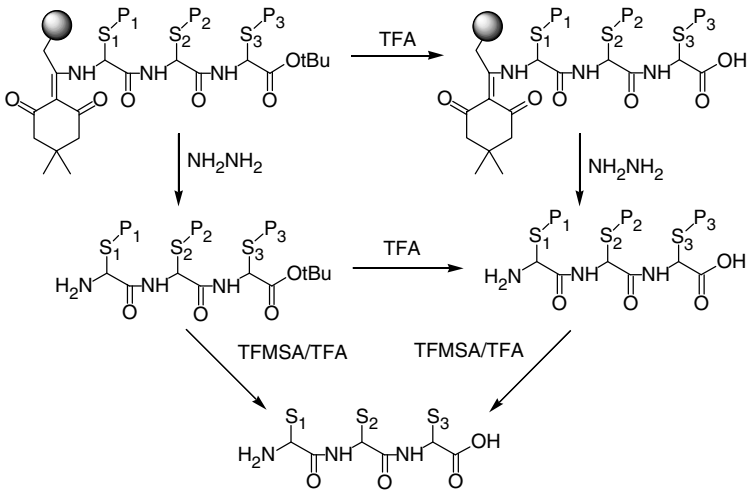
This report describes a Dde resin attachment strategy for *t*-butyl ester based ISPPS of short protected and deprotected peptides and peptide mimetics in good yield and purity, and with a low degree of epimerization. The utility of this approach for the preparation of protected peptide fragments is significant, since such protected peptide fragments could be employed in fragment condensation or convergent peptide synthesis strategies [13], or as synthetic intermediates in other applications. ISPPS also provides the synthetically versatile peptide *C*-terminal carboxyl group for further modification into *C*-terminally modified peptide mimetics. These are of general interest as potential bioactive agents, and the ability to elaborate the *C*-terminus on the solid-phase would greatly facilitate the synthesis of *C*-terminally modified peptide mimetics. The use of the Dde attachment strategy was therefore also demonstrated for the on-resin synthesis of peptide trifluoromethylketones.

MATERIAL AND METHODS

Materials

Dde resin (R-Dde) (0.87 mmol/g, 100–200 mesh) was purchased from Calbiochem-Novabiochem AG (Switzerland), and

* Correspondence to: Dr William G. Gutheil, Department of Pharmaceutical Sciences, University of Missouri-Kansas City, 5005 Rockhill Road, Kansas City, Missouri 64110-2265, USA; e-mail: gutheilw@umkc.edu



S_1, S_2, S_3 are amino acid side chains.
P_1, P_2, P_3 are Boc/tBu chemistry compatible side chain protecting groups.

Scheme 1 Conversion pathways possible through Dde based ISPPS.

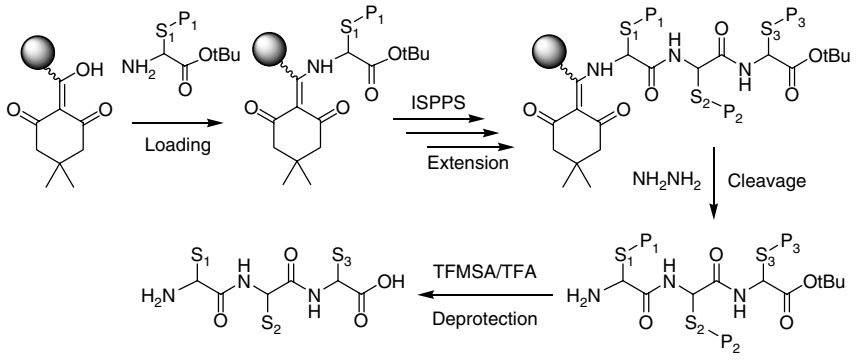
amino acid *t*-butyl esters were purchased from Bachem AG (King of Prussia, PA). HATU (O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluroniumhexafluorophosphate), TMP (2,4,6-trimethylpyridine), anhydrous NMP (N-methypyrrolidinone), TFA (trifluoroacetic acid), TFMSA (trifluoromethanesulfonic acid) and hydrazine were from Aldrich (Milwaukee, WI, USA). Anhydrous DMF (N,N-dimethyformamide) and DCM (dichloromethane) were from Acros (New Jersey, USA). Marfey's reagent (N^{α}-(2,4-dinitro-5-fluorophenyl)-L-alaninamide) was from Sigma (St Louis, MO, USA).

Methods

Loading of the first amino acid. The overall synthesis strategy is outlined in Scheme 2. To load the first amino acid, Dde resin (R-Dde) was swollen in DMF for 2 h and then washed with DMF. To the resin was added a freshly prepared solution of an amino acid *t*-butyl ester (AA-OtBu·HCl) (5 eq) and TMP (10 eq) in DMF, and mixed for 2 h. The resin was filtered and washed with DMF, and another solution of freshly prepared AA-OtBu·HCl (5 eq) and TMP (10 eq) in DMF was again added to the resin and mixed for 12 h (double loading). The resin was then washed and dried. To determine the loading efficiency with this method, Tyr-OtBu was loaded onto the resin. After washing and drying Tyr-OtBu was cleaved from the resin with 5% hydrazine, and the amount of Tyr-OtBu determined by HPLC. A yield of greater than 90% was obtained with double loading, whereas single loading only gave a yield of about 80%. Since double loading was used to completely saturate the resin, no capping reaction was necessary, and deletion peptides were not observed.

Inverse peptide synthesis. Inverse peptide synthesis cycles were performed as summarized in Table 1, using an HATU/TMP based coupling method with *in situ* activation (all reagents mixed together nearly at once) [7,9]. To a freshly prepared solution of AA-OtBu·HCl (5 eq) and TMP (10 eq) in DMF, HATU (5 eq) was added and stirred. This mixture was then added to the resin and the coupling reaction allowed to proceed for 12 h. The resin was then washed and dried before proceeding to the next cycle.

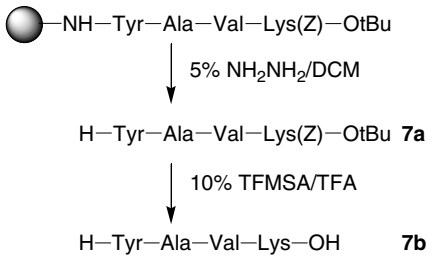
Cleavage of peptides from resin. Peptide-resin samples (10 mg) were treated with 5% hydrazine/DCM (400 µl) for 60 min. The cleavage solution was filtered, dried and analysed by HPLC and LC/MS.



Scheme 2 Inverse solid-phase peptide synthesis.

Table 1 ISPPS Protocol

Description	Reagent	Repetition and duration
OtBu	25% TFA/DCM	1 × 5 s
Deprotection	50% TFA/DCM	1 × 30 min
Washes	DCM	3 × 5 s
	NMP	2 × 5 s
	DCM	3 × 5 s
Activation/Coupling	5 × HATU	12 h
	5 × AA-OtBu·HCl	
	10 × TMP in DMF	
Washes	DCM	3 × 5 s
	DMF	3 × 5 s



Scheme 3 Cleavage of Tyr-Ala-Val-Lys(Z)-OtBu **7a** from resin and off-resin treatment with 10% TFMSA/TFA to give unprotected Tyr-Ala-Val-Lys-OH **7b**.

Side chain deprotection. Off-resin treatment of a protected peptide Tyr-Ala-Val-Lys(Z)-OtBu **7a** with 10% TFMSA/TFA for 30 min was used to quantitatively to deprotect peptide to give Tyr-Ala-Val-Lys-OH **7b** (Scheme 3). After treatment with 10% TFMSA/TFA, the reaction mixture was dried under vacuum to remove volatile TFMSA and TFA. The residue was dissolved in 1 : 1 H_2O : acetonitrile and analysed by HPLC and LC/MS.

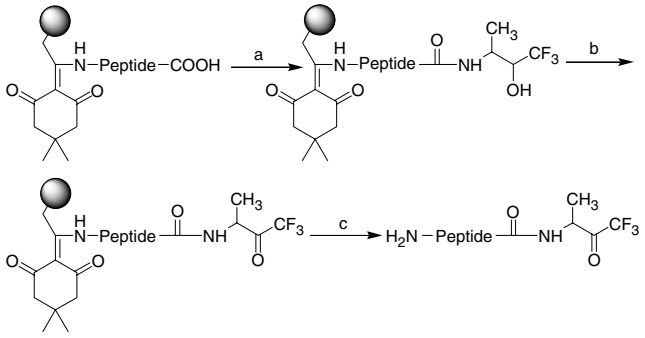
HPLC. HPLC was performed on a Hewlett-Packard series 1050 system equipped with a diode array detector and a C18 column (solvent miser, 2.1 × 250 mm, 5.0 μm particles). Compounds were separated by gradient elution; 100% solvent A (0.1% TFA in water) for 1 min, then 0% to 100% of solvent B (0.1% TFA in 30 : 70 water : acetonitrile) in 10 min, and then 0% to 100% of solvent C (0.095%TFA in acetonitrile) in 5 min. LC/MS was carried out on ThermoQuest (Finnigan) system equipped with atmospheric-pressure ionization (API) electrospray source.

Determination of racemization by Marfey's reagent. The degree of racemization of amino acids in product peptides was determined using Marfey's reagent [16]. A 2 μl aliquot of a 50 mM solution of peptide was hydrolysed with 100 μl 6 N HCl for 4 h at 110 °C in sealed vials, and the hydrolysed mixture dried under vacuum. To this was added 14.3 μl (5 eq) of a 1% solution of Marfey's reagent in acetone, 4 μl of 1 M $NaHCO_3$ and 6 μl of water, all on a per amino acid basis, and the mixture was kept at 35°–40 °C for 90 min. The reaction was quenched by the addition of 4 μl of 1 M HCl (per amino acid). Solvent was removed under vacuum and the residue dissolved in 400 μl of 1 : 1 water : acetonitrile. A 10 μl injection was made for HPLC analysis (detection at 340 nm). The same procedure except for hydrolysis was followed for standards (50 mM solution of amino acids). The percentage of D-isomer for each amino acid in each hydrolysed sample was determined by comparison of peak areas.

Solid-phase synthesis of two peptide trifluoromethylketones; Tyr-Ala-Val-NHCH(CH_3)C(O)CF_3 and Gly-Val-NHCH(CH_3)C(O)CF_3. To demonstrate the potential of a Dde resin based attachment strategy for solid-phase peptide mimetic synthesis, two peptide trifluoromethylketones were synthesized based on the approach outlined in Scheme 4 [9]. R-Dde-Tyr-Ala-Val and R-Dde-Gly-Val (100 mg each) were subjected to HATU/TMP coupling with racemic 1-trifluoromethyl-2-amino-1-propanol ($HCl{\cdot}NH_2CH(CH_3)CH(OH)CF_3$) in DMF for 6 h [20,9]. This procedure was repeated once, and the resins were washed and dried to provide R-Dde-Tyr-Ala-Val-NHCH(CH_3)CH(OH)CF_3 and R-Dde-Gly-Val-NHCH(CH_3)CH(OH)CF_3. R-Dde-Tyr-Ala-Val-NHCH(CH_3)CH(OH)CF_3 and R-Dde-Gly-Val-NHCH(CH_3)CH(OH)CF_3 were treated with a solution of DCC/$CHCl_2COOH$/DMSO/toluene (10 eq/1 eq/0.2 ml/0.2 ml) for 18 h. After filtration and washing this procedure was repeated once. After filtration, washing and drying the resins were treated with 400 μl 5% hydrazine/DCM for 60 min. The cleavage solution was filtered, dried and analysed by HPLC and LC/MS.

RESULTS AND DISCUSSION

Seven short peptides were synthesized using the Dde attachment strategy. All seven peptides were identified by LC/MS (Table 2) and were obtained in good yield and purity (Table 3, Figure 1A). Longer peptides can be synthesized but some yield is lost at each cycle. It is expected that the proposed approach will be suitable for peptides/mimetics up to at least six residues in length, which is suitable for most peptide mimetic based studies. All seven peptides were analysed for degree of amino acid racemization using Marfey's reagent [16]. The degree of racemization was less than 3% for each residue (Table 4). Off-resin treatment of a side chain



Scheme 4 Synthesis strategy for peptide trifluoromethylketones. (a) HATU/TMP, $NH_2CH(CH_3)CH(OH)CF_3$, in DMF for 6 h, then repeat once; (b) DCC/$CHCl_2COOH$/DMSO/toluene (10 eq/1 eq/0.2 ml/0.2 ml), 18 h, and then repeat once; (c) 5% v/v hydrazine/ DCM.

Table 2 Molecular Weights of the Synthesized Peptides and Peptide Mimetics

	Sample	Mol Wt $[M+H]^+$ Calc'd	Found[a]
1	Tyr-Ala-Val-OtBu	408.2	408.1
2	Gly-Pro-Leu-OtBu	342.2	342.5
3	Phe-Gly-Val-OtBu	378.2	378.3
4	Tyr-Ala-Leu-OtBu	422.3	422.0
5	Tyr-Gly-Orn(Z)-OtBu	543.3	543.0
6	Ala-Lys(Z)-Gly-OtBu	465.3	465.9
7a	Tyr-Ala-Val-Lys(Z)-OtBu	670.4	670.1
7b	Tyr-Ala-Val-Lys-OH	480.3	480.1
8	Tyr-Ala-Val-NHCH(CH_3)$COCF_3$·H_2O	493.2	492.9
9	Gly-Val-NHCH(CH_3)$COCF_3$·H_2O	316.1	316.0

[a] Determined on an aQa ThermoQuest (Finnigan) LC/MS instrument equipped with atmospheric-pressure ionization (API) electrospray source.

Table 3 Purity and Yields of Peptides and Peptide Mimetics

	Peptide/Peptide mimetic	Purity[a] (%)	Yield[b] (%)
1	Tyr-Ala-Val-OtBu	95	85
2	Gly-Pro-Leu-OtBu	95	85
3	Phe-Gly-Val-OtBu	85	80
4	Tyr-Ala-Leu-OtBu	80	75
5	Tyr-Gly-Orn(Z)-OtBu	80	70
6	Ala-Lys(Z)-Gly-OtBu	95	85
7a	Tyr-Ala-Val-Lys(Z)-OtBu	90	75
7b	Tyr-Ala-Val-Lys-OH	95	95[c]
8	Tyr-Ala-Val-NHCH(CH_3)$COCF_3$·H_2O	85	80
9	Gly-Val-NHCH(CH_3)$COCF_3$·H_2O	85	80

[a] Determined by HPLC of cleaved peptide/peptide mimetic.
[b] Determined by amount of peptide obtained after cleavage.
[c] From **7a**.

protected peptide with 10% TFMSA/TFA for 30 min can be used to deprotect the peptide, as demonstrated by off-resin treatment of Tyr-Ala-Val-Lys(Z)-OtBu to give Tyr-Ala-Val-Lys-OH (Scheme 3, Table 3 entry **7b**). An effort to perform side chain deprotection on-resin was not successful. We believe that the Dde group rearranged in strong acid in a way that trapped the peptide fragment on the resin.

Fluoromethylketones are of interest as inhibitors of serine, cysteine and aspartic acid proteases, including elastase, Cathepsin B, renin and HIV protease [9,18]. To demonstrate the potential of the Dde resin based attachment strategy for peptide mimetic syntheses, two peptide trifluoromethylketones were synthesized. R-Dde-Tyr-Ala-Val and R-Dde-Gly-Val were coupled with

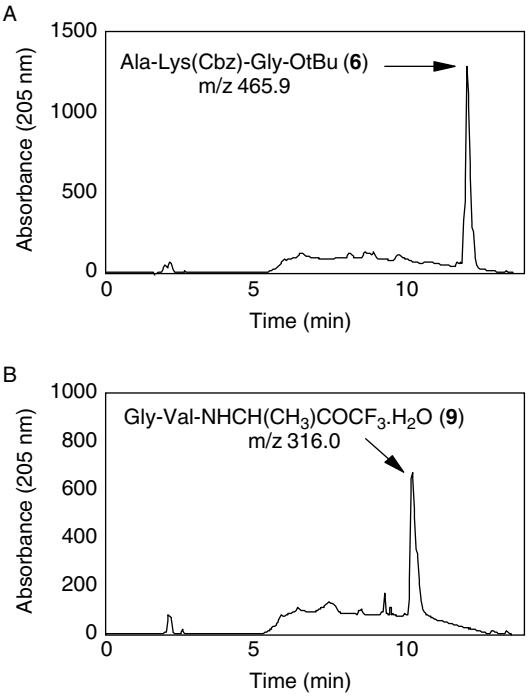


Figure 1 Representative HPLC chromatograms for peptides/peptide mimetics.

Table 4 Percentage of D-Isomers Determined with Marfey's Reagent for the Indicated Peptides

Peptide	AA_1	AA_2	AA_3	AA_4
1	D-Tyr (1.3%)	D-Ala (1.6%)	D-Val (1.8%)	
2	Gly	D-Pro (2.0%)	D-Leu (2.1%)	
3	D-Phe (2.4%)	Gly	D-Val (2.2%)	
4	D-Tyr (1.2%)	D-Ala (1.9%)	D-Leu (2.3%)	
5	D-Tyr (1.3%)	Gly	D-Orn (2.1%)	
6	D-Ala (2.1%)	D-Lys (1.8%)	Gly	
7b	D-Tyr (1.1%)	D-Ala (1.5%)	D-Val (2.3%)	D-Lys (1.4%)

an aminotrifluoromethylalcohol to give the corresponding resin attached peptide trifluoromethylalcohols. Oxidation of the alcohol by Pfitzner-Moffat oxidation gave the corresponding resin attached peptide trifluoromethylketones. After cleavage the peptide trifluoromethylketones were obtained in good yield and high purity (Table 3, Figure 1B). In LC/MS they were detected as their hydrates (Table 2 entry 8 and 9). The lack of detectable peptide trifluoromethylalcohol indicated quantitative oxidation. Although both peptide trifluoromethylketones were obtained in good yield

and purity, efforts to prepare peptide boronic acids with this strategy were unsuccessful, indicating that the Dde attachment strategy is incompatible with some peptide mimetic functional groups.

In summary, a Dde resin based attachment strategy for ISPPS of short peptides and peptide mimetics using amino acid *t*-butyl esters has been developed, which provides amino terminated peptides without a linker ending up in the product peptides. This strategy was demonstrated for the synthesis of side chain and *C*-terminally protected and unprotected peptides. Finally, peptide trifluoromethylketones were also prepared using this strategy, demonstrating the potential of *t*-butyl ester based ISPPS on Dde resin for peptide mimetic synthesis. This method can therefore provide short amino terminated peptides/mimetics with side chains and *C*-termini either protected or deprotected.

Acknowledgement

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