Journal of Peptide Science
J. Pept. Sci. 2008; **14**: 299–306
Published online 15 October 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.928

JOURNAL OF PEPTIDE SCIENCE

The depsipeptide technique applied to peptide segment condensation: Scope and limitations‡

IRENE COIN,* PETER SCHMIEDER, MICHAEL BIENERT and MICHAEL BEYERMANN

Leibniz-Institut für Molekulare Pharmakologie, Robert-Rössle-Str. 10, 13125 Berlin, Germany

Received 20 June 2007; Revised 25 July 2007; Accepted 27 July 2007

Abstract: A promising application of the depsipeptide technique has recently been proposed to provide ideal conditions for segment condensation, in that coupling of peptides bearing a *C*-terminal depsipeptide unit occurs without giving rise to epimerization at the activated amino acid. This is due to the low tendency of the activated depsipeptide units, in contrast to the corresponding peptide segments, to form optically labile oxazolones. In this work we demonstrate that coupling of depsipeptides via base-assisted activation using HBTU occurs not only without loss of configuration, but even much faster than the coupling of the corresponding all-amide segments. Nevertheless, when the coupling of long depsipeptide segments proceeds slowly, we uncovered the occurrence of β-elimination at the activated depsipeptide unit, in an extent dependent on the presence of base in the system and on the type of the solvent. Beta-elimination was completely suppressed by using carbodiimide/HOBt activation in a non-polar solvent (DCM), and in more polar media it was limited by substituting TMP for DIEA during HBTU activation, or using particular solvent mixtures (such as DMSO/toluene) for activation via carbodiimide. Finally, we show the application of *C*-terminal pseudoprolines, in comparison with that of depsipeptide units, to segment coupling. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: segment coupling; depsipeptide; epimerization; beta-elimination

INTRODUCTION

The chemosynthesis of peptides is routinely performed via step-wise assembly by successive couplings of urethane-protected amino acids. If step-wise synthesis fails, it may be advantageous to join two or more segments, in order to reduce the incidence of deletion sequences in the crude product and therefore facilitate purification. While current activation methods of the carboxyl group [1] allow fast and chirally safe coupling of urethane-protected amino acids, condensations of peptide segments are always affected by the danger of epimerization. An epimerization-free segment coupling can in principle be achieved in a non-polar solvent using a carbodiimide with acidic additives like HOBt [2] or HOAt [3]. Unfortunately, most peptide segments are poorly soluble in non-polar solvents, so that the use of polar media is normally necessary. Here activation via carbodiimide proceeds much more slowly, and stereomutation can occur [4–6]. In polar solvents a fast activation is achieved via uronium salts [7], but it is accompanied by serious loss of optical purity [8].

Loss of chiral integrity at the activated residue is due to formation of the oxazolone [9–11]. Its extent depends on the method used for coupling, the polarity of the solvent and the presence of bases as well as coupling additives in the system [12]. In general, the tendency of acylamino acids towards oxazolone formation is much higher with respect to that of urethane-protected amino acids, the oxazolones of which are moreover much more reactive and chirally stable.

Kiso and co-workers have recently presented a 'racemization-free segment condensation' [13], achieved through the application of the depsipeptide technique [14–16]. The depsipeptide technique is a recently developed methodology, which aims at interfering with peptide folding and association by interrupting the regular pattern of amide bonds with ester bonds, introduced at the level of Ser or Thr residues. With respect to the native peptides, the corresponding depsipeptides show a reduced tendency to aggregate and a higher solubility in aqueous media. Moreover, they can be easily converted into the amide form by an *O*,*N*-acyl shift [17], a reaction that occurs rapidly and quantitatively in aqueous media at mildly basic pH (Figure 1).

Therefore, depsipeptides are successfully used not only as 'easier' intermediates to achieve the synthesis

Abbreviations: aThr: allothreonine, ACN: acetonitrile, Boc: *t*-butyloxycarbonyl, DBU: diaza(1,3)bicyclo(5.4.0)undecane, DCHA: dicyclohexylamine, DCM: dichloromethane, DIC: diisopropylcarbodiimide, DIEA: diisopropylethylamine, DMAP: 4-dimethylaminopyridine, DMF: dimethylformamide, DMSO: dimethylsulfoxide, Fmoc: 9-fluorenemethyloxycarbonyl, HOAc: acetic acid; HOAt: 1-hydroxy-7-aza-1,2,3-benzotriazole, HOBt: 1-hydroxy-1,2,3-benzotriazole, NA: naphthylamide, HBTU: 1-[bis(dimethylamino)methylene]-*1H*-benzotriazolium hexafluorophosphate 3-oxide, NMI:*N*-methylimidazole, NMP: *N*-methylpyrrolidone, TFA: trifluoroacetic acid, TFE: trifluoroethanol, TMP: 2,4,6-trimethylpyridine, Z: benzyloxycarbonyl.

*Correspondence to: Irene Coin, Leibniz-Institut für Molekulare Pharmakologie, Robert-Rössle-Str. 10, 13125 Berlin, Germany;
e-mail: coin@fmp-berlin.de

‡ Dedicated to the outstanding scientist and dear friend Professor Louis A. Carpino, on the occasion of his 80th birthday.

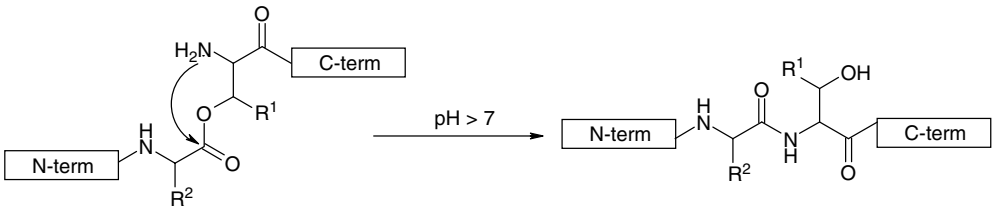

Figure 1 Conversion of a depsipeptide into the amide form through a base-promoted *O*,*N*-acyl shift. R^1: H/CH_3 (Ser/Thr).

of difficult peptides [14–16,18,19], but also as precursors of folded structures in conformational studies [14–16,20], besides finding a natural application to the development of pro-drugs [21–23].

A depsipeptide unit is conveniently incorporated into a sequence by coupling pre-formed depsidipeptide building blocks [18,24] (Figure 2(B)), a procedure that does not show the drawbacks related to *O*-acylation on solid phase (risk of epimerization at the activated amino acid, slow reaction, inapplicability of automated protocols). Contrary to the case of dipeptides, depsidipeptide blocks are not likely to form optically labile oxazolones when activated for coupling since their structure is analogous to that of protected Ser or Thr residues (Figure 2(A)), bearing urethane N^{α}-protection and acylated side chain. The same advantage is expected to be conserved when, instead of a simple depsidipeptide, a generic polypeptide bearing a depsipeptide unit at the *C*-terminus (Figure 2(C)) is coupled. This is indeed what has been shown by Kiso and co-workers [13], who coupled a 3-mer segment bearing a *C*-terminal depsipeptide unit onto a 2-mer segment bound to a solid phase without detecting any epimerization at the activated amino acid.

In the present work, by condensing in solution short model segments, we show the coupling of depsipeptides via HBTU to be not only epimerization-free, but also much faster than the coupling of peptide segments. Nevertheless, when coupling longer depsipeptide segments onto solid-phase-bound substrates, we observed the occurrence of a serious side reaction (β-elimination), which was confirmed by additional studies on small model peptides in solution, including characterization of products and side-products via MS analysis and NMR. Aiming at its minimization, we tested different solvents and activation methods, thereby determining the best conditions for the coupling of depsipeptide segments. Finally, we compared the use of *C*-terminal pseudoprolines [25,26] with that of depsipeptide units for segment coupling.

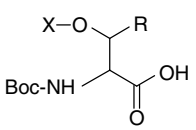

Figure 2 (A) X = protecting group; (B) X = Fmoc-Xaa; (C) X = polypeptide chain. R = H/CH_3 (Ser/ Thr).

RESULTS AND DISCUSSION

Efficiency of the Coupling of Depsidipeptide Units

At first, we studied a simple generic case of segment condensation in solution, and compared the coupling rates and optical purity of the products obtained by condensing with H-Ala-βNA either the dipeptide Fmoc-Val-Thr(*t*Bu)-OH or the corresponding depsidipeptide Boc-Thr(Fmoc-Val)-OH. We followed the kinetics of the reaction carried out by applying the same conditions that are usually employed in automated protocols for solid-phase peptide synthesis; that is, activation via HBTU (1 equiv.)/DIEA (2 equiv.) in DMF, with pre-activation for 1 min (Figure 3(A)).

As expected, the depsidipeptide couples very fast, providing ~90% of the product already after 30 s of coupling, whereas the coupling of the normal dipeptide occurs much more slowly (~85% product in 15 min), and it is moreover accompanied by racemization at the activated Thr (A% ratio in HPLC trace LDL/LLL ~1). The identity of the two diastereomers isolated from the crude product was confirmed by comparison of their retention times and NMR spectra with those of authentic compounds obtained by step-wise synthesis. Even by avoiding pre-activation or by reducing the amount of base to the minimum required for activation via HBTU (1 equiv.) the extent of epimerization remains substantial (~30% LDL epimer in the product). To check the eventual occurrence of epimerization for the depsidipeptide unit, the coupling mixture was at first diluted with water and lyophilized, and then treated with TFA to remove the Boc group. The unprotected depsipeptide was then shifted to the amide form by treatment with a solution 0.1 M $NaHCO_3$ in ACN/water. The resulting product gave a single peak in the HPLC trace, the retention time of which corresponded to that of the standard all-L Fmoc-Val-Thr-Ala-βNA, with no peak being detected at the retention time of the D-aThr epimer. (The two reference epimers were obtained by TFA treatment of the corresponding *t*Bu-protected standards step-wise assembled).

For comparison, we also studied the kinetics of the same coupling using base-free activation via DIC and HOBt (1/1 equiv.) in DCM (Figure 3(B)). Under these conditions the coupling occurs much more slowly, the two kinetic curves are similar and for both dipeptide and depsidipeptide we detected no epimerization. The

DOI: 10.1002/psc

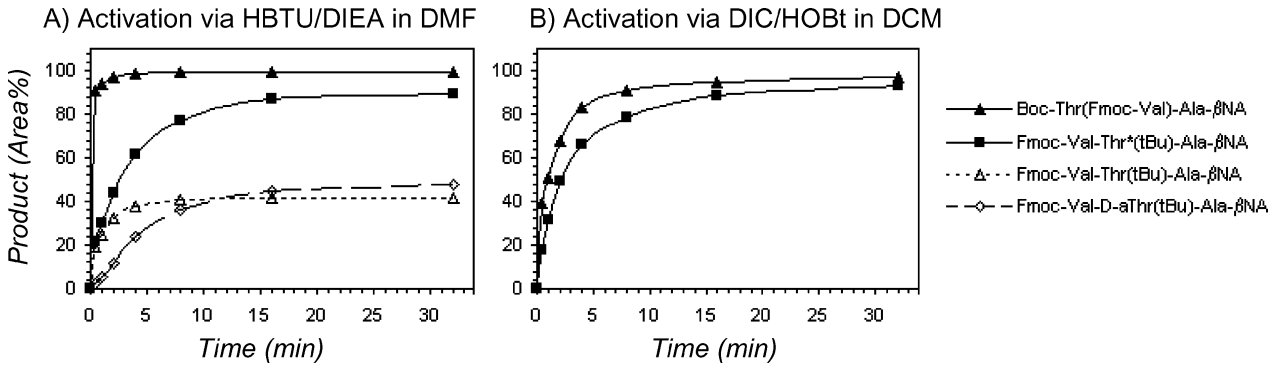

Figure 3 Kinetic curves for the coupling of either dipeptide Fmoc-Val-Thr(*t*Bu)-OH or depsidipeptide Boc-Thr(Fmoc-Val)-OH with H-Ala-βNA at a concentration of 0.01 M. The amount of product is represented by the area% of the corresponding peak in the HPLC trace of the reaction mixture at the indicated time. In (A) the curve marked by squares represents the sum of the two diastereomer products, whereas in (B) it represents the authentic curve of the all-L peptide, obtained as unique product.

addition of base can in principle speed up the reaction, but leads to loss of optical purity. In fact, the addition of 1 equiv. of DIEA to the reaction mixture (after the pre-activation) gave rise to the formation of ~10% epimerized product.

These results show that coupling of depsipeptide units, like coupling of urethane-protected residues, is efficiently achieved using base-assisted activation via HBTU. The reaction occurs so quickly that despite the presence of a tertiary amine in the system the chiral integrity is preserved. On the contrary, the same activation method applied to a normal peptide segment leads to a sluggish reaction and loss of configuration, owing to the low reactivity and the chiral instability of the oxazolone formed. An epimerization-free segment coupling was achieved in DCM using carbodiimide/HOBt activation, but nevertheless much more slowly than the coupling via HBTU/DIEA in DMF, so that in any case the coupling of a depsidipeptide unit via HBTU activation represents the most effective way to couple a two-residue block onto an amine.

Coupling of Depsipeptide Segments via HBTU

In the context of a research project on cell permeation [27], we wanted to prepare analogues of cell-penetrating peptides containing depsipeptide units. During the step-wise synthesis of the sequence

KLALKLA-depsiVT-ALKAALKLA-amide

the formation of the ester bond via DIC/NMI directly on the growing peptide chain happened to be very difficult, with low yields of esterification, even by using long reaction times and substituting the stronger catalyst DMAP for NMI.

We used then a convergent approach and condensed the protected depsipeptide *N*-terminal segment Boc-T[Fmoc-K(Boc)LALK(Boc)LAV]-OH with H-ALK(Boc)AALK(Boc)LA-Ram (Ram: rink amide resin). Taking into account the excellent result obtained in solution with the depsidipeptide, we used HBTU (1 equiv.)/DIEA (2 equiv.) activation, under the following conditions: two-fold excess of reagents over loading at a concentration of 0.05 M in DMF, 2 min pre-activation, 2 h coupling time. After coupling, the Fmoc group was removed and the *N*-terminus acetylated, in order to allow a correct estimation by UV detection at 220 nm of the relative amounts of product, eventual side-products and non-reacted starting material. The HPLC trace of the crude product (cleaved from the solid support with TFA/water 5%) showed a main peak (60%, A%) corresponding to the target product (identified by LC-MS), a small amount (5%) of non-reacted starting material and a third relevant peak (~40%), the mass of which corresponded to $M_{C-\mathrm{term}} + 84$, with $M_{C-\mathrm{term}}$ being the monoisotopic mass of the peptide H-ALKAALKLA-NH_2. (After cleavage from the solid support, crude products of segment coupling were treated with a solution of $NaHCO_3$ (0.1 M in ACN/water) in order to promote the shift to the all-amide form and thus to be able to compare HPLC and LC-MS data with those of standards of Ac-KLALKLAVTALKAALKLA-NH_2 and Ac-KLALKLAV-(D)-aT-ALKAALKLA-NH_2, *ad hoc* synthesized step-wise. No loss of configuration was observed.)

Aiming at the identification of the side-product, we decided at first to simplify our model, and studied the coupling of the simple depsidipeptide Boc-Thr(Fmoc-Val)-OH, corresponding to the *C*-terminal depsipeptide unit of the depsi-*N*-terminal segment, onto the resin-bound *C*-terminal segment. We used HBTU (1 equiv.)/DIEA (2 equiv.) activation, with 1 min pre-activation, 30 min coupling, a two-fold excess of reagents over loading, a concentration of 0.05 M and an identical procedure for work-up and cleavage as described above. Again, besides the peak of the target product (~85%, A%), in the HPLC trace we found another peak (~15%), hiding this time two coeluted side-products, the mass of which respectively corresponded to $M_{\mathrm{product}} - 101$ (equal to the mass of the

DOI: 10.1002/psc

des-Thr product) and, like in the previous experiment, $M_{C-\text{term}} + 84$.

Occurrence of β-elimination

In order to allow an easy structural identification of products and side-products via NMR, we turned back to the model reaction in solution and coupled the depsidipeptide Boc-Thr(Fmoc-Val)-OH onto H-Ala-βNA using increased pre-activation times with HBTU/DIEA, so as to mimic what happens to the activated species in the case of a slow coupling. We found that the longer the pre-activation the larger was the amount of two side-products, which were isolated and identified through MS analysis and NMR: Fmoc-Val-Ala-βNA and Boc-ΔThr-Ala-βNA. The product of dehydration is attributed to β-elimination on the *O*-acylated Thr [28], whereas the des-Thr product could arise via nucleophilic attack by H-Ala-βNA to the ester bond of the depsi unit, but more probably via the formation of a mixed anhydride between the two species generated during β-elimination at the Thr residue (Figure 4). The latter hypothesis would also explain why the amount of the two side-products increases almost symmetrically by increasing the time of pre-activation.

This finding gives a rationale for the side-products observed in the experiment on solid phase. While the analogy between the two des-Thr products is obvious, the side-product arising from coupling of Boc-ΔThr- onto the resin bound peptide is further modified during the work-up. In fact, when the peptide is cleaved from the resin, the Boc group is removed from the *N*-terminus, and an enamine is liberated. This species spontaneously tautomerizes to the corresponding imine [29,30], which undergoes rapid hydrolysis *in situ* [29,30] to give as final product a diketopeptide, the mass of which corresponds precisely to $M_{C-\text{term}} + 84$ (Figure 5).

These results identify β-elimination as an important side reaction that can occur by the coupling of peptide segments bearing a *C*-terminal depsipeptide unit via a base-assisted activation method in cases where, due to sterical hindrance or to arising of aggregation, the coupling is not fast enough to occur before the side reaction takes place.

Coupling of Depsipeptide Segments via DIC/HOBt

The simplest approach to prevent β-elimination is to avoid the presence of the base during coupling by using a carbodiimide as activating agent, and to carry out the reaction in a non-polar solvent. For instance, coupling of Boc-Thr(Fmoc-Val)-OH onto H-Ala-βNA via DIC/HOBt (1/1 equiv.) in DCM, with pre-activation for 2 h, showed no formation of side-products at all. Likewise, by coupling via DIC/HOBt in DCM the depsidipeptide Boc-Thr(Fmoc-Val)-OH onto H-ALK(Boc)AALK(Boc)LA-Ram, no products due to occurrence of β-elimination were detected.

The longer depsipeptide segment Boc-T[Fmoc-K(Boc)LALK(Boc)LAV]-OH is poorly soluble in DCM, but it easily dissolved in DMF. In our hands, its coupling via DIC/HOBt onto H-ALK(Boc)AALK(Boc)LA-Ram, under conditions analogous to those used by Kiso *et al.* [13] (2 equiv./loading, 0.05 M in DMF, 4 h reaction time), gave a yield of ~80% (A% in the HPLC trace) of the target product, identified by LC-MS. Surprisingly, despite the absence of base in the system, we found a significant amount (~15%) of side-product, arising from β-elimination ($M = M_{C-term} + 84$).

The occurrence of β-elimination during segment coupling via carbodiimide in DMF, even in the absence of base, indicates that the nature the solvent also has to be carefully evaluated. The unfavourable factor is probably constituted by the inefficiency of the activation via carbodiimide in polar media, which determines a low concentration of active ester and therefore a low coupling rate. It should also be considered that common commercial stocks of DMF, as well as of NMP, are notoriously contaminated with free amines. Nevertheless, since peptide segments are frequently not well soluble in DCM, the use of polar media is in most cases unavoidable. Looking for the best conditions to provide a convenient balance between efficiency of activation/coupling and β-elimination rate for depsipeptide segment condensation in polar media, we attempted two different approaches, reducing either the amount and the strength of the tertiary base for HBTU activation or the polarity of the solvent for carbodiimide activation. The results are summarized in Table 1.

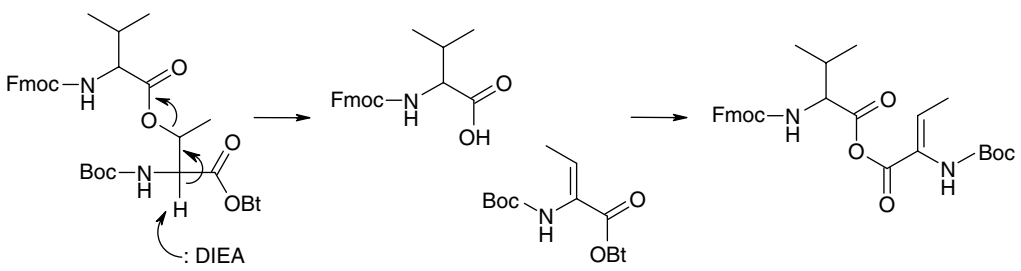

Figure 4 Mechanism of β-elimination occurring at the activated depsidipeptide unit in the presence of base, and subsequent formation of a mixed anhydride between the two products of reaction.

DOI: 10.1002/psc

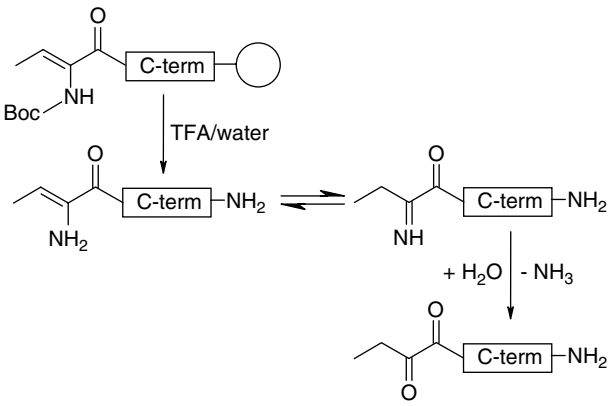

Figure 5 Pathway of reaction leading to the side-product having $M = M_{C-term} + 84$, with M_{C-term} indicating the monoisotopic mass of the peptide H-C_{term}-NH_2.

With respect to the standard activation via HBTU (1 equiv.)/DIEA (2 equiv.), the amount of product of β-elimination is actually halved by using a single equivalent of DIEA, but the reaction proceeds more slowly, so that the amount of the target product is essentially the same as that obtained by using 2 equiv. of the base. β-Elimination is more efficiently suppressed by using a weaker base for activation, such as collidine (TMP) [8], although this base is not strong enough to keep the amino component deprotonated for a fast coupling. Activation via carbodiimide without the base leads in general to a lower extent of β-elimination. With respect to the solvent, we found that the lower the polarity, the smaller is the extent of dehydration. On the other hand, the use of systems containing DMSO [31] or TFE [32], solvents which can interfere with the activation process, led also to reduced coupling yields.

The results show that activation via carbodiimide without base efficiently prevents the occurrence of β-elimination during the coupling of depsipeptide segments. In particular, the side reaction is completely suppressed by working in non-polar solvents, such as DCM. For depsipeptide segments not soluble in DCM, an efficient segment coupling was obtained using DIC/HOBt in DMF (80% yield in 4 h coupling). Good coupling yields (~70%) and a substantial suppression of β-elimination were achieved in DMF via HBTU/TMP activation (2 h coupling) and in DMSO/toluene via carbodiimide (4 h coupling).

In addition, it has to be mentioned that while the coupling of the all-amide *N*-terminus was accompanied by extensive epimerization at the activated residue, no loss of configuration was observed for couplings via the depsipeptide unit. (Coupling of the all-amide *N*-terminal segment Fmoc-K(Boc)LALK(Boc)LAVT-OH onto H-ALK(Boc)AALK(Boc)LA-Ram using DIC/HOBt activation in DMF (2 equiv./loading, coupling overnight) gave only a 50% yield (A% of the target peptide in the HPLC trace of the crude product), besides giving rise to extensive epimerization. The activation of the peptide in DMF gave a jelly solution.) Moreover, the depsipeptide segment showed a better solubility than the parent all-amide peptide, which can be an important advantage, particularly when working with long sequences.

Use of Pseudoprolines

A possible alternative to the use of *C*-terminal depsipeptide units for segment coupling is the use of *C*-terminal pseudoproline units [25,26]. In fact, like proline, pseudoprolines also cannot give rise to oxazolone formation when activated, and should therefore provide segment coupling the same advantages as the depsipeptide units. As expected, we found the coupling of the pseudoproline unit Fmoc-Val-Thr($\Psi^{Me,Me}$pro)-OH onto H-Ala-βNA via HBTU (1 equiv.)/DIEA (2 equiv.) in DMF to be more efficient in comparison to dipeptide coupling (>90% product in 4 min, no epimerization detected). With respect to the occurrence of β-elimination, by pre-activating the same pseudoproline dipeptide with HBTU (1 equiv.)/DIEA (2 equiv.) in DMF for up to 4 h, no formation of side-products was detected. β-Elimination did not occur even by coupling Fmoc-K(Boc)LALK(Boc)LAVT($\Psi^{Me,Me}$pro)-OH

Table 1 Study of different systems for the coupling of Boc-T[Fmoc-K(Boc)LALK(Boc)LAV]-OH onto H-ALK(Boc)AALK(Boc)LA-Ram via either HBTU/base 1/n equiv. or DIC/HOBt 1/1 equiv. at a concentration of 0.05 M, with reaction time 2 h for the activation via HBTU and 4 h for the activation via carbodiimide. After coupling, Fmoc was removed and the samples were acetylated. Cleavage via TFA/water 5%, 1 h

Activation	Solvent	Pre-activation Min	Products (A% in the HPLC trace)
HBTU/DIEA 1/2	DMF	2	58% target, 5% starting peptide, 37% ΔThr product
HBTU/DIEA 1/1	DMF	2	58% target, 21% starting peptide, 17% ΔThr product
HBTU/TMP 1/2	DMF	2	69% target, 26% starting peptide, 6% ΔThr product
DIC/HOBt	DMF	15	77% target, 7% starting peptide, 14% ΔThr product
DIC/HOBt	NMP	15	59% target, 16% starting peptide, 15% ΔThr product
DIC/HOBt	DMSO/toluene 1/3	10	70% target, 25% starting peptide, 4% ΔThr product
DIC/HOBt	TFE/DCM 1/3	3	33% target, 67% starting peptide

DOI: 10.1002/psc

onto H-ALK(Boc)AALK(Boc)LA-Ram via DIC/HOBt 1/1 equiv. in DMF/DMSO 2/1 (same work-up as described for the depsipeptide case). DMSO as co-solvent was required by the low solubility of the pseudoproline segment in DMF, so that the use of HBTU for activation was not possible. The yield of coupling can be estimated to be around 70% (A% of the product in the HPLC trace of the cleaved product).

CONCLUSIONS

In this work, the scope and limitations of the depsipeptide technique applied to segment coupling have been investigated. In particular, it has been demonstrated that the coupling of depsidipeptide units, owing to their low propensity to form oxazolone in comparison to activated dipeptides, is exceptionally efficient in that it is faster than a peptide segment coupling and occurs without loss of configuration, even using activation methods that require the presence of base. In general, for relatively fast couplings, the application of depsidipeptide blocks to standard procedure of solid-phase peptide synthesis and in particular to automated protocols looks to be safe.

By using *C*-terminal depsipeptide units for segment coupling, the occurrence of β-elimination at the activated residue was uncovered, depending on the presence of base in the system and the type of solvent. This side reaction was completely suppressed by using carbodiimide/HOBt activation in non-polar solvent (DCM), conditions which should be applied to the coupling of segments bearing a *C*-terminal depsipeptide unit, but should also be used to couple depsidipeptide units when hindrance or aggregation factors determine the coupling via HBTU/DIEA to be slow. In more polar media, the occurrence of β-elimination is limited by substituting TMP for DIEA for HBTU activation, or using carbodiimide activation, especially when working in solvent mixtures other than DMF (for instance DMSO/toluene).

Finally, the use of pseudoprolines as an alternative to that of depsidipeptide units has been illustrated: pseudoproline blocks have been shown not to undergo β-elimination even by long activation times in the presence of base, being at the same time equally able to prevent loss of configuration. Nevertheless, in the case of our model, segment coupling via depsipeptide gave a higher yield than that via pseudoproline because of the low solubility of the pseudoproline segment, so that the convenience of one method with respect to the other has to be evaluated from case to case.

EXPERIMENTAL

General Procedures for SPPS

Step-wise SPPS was performed on a peptide synthesizer using a standard Fmoc/*t*Bu protocol (0.25 M), comprising double coupling at every cycle. Acetylations were performed via coupling of acetic acid (HOAc) (HBTU/DIEA/DMF). A TentaGel-S-Ram resin (capacity 0.26 mmol/g) was used as solid support for peptide amides, which were cleaved from the resin with TFA/water 5% (1 h). After cleavage, the reference peptides were precipitated from diethyl ether, whereas for products of segment coupling the diluted samples were directly analysed.

Peptide- and depsipeptide acids were assembled on a 2-chlorotrityl chloride resin (capacity max 1.4 mmol/g). The linkage was performed using an amount of Fmoc amino acid equal to 0.7 mmol/g (1 equiv.) and 1.5 equiv. of DIEA in dry DCM (1 h 30 min); capping with MeOH/DIEA 9/1 (20 min) followed. Cleavage was carried out with a mixture of HOAc/TFE/DCM 1/1/3 for 1h. The samples were lyophilized from dioxane and precipitated with hexane. Depsipeptide and pseudoproline peptide segments were sufficiently soluble in ACN/water to be purified via HPLC.

Depsipeptide units were assembled on solid phase via *O*-acylation of N^{α}-Boc-protected Thr residues. The reaction was carried out at a concentration of 0.25 M in dry DCM for a time of 2×2 h, using DIC/NMI activation (1/1 equiv.) without pre-activation. After *O*-acylation, an acetylation step in the presence of NMI was performed using Ac_2O/NMI (1/1 equiv.) at a concentration of 0.25 M in DCM (2×90 min).

Kinetics of Coupling

Activation via HBTU/DIEA in DMF. Fmoc-Val-Thr(*t*Bu)-OH (2.48 mg, 5×10^{-3} mmol) and HBTU (1.90 mg, 5×10^{-3} mmol) were weighed in a small vial, and 500 µl of a 0.02 M solution of DIEA in DMF was added. After 1 min, the solution was added to 1.07 mg (5×10^{-3} mmol) of H-Ala-βNA, and the mixture was kept under stirring. To follow the progress of the reaction, 50 µl samples were taken from the reaction mixture (reaction times: 30 s, 1 min, 2 min, 4 min, 16 min, 32 min, 1 h, 2 h), diluted to 1 ml with ACN/water/TFA 0.1%, and injected into an HPLC column C_{18}, using the following solvent system: buffer A = water (0.1% TFA); buffer B = 80% acetonitrile in water (0.1% TFA); linear gradient 40 to 95% B in 40 min. The retention times of the LDL and of the all-L product were 34.4 min and 38.2 min, respectively. The coupling of Boc-Thr(Fmoc-Val)-OH (2.70 mg, 5×10^{-3} mmol) was studied using exactly the same procedure. The product Boc-Thr(Fmoc-Val)-Ala-βNA has a retention time of 38.3 min.

Activation via DIC/HOBt in DCM. Fmoc-Val-Thr(*t*Bu)-OH (2.48 mg, 5×10^{-3} mmol) and HOBt (0.76 mg, 5×10^{-3} mmol) were weighed in a small vial, and 500 µl of a 0.01 M solution of DIC in DCM was added. After 2 min, the solution was added to 1.07 mg (5×10^{-3} mmol) of H-Ala-βNA and the mixture kept under stirring. The progress of the reaction was followed as described above. The same procedure was applied for coupling of Boc-Thr(Fmoc-Val)-OH.

Synthesis of the Reference Peptides

Fmoc-Val-Thr(tBu)-Ala-βNA. To a stirred suspension of Z-Thr(*t*Bu)-OH × dicyclohexylamine (DCHA) (49.0 mg, 0.1 mmol) and HOBt (15.3 mg, 0.1 mmol) in DCM (2 ml), EDC (19.1 mg, 0.1 mmol) was added. Addition of H-Ala-βNA followed after 5 min, and the solution was allowed to react at room

DOI: 10.1002/psc

temperature for 2 h. Then, the solvent was removed under vacuum and the residue dissolved in AcOEt. The solution was washed with 10% aq. $KHSO_4$, brine, 5% aq. $NaHCO_3$, brine and water, dried over Na_2SO_4 and evaporated to dryness. LC-MS (ESI-TOF): A% main peak ~95%, $[M+H]^+$ calculated: 506.26, found: 506.23. The dipeptide was dissolved in MeOH and *Z* removed by hydrogenation in the presence of Pd(C) (~10 mg), 1 h. The solution was filtered and evaporated to dryness. Coupling of Fmoc-Val-OH (33.9 mg, 0.1 mmol) followed, with the same procedure indicated above. The final product was purified in HPLC. LC-MS (ESI-TOF): $[M+H]^+$ calcd: 693.36, found: 693.32. ^{1}H NMR (300 MHz, DMSO) δ10.02 (s, 1H), 8.23 (s, 1H), 8.00 (d, 1H), 7.89–7.58 (m, 10H), 7.48–7.28 (m, 6H), 4.47 (m, 1H), 4.36–4.20 (m, 4H), 3.99–3.94 (m, 2H), 2.04 (m, 1H), 1.37 (d, 3H), 1.14 (s, 9H), 1.06 (d, 3H), 0.86 (dd, 6H).

Fmoc-Val-D-aThr(tBu)-Ala-βNA. To a stirred suspension of Fmoc-D-aThr(*t*Bu)-OH (39.7 mg, 0.1 mmol) and HOBt (15.3 mg, 0.1 mmol) in DCM (2 ml), EDC (19.1 mg, 0.1 mmol) was added. Addition of H-Ala-βNA followed after 5 min, and the solution was let to react at room temperature for 2 h, with 1.71 µl of DIEA (0.1 mmol) added after the first 20 min. Then, the solvent was removed under vacuum and the residue dissolved in AcOEt. The solution was washed with 10% aq. $KHSO_4$, brine, 5% aq. $NaHCO_3$, brine and water, dried over Na_2SO_4 and evaporated to dryness. LC-MS (ESI-TOF): A% main peak ~90%, $[M+H]^+$ calcd: 594.30, found: 594.26. The dipeptide was dissolved in DCM and Fmoc deblocked by addition of 0.1 mmol of DBU (14.9 µ*L*) partitioned in two portions, for a total reaction time of 30 min. After evaporation of DCM, the residue was dissolved in ACN/water/TFA 0.1% and injected into a preparative HPLC column. The product H-D-aThr(*t*Bu)-Ala-β-NA was identified via LC-MS ((ESI-TOF): $(M+H)^+$ calcd: 372.23, found: 372.20), isolated and lyophilized. Coupling of Fmoc-Val-OH (33.9 mg, 0.1 mmol) followed, by using the same procedure indicated above. The final product was purified in HPLC. LC-MS (ESI-TOF): $(M+H)^+$ calcd: 693.36, found: 693.30. ^{1}H NMR (300 MHz, DMSO) δ9.96 (s, 1H), 8.29 (s, 1H), 8.12–8.09 (m, 2H), 7.89–7.62 (m, 8H), 7.47–7.26 (m, 7H), 4.43–4.35 (m, 2H), 4.27–4.18 (m, 3H), 4.06–3.96 (m, 2H), 1.97 (m, 1H), 1.34 (d, 3H), 1.11 (s, 9H), 1.07 (d, 3H), 0.90 (dd, 6H).

Isolation and Identification of the Products of β-elimination

In a small flask, 9.7 mg of Boc-Thr(Fmoc-Val)-OH (0.018 mmol) and 6.8 mg of HBTU (0.018 mmol) were weighed. To this 6.16 µl (0.036 mmol) of DIEA dissolved in 1 ml DMF were added and the mixture stirred at room temperature for 5 h. Then, 4.28 mg (0.020 mmol) of H-Ala-βNA was added. After 30 min the solution was diluted with ACN/water/TFA 0.1% and injected into a preparative HPLC column using a linear gradient 30–70% B in 70 min. Two products were isolated (peaks at t_R 31 and 52 min), lyophilized and identified. The peak at $t_R = 31$ min was attributed to Boc-ΔThr-Ala-βNA: LC-MS (ESI-TOF): $[M+H]^+$ calcd: 398.21, found: 398.16. ^{1}H NMR (600 MHz, 310 K, DMSO) δ9.83 (s, 1H), 8.46 (s, 1H), 8.27 (s, 1H), 8.03 (s, 1H), 7.86–7.68 (m, 4H), 7.48–7.39 (m, 2H), 6.11 (q, 1H), 4.49 (m, 1H), 1.67 (d, 3H), 1.42–1.39 (m, 12H). The peak at $t_R = 52$ min was attributed to Fmoc-Val-Ala-βNA: LC-MS (ESI-TOF): $[M+H]^+$ calcd: 536.25, found: 536.24. ^{1}H NMR (600 MHz, DMSO) δ10.16 (s, 1H), 8.26–8.22 (m, 2H), 7.89–7.32 (m, 14H), 4.48 (m, 1H), 4.30–4.23 (m, 3H), 3.93 (m, 1H), 2.00 (m, 1H), 1.35 (d, 3H), 0.89 (dd, 6H).

REFERENCES

1. Montalbetti CAGN, Falque V. Amide bond formation and peptide coupling. *Tetrahedron* 2005; **61**: 10827–10852.
2. König W, Geiger R. Racemization in peptide syntheses. *Chem. Ber.-Rec.* 1970; **103**: 2024–2033.
3. Carpino LA. 1-Hydroxy-7-azabenzotriazole – an efficient peptide coupling additive. *J. Am. Chem. Soc.* 1993; **115**: 4397–4398.
4. Beyermann M, Henklein P, Klose A, Sohr R, Bienert M. Effect of tertiary amine on the carbodiimide-mediated peptide-synthesis. *Int. J. Pept. Protein Res.* 1991; **37**: 252–256.
5. Hudson D. Methodological implications of simultaneous solid-phase peptide-synthesis. Comparison of different coupling procedures. *J. Org. Chem.* 1988; **53**: 617–624.
6. Carpino LA, El Faham A. The diisopropylcarbodiimide/1-hydroxy-7-azabenzotriazole system: segment coupling and stepwise peptide assembly. *Tetrahedron* 1999; **55**: 6813–6830.
7. Knorr R, Trzeciak A, Bannwarth W, Gillessen D. New coupling reagents in peptide chemistry. *Tetrahedron Lett.* 1989; **30**: 1927–1930.
8. Carpino LA, El Faham A. Effect of tertiary bases on O-benzotriazolyluronium salt-induced peptide segment coupling. *J. Org. Chem.* 1994; **59**: 695–698.
9. Kemp DS. In *The Peptides: Analysis, Synthesis, Biology*, vol. 1, Gross E, Meienhofer J (eds.). Academic Press: New York, 1979; 315–383.
10. Benoiton NL. In *The Peptides: Analysis, Synthesis, Biology*, vol. 5, Gross E, Meienhofer J (eds.). Academic Press: New York, 1983; 217–284.
11. Bodanszky M. *Principles of Peptide Synthesis*. Springer-Verlag: Berlin, 1984; 159.
12. Benoiton NL. 2-alkoxy-5(4H)-oxazolones and the enantiomerization of N-alkoxycarbonylamino acids. *Biopolymers* 1996; **40**: 245–254.
13. Yoshiya T, Sohma Y, Kimura T, Hayashia Y, Kiso Y. 'O-acyl isopeptide method': racemization-free segment condensation in solid phase peptide synthesis. *Tetrahedron Lett.* 2006; **47**: 7905–7909.
14. Carpino LA, Krause E, Sferdean CD, Schumann M, Fabian H, Bienert M, Beyermann M. Synthesis of 'difficult' peptide sequences: application of a depsipeptide technique to the Jung-Redemann 10- and 26-mers and the amyloid peptide A beta(1–42). *Tetrahedron Lett.* 2004; **45**: 7519–7523.
15. Mutter M, Chandravarkar A, Boyat C, Lopez J, Dos Santos S, Mandal B, Mimna R, Murat K, Patiny L, Saucede L, Tuchscherer G. Switch peptides in statu nascendi: Induction of conformational transitions relevant to degenerative diseases. *Angew. Chem. Int. Ed.* 2004; **43**: 4172–4178.
16. Sohma Y, Sasaki M, Hayashi Y, Kimura T, Kiso Y. Novel and efficient synthesis of difficult sequence-containing peptides through O-N intramolecular acyl migration reaction of O-acyl isopeptides. *Chem. Commun.* 2004; **7**(1): 124–125.
17. Bergmann M, Brand E, Weinmann F. Umlagerung peptidähnlicher Stoffe II. Derivate der γ-Amino-β-oxybuttersäure. *Hoppe-Seyler's Z. Physiol. Chem.* 1923; **131**: 1–17.
18. Coin I, Dolling R, Krause E, Bienert M, Beyermann M, Sferdean CD, Carpino LA. Depsipeptide methodology for solid-phase peptide synthesis: Circumventing side reactions and development of an automated technique via depsidipeptide units. *J. Org. Chem.* 2006; **71**: 6171–6177.
19. Sohma Y, Sasaki M, Hayashi Y, Kimura T, Kiso Y. Design and synthesis of a novel water-soluble A beta 1–42 isopeptide: an efficient strategy for the preparation of Alzheimer's disease-related

DOI: 10.1002/psc

peptide, A beta 1-42, via O-N intramolecular acyl migration reaction. *Tetrahedron Lett.* 2004; **45**: 5965–5968.
20. Taniguchi A, Sohma Y, Kimura M, Okada T, Ikeda K, Hayashi Y, Kimura T, Hirota S, Matsuzaki K, Kiso Y. "Click peptide" based on the "O-acyl isopeptide method": control of A beta 1-42 production from a photo-triggered A beta 1-42 analogue. *J. Am. Chem. Soc.* 2006; **128**: 696–697.
21. Oliyai R, Stella VJ. Structural factors affecting the kinetics of O,N-acyl transfer in potential O-peptide prodrug. *Bioorg. Med. Chem. Lett.* 1995; **5**: 2735–2740.
22. Hamada Y, Ohtake J, Sohma Y, Kimura T, Hayashi Y, Kiso Y. New water-soluble prodrugs of HIV protease inhibitors based on O – N intramolecular acyl migration. *Bioorg. Med. Chem.* 2002; **10**: 4155–4167.
23. Kazmierski WM, Bevans P, Furfine E, Spaltenstein A, Yang HB. Novel prodrug approach to amprenavir-based HIV-1 protease inhibitors via O – N acyloxy migration of P1 moiety. *Bioorg. Med. Chem. Lett.* 2003; **13**: 2523–2526.
24. Sohma Y, Taniguchi A, Skwarczynski M, Yoshiya T, Fukao F, Kimura T, Hayashi Y, Kiso Y. 'O-Acyl isopeptide method' for the efficient synthesis of difficult sequence-containing peptides: use of 'O-acyl isodipeptide unit'. *Tetrahedron Lett.* 2006; **47**: 3013–3017.
25. Mutter M, Nefzi A, Sato T, Sun X, Wahl F, Wahl T. Pseudo-prolines for accessing 'inaccessible' peptides. *Pept. Res.* 1995; **8**: 145–153.
26. Wohr T, Wahl F, Nefzi A, Rohwedder B, Sato T, Sun XC, Mutter M. Pseudo-prolines as a solubilizing, structure-disrupting protection technique in peptide synthesis. *J. Am. Chem. Soc.* 1996; **118**: 9218–9227.
27. Oehlke J, Lorenz D, Wiesner B, Bienert M. Studies on the cellular uptake of substance P and lysine-rich, KLA-derived model peptides. *J. Mol. Recognit.* 2005; **18**: 50–59.
28. Somlai C, Lovas S, Forgo P, Murphy RF, Penke B. Dehydration of threonine esters during tosylation. *Synth. Commun.* 2001; **31**: 3633–3640.
29. March J. In *Advanced Organic Chemistry: Reactions, Mechanisms and Structure*, 4th edn. John Wiley & Sons: New York, 1992; 73.
30. March J. In *Advanced Organic Chemistry: Reactions, Mechanisms and Structure*, 4th edn. John Wiley & Sons: New York, 1992; 884.
31. Rabinovich AK, Rivier JE. Accelerated protocols for solid-phase peptide-synthesis at elevated-temperature. *Am. Biotechnol. Lab.* 1994; **12**: 48.
32. Kuroda H, Chen YN, Kimura T, Sakakibara S. Powerful solvent systems useful for synthesis of sparingly-soluble peptides in solution. *Int. J. Pept. Protein Res.* 1992; **40**: 294–299.