Review

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The depsipeptide method for solid-phase synthesis of difficult peptides‡

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After about one century of peptide chemistry, the main limitation to the accessibility of peptides and proteins via chemosynthesis is the arising of folding and aggregation phenomena. This is true not only for sequences above a critical length but also for several biologically relevant substrates that are relatively short yet form either highly folded structures (e.g. WW domains) or fibrils and aggregates after final deprotection (β-amyloid peptide). Such so-called difficult sequences may be more easily obtained via their corresponding depsipeptides (*O*-acyl isopeptides), ester isomers that are often easier to assemble and purify, and are smoothly converted to the parent amides under mild conditions. The depsipeptide method is the most recent technique to improve the outcome of difficult syntheses, applicable to sequences containing residues of serine or threonine. A brief overview is presented about chemical aspects of the method, the steps that have been undertaken for its optimization, and the evaluation of its efficiency. Further applications of analogous principles to other critical topics in peptide synthesis such as condensation of peptide segments and solid-phase synthesis of naturally occurring cyclodepsipeptides are addressed as well.

Keywords: difficult sequences; depsipeptides; *O*-acyl isopeptides; diketopiperazine formation; segment condensation; DBU; cyclization; cotransin

Background

A fundamental characteristic of peptides and proteins is their inherent ability to adopt unique three-dimensional structures. In general, a polypeptide chain tends to fold so as to form an internal core from which the solvent is excluded and where hydrophobic AA side chains, pairs of charged groups, and hydrogen bond donors and acceptors build an optimal interaction network. This phenomenon, crucial for peptides and proteins to exert their biological function, is at the same time the main reason for difficulties during their chemical assembly.

Since the very beginning of peptide synthesis about one century ago when Emil Fischer achieved the first coupling of two AAs, with the breakthrough of the concept of solid-phase synthesis by Bruce Merrifield, a multitude of peptides and short proteins have been prepared via chemical methods. Nowadays, a broad choice of protecting groups and activation methods, polymeric support systems and linkers, and the development of versatile automated synthesizers joined to the availability of extremely efficient chromatographic techniques, allow the chemist to prepare medium length polypeptides (30–50 residues) quickly and in high purity using standardized solid-phase synthesis protocols [1]. However, even the use of the most sophisticated chemical tools is not sufficient to overcome the difficulties that arise as the peptide chains, growing onto the solid support, start folding and aggregating. At this point, the solvent is excluded from the peptide–resin, the polymer matrix shrinks, the substrate is inaccessible to solvents and reagents, coupling and deprotection reactions become slow and incomplete, and the synthesis fails.

The arising of aggregation phenomena during assembly and/or purification sets the real boundary to the accessibility of peptides and proteins via chemosynthesis. This is true not only for polypeptide chains above a critical length (~70 residues) but also for several biologically interesting substrates that are relatively short yet form either highly folded structures (e.g. WW domains, <40 residues, some of those inaccessible via standard SPPS) or fibrils and aggregates after final deprotection (e.g. β-amyloid peptide).

Aggregates forming during synthesis are stabilized by an interplay of inter- and intramolecular hydrogen bonding and hydrophobic interactions within the peptide chain, between adjacent chains and between the peptide and the solid support. Aggregation of resin-bound peptides has been observed by solid-state NMR, where static-aggregated systems give signal patterns clearly distinguishable from those given by well-solvated, isotropically mobile chains [2,3]. Characteristic adsorption bands observed in Fourier-transform infrared and Raman spectroscopy have shown that the secondary structure of resin-bound peptides is of the β-sheet type [4–6]. With respect to the polymer network consisting of the peptide and the solid support, the arising of

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Abbreviations used: *AA, amino acid; Bsmoc, 1,1-dioxobenzo[b]thiophene-2-ylmethyloxycarbonyl; DBU, diaza(1,3)bicyclo[5.4.0]undecane; DIEA, diisopropylethylamine; DKP, diketopiperazine; DMAP, 4-dimethylaminopyridine; HATU, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide; HBTU, 1-[bis(dimethylamino)methylene]-1H-benzotriazolium hexafluorophosphate 3-oxide; HOBt, 1-Hydroxy-1,2,3-benzotriazole; Hmb, 2-Hydroxy-4-methoxybenzyl; NMI, N-methylimidazole; SPPS, solid phase peptide synthesis, TBAF, tetrabutylammonium fluoride; TFA, trifluoroacetic acid.*

Biography

Irene Coin was born in Padua, Italy. After completing her studies at the Conservatory of Music of Vicenza, she graduated in Chemistry at the University of Padua in 2003 under the guidance of Claudio Toniolo, with a thesis on the synthesis in solution of alamethicin analogs. She carried out her graduate studies at the Leibniz-Institute of Molecular Pharmacology (FMP) in Berlin, Germany, under the supervision of Michael Beyermann, with a methodological study on the use of depsipeptides for the synthesis of difficult sequences, and obtained her Ph.D. at the University of Leipzig. After 10 months in the laboratory of Sidney Hecht at the Biodesign Institute in Tempe, Arizona, she is currently continuing her postdoctoral studies at the Salk Institute for Biological Studies, La Jolla, California, where she is working on the incorporation of non-natural amino acids into proteins in living cells.

aggregated structure corresponds to an effective increase of the cross-linking, which reduces the swelling capacity [7,8]. General strategies to prevent the onset of such interaction networks include the use of highly flexible solid supports with enhanced swelling properties, the limitation of loading, the use of special solvent mixtures and chaotropic salts, the application of solubilizing protecting groups, as well as the use of elevated temperatures and microwave irradiation, with each approach demonstrating a different extent of success. However, most modern and efficient strategies to hamper the formation of aggregating structures during synthesis apply specific reversible modifications directly into the peptide backbone during assembly.

An effective modification is the introduction of temporary tertiary amide bonds into the growing sequence, either using N^{α}-alkyl protecting groups based on the acid labile benzylic structure (Hmb group as proposed by Sheppard and associates [9,10]) or by the use of the so-called pseudoprolines of Mutter and coworkers [11,12]. The latter are derivatives of serine or threonine in which the β-hydroxyl group is reversibly linked to the α-amino group through an alkyl bridge (acid labile as well) in a ring that resembles the structure of proline. The lack of hydrogen in tertiary amides interrupts the continuity of hydrogen bonding, and the preferred *cis*-conformation of the amide bond characteristic of these proline-like residues determines a kink into the structure. In this way, peptide folding in β-sheets is destabilized and the tendency toward aggregation is greatly reduced. Pseudoprolines are very efficient and can be easily introduced into a growing sequence using standard protocols thanks to the commercial availability of suitable building blocks, so that they are currently the method of choice for the automated assembly of difficult sequences.

Depsipeptide Strategy

The 'depsipeptide technique', also reported as the '*O*-acyl isopeptide method' [13–15], is based on a different principle. Without introducing special protections into the amide backbone, the technique circumvents difficulties in synthesis by assembling onto the solid support, instead of the difficult peptide itself, its depsipeptide analog (*O*-acyl isopeptide [16]). This is built by extending the peptide chain, from a suitable point on, via the β-hydroxyl group of a serine or threonine residue. As in standard Fmoc-strategy, the depsipeptide is deprotected and cleaved from the resin with TFA and purified. Only at the very end, the *O*-acyl isomer is converted into the target peptide through an *O*,*N*-acyl shift, a long known reaction which occurs smoothly in aqueous environment at mildly basic conditions [17–20] (Figure 1). With respect to the all-amide form, depsipeptide isomers are often more easily synthesized and handled: by extending the backbone through an ester bond via a side chain, the regular polyamide structure - and thereby a possible regular hydrogen bond pattern - is interrupted, and elements of flexibility are introduced (the $C^{\alpha}-C^{\beta}$ bond and the C–O bond). This hampers peptide folding and facilitates the assembly. After cleavage from the solid support, as long as the amino function of the Ser/Thr residue - now deprotected - remains protonated, no *O*,*N*-shift occurs and the structure disrupting effect exerted by the backbone modification is retained, being at the same time an additional ionizable moiety provided to the molecule. These factors confer to depsipeptides a better solubility in acidic aqueous media compared to the parent amides, and facilitate their purification. Compared to the use of pseudoprolines or Hmb-N^{α}-protection, which are removed by the TFA treatment used for final deprotection, this is a fundamental advantage of the depsipeptide technique. In fact, difficult sequences show mostly poor solubility in aqueous systems, what makes their purification and characterization difficult or sometimes even impossible [15,16].

Besides being used as intermediates to achieve the synthesis of difficult peptides, depsipeptides found important application as models for the studies of folding and association processes (the so-called switch- or click-peptides) [21–23] and for the development of prodrugs with enhanced water-solubility [24–26].

The idea of synthesizing an *O*-acyl isomer that was subsequently converted into a target amide as a tool to bypass synthetic difficulties was illustrated for the first time in the late 1990s when applied to the assembly of highly hindered analogs of Leu-enkephalin [27]. Recently, the technique has been rediscovered almost simultaneously by three independent research groups (Mutter [13], Kiso [14], and Beyermann-Carpino [15]), with

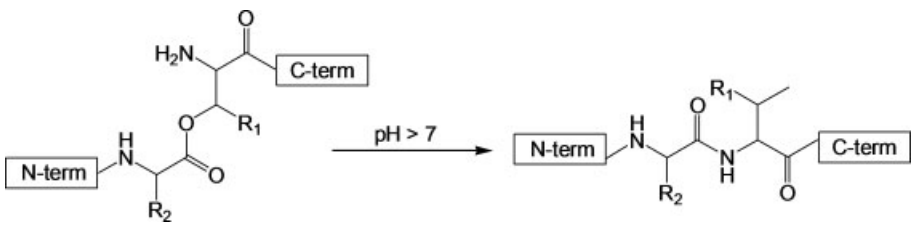

Figure 1. Conversion of a depsipeptide to the amide form, through a pH-promoted *O*,*N*-acyl shift. R_1, H/CH_3.

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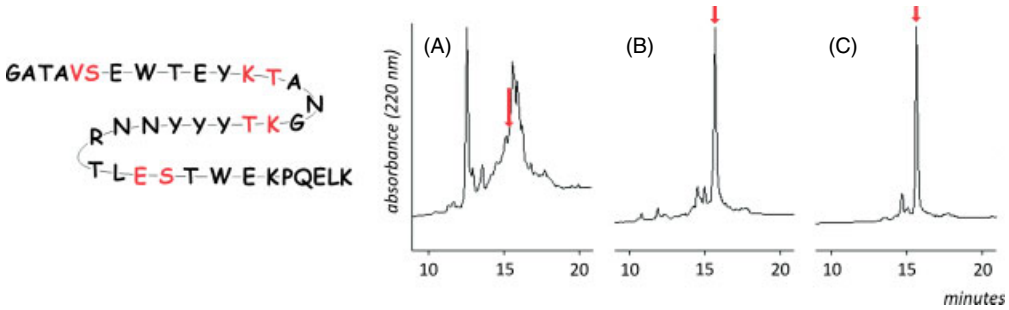

Figure 2. Left: [Asn[15]]FBP28-WW, sequence and draft of the folded structure. Right: Synthesis of [Asn[15]]FBP28-WW, HPLC profiles of crude products. The position of the correct product is indicated by the red arrow. (A) Product of standard SPPS protocol. (B) Product obtained via synthesis of a tetradepsipeptide analog. Sites of introduction of the despi-units are indicated in color on the left draft. (C) Product synthesized using pseudoprolines.

examples of application to the Jung–Redemann peptide, a short sequence very hard to assemble via standard SPPS, and the amyloid peptide Aβ(1–42), an emblematic case of a highly aggregating natural system. Nowadays, the use of depsipeptide analogs to achieve the synthesis of difficult sequences is well established in the peptide chemistry community. This has been made possible by the systematic investigation of several chemical aspects related to the technique, including the establishment of reliable procedures for the introduction of depsipeptide bonds into a growing sequence and analysis of their stability using Fmoc-based chemistry, the uncovering of possible side reactions related to the presence of an ester bond – first of all DKP formation – and the development of methods for their suppression. In addition, the efficacy of the depsipeptide approach in improving difficult syntheses as compared to the most consolidated use of pseudoprolines had to be evaluated.

Depsipeptide Technique Ready for a Broad Application to Difficult Sequences

In 2004, after the seminal works on the use of depsipeptides analogs of difficult sequences had been published, several questions were still open about the applicability of the depsipeptide method as a general tool to improve yields in the synthesis of aggregating systems. Of major concern was the discovery of sequences truncated *C*- and *N*-terminally at the level of the ester bond in crude mixtures, which we observed in our lab and was also reported by Kiso and coworkers, who hypothesized the occurrence of TFA-mediated hydrolysis of the ester bond during final deprotection [16]. In regard to this, besides the need of evaluating the actual stability of depsipeptide units under Fmoc-chemistry conditions, reliable protocols for the formation of ester bonds onto a resin-bound peptide substrate still had to be established. In fact, in these first publications depsipeptide units were built via direct esterification of the β-alcoholic function of a Ser/Thr residue – previously introduced with N^{α}-Boc protection and unprotected side chain – using carbodiimide as activating agent and either NMI [28,29] or DMAP [30,31] as catalyst. However, we had indication that the yield of such a procedure is not always quantitative, and it was moreover unknown to which extent the AA activated during *O*-acylation is affected by epimerization. This could well be expected, because the catalysts used in this reaction are known for not guaranteeing the optical integrity.

We investigated the stability of depsipeptide bonds toward repeated treatment with the standard reagents involved in solid-phase synthesis on the homooligopeptide $(Val\text{-}Thr)_{10}$, a difficult sequence forming β-structures [32]. By building a despipeptide bond at each Thr site, we were able to assemble a deca-depsipeptide that as crude product gave a MS spectrum attributable to a single component [33]. This showed that depsipeptide units are generally stable toward both piperidine and TFA treatment, when they are used for Fmoc removal and final deprotection/cleavage step, respectively. Instead, we demonstrated that the main origin of possible truncation at the level of the depsipeptide bond is the occurrence of intramolecular aminolysis (formation of DKP) during Fmoc removal from the second AA residue following the ester bond at the *N*-terminal side. This is a well-known side reaction in peptide synthesis when ester bonds are present [34,35], and is often observed in the case of non-hindered ester protecting group for the *C*-terminal residue (e.g. methyl ester) or when using a hydroxy linker to the polymeric support (Wang resin). We uncovered the formation of DKP during the synthesis of depsipeptide isomers of the WW domain of the formin binding protein (FBP) 28, a 37-mer difficult sequence that folds in triple stranded antiparallel β-sheet [36], and was otherwise impossible to assemble using standard SPPS [37] (Figure 2A). In the synthesis of multidepsi-isomers of FBP28-WW, DKP formation occurred at different positions to various extents depending on the nature of the AAs close to the ester bond. We were able to suppress DKP formation by using the Bsmoc [38,39] group instead of Fmoc for N^{α}-protection at the sensitive sites. Like Fmoc, the Bsmoc group is base labile, but it is removed more quickly and under milder conditions than Fmoc is.

During the synthesis of depsi-analogs of FBP28-WW other truncated products were found, namely sequences starting *C*-terminally with the AA expected to form an ester bond. We have shown that those sequences were originated during the *O*-acylation step, which involves an exceptionally strong activation method. This leads to undesired acylation of functional groups remaining available on the solid support even after repeated cycles of standard coupling. Once a new AA is bound to the resin, new chains are extended during the following coupling cycles. By capping such residual functional groups in the presence of NMI after linking the first AA to the resin, the formation of *C*-terminally truncated sequences was suppressed [33].

The extra capping step under *O*-acylation conditions after the linking and the use of Bsmoc-protection for every second residue following a depsipeptide unit represented the first firm step toward the optimization of the depsipeptide method. Using this strategy we were able to prepare several depsipeptide analogs of FBP28-WW differing in number and position of *O*-acyl units inserted. As the best result, we achieved the synthesis of a tetradepsi-isomer with high purity of the crude product, which was smoothly

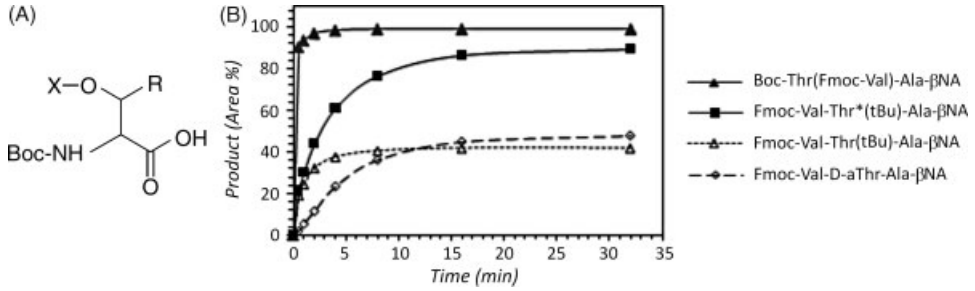

Figure 3. (A) Structure of either urethane protected amino acid (X = protecting group) or despidipeptide building block (X = Fmoc-AA-) or peptide segment bearing *C*-terminal depsipeptide unit (X = polypeptide chain); R = H/CH_3 for Ser/Thr; (B) coupling of either dipeptide Fmoc-Val-Thr(*t*Bu)-OH or depsidipeptide Boc-Thr(Fmoc-Val)-OH with H-Ala-βNA (NA = naphtylalanine) at a concentration of 0.01 M. Activation via HBTU/DIEA in DMF. 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. *The curve marked by squares represents the sum of those of the two diastereomer products Fmoc-Val-Thr(tBu)-Ala-βNA and Fmoc-Val-D-aThr(tBu)-Ala-βNA.

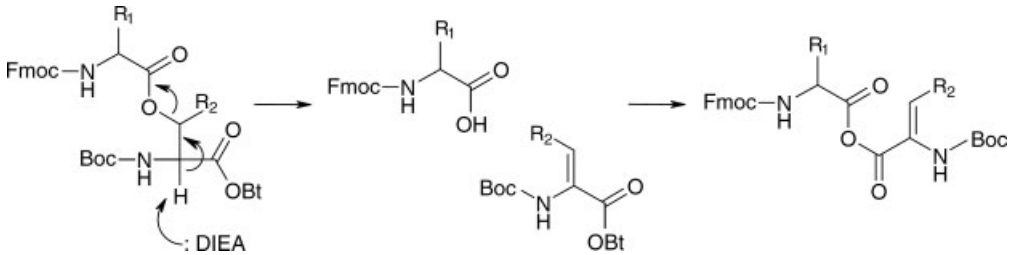

Figure 4. Mechanism of β-elimination at an activated depsidipeptide unit in the presence of base, and subsequent formation of a mixed anhydride between the two products of reaction. Side products deriving from β-elimination include sequences corresponding to the coupling of the dehydro-amino acid, and sequences missing the Ser/Thr residue.

shifted to the all-amide peptide in aqueous bicarbonate within 1 h (Figure 2B). This demonstrated that depsipeptide units are indeed able to prevent the onset of folded structures during the assembly of sequences prone to aggregate.

As mentioned earlier, depsipeptide units can be formed stepwise on a resin-bound peptide by acylating the β-hydroxyl group of Ser or Thr residues with the following AA. In general, this procedure allows the introduction of every desired depsipeptide unit into a growing chain in a relatively short time and at low cost. However, we have observed that - depending on the sequence, and both when using NMI or DMAP as a catalyst - *O*-acylations can occur slowly and give low yields, which make it necessary to perform an analytical control at this step. In addition, *O*-acylations are often accompanied, although to extents that for most applications could be acceptable (0.2–1.5%), by epimerization of the highly activated AA [33]. Consequently, a more convenient way to introduce a depsipeptide unit into a growing sequence has been shown to be the coupling of preformed depsidipeptide building blocks [33,40] (Figure 3A, X = Fmoc-AA-), which are easily prepared from commercially available starting materials, and purified so as to guarantee the optical purity of the esterified AA. Although being dipeptides, depsidipeptide units are not affected by the risk of epimerization when activated for coupling [41], because their structure is analogous to that of urethane protected AAs (Figure 3A, X = protecting group), which have a low propensity to form optically labile oxazolones [42]. In fact, the coupling of a depsidipeptide is exceptionally efficient, in that it is much faster than the coupling of the corresponding amide-dipeptide and occurs without loss of configuration, even using base-assisted activation methods (Figure 3B). Such building blocks are therefore well suited to be applied to standard solid-phase synthesis protocols, and open up the possibility of automating the assembly of depsipeptides. Indeed, we have demonstrated the fully automated assembly of a depsipeptide analog of the 31-mer *C*-terminal segment of the globular protein crambin [33], a difficult sequence previously obtained via native ligation techniques [43].

Based on analogous considerations, the application of depsipeptide units has also been proposed first by Kiso and coworkers to provide ideal conditions for segment coupling [44]. Indeed, the coupling of a peptide segment bearing a *C*-terminal depsipeptide unit (Figure 3A, X = polypeptide chain) is much more efficient than that of the corresponding regular peptide segment, and is chirally safe even under conditions (activation via HBTU/DIEA in DMF) that give rise to total epimerization in the case of the all-amide segment [41]. However, it has been shown that during activation of depsipeptide units β-elimination at the activated *O*-acylated Ser/Thr residue can occur to extents that depend on the presence of base in the system and the type of the solvent [41,45] (Figure 4). The occurrence of β-elimination was completely suppressed by using carbodiimide/HOBt activation in nonpolar solvent (DCM), and it was substantially reduced by substituting collidine for DIEA using HBTU activation in DMF. Thus, a segment coupling that gave 50% of an epimerized product after reaction overnight was achieved with a yield of 70% within 2 h, without detectable epimerization, and with minimal formation of side products. In principle, the possible occurrence of β-elimination has to be taken into account also when using depsidipeptide blocks. However, our results have shown that, if the coupling is fast, depsidipeptide blocks can be safely coupled via HBTU/DIEA activation and, as already demonstrated with the successful synthesis of crambin, are well applicable to automated protocols of SPPS. On the other hand, base-free activation in nonpolar solvent is required when, due to steric hindrance or early arising of aggregation, the coupling is not fast enough to occur before the side reaction takes place [41].

As part of this work, a comparison was made between the depsipeptide technique and the use of pseudoprolines,

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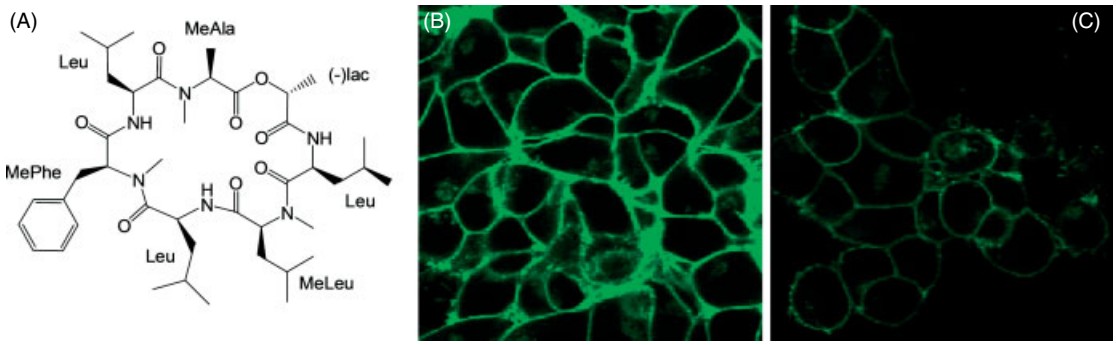

Figure 5. A) Structure of cotransin; (B-C) Laser Scanning Microscopy images of stably transfected HEK293 cells expressing CRFR1-GFP treated with (B) DMSO 1%, 24 h (control experiment), (C) cotransin 10 μM, DMSO 1%, 24 h. Pictures were taken using identical optical parameters. Inhibition of CRFR1 expression by cotransin was confirmed by western blot and analysis by fluorescence-assisted cell sorting [47]. Cell viability was assessed by trypan blue staining.

which is currently the most applied method to improve the synthesis of difficult peptides. Thus, most of the peptides synthesized via depsipeptide analogs were prepared in parallel using pseudoprolines. The results obtained by the assembly of FBP28 (Figure 2C) and crambin have shown the two methods to be equally efficient in improving the yield of the synthesis and purity of the crude products [33]. Likewise, we have shown that depsidipeptide units, such as pseudoprolines, can also be introduced in a growing chain using building blocks applicable to automated protocols. It is worth mentioning that during the progress of our work some depsidipeptide building blocks have become commercially available. With respect to the application of the two techniques to segment coupling, both *C*-terminal depsipeptide units and pseudoprolines prevented epimerization at the activated residue and increased the rate of coupling to comparable extents [41].

Naturally Occurring Depsipeptides

Naturally occurring depsipeptides, in particular cyclo-depsipeptides, are of increasing interest for pharmacological research because of their wide range of biological activities. The synthesis of such compounds may be very laborious, due to the presence of non-coded AAs (*N*- or *C*-alkylated AAs, D-AAs, AAs bearing structurally elaborated groups on the side chain) in combination with hydroxy acids. In particular, the stepwise assembly of such compounds is expected to be extraordinarily difficult when *N*-alkyl AA residues are in the two positions following *N*-terminally the ester bond, as DKP formation can be extremely fast in such cases [35]. On the other hand, the high potential of these substances for drug discovery, and the related necessity of performing extended screening and structure–function relationship studies, prompts the search for stepwise strategies on solid phase capable of making accessible a large number of analogs within a short time. With the intention of exploiting the synthetic methodologies developed for the preparation of depsipeptide analogs of difficult sequences, we studied the synthesis of the cyclodepsipeptide 'cotransin' [46], where an *N*-methyl-alanine residue is bound to the hydroxy group of a lactic acid unit (Figure 5A).

As expected, by the stepwise assembly of cotransin onto a chlorotrityl resin, DKP formed dramatically fast during removal of the Fmoc protection from the leucine residue that follows the *N*-methyl-alanine at the second position after the ester bond [48]. At this position DKP formation did not diminish even by using

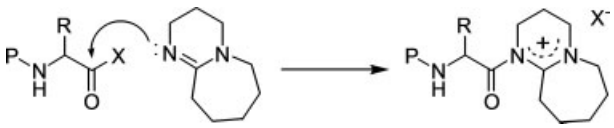

Figure 6. Reaction between an activated amino acid and DBU, product isolated via HPLC and identified via ESI-TOF-MS and NMR for Bsmoc-Val-X. P, protecting group; X, leaving group of the active species.

the Bsmoc protecting group, although for its deprotection only a brief treatment with diluted piperidine was applied. Therefore, we attempted to develop a deprotection-coupling tandem system [49,50] (deprotection in the presence of an orthogonally protected, activated AA) based on the use of the Bsmoc and Fmoc groups. By testing the stability of the two groups toward different bases, we discovered new conditions for their orthogonal use, among which the complete stability of the Bsmoc group toward TBAF (which removes Fmoc in a short time [51]) is of practical interest. However, the water bound to TBAF provoked fast hydrolysis of the activated AA and consequent failure of the tandem system approach. Selective Fmoc removal was achieved also using diluted DBU [52], but this base was shown to be not suitable for a tandem system, as it undergoes rapid acylation by activated species (Figure 6). This was a quite surprising result, as DBU is a tertiary amine, and traditionally considered as a non-nucleophile [53]. Abandoning the one-pot approach, we succeeded in limiting DKP formation simply by performing Fmoc removal by either a 'flash' treatment with concentrated DBU, which allows deprotection in few seconds, or using TBAF instead of piperidine, which is a known catalyst for this side reaction [54]. Facile-optimized protocols for the use of these two alternative deblocking agents eventually allowed the assembly of linear cotransin, otherwise not accessible via Fmoc-chemistry [48].

With respect to the cyclization step, we reasoned that the same principle underlying the advantageous use of depsipeptide units for segment coupling could be fruitfully applied to substrates containing α-hydroxy acids within the backbone, as such residues do not form optically labile oxazolones when activated for coupling [55]. Thus, by appropriately disconnecting the ring at such a position, the cyclization reaction is expected to be fast and epimerization-free. The efficacy of this approach was demonstrated for the cyclization of linear cotransin bearing the lactic acid unit at the *C*-terminus. By using HATU as an activating agent [56], the ring closure occurred quickly (it was almost complete within 15 min) and, as expected, was not accompanied

by epimerization. Moreover, despite the presence of base in the system, no products due to β-elimination were detectable. Almost at the same time, another group reported about epimerization-free head-to-tail cyclization of small Ser/Thr containing cyclopeptides through the use of depsipeptide units [57].

Cotransin, like its natural parent compound HUN-7293 [58], shows a very interesting biological activity, being able to suppress, selectively and reversibly, the expression of a subset of secreted and membrane proteins in mammalian cells. Among them, studies carried out in a cell-free translation system have shown that cotransin inhibits the expression of the corticotropin-releasing factor receptor (CRFR-1) [46]. By treating stably transfected HEK293 cells overexpressing GFP-tagged CRFR-1 with cotransin, we have demonstrated that the inhibitory effect on CRFR-1 expression that has been observed *in vitro* is reproducible in living cells [47] (Figure 5B-C). HUN-7293 and its analogs act at the level of the endoplasmic reticulum, by perturbing the interaction between the nascent chain–ribosome and the Sec61 complexes [46,59]. This prevents the recognition of the signal sequence by the translocation channel and the feeding of the nascent chain into the lumen, thus stopping the synthesis at the initial stage. This represents a completely new way of inhibiting protein expression, which appears an attractive alternative to permanent genetic knock out and the use of RNA interference methods, and might have a great pharmacological potential. However, it is not yet completely understood how these compounds exert their selectivity [60], and – most importantly – none of the analogs of HUN-7293 known so far shows high specificity in suppressing the expression of a single protein only. On this basis, further studies are being carried out, which involve cotransin and a broad set of its analogs, now easily accessible by SPPS thanks to our newly developed synthesis strategy.

Conclusion

In conclusion, it has been clearly demonstrated that depsipeptide isomers can be successfully used as intermediates to achieve the synthesis of peptides that are otherwise 'inaccessible' using standard Fmoc-chemistry protocols because of the arising of aggregation phenomena. We have shown that depsipeptide bonds are in general stable toward standard reagents used in Fmoc-based SPPS, and that if products truncated at the level of the ester bond are formed, these are due to the occurrence of side reactions, especially DKP formation during Fmoc removal. However, we were able to suppress DKP formation either using a protecting group that is more easily removed than Fmoc by piperidine like the Bsmoc group or bases alternative to piperidine for Fmoc removal. The efficiency of the depsipeptide technique in improving the outcome of difficult syntheses was shown to be comparable to that of the pseudoproline method. By designing depsidipeptide building blocks, assembly of depsipeptides via automated protocols has been made possible. It should also be mentioned that during the proceeding of our work some depsidipeptide building blocks have become commercially available. Taken together, these results provide a solid basis for the wide application of the depsipeptide method to the synthesis of difficult peptides as a valid possible alternative to the use of pseudoprolines.

Optimized conditions have been established for the advantageous application of depsipeptide units to segment coupling. This also extends the possibility to achieve efficient and epimerization-free couplings to segments bearing *C*-terminal serine or threonine residues, which up to now has been possible only with *C*-terminal glycine or proline residues.

Finally, based on the experience acquired by studying the depsipeptide technique, a solid-phase protocol based on Fmoc-chemistry has been developed for the assembly of the difficult cyclodepsipeptide cotransin. This method seems to represent a suitable approach for the solid-phase synthesis of cyclic depsipeptides in general, making extensive structure–activity relationship studies of such biologically very interesting molecules now feasible.

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