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Depsipeptide Methodology for Solid-Phase Peptide Synthesis: Circumventing Side Reactions and Development of an Automated Technique via Depsidipeptide Units[†,‡]

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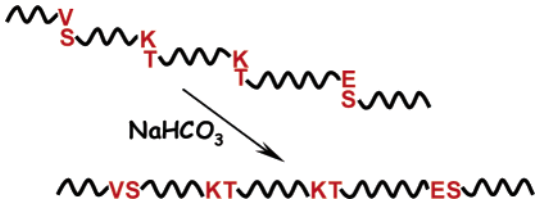

The depsipeptide technique is a recently developed method for peptide synthesis which is applicable to difficult sequences when the synthetic difficulty arises because of aggregation phenomena. In the present work, application of the depsipeptide method to extremely difficult sequences has been demonstrated and a serious side reaction involving diketopiperazine formation uncovered and subsequently avoided by the appropriate use of the Bsmoc protecting group. Many other aspects of the technique have been investigated, such as the stability of the depsi units during assembly and workup procedures, the completeness of the O-acylation step, the occurrence of epimerization of the amino acid activated during O-acylation, and the nature of side products formed. In addition, the method was modified so as to allow for completely automated syntheses of long-chain depsipeptides without the need for any interruption by manual esterification procedures. Finally, the synthesis efficiency of the new depsipeptide technique was shown to be comparable to that of the well-known pseudoproline technique.

Introduction

A main obstacle to the chemical synthesis of long peptides and proteins is the occurrence of aggregation phenomena, which cause both coupling and deprotection reactions during the assembly of peptide chains to be slow and incomplete and the purification of the resulting products to be extremely difficult. Current strategies to prevent difficulties in synthesis aim at interfering with peptide folding and association by introducing specific reversible modifications at the peptide backbone, using N-alkylation systems (e.g., Hmb-protected amino acids[1]), pseudoprolines,[2] or most recently depsipeptide units.

Insertion of a depsipeptide unit in a sequence interrupts the regular pattern of amide bonds at the site of a Ser/Thr unit, with the peptide chain being extended from that point on via the β-hydroxyl function. Each depsipeptide unit provides an additional ionizable moiety, thereby increasing solubility and facilitating purification, as reported for the case of the β(1−42) amyloid peptide.[3,4] The conversion to the target amide

[†] Dedicated to the memory of Professor Bruce Merrifield, a warm human being and a great scientist.

[‡] Abbreviations used: Bn, benzyl; Boc, *tert*-butyloxycarbonyl; Bsmoc, 1,1-dioxobenzo[*b*]thiophene-2-ylmethyloxycarbonyl; DCM, dichloromethane; DIC, N,N′-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMAP, 4-(dimethylamino)pyridine; DMF, *N*,*N*-dimethylformamide; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride; Fm, 9-fluorenemethyl; Fmoc, 9-fluorenemethyloxycarbonyl; Hmb, 2-hydroxy-4-methoxybenzyl; NEt_3, triethylamine; *N*-HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium hexafluorophosphate 3-oxide; *N*-HBTU, 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide; NMI, *N*-methylimidazole; NMP, *N*-methylpyrrolidone; O-Su, succinimido ester; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl.

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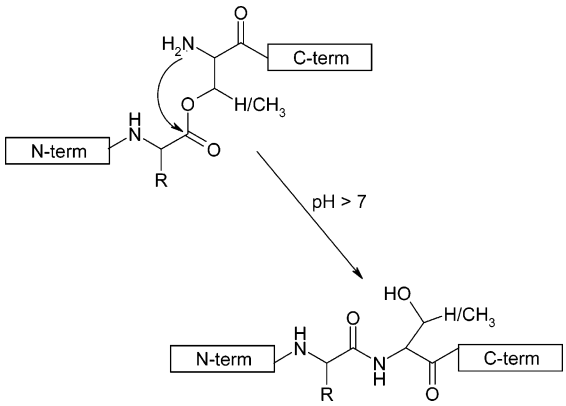
SCHEME 1. Conversion of a Depsipeptide to the Amide Form through a Base-Promoted *O,N*-Acyl Shift



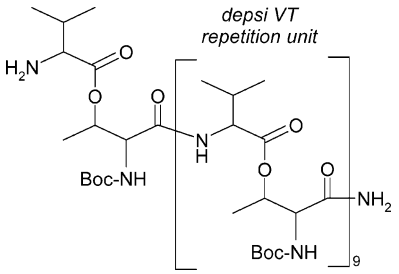
peptide is smoothly achieved through an *O,N*-acyl shift,[5] which occurs quantitatively under mildly basic conditions over a short period of time (Scheme 1).

The idea of synthesizing an *O*-acyl isomer that could be subsequently converted to a target amide as a means of circumventing synthetic difficulties was first described in 1998 for the assembly of some highly hindered α-methylserine analogues of leucine-enkephalin.[6] More recently, the technique was rediscovered almost simultaneously by three research groups,[3,4a,7a] and the depsipeptide method (otherwise called the *O*-acyl isopeptide method[4]) began to be generalized for the synthesis of difficult sequences where the difficulty arises because of aggregation phenomena. Relative to the pseudoproline technique[2] for solving similar problems via the same two amino acids (Ser, Thr), the depsipeptide methodology has the advantage of providing an isomer of the native amide which needs only to be isomerized[5] under essentially physiological conditions to the native amide species. This methodology lends itself to the generation of highly soluble prodrugs[8,9] for medicinal purposes and to the study of peptide folding and association for highly aggregating natural systems (so-called switch-peptides[7] or click peptides[4b]).

Results and Discussion

In the present work, application of the depsipeptide method to an extremely difficult sequence has been demonstrated and a serious side reaction involving diketopiperazine (DKP)

SCHEME 2. Structure of Depsi-(VT)$_{10}$-NH$_2$ (1a)



formation[10] uncovered and subsequently avoided by judicious use of a new protecting group. Many other questions that have arisen during studies of the application of the technique were addressed, for example, questions concerning the stability of the depsi units during assembly and workup procedures, the completeness of O-acylation, the occurrence of epimerization of the amino acid activated during the O-acylation step,[11] and the eventual role played by the choice of solid support.[4] In addition, the method has been modified so as to allow for completely automated syntheses of long-chain depsipeptides without the need for any interruption by manual esterification procedures.

The synthesis efficiency of the new depsipeptide method was compared to that of the pseudoproline technique. For this purpose, most of the peptides obtained via depsipeptide analogues were synthesized also via pseudoproline, and the purities of the crude products respectively obtained were compared.

Stability of Depsipeptide Units toward the Standard Conditions of Solid-Phase Peptide Synthesis. Our first aim was to check the stability of the depsipeptide bonds toward repeated treatment with the standard reagents involved in solid-phase synthesis. We took as a model the homooligopeptide (VT)$_{10}$, a difficult sequence[12] forming β-structures,[13] and built a depsi bond at each Thr site, thus obtaining a poly-depsipeptide chain made up of a series of alternate ester−amide units (Scheme 2).

Figure 1 reports the MALDI-MS spectra of the crude (VT)$_{10}$-NH$_2$ (**1**) obtained via standard automated synthesis and

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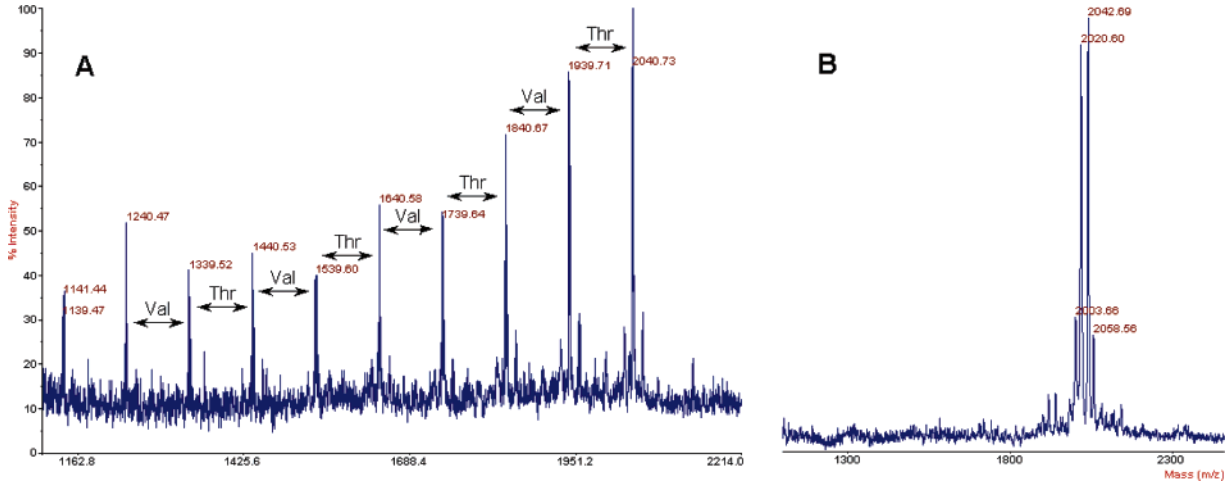

FIGURE 1. MALDI-MS spectra of crude products. (A**)** $(VT)_{10}-NH_2$ (**1**). Mass differences between peaks identify a series of Val and Thr deletions. (B**)** depsi-$(VT)_{10}$-NH_2 (**1a**). Calculated values *m*/*z* (average mass, the same in both cases): $[M + H]^+_{calc}$= 2020.4; $[M + Na]^+_{calc}$= 2043.4; $[M + K]^+_{calc}$= 2059.4. $[M + H - 17]^+_{calc}$ = 2003.4 (neutral loss of NH_3 from a side chain amino group).

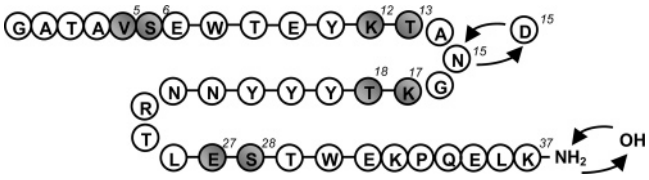

FIGURE 2. [Asn[15]]FBP28-WW-amide (**3**). Native Asp[15] was substituted by Asn in order to avoid byproducts due to aspartimide formation.[16] Replacing the native free acid form with an amide at the C-terminal position results in sharper peaks during HPLC analysis. Colored circles highlight sites chosen for the introduction of depsi units.

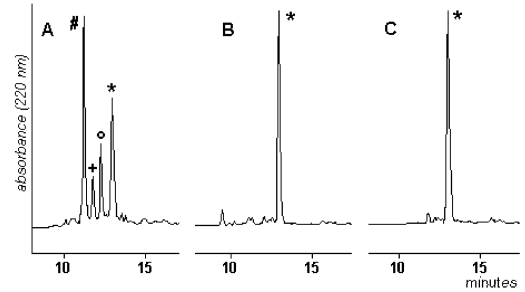

FIGURE 3. HPLC profiles of the crude Y^{19}-K^{37} segment: (A**)** standard synthesis (**2**); (B**)** synthesis via the depsi analogue after shift (**2a**); (C**)** synthesis via pseudo-proline (**2b**). Key: *, target product **2**, in **A** coeluted with des-N; #, N^{23}-K^{37} (43%); +, des-Y,Y,N; ○, des-Y,N.

of the crude depsi-$(VT)_{10}$-NH_2 (**1a**), manually assembled as described in the Experimental Section. In both cases, a TentaGel SRam resin was used as solid support and the final cleavage was carried out with TFA in the presence of scavengers.

The signals detected in the MALDI-MS spectrum of depsi-$(VT)_{10}$-NH_2 are all attributable to the target depsipeptide and do not give any indication for the presence of byproducts, thus confirming that ester bonds are in general stable both toward piperidine and under TFA cleavage conditions.

Application of the Depsipeptide Methodology to the Extremely Difficult WW Domain FBP28. As a suitable model to study the depsipeptide technique, we chose an appropriate analogue of the WW domain FBP28 (Figure 2), a small, 37-residue peptide, exhibiting a triple-stranded, antiparallel β-sheet structure,[14] which is impossible to synthesize using standard SPPS[15] (see Figure 5A).

Even the shorter C-terminal segment Y^{19}-K^{37} (**2**) proved to be a difficult sequence, with a standard SPPS via a TentaGel SRam resin (amino linker) giving less than 25% of the target peptide (Figure 3A). By introducing a depsipeptide unit at ES 27/28, the synthetic difficulties were completely overcome, and the crude product obtained (**2a**) was of excellent quality according to HPLC (Figure 3B).

$$Y^{19}YYNNRTLESTWEKPQELK^{37}\text{-}NH_2$$

2

On the other hand, synthesis with the same resin of the complete sequence [Asn[15]]FBP28-NH_2 via the introduction of two depsi units (**3a**), at positions ES 27/28 and KT 17/18, gave less than 20% of the target depsipeptide. By MALDI-MS analysis, among the side products we found sequences arising from apparent cleavage at the depsi bonds: a group of segments having Glu^{27} as the C-terminal unit, and a product involving truncation at Thr^{18}.

$$GATAVSEWTEYKTANG\text{-}K^{17}T^{18}\text{-}YYYNNRTL\text{-}E^{27}S^{28}\text{-}TWEKPQELK\text{-}NH_2$$

3a

Detection of Diketopiperazine Formation, Circumvented by Use of Bsmoc Chemistry. To investigate the origin of truncation at Thr^{18}, the assembly was monitored step by step after introduction of the second depsi unit at residues KT 17/18, by cleaving and analyzing a small amount of sample at each step. DKP formation was found to occur during removal of the Fmoc group from Gly^{16}, leading to 20% (according to HPLC trace) of material missing the first two amino acids, product **4**:

$$G\text{-}K^{17}T^{18}\text{-}YYYNNRTL\text{-}E^{27}S^{28}\text{-}TWEKPQELK\text{-}NH_2$$

and T^{18}-YYYNNRTL-$E^{27}S^{28}$-TWEKPQELK-NH_2, 20%

As no evidence of DKP formation was observed after standard deprotection of the Leu^{26} residue (next to the depsi unit ES 27/28), the occurrence of such intramolecular aminolysis is

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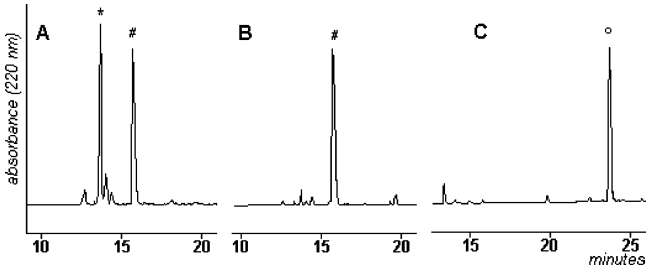


FIGURE 4. HPLC profiles of crude products. To avoid artifacts in estimating the extent of DKP formation, due to possible degradation of the samples during workup and/or storage,[18] removal of the N^{α}-protection from Leu^{26} was immediately followed by acetylation under acidic conditions with Ac_2O 50% v/v in DMF (**5a**, **5b**). Identification of the products was assessed by LC−MS analysis: (A**)** product of standard Fmoc removal, **5a**; (B**)** product of Bsmoc removal, **5b**; (C**)** product of standard coupling after Bsmoc removal, **6**. Key: *, Ac-lac-TWEKPQELK-NH_2 **5a**; #, Ac-LE-lac-TWEKPQELK-NH_2 **5b**; ○, Fmoc-TLE-lac-TWEKPQELK-NH_2 **6**.

clearly dependent on the nature of the two amino acids adjacent to the depsi bond (at the N-terminal side) and probably also on their position within the sequence. To solve the problem of truncation at Thr^{18} we used for α-amino protection the $Bsmoc^{17}$ group, which is removed under milder conditions than is the Fmoc residue.

Upon consideration of short DKP-prone sequences suitable for a preliminary study, it was found that substitution of Ser^{28} with L-lactic acid in the C-terminal segment of the WW domain provoked Fmoc removal from Fmoc-LE-(+)lac-TWEKPQELK to take place with a 50% yield of product missing the first two N-terminal amino acids (**5a**, Figure 4A). The amount of DKP formation was markedly reduced by using the Bsmoc group, which was quantitatively removed from Leu^{26} using 2% piperidine v/v in DMF for 3 min (**5b**, Figure 4B), and coupling of the following Thr residue could be performed via the standard procedure, leading to a product of high purity (**6**, Figure 4C).

Suppression of Sequences Truncated at Glu^{27}: Role of the Solid Support. The formation of sequences of the type $Xxx^{n}\rightarrow Glu^{27}$ was circumvented by performing an acetylation step in the presence of NMI before introduction of the first depsi unit. This indicates that the stronger activation process employed for O-acylation (via DIC/NMI, 4 h) can give rise to undesired additional acylation of free amino groups remaining available on the resin after repeated cycles of standard couplings (*N*-HBTU, 20 min), thus leading to the birth of new chains having as the C-terminus an amino acid belonging to a depsi unit.

When assembling on a preloaded TentaGelA resin a depsi analogue of Aβ(1−42) containing a depsi unit at Gly-Ser 25/26,[4] Kiso and co-workers found large amounts of undesired Aβ(1−25) and Aβ(26−42) (20% and 30%, respectively, according to the HPLC trace), whereas performing the same synthesis on a chlorotrityl resin gave no Aβ(1−25) and only a small amount (1.6%) of Aβ(26−42). To explain these differing results based on the use of the two resins, it was suggested that the TFA-cocktail used for the final cleavage promotes hydrolysis of the ester bond, which should occur only when the depsipeptide is still bound to the resin. To avoid such ester bond hydrolysis, the use of an extremely acid-labile resin such as the chlorotrityl resin appeared to be necessary. In light of our results an alternative explanation can be proposed, according to which the byproduct Aβ(1−25) is generated by acylation of hydroxyl functions still available on the TGA resin when Gly^{25} is coupled to the hydroxyl function of Ser^{26} under O-acylation

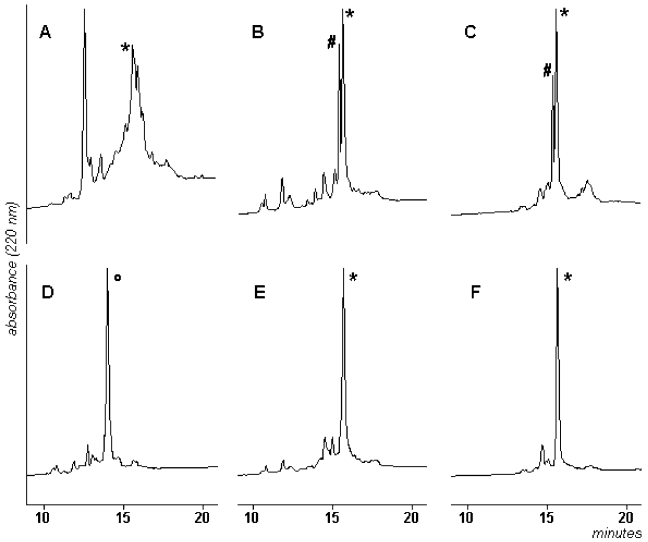


FIGURE 5. HPLC profiles of the crude [Asn^{15}]FBP28-NH_2: (A**)** standard synthesis (**3**); (B**)** synthesis via two depsi units (ES 27/28, KT 17/18) after shift, peptide **3b**; (C**)** synthesis via two pseudoproline units (ES 27/28, KT 17/18), peptide **3d**; (D**)** depsi analogue containing four depsi units (**depsi-3c**); (E**)** shifted depsi analogue (**3c**); (F**)** synthesis via three pseudo-proline units, (ES 27/28, KT 12/13 coupled via HATU, 4 h, VS 5/6), peptide **3e**. Key: *, target product **3**, in **A** coeluted with byproducts; #, des-Thr^{3}; ○, depsi-3c.

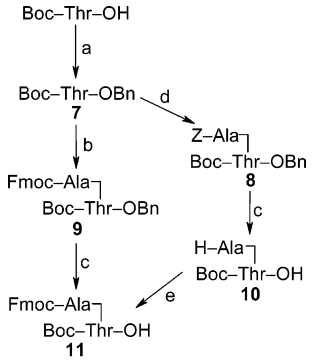
conditions. This cannot occur in the case of the chlorotrityl resin, since even if some chloride functions remained uncapped after the treatment with MeOH which usually follows the loading step, they are anyway unreactive toward the acylating species that are formed during O-acylation. The other byproduct, segment Aβ(26−42), may arise via DKP formation.

Based on these considerations, we can propose an optimized strategy for application of the depsipeptide technique, according to which a capping step in the presence of NMI is performed after linking of the first amino acid to the resin, and every second residue following a depsi unit is introduced with Bsmoc N^{α}-protection. Following this procedure we synthesized some depsi analogues of FBP28-WW, differing in the number and positions of the depsi units introduced. We obtained a crude product of high purity by introduction of four depsi units: ES 27/28, KT 17/18, KT 12/13, and VS 5/6. In the analysis of the crude depsipeptide product obtained by this synthesis (**depsi-3c**, Figure 5D) no sequences ending at Glu^{27} were found and no indication of DKP formation was detected. In addition the subsequent simultaneous O,N-shift at the four ester units was completed smoothly in aqueous bicarbonate within 1 h (**3c**, Figure 5E).

To evaluate the occurrence of epimerization of the amino acid activated during O-acylation, after shifting from the four-depsi analogue, crude peptide **3c** was hydrolyzed, and the derivatized amino acid mixture analyzed in a GC−MS system. The quantity of D-enantiomers found was 0.13% for Glu, 1.21% for Val, and 1.66% for Lys, the last value being the sum of two contributions (depsi units KT 17/18 and 12/13).

With regard to the synthesis of the other depsi analogues containing smaller numbers of depsi units the appearance of products due to incomplete O-acylation is notable, whereas all ester bonds of the 4-depsi-[Asn^{15}]FBP28-NH_2 system were smoothly formed using a standard protocol, a fact which is indicative of an important role for conformational effects. In particular, synthesis of the depsi peptide containing depsi units at ES 27/28, KT 17/18, and VS 5/6 gave as the main byproduct

SCHEME 3. Method for the Synthesis of the Ala/Thr Depsidipeptide Building Block[a]



[a] Key: (a) Cs_2CO_3/MeOH, followed by $C_6H_5CH_2Br$/DMF;[19] (b) Fmoc-Ala-OH/EDC/DMAP/DCM;[20] (c) H_2/Pd(C)/EtOH; (d) Z-Ala-OH/EDC/DMAP/DCM;[20] (e) Fmoc-OSu/NEt_3/CH_3CN/H_2O. The longer pathway avoids possible difficulties due to the incomplete orthogonality between Fmoc and OBn systems toward hydrogenolysis. The overall yields are comparable (~50%).

S^6(Ac)-K^{37} and incomplete esterification was also detected when the second depsi unit was introduced at KT 12/13 instead of KT 17/18. Replacing NMI by the stronger acylation catalyst DMAP did not help to improve the yield, and even worse results were found using structure disrupting solvents such as DMF, NMP, and DMSO, probably because of inefficient activation of the carboxylic group via the carbodiimide. In the routine procedure, O-acylation was always followed by a capping step (Ac_2O/NMI), thus allowing one to distinguish the products of incomplete acylation from those of DKP formation, for which the alcoholic function on the side chain is not acetylated.

Comparison of the Depsipeptide and Pseudoproline Techniques. As part of the present work a comparison was made between the new depsipeptide technique and the well-known pseudoproline technique of Mutter and co-workers.[2] This was done for most of the depsipeptides examined in the case of both the FBP28 domains and the Crambin sequence which is described below and in all cases both methods were shown to be equally efficient. Figures 3 and 5 show the results obtained for the synthesis of the WW domain (the results for the synthesis of Crambin are reported in the Supporting Information). It may be noted that during the synthesis of peptide **3e** (Figure 5F) coupling of the pseudoproline at KT 12/13 had to be performed manually (via HATU/DIEA/NMP/2 × 2 h, following standard acetylation), since the standard procedure led to incomplete coupling of about 40%.

Fully Automated Solid-Phase Synthesis via the Use of Depsidipeptide Units. The necessity of performing an O-acylation step represents a possible drawback of the depsipeptide method in comparison with the pseudoproline technique, since N-acylation is notoriously more efficient and more likely to be applied to automated protocols. Therefore, we prepared suitable Fmoc derivatives of depsidipeptides in order to study their use in the simultaneous introduction of two adjacent amino acids. Scheme 3 shows two alternative methods for the synthesis of the Ala/Thr system. The optical purity of depsidipeptide **8** was investigated both by comparison of its NMR spectrum with that of its authentically synthesized diastereoisomer Boc-Thr(Z-D-Ala)-OBn (**8a**) and via an HPLC-based derivatization technique using the Yamada reagent (Z-Val-Aib-Gly-OH)[25] (see the Supporting Information). No significant loss of configuration was detected.

For the first model of an automated synthesis the 31-mer C-terminal segment (**12**) of the small, hydrophobic, globular protein Crambin-46[21] was chosen.

$$C^{16}RLPG^{20}TPEAL^{25}CATYT^{30}GCIII^{35}PGATC^{40}PGDYA^{45}N^{46}$$
12

The full 46-mer has been cited as a difficult sequence by Kent and co-workers,[21] who reported an excellent total synthesis by a unique one-pot native chemical ligation technique. Synthetic difficulties were also described for the 31-mer. From a preliminary standard synthesis of the native 31-mer the UV deblocking trace showed difficulties at positions 27−28 (Figure 6A inset), which were completely eliminated by introduction of a depsi unit at AT 27/28 (Figure 6B inset). The depsipeptide CRLPGTPEALC-*AT*-YTGCIIIPGATCPGD was assembled using a standard automated protocol and introducing the depsi unit via the depsidipeptide block **11**. Synthesis of the double depsi analogue CRLPGTPEALC-*AT*-YTGCIIIPG-*AT*-CPGDYAN gave a comparable result, without showing any further improvement.

Conclusions. In conclusion, this work has demonstrated the general stability of depsipeptides during the normal operations of solid-phase peptide synthesis. By study of the very difficult FBP28-WW sequence a serious side reaction due to diketopiperazine formation was identified and suppressed by substitution of Bsmoc for Fmoc amino acids at appropriate positions. By prior synthesis of a particular depsidipeptide, completely automated syntheses of difficult long chain sequences was possible. Finally, the synthesis efficiency of the new depsipeptide technique was shown to be comparable to that of the well-known pseudoproline technique.

Experimental Section

General Procedures for the Synthesis of $(VT)_{10}$ Sequence and [Asn^{15}]FBP28-WW. Standard solid-phase peptide synthesis was performed on a peptide synthesizer using a standard Fmoc/*t*Bu protocol (0.25 M). A TentaGel-S-Ram resin (capacity 0.26 mmol/g) was used as solid support. Each cycle comprised double couplings, unless otherwise stated. Manual standard couplings of amino acid were carried out at a concentration of 0.25 M in DMF using *N*-HBTU/DIEA activation (1:2 equiv) without preactivation, couplings via DIC/HOBt (1:1 equiv) were carried out in DCM, with a 5 min preactivation period. Fmoc removal was achieved by piperidine 20% v/v in DMF (2 × 5 min). Standard O-acylations were carried out at a concentration of 0.25 M in dry DCM for a

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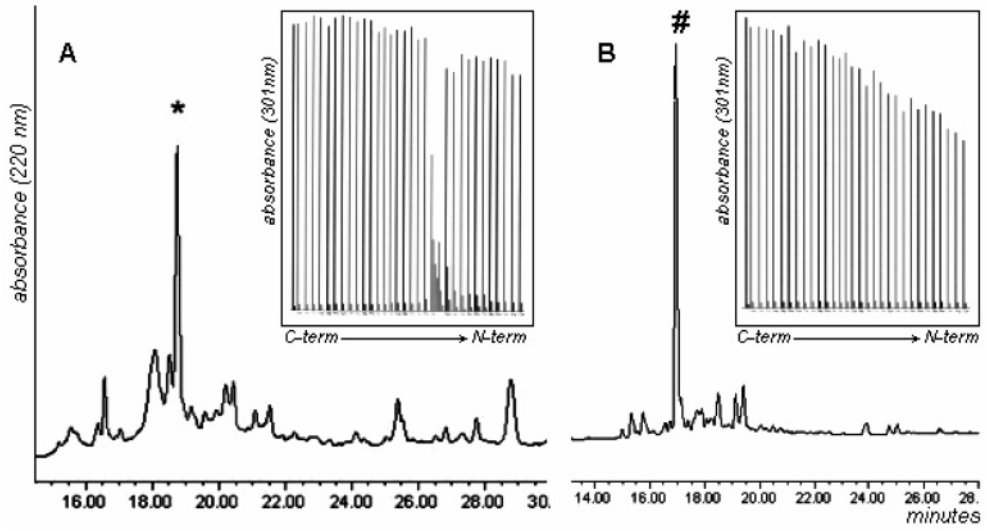

FIGURE 6. Syntheses of crambin (16−46): HPLC profiles of crude products and UV monitoring traces for deblocking steps (insets). The UV monitoring data measure, for each coupling cycle, the absorbance of the solution containing the Fm-piperidine adduct released during the Fmoc removal step, which is an indication both of the efficiency of the previous coupling cycle and of the efficiency of the deblocking step itself. For aggregated systems, where deblocking is slow, the instrument is programmed to repeat the deblocking step until the process is essentially complete. For the full-size monitoring traces, see the Supporting Information: (A**)** standard synthesis (**12a**); (B**)** automated synthesis of **depsi-12b** (depsidipeptide AT at 27/28). Key: *, target peptide, 27% purity; #, target depsipeptide, 44% purity.

time of 2 × 2 h, using DIC/NMI activation (1:1 equiv) without preactivation. Standard acetylation was carried out using a solution of 20% Ac_2O, 10% DIEA in DMF (15 min). Acetylations in the presence of NMI were performed using Ac_2O/NMI (1:1 equivalents) at a concentration of 0.25 M in DCM (2 × 90 min). All peptides and depsipeptides were cleaved from the resin with TFA over a period of 1−3 h in the presence of scavengers (standard cleavage solution: TFA 88%, water 5%, TIS 2%, phenol 5%) and precipitated in diethyl ether. O,N-Shifting of the depsipeptide to the amide form was achieved by treatment in aqueous buffer ($NaHCO_3$ 0.1 M, pH 8.5) for 1 h.

$(VT)_{10}$-NH_2 (1) was automatically assembled on a TentaGel-SRam resin (capacity: 0.26 mmol/g), using a standard 0.25 M Fmoc protocol, performing double coupling at each step. MALDI-MS spectrum is reported in the text.

Depsi-$(VT)_{10}$-NH_2 (1a). Boc-Thr-OH was linked to a TentaGel-SRam resin (capacity: 0.26 mmol/g) by standard coupling. The esterification of the hydroxyl group with Fmoc-Val-OH was carried out according to the general procedure. A capping step in the presence of NMI followed. Fmoc was deblocked with 20% piperidine in DMF and Boc-Thr-OH coupled. These steps were repeated in the same way up to completion of the sequence. Cleavage was performed in standard TFA solution for 3 h. MALDI-MS spectrum is reported in the text.

[Asn^{15}]FBP28-NH_2 (3b, via Two Depsi Units: ES 27/28, KT 17/18). Fmoc-Lys(Boc)-OH was linked to a TentaGel-SRam resin (capacity: 0.26 mmol/g) by standard coupling, followed by acetylation in the presence of NMI. The sequence was extended to Y^{19} with introduction of the first depsi unit at ES 27/28 as described for peptide **2a**. Boc-Thr-OH was manually coupled and O-acylated by Fmoc-Lys(Boc)-OH. Acetylation in the presence of NMI followed. The Fmoc group was deblocked and Bsmoc-Gly-OH coupled by the standard procedure. Bsmoc removal was carried out with 2% piperidine v/v in DMF (3 × 1 min), the peptide-resin was washed with DMF (1 min) and Fmoc-Asn(Trt)-OH coupled in the standard way. The rest of the sequence was completed automatically. Cleavage was carried out with standard TFA solution (3 h). The depsi peptide obtained, GATAVSEWTEYKTANG-*KT*-YYYNNRTL-*ES*-TWEKPQELK-NH_2, was shifted to the native amide form in $NaHCO_3$ 0.1M (1h). MALDI-MS: $[M + H]^+_{calc}$ = 4358.70 (average), found 4358.42.

[Asn^{15}]FBP28-NH_2 (3c, via Four Depsi Units: ES 27/28, KT 17/18, KT 12/13, VS 5/6). Fmoc-Lys(Boc)-OH was linked to a TentaGel-SRam resin (capacity: 0.26 mmol/g) by standard coupling, followed by acetylation in the presence of NMI. The sequence was extended to Y^{19} with introduction of the first depsi unit at ES 27/28 as described for peptide **2a**. Boc-Thr-OH was manually coupled and O-acylated by Fmoc-Lys(Boc)-OH. Acetylation in the presence of NMI followed. The Fmoc group was removed and Bsmoc-Gly-OH coupled by the standard procedure. Bsmoc removal was carried out with 2% piperidine v/v in DMF (3 × 1 min), the peptide-resin was washed with DMF (1 min) and Fmoc-Asn(Trt)-OH coupled in the standard way. Fmoc-Ala-OH and Boc-Thr-OH were successively coupled by the standard procedure. O-Acylation by Fmoc-Lys(Boc)-OH and acetylation in the presence of NMI followed. The Fmoc group was removed and Bsmoc-Tyr(*t*Bu)-OH coupled by the standard procedure. Bsmoc removal was carried out with 2% piperidine v/v in DMF (3 × 1 min), the peptide-resin was washed with DMF (1 min), and Fmoc-Glu(O*t*Bu)-OH was coupled by the standard procedure. The sequence was extended up to Glu^7 by the standard protocol. Boc-Ser-OH was manually coupled and O-acylated by Fmoc-Leu-OH. Acetylation in the presence of NMI followed. The Fmoc group was removed and Bsmoc-Ala-OH coupled by the standard procedure. Bsmoc removal was carried out with 2% piperidine v/v in DMF (3 × 1 min), the peptide-resin was washed with DMF (1 min), and the last amino acids subsequently coupled via the standard procedure. Cleavage was carried out with a standard TFA solution (3 h). The depsi peptide obtained GATA-*VS*-EWTEY-*KT*-ANG-*KT*-YYYNNRTL-*ES*-TWEK-PQELK-NH_2 was shifted to the amide form in 0.1 M aqueous bicarbonate (1 h). MALDI-MS: $[M + H]^+_{calc}$ = 4358.70 (average), found 4358.61.

Ac-LE-(+)lac-TWEKPQELK-NH_2 (Peptide 5a, Standard Procedure). The sequence TWEKPQELK-NH_2 was automatically assembled on a TentaGel-SRam resin (capacity: 0.26 mmol/g), using a standard 0.25 M Fmoc protocol. Lactic acid was coupled by the standard method and acylated by means of Fmoc-Glu(O*t*Bu)-OH, with a subsequent acetylation step in the presence of NMI. Fmoc was removed in the standard way and Fmoc-Leu-OH coupled. The Fmoc protected peptide was treated with 20% piperidine v/v in DMF (2 × 5 min), washed with DMF (1 min), and immediately acetylated using a solution of acetic anhydride 50% v/v in DMF

(1 h). Peptide **5a** was cleaved from the resin under standard cleavage conditions (1 h). LC−MS: retention time 18.0min, $[M + H]^+_{calc}$ = 1513.766 (monoisotopic) for **5a**, found 1513.87; retention time 16.2 min: $[M + H]^+_{calc}$ = 1271.640 (monoisotopic) for Ac-lac-TWEKPQELK-NH_2, found 1271.73.

Synthesis of Depsidipeptide 8. *N*-(*tert*-Butyloxycarbonyl)-threonine Benzyl Ester (7). Following the method of Halcomb and Cohen,[22] 13 g (59.3 mmol) of *N*-(*tert*-butyloxycarbonyl)-threonine was dissolved in 250 mL of methanol and treated with 11 g (33.7 mmol) of Cs_2CO_3. The methanol was removed with a rotary evaporator and the resulting oil was treated with 100 mL of ethyl ether and the ether evaporated. The residual white solid was redissolved in 125 mL of dry DMF and 8 mL (67.4 mmol) of benzyl bromide was added slowly. The reaction mixture was stirred at room temperature and the solvent removed by distillation under high vacuum. During distillation of solvent the temperature of the oil bath was kept at about 50 °C. The reaction mixture was partitioned between ethyl acetate and water, and the organic layer was collected, washed with 10% sodium bicarbonate solution and brine, and dried over $MgSO_4$. The crude benzyl ester was purified by column chromatography on silica gel using ethyl acetate/ hexane 3:2 as eluant to give 14.8 g (81%) of **7** as a colorless oil which was used as such in the next step: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.24 (d, 3), 1.44 (s, 9), 2.1 (s, 1), 4.3 (m, 2), 5.2 (q, 2), 5.32 (d, 1), 7.3−7.39 (m, 5); IR (DCM) 1741, 1714 cm^{-1}.

***O*-[(*N*-Benzyloxycarbonyl)alanyl]-*N*-(*tert*-butyloxycarbonyl)-threonine Benzyl Ester (8).** To an ice-cold solution of 3 g (9.708 mmol) of **7**, 4.5 g (20.18 mmol) of Z-Ala-OH, and 0.355 g (2.9 mmol) of DMAP in 100 mL of DCM was added in portions 5.6 g (29 mmol) of EDC. The reaction mixture was stirred at 0 °C for 1 h and then at room temperature for 4 h. The reaction mixture was diluted with 750 mL of ethyl acetate, washed with 5% citric acid, 10% sodium bicarbonate solution, and brine, and dried over $MgSO_4$. After removal of the solvent with a rotary evaporator, the crude product was sampled by ^{1}H NMR for chiral purity (see later) and the main portion purified by column chromatography on silica gel using as eluant ethyl acetate/hexane (at first 3:7 and increasing the ethyl acetate component gradually to 1:1) to give 4.5 g (97%) of the pure ester as an oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.27 (d, 3), 1.31 (d, 3), 1.46 (s, 9), 4.18 (m, 1), 4.48 (dd, 1), 5.07−5.2 (m, 6), 5.25 (d, 1) 5.45 (m, 1), 7.26−7.37 (m, 10); IR (DCM) 1719 cm^{-1}. Anal. Calcd for $C_{27}H_{34}N_2O_8$: C, 63.02; H, 6.66; N, 5.44; Found: C, 63.04; H, 6.68; N, 5.42.

General Procedures for the Synthesis of Crambin (16−46). All of the syntheses were carried out on a peptide synthesizer using an UV detector for monitoring the deblocking step and equipped with a 125-μm measuring loop. The peptides were assembled onto an Fmoc-Amide resin with a loading of 0.67 mmol/g via Fmoc chemistry on a 20-μmol scale with 5 equiv of the appropriate Fmoc-AA-OH and 10 equiv of DIEA using standard instrument files. The protected amino acids were dissolved in 125 μL of dry NMP, *N*-HATU was dissolved in DMF (0.38 M), and DIEA (1.6 M) was dissolved in NMP. The final concentration of the amino acid was 0.2 M. The activator and the base were delivered to the amino acid cartridge, the mixture was preactivated for 30 s, the resulting mixture was delivered to the reaction vessel, and the mixture was shaken for 30 min. The deblocking step was carried out with 30% piperidine in NMP for 2 × 3.5 min. After the final deblocking step, the resin was washed with DCM and ethyl ether and dried under vacuum and the peptide removed from the resin by treatment with TFA in the presence of Reagent R[24] (TFA-thioanisole-1,2-ethanedithiol-anisole, 90:5:3:2) for 1 h. The TFA was removed with a rotary evaporator, the peptide precipitated with cold ethyl ether and lyophilized from acetic acid/water. Syntheses of analogues of Crambine via use of depsidipeptide or pseudoproline units were carried out under the same conditions given above, introducing the building block in place of the threonine cartridge. Crude peptides were dissolved in acetonitrile/water and injected onto an HPLC column for HPLC analysis.

12a, Crambin (16−46) (standard synthesis): ESI-MS $[M + 2H]^{2+}_{calc}$ = 1571.82, found 1571.7, $[M + 3H]^{3+}_{calc}$ = 1048.21, found 1047.9 $[M + 4H]^{4+}_{calc}$ = 786.4, found 784.6. See Figure 1 in the Supporting Information (p S25).

12b, Crambin (16−46) (via depsi unit AT 27/28, after shift): ESI-MS reduced form $[M + 2H]^{2+}_{calc}$ = 1571.82, $[M + 3H]^{3+}_{calc}$ = 1048.21, oxidized form $[M + 2H]^{2+}_{calc}$ = 1569.82, $[M + 3H]^{3+}_{calc}$ = 1046.88, found $[M + 2H]^{2+}$= 1569.0, $[M + 3H]^{3+}$=1046.6. See Figure 6 in the Supporting Information(p S34).

Determination of Loss of Configuration during the Synthesis of Depsidipeptide 8 by O-Acylation of Boc-Thr-OBn with Z-Ala-OH via an NMR Technique. A small sample of the crude product **8** described in the text was removed prior to purification by column chromatography, dissolved in $CDCl_3$, and the ^{1}H NMR spectrum recorded at 400 Hz (462 scans). Examination of the spectrum at δ 1.19 showed no significant amount of the LD-form to be present (see pp S20−21 (Supporting Information) for the ^{1}H NMR spectral data).

Determination of Loss of Configuration during the Synthesis of Depsidipeptide 8 via an HPLC-Based Derivatization Technique. A. Removal of Boc Protection To Give H-Thr(Z-Ala)-OBn. A 0.5 g (0.971 mmol) sample of crude **8** was dissolved in 10 mL of DCM/TFA (1:1, v/v), and the mixture was allowed to stand at room temperature for 2 h. The solvent was removed by means of a rotary evaporator. Traces of TFA were removed by repetitive treatment with DCM followed by evaporation of the solvent. Finally, the oil was dried under high vacuum and the free amino depsidipeptide used without further purification.

B. Derivatization of H-Thr(Z-Ala)-OBn. To an ice-cold solution of 120 mg (0.24 mmol) of H-Thr(Z-Ala)-OBn, obtained as described in A above, 120 mg (0.3 mmol) of the Yamada derivatization reagent Z-Val-Aib-Gly-OH,[25] and 0.156 mL (0.89 mmol) of DIEA in 1 mL of DMF was added in one portion 114 mg (0.3 mmole) of *N*-HATU and the mixture stirred at room temperature for 2 h. From the reaction mixture a small sample (0.1 mL) was withdrawn and diluted with acetonitrile to 2 mL, and from this solution 20 μL was injected onto an HPLC column. The same procedure was applied for the preparation of the LL(D)-isomer from H-Thr(Z-D-Ala)-OBn in order to determine the appropriate retention times of the two depsipentapeptides. Examination of the HPLC trace derived from crude **8** showed that no more than 1% of the LL(D)-isomer could have been present. The identity of the LL(L)- and LL-(D)-isomers was confirmed by collection of the appropriate HPLC fractions and subjecting each isomer to MALDI-MS analysis: LL-(L)- calcd $[M + Na]^+$ 812.348, found 812.320; LL(D)- calcd $[M + Na]^+$ 812.348, found 811.969. See pp S38−S40 in the Supporting Information. The synthesis of the authentic LL(D)--form was carried out using a sample of **8a** following the derivatization technique given for **8**.

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Supporting Information Available: Description of general experimental techniques. Detailed procedure of synthesis of compounds **2**, **2a**,**b**, **3**, **3a**,**d**,**e**, **4**, **5b**, **6**, **8a**, **10**, **11**, and **9**. Spectral data for all new compounds. Monitoring traces and HPLC data for automated Crambin (16−46) syntheses. Detailed evidence demonstrating the lack of loss of configuration during the synthesis of the Thr/Ala depsidipeptide. This material is available free of charge via the Internet at http://pubs.acs.org.

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