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FULL PAPER

Development of a general Fmoc-based solid phase methodology for the synthesis of complex depsipeptides by circumventing problematic Fmoc removal

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Abstract: Development of an Fmoc-based solid phase depsipeptide methodology has been hampered by base-promoted fragmentation and diketoperazine formation upon Fmoc group elimination. Such an strategy would be a useful tool given the number of commercially available Fmoc-protected residues. Herein we report that the addition of small percentages of organic acids to the Fmoc-removal cocktail proves effective to circumvent these drawbacks and most importantly, allowed the development of an exclusively solid phase stepwise methodology to prepare a highly complex depsipeptide with multiple and consecutive esters bonds. Alongside, the optimal protecting group scheme for residue incorporation, which is not as straightforward as it is for traditional peptide synthesis, was explored. The developed stepwise strategy proved effective for the synthesis of a highly complex cyclodepsipeptide, being comparable to the yields obtained when using traditional combined chemistry approaches.

Introduction

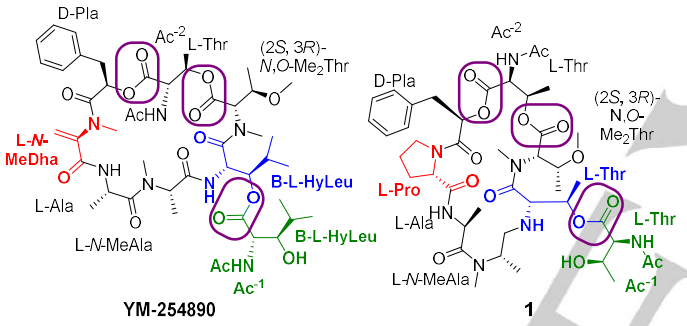
Depsipeptides are biomolecules commonly found in nature that are characterized by the presence of at least one ester bond within the peptide backbone.[1-10] Despite growing interest in the exploitation of naturally-occurring depsipeptides as therapeutic agents,[6,10–15] the difficulties encountered during the isolation and purification of large quantities from natural sources, as well as their challenging chemical synthesis, have hampered their growth in the drug market. Up to date, the most general and effective strategy for the preparation of complex depsipeptides combines solid phase synthesis with solution chemistry approaches, where building blocks containing the ester moieties are prepared in solution and later incorporated onto the polymeric support.[16-19] In these hybrid strategies the chemical and chiral purities of the different intermediates can be rigorously controlled, and crude purities tend to be higher when compared to fully stepwise strategies, usually leading to higher post-purification yields. This is especially true for high molecular weight peptides. For this reason, combined strategies are regarded as the best alternative for the large scale synthesis of peptide-based Active Principle Ingredients (APIs) in the pharmaceutical industry, where the economic viability of the products is paramount.[20] Several peptide APIs are being produced using hybrid strategies after observing the benefits in the overall yield obtained after purification.[21-22]

However these strategies also present some disadvantages. For instance, the synthetic route must be designed and optimized for each particular case, and therefore a general synthetic method cannot be outlined. This can be a limitation in screening processes, where only small amounts of products are required and fast-delivery of multiple analogues is more important than high yields. Furthermore, the synthesis of rather short peptides and depsipeptides (less than 20 amino acids) might not benefit from the advantages of hybrid strategies, since the drop in yields is still more than acceptable even for typical low purity ranges.[20]

An exclusive solid phase strategy for the preparation of complex depsipeptides would become a valuable tool to rapidly generate numerous synthetic analogues for structure-activity relationship studies (SARs), screening drug candidates and in general working in projects where time limits the possibility of process optimization. Among the advantages of solid phase synthesis are included the convenient elimination of excess reagents after each step by simple washing and filtration processes, and stepwise incorporation of orthogonally protected amino and hydroxy acid monomer units. In this context, it would be extremely useful the use of commercially available derivatives to avoid building block preparation in solution. However, ester bond instability upon removal of certain protecting groups has been reported. Whereas the strong acidic conditions required to eliminate the *tert*-Butyloxycarbonyl (Boc) group might lead to fragmentation,[23] the basic conditions needed to remove the Fluorenylmethyloxycarbonyl (Fmoc) group can trigger the following side-reactions: (i) racemization; (ii) depsipeptide fragmentation; and (iii) formation of undesired α,β-elimination side-products. Concomitantly, diketopiperazine (DKP) formation, which ultimately leads to lower yields due to dipeptide loss, is a prevalent drawback encountered during Fmoc removal of the second residue.[24-29] By contrast, Allyloxy carbonyl (Alloc) removal under neutral conditions is likely to prevent fragmentation. Unfortunately, there are not many commercially available Alloc-derivatives. Given the number of Fmoc-protected residues compared to Boc-derivatives commercially available in the market, we put great efforts into developing a convenient Fmoc-based

stepwise strategy for the synthesis of complex depsipeptides as well as circumventing the problems associated with the use of piperidine when using an Fmoc/*t*Bu strategy. With that purpose, we used a close analogue of naturally-occurring cyclodepsipeptide YM-254890 (**1**, Figure 1) as a model peptide to address our research.[30] YM-254890 is a synthetically challenging depsipeptide that presents a head-to-side-chain cyclic arrangement, an overall of three ester bonds (two of them being consecutive) and a highly *N*-methylated structure. In 2005, 228 applicants participated in a worldwide contest where a $100.000 reward was offered for the preparation of at least 1 mg of this compound, however, none of the candidates succeeded in this task.[31] It was not until 2016 that the total synthesis of YM-254890 was achieved for the first time.[29] A combined solid phase and solution chemistry approach was applied to prepare the so-called molecule. Development of an exclusive Fmoc/*t*Bu strategy was hampered by the presence of the two consecutive ester bonds and undesired α,β-elimination side reactions arising from Fmoc removal of the *N,O*-Me$_2$Thr-OH residue. In fact, another group reported complete α,β-elimination at the same point of the synthesis when attempting the synthesis.[28]

Figure 1. Structure of our proposed model cyclodepsipeptide (**1**) and naturally-occurring depsipeptide YM-254890.



The work presented herein describes the development of a fully Fmoc-based solid phase methodology for the preparation of complex depsipeptides. The synthetic complexity conferred by the presence of two consecutive ester bonds and the overall chemical diversity provided by depsipeptide **1**, allowed Fmoc removal studies after ester bond formation in diverse chemical environments as well as evaluation of the optimal protecting group scheme for each residue incorporation *via* ester bond formation. Additionally, Fmoc elimination conditions to fully prevent or minimize DKP formation were evaluated. Note that the proposed sequence modifications (**1**) preserve the synthetic challenges whilst making the methodological studies more viable, since (i) higher coupling rates were expected due to the less hindered environment observed when replacing β–hydroxyleucine (β-HyLeu) residues by Thr, and (ii) the constrained dehydroalanine moiety, which is prone to undergo Michael addition secondary reactions and therefore must be selectively protected, was substituted by a likewise residue, Pro.

Results and Discussion

The starting point of the synthesis was incorporation of *N*-MeAla-OH onto the 2-Chlorotrityl chloride (2-CTC) resin, which renders the *C*-terminus as a free carboxylic acid function and allows cyclization in solution at the latest stage of the synthesis (Scheme 1). The peptide chain elongation consisted of coupling and deprotection repetitive cycles following the well-known Fmoc/*t*Bu strategy. Couplings were carried out using as coupling system Fmoc-protected amino acid (Fmoc-AA),*N,N'*-Diisopropylcarbodiimide (DIC) and Ethyl(hydroxyimino)cyanoacetate (usually known as OxymaPure), in which the residue was incorporated upon mild and neutral conditions, hence preventing epimerization. Strong coupling conditions were required for residue incorporation onto secondary amines, which present lower reactivity compared to primary amines. Couplings onto secondary amines such as Pro or *N*-methyl amino acids were performed using as coupling system the Fmoc-AA, 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), 1-Hydroxy-7-azabenzotriazole (HOAt) and *N,N*-diisopropylethylamine (DIEA).

Fmoc removal studies to minimize DKP formation

A good approach to determine the extent of this secondary reaction is to estimate the resin loading after the third residue incorporation and compare it to the substitution level after the first amino acid incorporation. A decrease in this value implies dipeptide loss due to DKP formation. The tested conditions for Fmoc elimination from **2** are summarized in Table 1. In all cases, a quick treatment (2 x 1 min) shorter than the conventional procedure was performed.

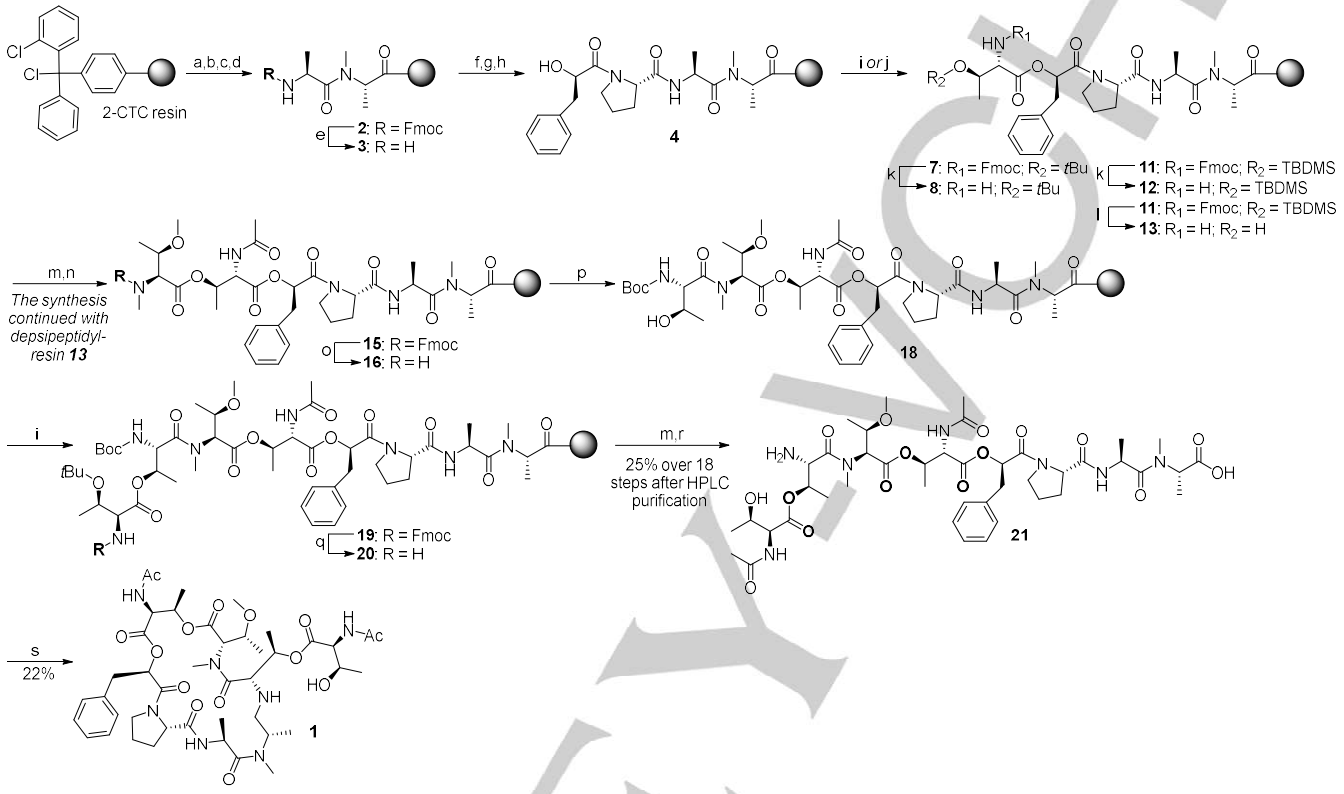
Table 1. Study of the optimal Fmoc removal conditions to minimize DKP formation.

#	Fmoc removal conditions	% DKP formation[a]
1	Piperidine–DMF (1:4 v/v) (2 × 1 min)	18
2	0.1 M DBU in DMF (2 × 1 min)	11
3	0.1 M HOBt in piperidine–DMF (1:4 v/v) (2 × 1 min)	15
4	0.1 M OxymaPure in piperidine–DMF (1:4 v/v) (2 × 1 min)	23
5	**0.1 M HOBt, 0.13 M DBU in DMF (2 × 1 min)**	**5**
6	0.1 M OxymaPure, 0.13 M in DMF (2 × 1 min)	15

[a]The DKP formation percentage was determined by the difference between the loading level after the first and third residue incorporation. The loading level was determined by Fmoc-UV quantification at 290 nm.

An improvement in the Fmoc removal outcome was observed when replacing the traditional piperidine system by the 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) cocktail (entries #1 and #2, Table 1). Addition of small percentages of slightly acidic molecules such as Hydroxybenzotriazole (HOBt) or OxymaPure to the Fmoc removal cocktail has proven effective to minimize aspartimide formation during Fmoc removal.[32] Inspired by this principle, we assessed whether addition of HOBt and OxymaPure could further reduce DKP formation. Surprisingly, OxymaPure increased the secondary reaction when added to either cocktail

(entries #4 and #6, Table 1). Contrary, HOBt slightly reduced DKP formation when added to the traditional piperidine mixture and halved undesired dipeptide loss when added to the DBU solution (entries #3 and #5, Table 1). Remarkably, Fmoc removal with a 0.1 M HOBt, 0.13 M DBU in dimethylformamide (DMF, 2 x 1 min) solution seemed to be the most effective treatment, resulting in a small DKP formation compared to the DKP formed when using the traditional piperidine–DMF (1:4 v/v) or the alternative DBU–DMF (2:98 v/v) treatments. However, these optimized conditions (0.1 M HOBt, 0.13 M DBU in DMF (2 x 1 min)) become a good alternative to considerably reduce the undesired side-reaction.



Scheme 1. Reaction conditions: a) Fmoc-*N*MeAla-OH, DIEA, DCM, 50 min; b) MeOH, 10 min; c) piperidine–DMF (1:4 v/v) (1 × 1 min + 2 × 10 min); d) Fmoc-Ala-OH, HATU, HOAt, DIEA, DMF, 1 h; e) 0.1 M HOBt, 0.13 M DBU in DMF (2 × 1 min); f) Fmoc-Pro-OH, OxymaPure, DIC, DMF, 40 min; g) piperidine–DMF (1:4 v/v) (1 × 1 min + 2 × 5 min); h) D-Pla, HATU, HOAt, DIEA, DMF, 1 h; i) Fmoc-Thr(*t*Bu)-OH, DIC, DMAP, DCM–DMF (9:1 v/v), 35 °C, 2 h 30 min; j) Fmoc-Thr(TBDMS)-OH (**9**, see SI for details), DIC, DMAP, DCM–DMF (9:1 v/v), 35 °C, 2 h 30 min; k) 0.1 M OxymaPure, 0.13 M DMF in DMF (1 × 1 min); l) 1.0 M TBAF in THF, 10 min, N_2 atm., this step was repeated twice; m) AcOH, OxymaPure, DIC, DCM, 40 min; n) Fmoc-*N,O*-Me_2Thr-OH (**14**, see SI for details), DIC, DMAP, DCM–DMF (9:1 v/v), 35 °C, 2 h 30 min; o) DBU–DMF (2:98 v/v) (1 × 1 min); p) Boc-Thr-OH, HATU, HOAt, DIEA, rt, 1 h, this step was repeated twice; q) 0.1 M HOBt, 0.13 M DBU in DMF (2:98 v/v) solution (1 × 1 min); r) TFA–TIS–DCM (90:5:5 v/v), 25 °C, 30 min, anhydrous conditions; s) HATU, 2,4,6-collidine, DMF, 2 h.

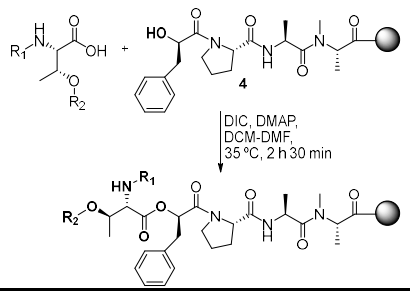
Selection of the protecting group scheme for the formation of the first ester linkage and subsequent Fmoc removal studies

Ester bond formation between the α-hydroxyl group of D-phenyllactic acid (Pla, **4**) and the carboxylic acid of the subsequent residue, Ac-Thr(OH)-OH, requires protection of the α-amino and

β-hydroxyl moiety of the Thr derivative. Selection of the alcohol protecting group entails significant importance, since the Barlos resin does not allow deprotection under acidic conditions. Moreover, the formed ester linkage must be stable to hydroxyl deprotecting conditions. On the basis of the above considerations, incorporation of the Thr derivative as Ac-Thr(Silyl)-OH turns into the smartest choice. Note that silyl protecting groups are orthogonal to the Fmoc/*t*Bu strategy and can be removed by treatment with tetrabutylammonium fluoride (TBAF).[33] However, Kaur *et al*. reported the low reactivity of the Ac-Thr(Silyl)-OH residue upon esterification conditions. It was suggested that the acetyl moiety might deactivate the carbonyl function resulting in no residue incorporation.[28] In order to develop a fully stepwise strategy, we explored this residue incorporation by evaluating the effect of the protecting group scheme for both the amine and the hydroxyl functions on the esterification extent (see Table 2). As a first approach to considerably simplify the screening process, *t*Bu was selected as the hydroxyl protecting group (note that Fmoc-Thr(*t*Bu)-OH is readily used in peptide synthesis and can be purchased at low prices) and the α-amino function was protected with two different orthogonal protecting groups (Fmoc and Ac). Unfortunately, ester bond formation with the conveniently acetylated Thr derivative (Ac-Thr(*t*Bu)-OH, **5**) was not accomplished (entry #1, Table 2), complying with previous reported experiments.[28] Therefore, the amine function of the Thr derivative must be acetylated after residue incorporation. Surprisingly, the esterification product, **7**, with commercially available Fmoc-Thr(*t*Bu)-OH (**6**) was obtained with a quantitative conversion according to High Performance Liquid Chromatography – Mass Spectrometry (HPLC-MS) analysis (entry #2, Table 2), confirming that the amine protecting group plays an important role in the reaction outcome. With this promising result in hands, optimal Fmoc removal conditions of the model depsipeptide (**7**) to afford **8** were extensively studied and

are summarized in Table 3. In all cases, a quick treatment of 1 min was performed and the deprotection efficiency was evaluated by HPLC-MS analysis. Treatment with the traditional piperidine cocktail resulted in high epimerization rates (54%, entry #1, Table 3). Unfavorable elimination of the Fmoc group was also observed with the milder DBU cocktail. At this stage, we evaluated whether addition of HOBt and OxymaPure to the Fmoc removal cocktail could reduce racemization of **8**. Whilst HOBt had a negative effect when used as additive, incorporation of OxymaPure to the DBU cocktail proved effective to fully prevent epimerization and afford the unprotected model depsipeptidyl-resin **8** (entry #5, Table 3).

Table 2. Study of the optimal protecting group scheme of the Thr derivative for the formation of the first ester linkage.



#	Amino acid	HPLC conversion (%)[a]
1	Ac-Thr(*t*Bu)-OH (**5**)	0
2	Fmoc-Thr(*t*Bu)-OH (**6**)	100
3	Fmoc-Thr(TBDPS)-OH (**9**)	0
4	Fmoc-Thr(TBDMS)-OH (**10**)	100

[a] Reaction monitoring was carried out by HPLC-MS analysis. HPLC data processed at 220 nm. See all the chromatograms in the SI.

Once Fmoc was selected as the optimal protecting group for the amine moiety, assessment of the most suitable protecting group for the β-hydroxyl function was carried out with *tert*-butyldimethylsilyl (TBDMS) and *tert*-butyldiphenylsilyl (TBDPS, which allowed evaluation of the bulkiness effect on the esterification rates (Table 2). Whereas incorporation of bulkier Fmoc-Thr(TBDPS)-OH (**9**) resulted in failed attempts to introduce the Thr derivative (entry #3, Table2), incorporation of Fmoc-Thr(TBDMS)-OH (**10**) was successfully achieved with a quantitative incorporation yield (entry #4, Table2). Although incorporation of Fmoc-Thr(TBDMS)-OH (**10**) was a good starting point, further studies on TBDMS and Fmoc removal of **11** needed to be carried out. Initial attempts consisted of Fmoc elimination with the already optimized conditions (treatment with a 0.1 M OxymaPure, 0.13 M DBU in DMF solution for 1 min), which fully prevented epimerization, and subsequent acetylation with the AcOH–OxymaPure–DIC system. Next, stability of the ester linkage upon treatment with a 1.0 M TBAF solution in tetrahydrofuran (THF) under anhydrous conditions was studied. Unfortunately, treatment of the peptidyl-resin **12** with TBAF failed to afford the desired product, and depsipeptide fragmentation was observed instead yielding **4** with a quantitative HPLC conversion. In order to avoid depsipeptide fragmentation, simultaneous Fmoc and TBDMS removal was explored by treating depsipeptidyl-resin **11** with a 1.0 M TBAF solution in THF. This strategy led to partial elimination of the Fmoc and TBDMS protecting groups. An additional treatment and subsequent acetylation resulted in the formation of the desired product **13** with quantitative yields and no evidence of fragmentation according to HPLC analysis.

Table 3. Study of the optimal Fmoc removal conditions to deprotect depsipeptidyl-resin **7** to afford **8**.

#	Fmoc removal conditions	**8:7** ratio	Epimerization of **5**[a]
1	Piperidine–DMF (1:4 v/v) (2 × 1 min)	95:5	54%
2	0.1 M HOBt in Piperidine–DMF (1:4 v/v) (1 × 1 min)	95:5	60%
3	DBU–DMF (2:98 v/v) (2 x 1 min)	100:0	Broad peak
4	0.1 M HOBt, 0.13 M DBU in DMF (2 × 1 min)	100:0	56%
5	**0.1 M OxymaPure, 0.13 M DBU in DMF (2 × 1 min)**	**100:0**	**0%**

[a] Epimerization yields were determined by HPLC-MS analysis. HPLC data processed at 220 nm. See all the chromatograms in the SI.

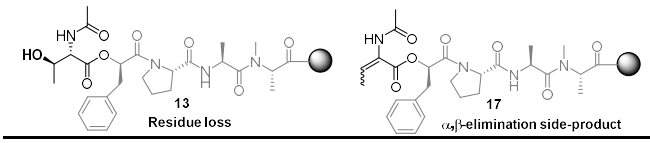
Formation of the second ester linkage and subsequent Fmoc removal studies

With pentadepsipeptidyl-resin **13** in hand, esterification of the Fmoc-*N,O*-Me$_2$Thr-OH (**14**, see SI for details) residue was carried out, and the incorporation yield was quantitative according to HPLC-MS analysis. However, 22% of epimerization was observed, probably due to the presence of an *N*-alkylated residue, which is prone to undergo epimerization at the α-carbon.[34] Further efforts to minimize epimerization resulted in failure. It has been reported that Fmoc removal after incorporation of the Fmoc-*N,O*-Me$_2$Thr-OH residue (for the synthesis of the natural analogue, YM-254890) completely failed to afford the desired product, with the α,β-elimination side-product formed instead.[28,29] We hypothesized that the presence of two consecutive ester bonds might enhance the α,β-elimination side-reaction. In order to establish the best conditions for this particular deprotection step, the previously tested Fmoc removal conditions were applied to the present system. In all cases, a quick treatment with the corresponding deprotection cocktail was performed. The HPLC yields as well as the structures of the desired product (**16**) and the two main side-products corresponding to the *N,O*-Me$_2$Thr-OH residue loss (**13**) and the α,β-elimination product (**17**) are shown in Table 4. As expected, Fmoc removal with the traditional piperidine–cocktail mainly led to the formation of the α,β-elimination product (**17**), but also to *N,O*-Me$_2$Thr-OH residue loss (**13**) and only 37 % of the desired product **16** (entry #1, Table 4). Remarkably, the best conditions were obtained with the DBU–DMF (2:98 v/v) system, where the desired product (**16**) was obtained with a 57% HPLC yield, and we were able to significantly decrease side-products **13** and **17** formation (entry #3, Table 4). Although small percentages of HOBt and OxymaPure were added to the Fmoc removal cocktail, the deprotection outcome could not be further improved. To the best of our knowledge, a big step forward compared to the results reported in the literature (where only traces of the desired product **16** were detected and **17** was obtained as a major product) was made, since formation of the α,β-elimination side-product (**17**) was considerably minimized.

We were able to prevent complete α,β-elimination, and obtain the desired Fmoc-unprotected depsipeptide in a good enough HPLC yield (57%) to carry on with the synthesis.

Table 4. Study of the optimal Fmoc removal conditions to deprotect depsipeptidyl-resin **15**, which afforded the desired product **16** and side-products **13** and **17**.

Side products derived from the Fmoc removal step



#	Fmoc removal conditions	**15** (%)	**16** (%)	**13** (%)	**17** (%)[a]
1	Piperidine–DMF (1:4 v/v) (2 × 1 min)	0	37	15	48
2	0.1 M HOBt in Piperidine–DMF (1:4 v/v) (1 × 1 min)	32	8	11	49
3	**DBU–DMF (2:98 v/v) (2 × 1 min)**	**0**	**57**	**23**	**19**
4	0.1 M HOBt, 0.13 M DBU in DMF (2 × 1 min)	0	43	26	21
5	0.1 M OxymaPure, 0.13 M DBU in DMF (2 × 1 min)	51	22	18	9

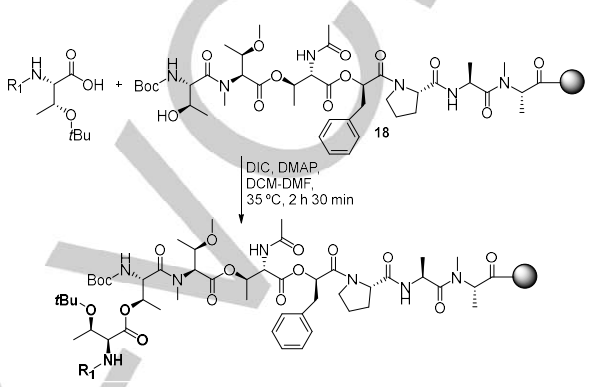
[a] Product percentages were determined by HPLC-MS analysis. HPLC data processed at 220 nm. See all the chromatograms in the SI

Selection of the optimal N$^{\alpha}$-protecting group for the formation of the third ester linkage and subsequent Fmoc removal studies

Peptide coupling of Boc-Thr-OH was followed by assembly of the Ac-Thr(*t*Bu)-OH residue *via* an ester bond formation between its carboxylic acid and the β-hydroxyl group of **18**. Two N$^{\alpha}$-protecting groups, Ac and Fmoc (see Table 5), were explored for this esterification reaction. Complying with previously obtained results for the first esterification, formation of the ester bond was only accomplished with the Fmoc derivative (**6**) (entry #2, Table 5). As a part of the Fmoc removal studies after formation of all three ester linkages, efficient Fmoc removal from **19** was extensively studied. Again, a quick treatment was performed and the deprotection efficiency was evaluated by HPLC-MS analysis. The same conditions as the ones used for the first and second ester deprotection were tested and are summarized in Table 6. The α,β-elimination side-product (**17**) was formed in high percentages (90%) when piperidine was used as base (entry #1, Table 6). Depsipeptide fragmentation was also observed when using 0.1 M HOBt in piperidine–DMF (2:98 v/v), DBU–DMF (2:98 v/v) and 0.1 M OxymaPure, 0.13 M in DMF (entries #2-4, Table 6). Optimal Fmoc removal conditions after formation of the third ester linkage were accomplished by treatment of **19** with a 0.1 M HOBt in DBU–DMF (2:98 v/v) solution, in which formation of the α,β-elimination product was fully suppressed (entry #5, Table 6). Again, we were able to improve the Fmoc removal outcome when adding small percentages of organic acids.

Lastly, acetylation of the free amine was carried out prior to depsipeptide cleavage and global deprotection with a trifluoroacetic acid (TFA) – triisopropyl silane (TIS) – dichloromethane (DCM) (90:5:5 v/v) cocktail under anhydrous conditions. The linear precursor **21** was subjected to HPLC purification and obtained with a yield of 25% (over 18 steps). Considering the synthetic complexity of the target molecule and the numerous steps required for its preparation, **21** was prepared with an outstanding overall yield. Obtention of the final cyclic analogue (**1**) was accomplished by treatment of the pure linear unprotected depsipeptide chain with the HATU–collidine coupling system.

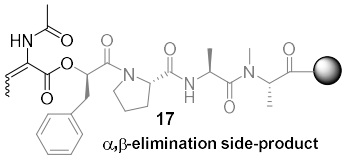
Table 5. Study of the optimal protecting group scheme of the Thr derivative for the formation of the third ester linkage.



#	Amino acid	HPLC conversion (%)[a]
1	Ac-Thr(*t*Bu)-OH (**5**)	0
2	Fmoc-Thr(*t*Bu)-OH (**6**)	100

[a] Reaction monitoring was carried out by HPLC-MS analysis. HPLC data processed at 220 nm. See all the chromatograms in the SI.

Table 6. Study of the optimal Fmoc removal conditions to deprotect depsipeptdiyl-resin **19**, which rendered the desired product **20** and side-product **17**.



Side product derived from the Fmoc removal step

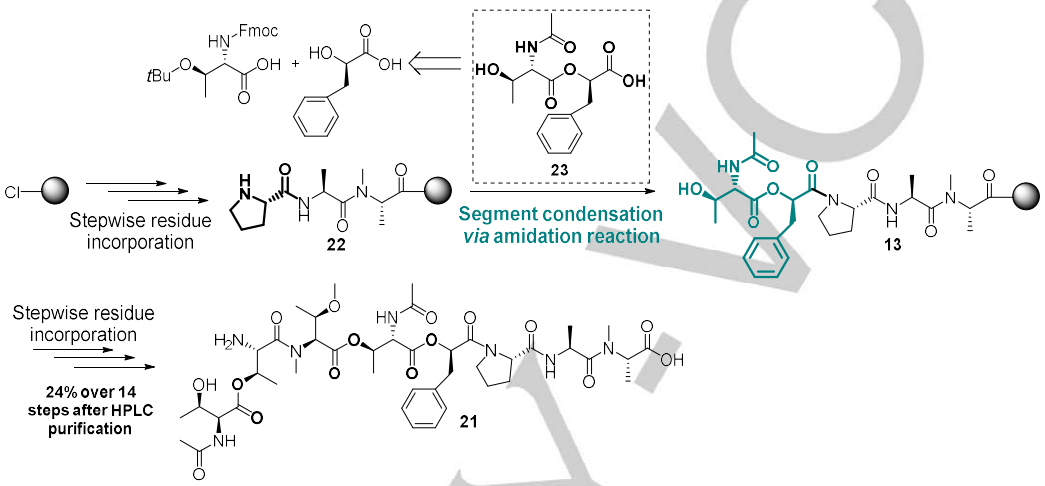
#	Fmoc removal conditions	**19** (%)	**20** (%)	**17** (%)[a]
1	Piperidine–DMF (1:4 v/v) (2 × 1 min)	0	10	90
2	0.1 M HOBt in Piperidine–DMF (1:4 v/v) (1 × 1 min)	0	12	88
3	DBU–DMF (2:98 v/v) (2 × 1 min)	15	45	22
4	0.1 M OxymaPure in DBU–DMF (2:98 v/v) (2 × 1 min)	30	26	9
5	**0.1 M HOBt, 0.13 M DBU in DMF (2 × 1 min)**	**0**	**100**	**0**

[a] Product percentages were determined by HPLC-MS analysis. HPLC data processed at 220 nm. See all the chromatograms in the SI.

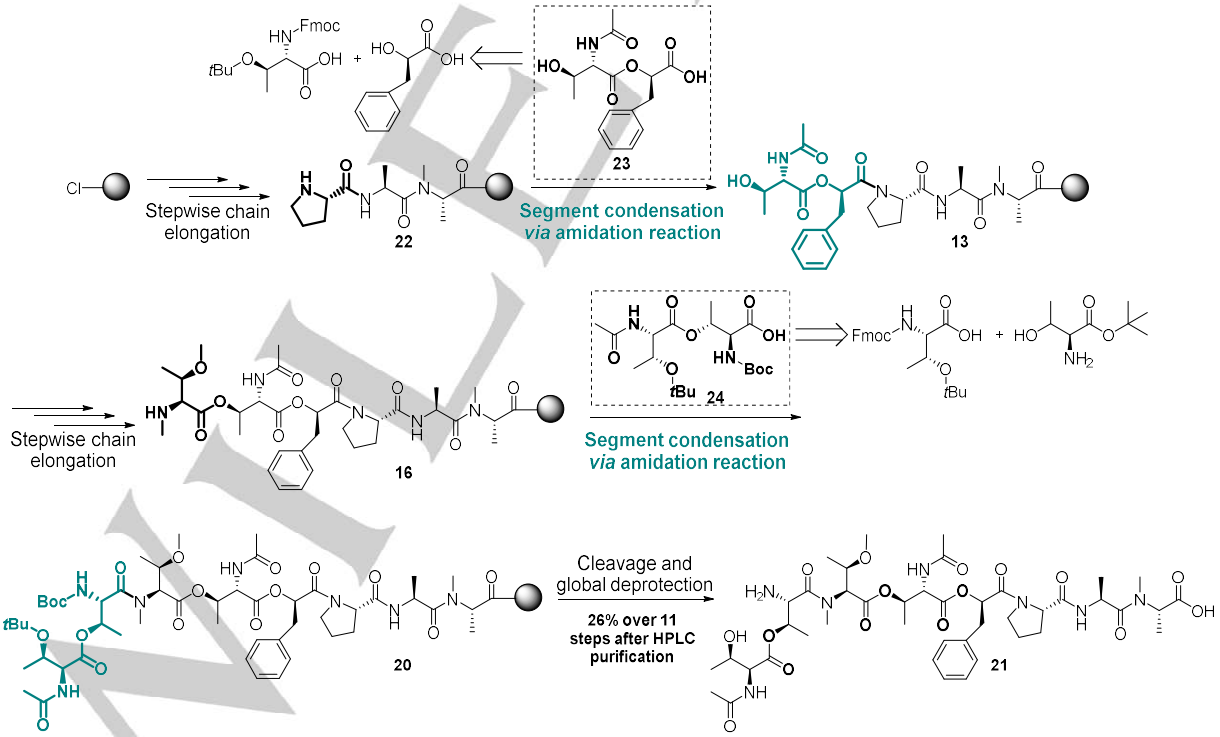
Evaluation of the stepwise strategy efficiency by comparison with traditional combined chemistry approaches

In order to evaluate whether it is more efficient to incorporate the residues in an exclusive stepwise manner or following traditional segment condensation approaches, two synthetic strategies parallel to the synthesis of natural YM-254890 were developed and optimized for the preparation of the target linear depsipeptide (**21**), including (i) a strategy containing one depsipeptide segment condensation (Scheme 2, see SI for details) and (ii) a strategy containing two depsipeptide segment condensations (Scheme 3, see SI for details). All three strategies led to comparable yields (between 24-26%) and therefore prove effective for the preparation of the target molecule. However, the fully stepwise strategy presents some advantages over segment condensation approaches.

Scheme 2. Synthetic strategy containing one-segment condensation. Yield: 24% over 14 steps.



Scheme 3. Synthetic strategy containing two-segment condensations. Yield: 26% over 11 steps.



A major advantage of this strategy is that the excesses of reagents can be washed away by simple suction, and therefore only one purification step is required throughout the synthesis, thus the synthesis is less time-consuming and laborious. Additionally, the numerous commercially available Fmoc-protected amino acid derivatives facilitate the synthetic process.

Conclusion

In conclusion, a stepwise Fmoc-based solid phase methodology for the synthesis of a highly complex depsipeptide was developed by selecting the most suitable protecting group scheme for each residue incorporation as well as circumventing problematic Fmoc group elimination of the second residue or after ester bond formation. In the latter, replacement of the traditional piperidine-based Fmoc removal cocktail by a DBU solution led in all cases to better deprotection outcomes. Noteworthy, the addition of small percentages of organic acids (HOBt or OxymaPure) to the deprotection cocktail resulted in most cases in an improvement in this step, being secondary reactions fully prevented or significantly minimized. Nevertheless, the best deprotection conditions (HOBt, OxymaPure or no additive) are highly chemical environment-dependent and therefore should be evaluated for each problematic Fmoc elimination step. This newly developed methodological study is a valuable tool for the preparation of synthetically challenging depsipeptides and the rapid generation of numerous analogues.

Experimental Section

Depsipeptide assembly on solid-phase using a fully stepwise strategy

2-CTC resin (25 mg, 1.6 mmol/g resin) was placed in a 2 mL-polypropylene syringe fitted with two polyethylene filter discs. The conditioning of the resin and incorporation of the first amino acid, Fmoc-*N*MeAla-OH (6 mg, 0.02 mmol, 1.0 eq), was carried out by standard means. The Fmoc group was removed by treatment with a piperidine–DMF solution (1:4 v/v) (1 x 1 min + 2 x 10 min). The peptidyl-resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min), and a solution of Fmoc-Ala-OH*H_2O (17 mg, 0.05 mmol, 3.0 eq), HATU (20 mg, 0.05 mmol, 3.0 eq), HOAt (7 mg, 0.05 mmol, 3.0 eq) and DIEA (18 µL, 0.11 mmol, 6.0 eq) in DMF (500 µL) was added to the peptidyl-resin and the coupling was shaken for 1 h. In order to minimize diketopiperazine formation, the Fmoc group of the second residue was eliminated by treatment with a 0.1 M HOBt in DBU–DMF (2:98 v/v) solution (2 x 1 min). A mixture of Fmoc-Pro-OH (18 mg, 0.05 mmol, 3.0 eq), OxymaPure (8 mg, 0.05 mmol, 3.0 eq) and DIC (8 µL, 0.05 mmol, 3.0 eq) in DMF (500 µL) was added to the peptide-bound and the resin was shaken for 40 min, and then the Fmoc group was subsequently removed by treatment with a piperidine–DMF (1:4 v/v) (1 x 1 min + 2 x 5 min) solution. A mixture of D-(+)-phenyllactic (9 mg, 0.05 mmol, 3.0 eq), HATU (20 mg, 0.05 mmol, 3.0 eq), HOAt (7 mg, 0.05 mmol, 3.0 eq) and DIEA (18 µL, 0.11 mmol, 6.0 eq) in DMF (500 µL) was added to the resin and the peptidyl-resin was shaken for 1 h.

Next, the peptidyl-resin was washed with dry DCM (3 x 1 min) and it was dried under *vacuum* for 20 min. The peptide-resin was transferred to a pyrex® culture tube provided with a magnetic stirrer. A solution containing Fmoc-Thr(TBDMS)-OH, (**10**, 64 mg, 0.14 mmol, 8.0 eq), DIC (22 µL, 0.14 mmol, 8.0 eq) and 4-dimethytlaminopyridine (DMAP) in dry DCM was pre–activated for 5 min before it was added to the tube. A solution of DMAP (1 mg, 0.01 mmol, 0.5 eq) in dry DMF was also added to the tube and the reaction was shaken for 2 h and 30 min at 35 °C. The reaction mixture was cooled to 25 °C, transferred to a polypropylene syringe fitted with two polyethylene discs and washed with dry DCM (3 x 1 min), DMF (3 x 1 min) and DCM (3 x 1 min). Esterification completion was monitored by HPLC-MS. Next, the TBDMS and Fmoc groups were simultaneously removed by treatment with a 1.0 M TBAF (36 µL, 0.02 mmol, 1.0 eq) (2 x 10 min) solution in THF (500 µL) under N_2 atmosphere. The resin was washed with dry THF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min), and the corresponding free amine was acetylated by treatment with a solution of AcOH (2 µL, 0.05 mmol, 3.0 eq), OxymaPure (8 mg, 0.05 mmol, 3.0 eq) and DIC (8 µL, 0.05 mmol, 3.0 eq) in DMF (500 µL) for 40 min.

The peptidyl-resin was washed with dry DCM (3 x 1 min), dried under *vacuum* for 20 min and transferred to a pyrex® culture tube provided with a magnetic stirrer. Esterification with Fmoc-*N,O*-Me_2Thr-OH (**14**, 52 mg, 0.14 mmol, 8.0 eq), DIC (22 µL, 0.14 mmol, 8.0 eq) and DMAP (1 mg, 0.01 mmol, 0.5 eq) was performed as before. Fmoc removal was achieved by treatment with a DBU–DMF (2:98 v/v) (1 x 1 min) solution. The peptidyl-resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) -and DMF (3 x 1 min).

A mixture of Boc-Thr-OH (12 mg, 0.05 mmol, 3.0 eq), HATU (20 mg, 0.05 mmol, 3.0 eq), HOAt (7 mg, 0.05 mmol, 3.0 eq) and DIEA (18 µL, 0.11 mmol, 6.0 eq) in DMF (500 µL) was added to the resin through an amidation reaction and the peptidyl-resin was shaken for 1 h. In this case, re-coupling was required to accomplish full incorporation of the Thr derivative. Incorporation of the last amino acid was achieved again *via* an esterification reaction using Fmoc-Thr(*t*Bu)-OH (56 mg, 0.14 mmol, 8.0 eq), DIC (22 µL, 0.14 mmol, 8.0 eq) and DMAP (1 mg, 0.01 mmol, 0.5 eq).

Incorporation of the last amino acid was achieved *via* a Steglich esterification reaction. Fmoc-Thr(*t*Bu)-OH (56 mg, 0.14 mmol, 8.0 eq), DIC (22 µL, 0.14 mmol, 8.0 eq) and DMAP (1 mg, 0.01 mmol, 0.5 eq) were added to the peptidyl-resin following the conditions reported above. Fmoc removal was achieved by treatment with a solution of 0.1 M HOBt in DBU–DMF (2:98 v/v) (1 x 1 min). The peptide resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min). The peptidyl-resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min), and the corresponding free amine was acetylated by treatment with a solution of AcOH (2 µL, 0.05 mmol, 3.0 eq), OxymaPure (8 mg, 0.05 mmol, 3.0 eq) and DIC (8 µL, 0.05 mmol, 3.0 eq) in DMF (500 µL) for 40 min. The peptide resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min). Treatment of the peptidyl-resin with a TFA–TIS–DCM (90:5:5 v/v) over 30 min furnished the crude linear depsipeptide. The lyophilized crude linear peptide was purified by HPLC using a XBridgeTM C18 reversed-phase column (3.5 µm x 4.6 mm x 42 mm) (purification gradient: C18 G0100t20T25) to afford pure **21** (4.0 mg, 25% over 16 steps) as white solid. XBridge™ C18 reversed-phase analytical column (3.5 µm x 4.6 mm x 42 mm) linear gradients (0% to 100%) of ACN over 9 min, with a flow rate of 1.0 mL/min, t_R = 10.07 min, purity (λ = 220 nm) = 96%.HRMS-ESI(+) characterization: *m/z* calculated for $C_{43}H_{65}N_7O_{16}$ 935.4566, found $[M + H]^+$ 936.4558

Depsipeptide assembly on solid-phase containing one depsipeptide building block segment condensation

2-CTC resin (25 mg, 1.6 mmol/g resin) was placed in a 2 mL-polypropylene syringe fitted with two polyethylene filter discs. The conditioning of the resin and incorporation of the first amino acid, Fmoc-*N*MeAla-OH (6 mg, 0.02 mmol, 1.0 eq), was carried out by standard means. The Fmoc group was removed by treatment with a piperidine–DMF solution (1:4 v/v) (1 x 1 min + 2 x 10 min). The peptidyl-resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min), and a solution of Fmoc-Ala-OH*H_2O (17 mg, 0.05 mmol, 3.0 eq), HATU (20 mg, 0.05 mmol, 3.0 eq), HOAt (7 mg, 0.05 mmol, 3.0 eq) and DIEA (18 µL, 0.11 mmol, 6.0 eq) in DMF (500 µL) was added to the peptidyl-resin and the coupling was shaken for 1 h. In order to minimize diketopiperazine formation, the Fmoc group of the second residue was eliminated by treatment with a 0.1 M HOBt in DBU–DMF (2:98 v/v) solution (2 x 1 min). A mixture of Fmoc-Pro-OH (18 mg, 0.05 mmol, 3.0 eq), OxymaPure (8 mg, 0.05 mmol, 3.0 eq) and DIC (8 µL, 0.05 mmol, 3.0 eq) in DMF (500 µL) was added to the peptide-bound and the resin was shaken for 40 min, and then the Fmoc group was subsequently removed by treatment with a piperidine–DMF (1:4 v/v) (1 x 1 min + 2 x 5 min) solution.

Segment condensation of **23** was performed as follows. A mixture of building block **23** (16 mg, 0.05 mmol, 3.0 eq), PyBOP (mg, 0.05 mmol, 3.0 eq), HOAt (7 mg, 0.05 mmol, 3.0 eq) and DIEA (18 µL, 0.11 mmol, 6.0 eq) in DMF (500 µL) was added to the resin and shaken for 24 h. The resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min). Next, the peptidyl-resin was washed with dry DCM (3 x 1 min) and it was dried under *vacuum* for 20 min. The peptide-resin was transferred to a pyrex® culture tube provided with a magnetic stirrer. A solution containing Fmoc-*N,O*-Me_2Thr-OH (**14**, 52 mg, 0.14 mmol, 8.0 eq), DIC (22 µL, 0.14 mmol, 8.0 eq) and DMAP in dry DCM was pre–activated for 5 min before

it was added to the tube. A solution of DMAP (1 mg, 0.01 mmol, 0.5 eq) in dry DMF was also added to the tube and the reaction was shaken for 2 h and 30 min at 35 °C. The reaction mixture was cooled to 25 °C, transferred to a polypropylene syringe fitted with two polyethylene discs and washed with dry DCM (3 x 1 min), DMF (3 x 1 min) and DCM (3 x 1 min). Esterification completion was monitored by HPLC-MS. Fmoc removal was achieved by treatment with a DBU–DMF (2:98 v/v) (1 x 1 min) solution. The peptide resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min).

A mixture of Boc-Thr-OH (12 mg, 0.05 mmol, 3.0 eq), HATU (20 mg, 0.05 mmol, 3.0 eq), HOAt (7 mg, 0.05 mmol, 3.0 eq) and DIEA (18 µL, 0.11 mmol, 6.0 eq) in DMF (500 µL) was added to the resin through an amidation reaction and the peptidyl-resin was shaken for 1 h. In this case, re-coupling was required to accomplish full incorporation of the Thr derivative. Incorporation of the last amino acid was achieved again via a esterification reaction using Fmoc-Thr(*t*Bu)-OH (56 mg, 0.14 mmol, 8.0 eq), DIC (22 µL, 0.14 mmol, 8.0 eq) and DMAP (1 mg, 0.01 mmol, 0.5 eq).

Incorporation of the last amino acid was achieved via a Steglich esterification reaction. Fmoc-Thr(*t*Bu)-OH (56 mg, 0.14 mmol, 8.0 eq), DIC (22 µL, 0.14 mmol, 8.0 eq) and DMAP (1 mg, 0.01 mmol, 0.5 eq) were added to the peptidyl-resin following the conditions reported above. Fmoc removal was achieved by treatment with a solution of 0.1 M HOBt in DBU–DMF (2:98 v/v) (1 x 1 min). The peptide resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min). The peptidyl-resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min), and the corresponding free amine was acetylated by treatment with a solution of AcOH (2 µL, 0.05 mmol, 3.0 eq), OxymaPure (8 mg, 0.05 mmol, 3.0 eq) and DIC (8 µL, 0.05 mmol, 3.0 eq) in DMF (500 µL) for 40 min. The peptide resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min). Treatment of the peptidyl-resin with a TFA–TIS–DCM (90:5:5 v/v) over 30 min furnished the crude linear depsipeptide. The lyophilized crude linear peptide was purified by HPLC using a XBridgeTM C18 reversed-phase column (3.5 □m x 4.6 mm x 42 mm) (purification gradient: C18 G0100t20T25) to afford pure **21** (3.9 mg, 24% over 14 steps) as white solid.

S3.2. Depsipeptide assembly on solid-phase using a synthetic strategy containing two depsipeptide building block segment condensations

2-CTC resin (25 mg, 1.6 mmol/g resin) was placed in a 2 mL-polypropylene syringe fitted with two polyethylene filter discs. The conditioning of the resin and incorporation of the first amino acid, Fmoc-*N*MeAla-OH (6 mg, 0.02 mmol, 1.0 eq), was carried out by standard means. The Fmoc group was removed by treatment with a piperidine–DMF solution (1:4 v/v) (1 x 1 min + 2 x 10 min). The peptidyl-resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min), and a solution of Fmoc-Ala-OH*H_2O (17 mg, 0.05 mmol, 3.0 eq), HATU (20 mg, 0.05 mmol, 3.0 eq), HOAt (7 mg, 0.05 mmol, 3.0 eq) and DIEA (18 µL, 0.11 mmol, 6.0 eq) in DMF (500 µL) was added to the peptidyl-resin and the coupling was shaken for 1 h. In order to minimize diketopiperazine formation, the Fmoc group of the second residue was eliminated by treatment with a 0.1 M HOBt in DBU–DMF (2:98 v/v) solution (2 x 1 min). A mixture of Fmoc-Pro-OH (18 mg, 0.05 mmol, 3.0 eq), OxymaPure (8 mg, 0.05 mmol, 3.0 eq) and DIC (8 µL, 0.05 mmol, 3.0 eq) in DMF (500 µL) was added to the peptide-bound and the resin was shaken for 40 min, and then the Fmoc group was subsequently removed by treatment with a piperidine–DMF (1:4 v/v) (1 x 1 min + 2 x 5 min) solution.

Segment condensation of **23** was performed as follows. A mixture of building block **23** (16 mg, 0.05 mmol, 3.0 eq), PyBOP (mg, 0.05 mmol, 3.0 eq), HOAt (7 mg, 0.05 mmol, 3.0 eq) and DIEA (18 µL, 0.11 mmol, 6.0 eq) in DMF (500 µL) was added to the resin and shaken for 24 h. The resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min). Next, the peptidyl-resin was washed with dry DCM (3 x 1 min) and it was dried under *vacuum* for 20 min. The peptide-resin was transferred to a pyrex® culture tube provided with a magnetic stirrer. A solution containing Fmoc-*N,O*-Me_2Thr-OH (**14**, 52 mg, 0.14 mmol, 8.0 eq), DIC (22 µL, 0.14 mmol, 8.0 eq) and DMAP in dry DCM was pre–activated for 5 min before it was added to the tube. A solution of DMAP (1 mg, 0.01 mmol, 0.5 eq) in dry DMF was also added to the tube and the reaction was shaken for 2 h and 30 min at 35 °C. The reaction mixture was cooled to 25 °C, transferred to a polypropylene syringe fitted with two polyethylene discs and washed with dry DCM (3 x 1 min), DMF (3 x 1 min) and DCM (3 x 1 min). Esterification completion was monitored by HPLC-MS. Fmoc removal was achieved by treatment with a DBU–DMF (2:98 v/v) (1 x 1 min) solution. The peptide resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min).

Segment condensation of **24** was performed as follows. A mixture of building block **24** (21 mg, 0.05 mmol, 3.0 eq), HATU (19 mg, 0.05 mmol, 3.0 eq) and 2,4,6-collidine (14 µL, 0.11 mmol, 6.0 eq) in DMF (500 µL) was added to the resin and shaken for 2 h. The resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min).

The peptide resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min). Treatment of the peptidyl-resin with a TFA–TIS–DCM (90:5:5 v/v) over 30 min furnished the crude linear depsipeptide. The lyophilized crude linear peptide was purified by HPLC using a XBridgeTM Peptide BEH C18 Prep reversed-phase 130Å column (5 □m x 10 mm x 100 mm) (purification gradient: C18G0100t20T25) to afford pure **21** (4.3 mg, 26% over 11 steps) as white solid.

Cyclization of 21 to afford 1:

21 (2.9 mg, $3.10 \cdot 10^{-3}$ mmol, 1.0 eq) was dissolved in DMF (3.10 mL) and 2,4,6-collidine (1.2 µL, $9.30 \cdot 10^{-3}$ mmol, 3.0 eq) and HATU (1.2 mg, $3.10 \cdot 10^{-3}$ mmol, 1.0 eq) were added. The reaction mixture was let to stir for 2 h until full consumption of the starting material was observed by HPLC analysis. The solvent was removed under reduced pressure, and the crude cyclic peptide was purified by HPLC using a XBridgeTM Peptide BEH C18 Prep reversed-phase 130Å column (5 µm x 10 mm x 100 mm) (purification gradient: C18 G0100t20T25) to afford pure **1** (1 mg, 22%) as white solid**.** XBridge™ C18 reversed-phase analytical column (3.5 µm x 4.6 mm x 42 mm) linear gradients (0% to 100%) of ACN over 9 min, with a flow rate of 1.0 mL/min, t_R = 11.42 min, purity (λ = 220 nm) = 98%. HRMS-ESI(+) characterization: calculated for $C_{43}H_{63}N_7NaO_{15}$.917.4382, found $[M + Na]^+$ 940.4265

Fmoc removal studies to minimize DKP formation

Determination of the DKP formation percentage:

DKP formation was assessed by comparison of the loading level after the first and the third amino acid incorporation. The following formula was used to calculate the DKP formation percentage for all six tested conditions.

$$DKP\ formation = \frac{Loading\ first\ amino\ acid - Loading\ third\ amino\ acid}{Loading\ first\ amino\ acid} \cdot 100$$

Where: Loading first amino acid (loading of the resin after the first amino acid incorporation (mmol/g resin)); Loading third amino acid (loading of the resin after the third amino acid incorporation (mmol/g)).

General protocol to evaluate DKP formation:

The resin conditioning and incorporation of the first amino acid, Fmoc-*N*MeAla-OH, were accomplished as described previously. The real loading after the first residue incorporation was determined by Fmoc UV quantification of the dibenzofulvene-piperidine adduct formed during Fmoc removal at λ = 290 nm. Next, a solution of Fmoc-Ala-OH*H_2O (3.0 eq), HATU (3.0 eq), HOAt (3.0 eq) and DIEA (6.0 eq) in DMF was added to the peptidyl-resin and the coupling was shaken for 1 h. Fmoc removal was accomplished with a short treatment of the peptidyl-resin with the corresponding deprotection cocktail (2 x 1 min) (see all six tested Fmoc removal conditions in Table S1). The resin was washed with DMF (3 x 1 min), DCM (3 x 1 min) and DMF (3 x 1 min). Quickly after, the third residue, Fmoc-Pro-OH (3.0 eq), was assembled with OxymaPure (3.0 eq) and DIC (3.0 eq) in DMF for 40 min. A Kaiser test was run to ensure full residue assembly. Finally, the real loading at this stage was determined by Fmoc UV quantification of the dibenzofulvene-piperidine adduct formed during Fmoc removal at λ = 290 nm.

Acknowledgements

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Keywords: Depsipeptides • Fmoc-based stepwise synthesis • natural products • peptides • solid phase synthesis •

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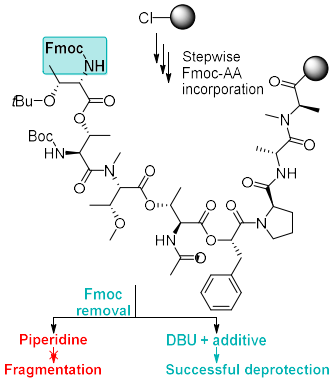
FULL PAPER

Entry for the Table of Contents

Key topic: Depsipeptides, Fully Stepwise Synthesis



Given the number of commercially available Fmoc-protected residues, an Fmoc-based solid phase depsipeptide methodology would be extremely useful. This is often hampered by side-reactions arising from the Fmoc removal step. Herein we explored the best conditions to circumvent these drawbacks, and applied these findings to the preparation of a highly complex linear depsipeptide exclusively on solid phase, opening the door to a general fully Fmoc-based strategy for complex depsipeptides