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Protection by Conformationally Restricted Mobility: First Solid-Phase Synthesis of Triostin A

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Triostin A (Figure 1) belongs to a family of peptide antibiotics produced by marine bacteria. Triostins (A, B, and C) were isolated in 1961 from a strain of *Streptomyces aureus*.[1] Differing only in one of the *N*Me residues, triostin A bears an *N*Me-Val, whereas triostin B and C contain an *N*Me-Ile and an *N*,γ-dimethyl-*allo*-Ile, respectively.[2] Triostin A, as well as other members of this family, which include echinomycin,[3] BE-22179,[4] and thiocoraline[5,6] show antibacterial and cytotoxic activity.[7]

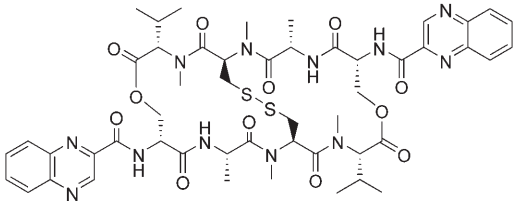

Figure 1. Structure of triostin A.

Triostin A binds to DNA by bisintercalation[8] through its two quinoxaline units and shows CpG selectivity.[9] The removal of the four *N*-methyl groups in the TANDEM analogue results in a change of selectivity to TpA sequences.[10] Later studies have shown that binding to specific bases is not only driven by hydrogen bonding, but that these compounds also search for the most favourable stacking interactions.[11]

The difficulty to synthesize triostin A is exemplified by the fact that since its discovery more than 30 years ago, only two elegant, but tedious, solution chemistry syntheses, which involved purification at every intermediate, have been performed more than 20 years ago.[12] To elucidate the mode of action of this family of molecules, further biological studies require an efficient synthetic protocol.[13]

The presence of consecutive *N*Me amino acids in triostin A, which intrinsically implies synthetic difficulty, and of the two ester bonds, which favour the formation of diketopiperazines (DKPs) as the main problem to be solved, makes the solid-phase approach of this peptide a challenge.[14] Herein we describe the first solid-phase synthesis of triostin A, applying a new concept in protection during synthesis. The present strategy allows fast and efficient access to the natural product, and could also be used to prepare libraries of analogues with modifications on the sequence and/or on the heterocyclic unit.

The solid-phase synthesis employs several coupling reagents, a number of orthogonal protecting groups, and the new concept of protection, referred to as conformationally restricted mobility, which is crucial for the minimization of DKP formation and for the success of the approach.

Stepwise elongation: Taking advantage of the C_2 symmetry of triostin and given its synthetic difficulty, the strategy of choice is a [4+4] fragment coupling, because it reduces the number of steps and therefore minimizes the risk of side-reactions. The 4-amino acid fragment contains two ester bonds (between the first amino acid and the resin and between the Val and the D-Ser) and therefore two points where DKPs could form. This risk is increasing because the two remaining amino acids are *N*Me-Val and *N*Me-Cys. The *N*Me increases the presence of the *cis*-configuration in the dipeptide, thereby making it prone to DKP formation.[15]

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Previous experience with analogues of this family prompted the selection of the 4-amino acid fragment shown in Figure 2 as a target intermediate and the following approach for its synthesis.

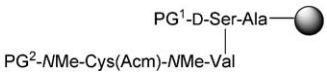


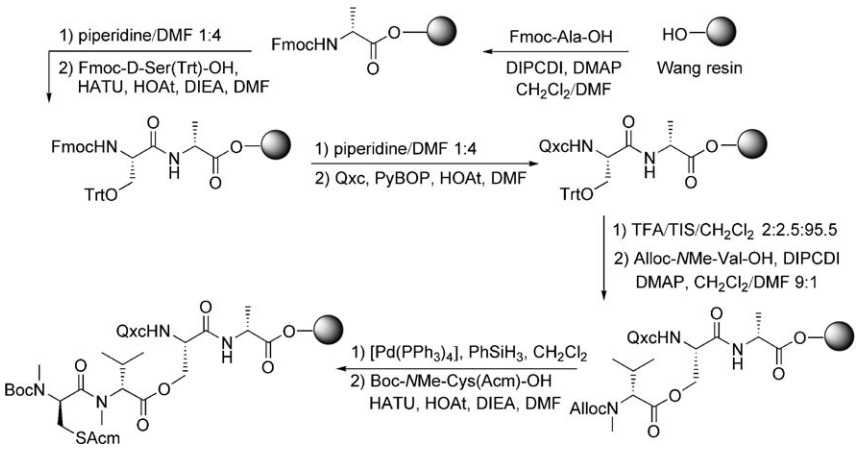
Figure 2. Sequence of the tetrapeptide moiety.

i) A Wang-type resin was used for the synthesis because the Cl-TrtCl-resin,[16] known to minimize DKP formation, is incompatible with Trt protection, used for temporary protecting group of the hydroxyl function of the D-Ser; ii) Ala was used as the starting point for the synthesis. Since the second amino acid is D-Ser, which is then elongated through its side-chain, the risk of DKP formation is kept to minimum. After introducing Fmoc-D-Ser(Trt)-OH, the Fmoc group was removed and the 2-quinoxaline carboxylic unit (Qxc) was introduced on solid-phase at the dipeptide level, prior to ester formation.[17] iii) To introduce the two remaining residues *N*Me-Val and *N*Me-Cys, different combinations of protecting groups were investigated. Removal of the Fmoc and pNZ groups by piperidine and $SnCl_2$, respectively, at the *N*Me-Val position resulted in partial cleavage of the ester bond, with recovery of only the dipeptide (Qxc-D-Ser-Ala-OH). Thus, at this position the Alloc derivative was introduced, which was removed under practically neutral conditions. For the introduction of *N*Me-Cys(Acm), the Boc group was selected, to allow concomitant cleavage with the release of the peptide from the support (Scheme 1).

To further detail the construction of the tetradepsipeptide, Fmoc-Ala-OH was coupled onto Wang resin by treatment with DIPCDI/DMAP. The Fmoc group was removed with piperidine-DMF (1:4) and Fmoc-D-Ser(Trt)-OH was coupled with HATU/HOAt/DIEA. After removing the Fmoc group, Qxc was introduced by reaction with PyBOP/HOAt/DIEA in DMF. The Trt was removed by washings with TFA/TIS/CH_2Cl_2 2:2.5:95.5 until colourless filtrates were obtained. After washing the resin with CH_2Cl_2, introduction of Alloc-*N*Me-Val-OH was accomplished by treatment with DIPCDI/DMAP in CH_2Cl_2/DMF 9:1. Removal of the Alloc-protecting group with $[Pd(PPh_3)_4]$ and $PhSiH_3$ in CH_2Cl_2, followed by introduction of Boc-*N*Me-Cys(Acm)-OH afforded the target tetradepsipeptide with 85% purity. Coupling of this second consecutive *N*Me amino acid was particularly difficult, therefore the coupling reaction using HATU/HOAt/DIEA was repeated.

Protection by conformationally restricted mobility:[18] Formation of the disulfide bridge: Synthetic organic chemistry is based on the concourse of reagents and catalysts to achieve clean formation of new bonds and appropriate protecting groups to prevent the formation of undesired bonds as well as side-reactions.

Conventional protection chemistry masks the properties of the reactive chemical groups. Thus, carbamates such as Fmoc, pNZ, or Boc, used in this study, reduce the nucleophilicity and basicity of the amines. A different approach for protection is by the introduction of steric congestion around the reactive group. A good example of this is the Trt group. Thus, the Cl-TrtCl-resin, in addition to linking the carboxyl group of the *C*-terminal amino acid to the resin, reduces DKP formation (attack of the amine of the second amino acid at the carboxyl group of the first amino acid) by steric hindrance. In this regard, Barlos has reported that the Cl at position 2 is crucial because simply by removing the Cl from the Cl-TrtCl-resin, this support is more susceptible to DKP formation.[19] Furthermore, Trt-amino acids, although difficult to couple to the peptide sequence, are practically free of racemization, again due to the steric hindrance induced around the α-H.[20] Moreover, backbone protection, such as the introduction of the 2-hydroxy-4-methoxy benzyl (Hmb) group[21] and the use of pseudoprolines[22] have enabled the synthesis of difficult peptides by disrupting secondary structure formation. Herein, we describe a new concept of protection based on conformational mobility restriction.



Scheme 1. Synthesis of the tetradepsipeptide of triostin A on solid-phase. HATU = *O*-(7-azabenzotriazol-1-yl)tetramethyluronium hexafluorophosphate; DIPCDI = *N*,*N*′-diisopropylcarbodiimide; Qxc = 2-quinoxaline carboxylic unit; PyBOP = benzotriazol-1-yloxy-tris (dimethylamino) phosphonium hexafluorophosphate; HOAt = 1-hydroxy-7-azabenzotriazole.

In previous syntheses of analogues, the removal of the protecting group of *N*Me-Cys (Boc in this case) was accompanied by immediate DKP formation. Regardless of the protecting groups used (Fmoc removed with a base, pNZ and Alloc removed under almost neutral conditions, and the acid labile Trt and Boc, removed by acid and neutralized), the formation of DKPs was extensive, and in most cases quantitative (Fig-

ure 3A). At this point, we considered that the only solution to solve this problem was to restrict the mobility of the peptide chain, by making the amino function of the *N*Me-Cys

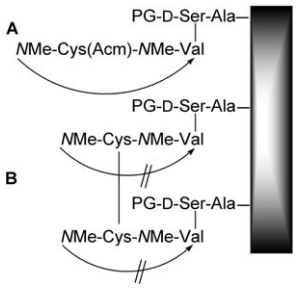
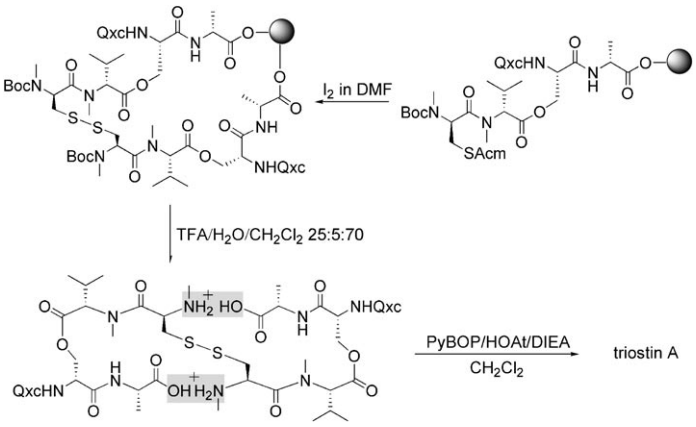


Figure 3. Protection by conformationally restricted mobility prevents DKP formation.



Scheme 2. Dimerization and cyclization to obtain triostin A.

unable to reach the carboxyl of the *N*Me-Val. In this case, this was achieved by the formation of the inter-chain disulfide bridge (Figure 3B) which restricts the mobility of the peptide chain and thereby effectively prevents DKP formation.

Scheme 2 shows the on-resin disulfide formation, followed by the cleavage, and the final double cyclization, which rendered triostin A (Scheme 2).

The tetrapeptidyl resin was subjected to two 10 min treatments of oxidation with iodine. The dimer was cleaved by treatment with $TFA/H_2O/CH_2Cl_2$ 25:5:70, evaporated, redissolved in H_2O/CH_3CN 1:1 and lyophilized. The challenging final macrocyclization (double and with an *N*Me-amino as a nucleophile) was performed by using PyBOP/HOAt/DIEA in CH_2Cl_2 for 4 h. After workup, 83 mg of crude triostin A was obtained with excellent purity (51 %, Figure 4B) for a crude peptide after 12 steps, using six protecting groups (Fmoc, Alloc and Boc for the α-amino, Trt for hydroxyl, Acm for Cys, and supported *p*-alkoxybenzyl for the *C*-terminal), an on-resin disulfide formation, and a double cyclization.

Analytical HPLC and HPLC-MS analysis revealed the presence of two peaks of the same mass in a 65:35 ratio, which were separated by semi-preparative reversed HPLC (17 % overall yield) (Figure 4C, D). The presence of two discrete conformers of triostin A in slow exchange, which may be separated chromatographically, has been reported.[23] ^{1}H NMR spectra in $CDCl_3$ of the two compounds were consistent with previously reported data,[24,25] where the *N*Me-Val adopts a trans conformation

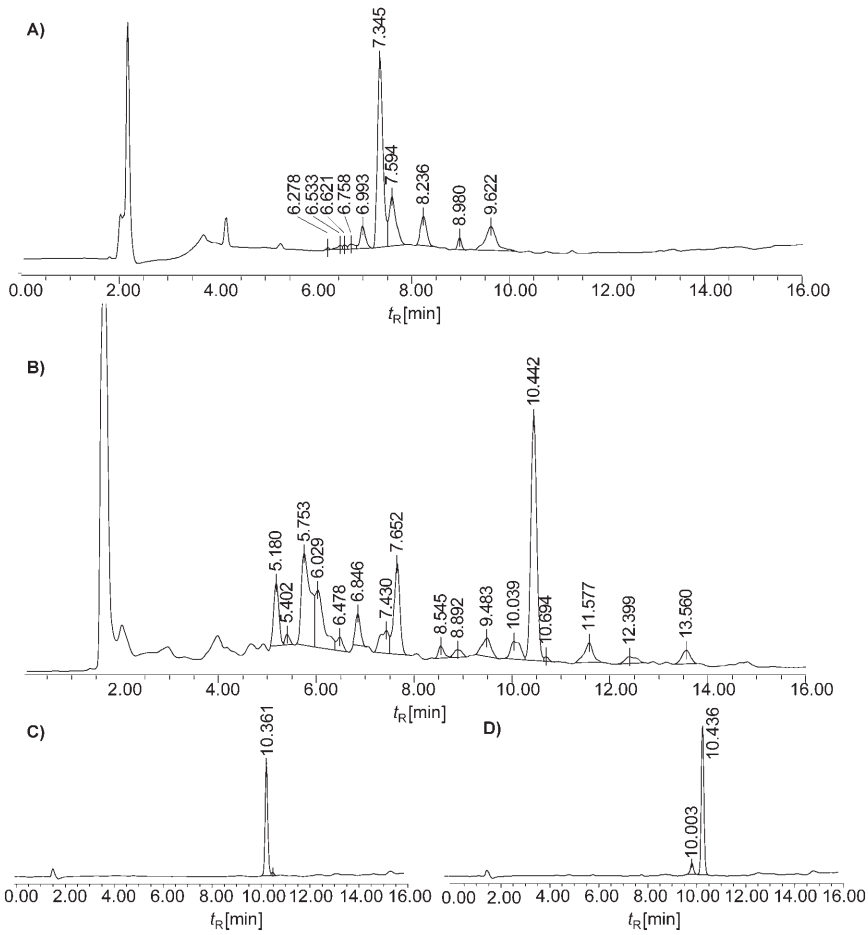


Figure 4. Analytical HPLC of A) crude dimer ($t_R = 7.34$ min), B) crude triostin A ($t_R = 10.44$ min), C) most polar and D) least polar conformer.

and the presence of the two conformers is a result of the isomerization *cis*/*trans* of the *N*Me groups of Cys.

The in vitro activity of the two conformers of triostin A was evaluated in three tumour cell lines (MDA-MB-231, A549, HT29). To the best of our knowledge, this is the first time that these two conformers have been assayed separately. In the three cell lines, the most polar conformer showed a higher inhibitory effect on cell growth (10^{-7} M) than the least polar one, which was one order of magnitude less active.

In conclusion, we have demonstrated that an optimized synthetic approach should allow the synthesis of any natural product, as the two triostins A. Furthermore, the new concept of conformationally restricted mobility can be key for the synthesis of other peptides as well as other complex natural products.

Acknowledgements

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Keywords: conformation analysis · peptides · protecting groups · solid-phase synthesis · triostin

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