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Efficient Solid-Phase-Based Total Synthesis of the Bisintercalator TANDEM

John P. Malkinson,* Michael K. Anim, Mire Zloh, and Mark Searcey*

Department of Pharmaceutical and Biological Chemistry, School of Pharmacy, University of London, 29-39 Brunswick Square, London WC1N 1AX, U.K.

Andrew J. Hampshire and Keith R. Fox

School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, U.K.

mark.searcey@ulsop.ac.uk; john.malkinson@ulsop.ac.uk

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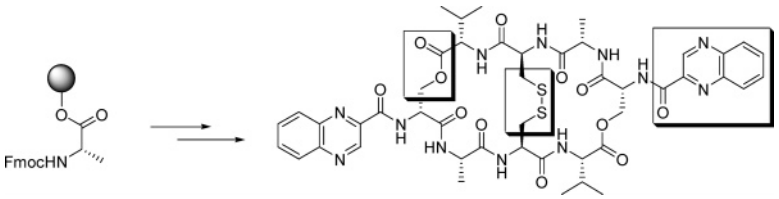

In this article, the first solid-phase-based total synthesis of TANDEM, a synthetic analogue of triostin A, is described. In initial studies, the synthesis incorporated depsipeptide formation, introduction of chromophores, and disulfide bond formation on the solid phase, prior to a final solution-phase macrolactamization, to give the target molecule. Although pure TANDEM was obtained in an overall yield comparable to those for all syntheses to date, the yield of the final cyclization was low (11%). A more efficient approach involved removal from the solid phase prior to disulfide bond formation. The resulting linear peptide underwent macrolactamization under mild conditions and high dilution. Final disulfide bond formation was essentially quantitative and gave the target molecule, TANDEM, in an overall yield of 18%. The final compound was assessed for its ability to bind to 5′-TpA sequences on DNA by DNase I footprinting. This efficient synthesis sets the stage for a study of the structure−activity relationship of TANDEM and the natural product triostin A, with analogues containing "point mutations" at every site within the cyclic compounds.

Introduction

Echinomycin (**1**)[1] and triostin A[2] (**2**) (Figure 1) are parent members of a family of antitumor antibiotics that have, at one stage, advanced into phase I clinical trials.[3] Numerous natural product analogues have been described, most recently thiocoraline **3**,[4] which is currently under preclinical development and has recently been synthesized by solution-phase methods.[5] Both echinomycin and triostin A bind to DNA by bisintercalation, inserting their chromophores into the duplex on either side of 5′-CG, forming a two base pair sandwich.[6] It has also been demonstrated that the bases on either side of the sandwich exert an effect on binding, such that the natural products effectively recognize a four base pair sequence.[7] **T**riostin **A** *N*-**Dem**ethylated (TANDEM **4**) is a synthetic analogue of triostin A in which secondary amides replace the tertiary amides of the natural product.[8] This small change results in a change in sequence selectivity, with TANDEM showing a preference for 5′-TA steps, particularly 5′-ATAT.[9] Such a change mirrors that seen with the incorporation of imidazole groups into the hairpin polyamides. However, few, if any, attempts

* Corresponding authors. Phone: 020-7753-5873 or 5862. Fax: 020-7753-5964.

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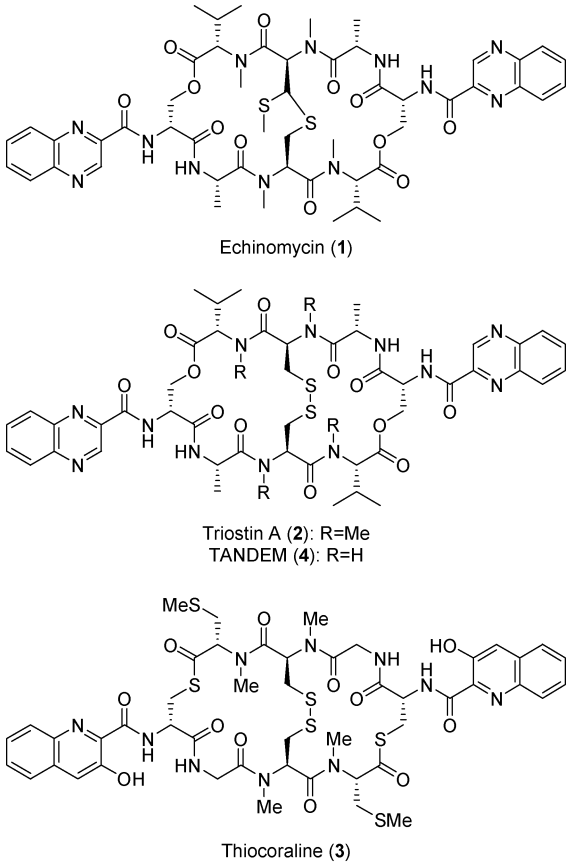

FIGURE 1. Structures of **1**−**4**.

have been made to exploit the effect seen in going from the natural products to the synthetic agents in the quinoxaline antibiotics. In part, we believe this stems from problems encountered in the solution-phase synthesis, which is relatively demanding, even though recent efforts have been disclosed that focused on developing methods for solution-phase combinatorial libraries.[10] If a solid-phase approach to these compounds could be developed, it would allow the rapid synthesis of analogues with different amino acids at each position. A solid-phase synthesis of an amide-linked analogue of TANDEM has recently been disclosed.[11] The linear nature of a solid-phase synthesis would allow the generation of unsymmetrical derivatives. In all, a parallel synthesis of analogues would allow the rapid development of a full structure−activity profile for these compounds. The investigation of extended analogues will also be straightforward and allow the determination of the suitability of these compounds for targeting longer sequences of DNA. With this reinvestigation of classical compounds in the light of new synthetic, high-throughput methods, we report here the first solid-phase-based total synthesis of TANDEM and a more efficient second generation synthesis that gives the highest reported yields of the target molecule. We also investigated the DNA binding ability of several of the intermediates in the synthetic pathway and found that only the final product, TANDEM, binds to DNA with high affinity.

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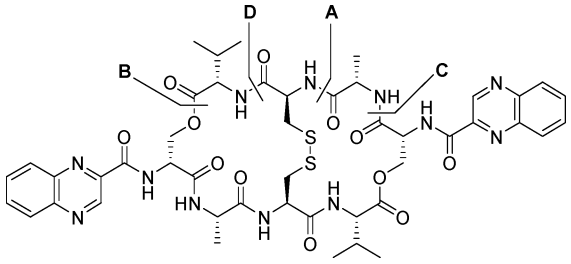

FIGURE 2. Potential sites for off-resin cyclization of **4**.

Results and Discussion

Design of the Solid-Phase Synthesis. The most attractive synthesis of TANDEM involved the incorporation of as many steps as possible on the solid phase prior to release, maintaining a minimum number of solution-phase steps to complete the synthesis. Thus, formation of the depsipeptides, incorporation of the chromophores, and preferably, closure of the disulfide bridge with the growing chain immobilized were all considered. The identification of the point of final ring closure was also key to the successful achievement of the synthesis of TANDEM. There are four potential sites for macrocyclization (A−D, Figure 2). All were carefully analyzed, and methodology was compared with published procedures. Olsen and co-workers used NHS-DCC to bring about macrolactamization at A of the bis-Cbz-protected cyclic peptide in the original synthesis of the depsipeptides, although the isolated yields were only of the order of 26−43%.[8] In our synthesis, this would involve immobilization of alanine and completion of the linear synthesis at the final cysteine, an attractive proposition as the starting resin bound Fmoc amino acid is readily commercially available and the distance from the depsipeptide bond is optimal. The formation of the depsipeptide bond (B, Figure 2) as the final step was less attractive, as the lower nucleophilicity of the alcohol would not be helpful in the final macrolactonization. The conditions required for such a ring closure would be a potential source of significant racemization of the terminal valine and/or dehydration and racemization of the serine residue. Diederichsen and co-workers have recently described a ring closure using DIC/HOAt between serine and alanine (C, Figure 2), which gave an excellent yield of 82% for the bis-Cbz-protected peptide.[12] Application to a solid-phase method would be difficult. It would require an orthogonally protected resin-bound D-serine that could be selectively N^{α}-deprotected to introduce first the chromophore and then side-chain *O*-deprotected to introduce the depsipeptide ester. Wang resin-bound Fmoc-D-Ser(Trt)-OH would be suitable but is not readily available in the U.K. Finally, formation of the cysteine-valine bond (D, Figure 2) is a possibility but would involve immobilization of an orthogonally protected cysteine residue onto the solid support and final macrolactamization via activation of the terminal cysteine carboxyl, both of which are known to lead to racemization.[13] Bearing these points in mind, we initially focused on a synthesis involving incorporation of both depsipeptide bonds, both chromophores and the disulfide bond, fol-

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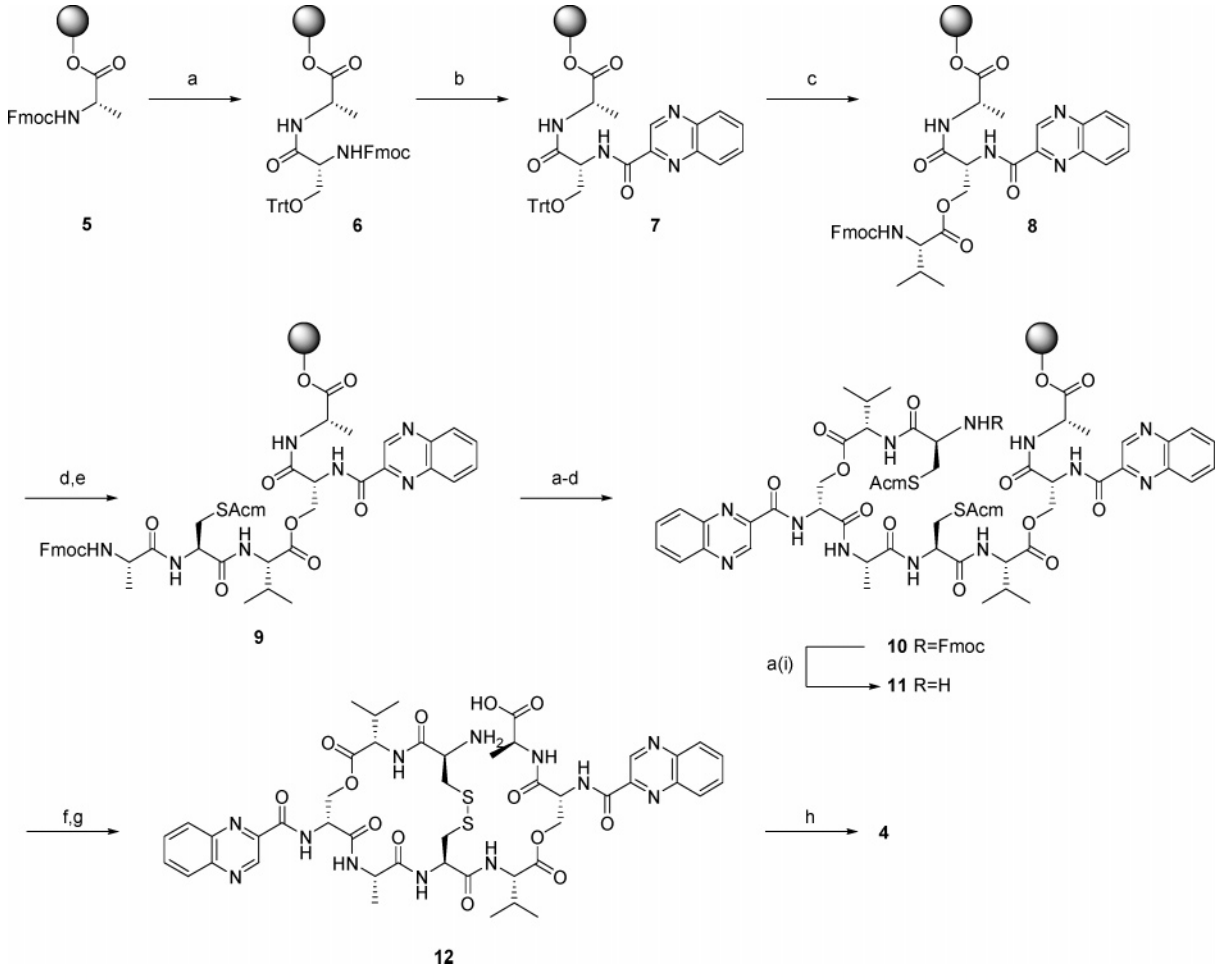
SCHEME 1[a]



[a] Reagents and conditions: (a) (i) 20% piperidine, DMF, rt, 2 × 10 min, (ii) Fmoc-D-Ser(Trt)-OH, HBTU, HOBt, *i*Pr$_2$NEt, DMF, rt, 2 × 30 min; (b) (i) 20% piperidine, DMF, rt, 2 × 10 min, (ii) quinoxaline-2-carbonyl chloride, *i*Pr$_2$NEt, DMF, rt, 2 × 30 min; (c) 2% TFA, 5% *i*Pr$_3$SiH, CH_2Cl_2, rt, 3 × 5 min then 1 × 20 min, (ii) Fmoc-Val-OH, DIC, DMAP, DMF, rt, 3 × 60 min; (d) (i) 20% piperidine, DMF, rt, 2 × 10 min, (ii) Fmoc-Cys(Acm)-OH, HBTU, HOBt, *i*Pr$_2$NEt, DMF, rt, 2 × 30 min; (e) (i) 20% piperidine, DMF, rt, 2 × 10 min, (ii) Fmoc-Ala-OH, HBTU, HOBt, *i*Pr$_2$NEt, DMF, rt, 2 × 30 min; (f) I_2, DMF, rt, 1 h; (g) TFA/H_2O/*i*Pr$_3$SiH (95:2.5:2.5), rt, 2 h; (31% overall yield of **12** from **5**); (h) EDC (5 equiv), HOBt (5 equiv), CH_2Cl_2/DMF 7:1, rt, 24 h, 11% yield.

lowed by release from the resin and solution-phase-based ring closure.

First Generation Synthesis. The linear depsipeptide precursor was assembled using N^{α}-Fmoc-based solid-phase peptide synthesis. N^{α}-Fmoc-alanine, immobilized onto a polystyrene solid support via the Wang linker (**5**), was deprotected using 20% piperidine (Scheme 1). Subsequent coupling of an N^{α}-Fmoc, side-chain *O*-trityl-protected D-serine derivative generated resin-bound dipeptide **6**. The introduction of this orthogonally protected trifunctional amino acid was key to the synthesis of the linear precursor. It allowed selective N^{α}-Fmoc deprotection, followed by introduction of the quinoxaline-2-carbonyl chromophore, which was coupled cleanly as its acyl chloride derivative. The side-chain *O*-trityl protection of **7** was then removed by very mild acidolysis (2% TFA, 5% TIS, CH_2Cl_2) to generate the free β-hydroxyl group necessary for formation of the first depsipeptide bond. Esterification was achieved using a 10-fold excess of N^{α}-Fmoc-valine in the presence of a 5-fold excess of DIC and catalytic (0.1 equiv) DMAP. The use of additional base was avoided to minimize the potential risk of epimerization and/or dehydration of the D-serine residue. Resin-bound tridepsipeptide **8** was then extended, coupling N^{α}-Fmoc, *S*-acetamidomethyl-cysteine, and N^{α}-Fmoc-alanine in turn. Repetition of the sequence described above (up to and including N^{α}-Fmoc deprotection of the second cysteine residue) gave the completed linear sequence **11**, immobilized on the solid phase. Simultaneous removal of the cysteine *S*-acetamidomethyl protecting groups and oxidation to form the disulfide bridge was achieved by treatment with iodine in DMF for 1 h at room temperature. Unlike solution-phase iodine oxidation, which must be performed under high-dilution conditions, on-resin oxidation proceeds without the risk of intermolecular disulfide formation. The disulfide-bridged peptide was removed from the solid support by acidolysis using 95% TFA (TFA/H_2O/TIS 95:2.5:2.5). The suspension was then filtered, the filtrate was concentrated in vacuo, and the

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crude peptide **12** (75 mg, 36% based upon resin loading) was isolated by precipitation with ether. Analytical RP-HPLC on a Zorbax C18 column revealed one major peak (R_t 14.6 min) with minor impurities. Preparative HPLC gave the pure peptide **12** (65 mg, 31% for 21 steps).

Head-to-tail cyclization of the pure **12** (0.8 mM, CH_2Cl_2/DMF 7:1, 24 h, room temperature (rt)) in solution using EDC gave a low (11%) yield of the ring-closed compound TANDEM (**4**). Efforts to increase the yield, including longer reaction times and increased temperature were not successful. Although the starting material was consumed to generate the active ester, further product could not be isolated, and it appeared by HPLC that unreacted active ester and, perhaps, some dimer peptide were present (although neither was isolated). The structure of the final product **4** was confirmed by NMR and mass spectrometry and compared with the literature data given by Olsen and co-workers.[14]

Although the yield of the final solution-phase cyclization was poor, the overall yield of the completed synthesis was 4.2%, which compares well with the 2.6−6.5% reported by Olsen for the same synthesis in solution.[8] Diederichsen and co-workers recently reported the synthesis of the bis-Cbz-protected cyclic peptide in which a solution-phase cyclization of the peptide incorporating the disulfide bond and the depsipeptide bonds resulted in an 82% yield, although their overall yield for this synthesis was only 3.6%.[12] The final cyclization in this case was between D-Ser and Ala, suggesting, perhaps, that the low yield in our case may be due to constraints imposed by the trajectory of attack on the cysteine that is already involved in the disulfide bond. A solution-phase structure of peptide **12** suggests that the molecule has a large hydrophobic core and a hydrogen bond that may also contribute to hindering the cyclization reaction. However, this structure was obtained in a solvent (CD_3OD) different from that of the reaction, as all attempts to obtain the NMR spectra in CD_2Cl_2/DMF-d_7 (7:1) gave poor resolution (data not shown), possibly as a consequence of aggregation and/or limited solubility.

Investigations of Depsipeptide Formation. The final yield of the peptide **12** represents an average of 94% for each step of the synthesis. Although this would be acceptable in a solution synthesis, and a yield of 31% for 21 steps would be remarkable, in the context of a solid-phase synthesis, yields of more than 99% would be expected for each peptide coupling. This would suggest that the introduction of the chromophore and the depsipeptide bond formation may be limiting the final yield. As a consequence, we briefly investigated the use of other ester-forming reagents and the assembly of an orthogonally protected didepsipeptide building block in solution prior to incorporation in the solid-phase synthesis in a convergent approach.

Depsipeptide bond formation during the original assembly of resin-bound linear precursor **11** was achieved essentially by activating N^{α}-Fmoc-valine (10 equiv) as the preformed symmetrical anhydride (PSA), using DIC (5 equiv) and catalytic DMAP. In situ activation using HBTU and HOBt in the presence of iPr$_2$NEt (i.e., the same conditions used for peptide chain elongation) had previously been found ineffective for esterification, based on the (indirect) observation of a negative or very weak Kaiser test after subsequent treatment of a sample of resin with 20% piperidine (indicating an absence of N^{α}-Fmoc-protected amino groups and, therefore, a failure of coupling). Direct monitoring of the presence (or absence) of hydroxyl groups on the solid phase[14] proved insufficiently sensitive or reliable in our hands. In situ formation of the OBt active ester (using HOBt in the presence of equimolar DIC and iPr$_2$NEt) and the use of a preformed N^{α}-Fmoc-valine pentafluorophenyl ester (with catalytic DMAP) proved similarly unsuccessful. Reduction of the molar excess of N^{α}-Fmoc-valine in the original depsipeptide bond-forming conditions (from 10 equiv to 5 equiv, effectively promoting activation as the *O*-acylisourea rather than primarily as the PSA) resulted in similar to slightly improved yields and purities of linear precursor **12** on resin cleavage. Increasing the catalytic amount of DMAP (from 0.1 to 0.5 equiv) resulted in a slightly reduced yield of crude depsipeptide of similar purity.

Finally, two other methods for depsipeptide bond formation were evaluated. The activation of N^{α}-Fmoc-protected amino acids using 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT) in the presence of 4-methylimidazole has been reported for the esterification of hydroxymethyl-derivatized solid supports with minimal racemization.[15] Activation of N^{α}-Fmoc-valine (5 equiv) using MSNT (5 equiv) and 4-methylimidazole (3.75 equiv) for the depsipeptide bond-forming steps was successful, but gave lower crude yields and purities of **12**. Mitsunobu conditions have also been used for loading hydroxymethyl-derivatized resins.[16] Esterification using PPh_3 and either DEAD or DIAD in the presence or absence of added base was unsuccessful (as indirectly monitored as described above). Analysis of the mass spectra of the crude cleavage products after attempted esterification indicated the presence of both unreacted **7** and the corresponding side chain dehydrated byproduct, in addition to a small amount of desired depsipeptide **8**.

The alternative convergent approach necessitated the solution-phase synthesis of a suitably orthogonally protected didepsipeptide building block. The primary feature of the solid-phase synthesis of TANDEM is to maximize the number of synthetic steps performed on the resin, thus exploiting the advantages of this approach. The incorporation of a preformed didepsipeptide building block (prepared in solution), however, serves several purposes: it removes two of the potentially significant sources of byproduct formation (i.e., failure of depsipeptide bond formation and epimerization/dehydration of the sensitive D-serine residue), depsipeptide bond formation is difficult to monitor directly in a satisfactory manner, and any byproducts resulting from undesirable or incomplete reaction(s) are likely to be difficult to separate from the desired product during final HPLC purification. The didepsipeptide building block can be prepared relatively

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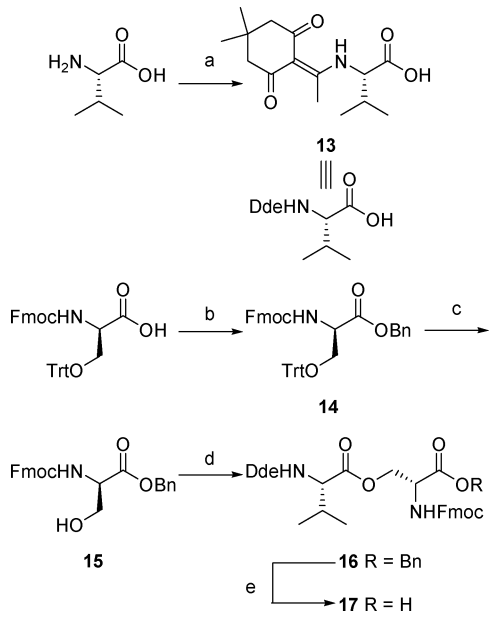
SCHEME 2[a]



[a] Reagents and conditions: (a) Dde-OH, NEt_3, abs. EtOH, reflux, 18 h, 70%; (b) (i) Cs_2CO_3, MeOH/H_2O, 0 °C to rt, 1 h, (ii) BnBr, DMF, rt, 4 h, 89%; (c) 5% TFA/CH_2Cl_2, *i*Pr_3SiH, rt, 5 min, 85%; (d) 10, DIC, DMAP, CH_2Cl_2, rt, 6 h, 85%; (e) 10% Pd(C), THF, H_2, rt, 48 h, 79%.

easily in large scale and with guaranteed diastereomeric purity. The depsipeptide product thus resulting from a convergent approach to synthesis would potentially be cleaner and more easily purified, and the yield (compared to the original synthesis) would give an indication of the loss of material attributable to formation of the depsipeptide bonds on the solid phase.

Quasi-orthogonal protection of the didepsipeptide building block was chosen such that the D-serine N^{α}-protection could first be removed, allowing selective introduction of the quinoxaline-2-carbonyl chromophore, with subsequent deprotection of the L-valine α-amino group allowing continuation of depsipeptide chain assembly. L-Valine was protected as its N^{α}-Dde derivative **13** (Scheme 2). The carboxyl group of commercially available N^{α}-Fmoc, side-chain *O*-trityl D-serine was protected as its benzyl ester derivative **14**, with subsequent removal of the *O*-trityl protection by mild acidolysis. DIC-mediated condensation of the carboxyl of the protected L-valine **13** and the free hydroxyl of the protected D-serine residue **15**, followed by catalytic hydrogenation, gave the desired didepsipeptide building block **17** in good overall yield.

The building block was incorporated smoothly into the growing depsipeptide chain using the standard coupling conditions for peptide chain assembly. After N^{α}-Fmoc deprotection and chromophore coupling, the N^{α}-Dde group was removed using 2% hydrazine hydrate in DMF (3 × 3 min) and the depsipeptide assembly continued.

Unfortunately, HPLC analysis of the crude peptide product after removal from the solid support demonstrated the presence of many uncharacterized byproducts in addition to the desired product. It is likely that the depsipeptide bonds were not completely stable to the very mild conditions used for N^{α}-Dde removal. This was unexpected since previous work in our laboratory (solid and solution phase) had demonstrated the stability of ester-based protecting groups to the conditions of Dde removal (in some cases up to 10% hydrazine hydrate was used), and the Dde group is routinely removed in the solid-phase synthesis of modified peptides without affecting ester-based protecting groups or resin handles.

In addition to the investigations of depsipeptide bond formation reported earlier in this article, we also briefly examined the conditions for incorporation of the quinoxaline-2-carbonyl chromophores and for disulfide formation on the solid phase. Acylation with quinoxaline-2-carbonyl chloride in the presence of HOBt and *i*Pr_2NEt, or with the corresponding quinoxaline-2-carboxylic acid in the presence of HBTU, HOBt, and *i*Pr_2NEt, provided no significant advantage over the original coupling conditions (quinoxaline-2-carbonyl chloride and *i*Pr_2NEt alone). Furthermore, disulfide formation using $Tl(tfa)_3$ (1.2 equiv) in DMF/anisole (19:1)[17] gave disulfide-bridged depsipeptide **12** in similar crude yields but reduced purity compared to the use of iodine.

Second Generation, Efficient Synthesis of TANDEM. Bearing in mind the problems encountered with the alanine-cysteine head-to-tail ring closure reaction, we reasoned that a more flexible, linear peptide may lead to higher yields in the macrolactamization step. This approach removes the conformational constraints imposed by the prior formation of the disulfide bridge, potentially promoting the desired intramolecular cyclization. We also reasoned that intramolecular disulfide bond formation would be highly favored (i.e., over competing intermolecular reactions) in the head-to-tail cyclic bis-Acm-protected depsipeptide so produced. If so, the final disulfide-forming step should proceed rapidly and cleanly with few unwanted byproducts. To this end, we repeated the solid-phase synthesis and reversed the final two steps of disulfide bond formation and macrolactamization.

The linear peptide was assembled in a manner similar to the first synthesis, utilizing N^{α}-Fmoc chemistry (Scheme 3). Depsipeptide bond formation was achieved using a 5-fold excess of N^{α}-Fmoc-valine in the presence of a 5-fold excess of DIC and catalytic (0.1 equiv) DMAP. Once assembly on the solid phase was complete, the resin-bound bis-Acm-protected linear precursor was removed from the solid support (i.e., without prior disulfide bond formation). The depsipeptide **18** was isolated in 65% yield (typically 48−65% yield) in crude form and 30% yield after purification by HPLC.

Preparative HPLC also allowed the isolation of the two most significant byproducts present in the crude cleavage mixture. Mass spectrometry, as well as extensive ^{1}H-, ^{13}C-, and 2D-homo- and heteronuclear NMR, identified these byproducts as the dipeptide chromophore conjugate **19** (8 mg isolated; 3% of crude product), in which the D-serine side chain had undergone dehydration to dehydroalanine (Dha) and, unexpectedly, a mono-Acm-protected linear precursor **20** (20 mg isolated; 6% of crude product). Careful examination of the TOCSY and NOESY NMR spectra in conjunction with the assigned ^{1}H- and ^{13}C-spectra identified the midchain cysteine, rather than

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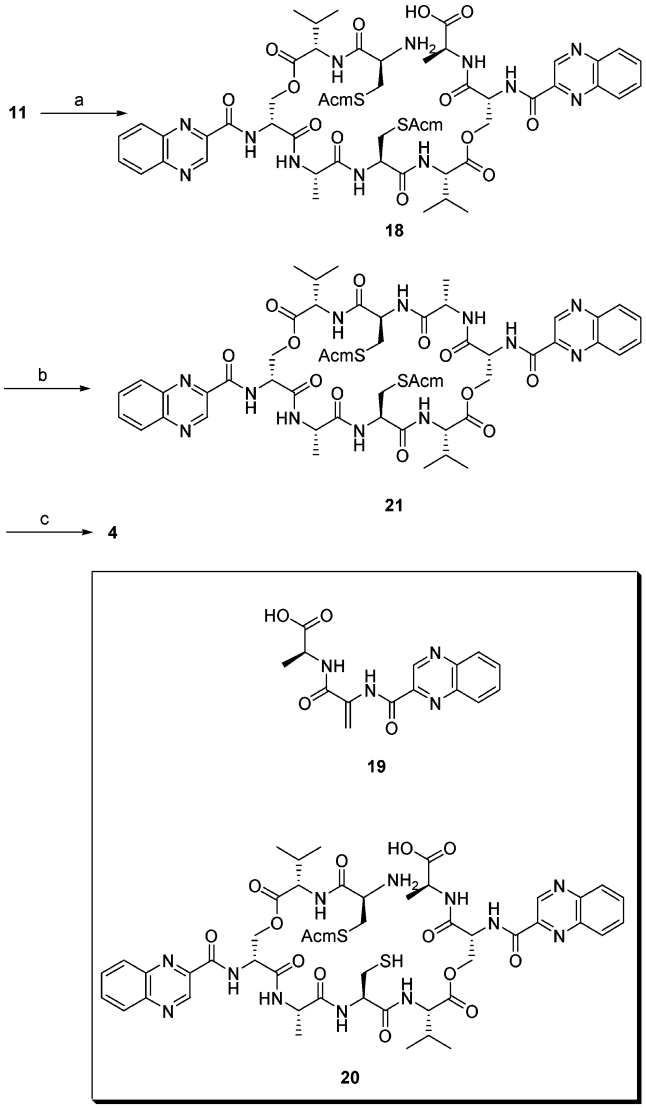
SCHEME 3[a]



[a] Reagents and conditions: (a) TFA/H_2O/iPr_3SiH (95:2.5:2.5), rt, 2 h; (b) EDC (5 equiv), HOSu (4 equiv), NMM (1 equiv), THF/DMF (10:1), 0 °C to rt, 5 h, 59%; (c) I_2 (10 equiv), abs. MeOH, rt, 1 h, quant.

the terminal cysteine, as the residue that had undergone *S*-Acm deprotection, presumably during TFA-mediated cleavage.[18] The structural assignment was made primarily on the basis of NOEs between the methylene protons of the Acm group and the NH proton of the valine residue (Val2) known to be adjacent to the terminal cysteine (Cys1) and between the Acm methyl group protons and the βH of Val2.

The linear peptide was then subjected to Olsen's methodology for macrolactamization in solution under high dilution conditions.[8] Head-to-tail cyclization of the pure depsipeptide **18** (1−2 mM, THF/DMF 10:1, 5 h, 0 °C to rt) using EDC (3 equiv) and HOSu (4 equiv) in the presence of NMM (1 equiv) gave the bis-Acm-protected cyclic depsipeptide **21** in 59% yield (typically 40−59% yield) after preparative HPLC purification. Increasing the reaction time (up to 48 h) did not significantly increase the yield of the macrolactamization step. Head-to-tail cyclization was also performed using EDC (5 equiv) and HOAt (5 equiv) under high dilution conditions in THF/DMF 4:1[10] for 24 h, but resulted in lower yields (up to 32%) of bis-Acm cyclic depsipeptide **21**.

Treatment of the protected cyclic depsipeptide (2 mM in abs. MeOH, 1 h, rt) with iodine (10 equiv) resulted in concomitant removal of the Acm protecting groups and disulfide bond formation affording TANDEM **4** in essentially quantitative yield. This is an overall yield of 18% (based on manufacturer's resin loading) and represents the most efficient synthesis of the synthetic bisintercalator reported to date.

DNA Binding and Sequence Selectivity of TANDEM and Intermediates. Unlike the natural products triostin A and echinomycin, TANDEM **4** has been shown to bind to DNA with a preference for 5′-TpA sequences, particularly 5′-ATAT.[9] This change in sequence selectivity on going to a synthetic agent lacking the tertiary amides of the natural products appears to be unique even among the cyclic depsipeptides. Thiocoraline, the latest cyclic depsipeptide to be discovered (actually a thiodepsipeptide), has only relatively nonsequence-selective DNA binding even though it contains similar tertiary amides to echinomycin.[5] The potential to exploit the sequence selectivity of triostin A and TANDEM in the design of potential DNA-reading agents remains largely unexplored despite extensive studies of the natural products. It was germane, therefore, to investigate the potential DNA interactions of the intermediates of the synthesis, as well as to establish that our synthetic TANDEM is similar to the previously described agent in its DNA binding and sequence selectivity. The sequence selectivity of TANDEM and its close derivatives has previously been determined by DNase I footprinting,[9,19] and representative footprinting gels showing the interaction with this synthetic TANDEM are shown in Figure 3. The left-hand panel shows the interaction with *tyr*T fragment, used in the original footprinting studies.[19d] This shows a single clear footprint at the sequence 5′-ATAT as indicated, with a weaker footprint at the 5′-CTAA halfway down the gel, located at the top of the oligopurine tract. The second and third panels show the interaction with fragments MS1 and MS2, which each contain all possible tetranucleotide sequences. The footprinting patterns are similar to those previously published and show inhibition of DNase I cleavage at most of the TpA steps, with the exception of 5′-TTAA as previously noted.[9] The final panel shows the interaction with fragment pAAD1, which contains different combinations of $(A/T)_4$ sites, separated by the sequence CGCGCG.[20] Again, footprints are apparent at the sequences containing TpA steps (ATAT and TATA), although there is no interaction with TTAA.[9] These results clearly demonstrate that this newly syn-

(18) Unexpected loss of Cys(Acm) protection during mild acidolytic cleavage has been previously reported. (a) Singh, P. R.; Rajopadhye, M.; Clark, S. L.; Williams, N. E. *Tetrahedron Lett.* **1996**, *37*, 4117−4120. (b) Engebretsen, M.; Agner, E.; Sandosham, J.; Fischer, P. M. *J. Pept. Res.* **1997**, *49*, 341−346.

(19) (a) Lavesa, M.; Olsen, R. K.; Fox, K. R. *Biochem. J.* **1993**, *289*, 605−607. (b) Waterloh, K.; Olsen, R. K.; Fox, K. R. *Biochemistry* **1992**, *31*, 6246−6253. (c) Low, C. M. L.; Fox, K. R.; Olsen, R. K.; Waring, M. J. *Nucleic Acids Res.* **1986**, *14*, 2015−2033. (d) Low, C. M. L.; Olsen, R. K.; Waring, M. J. *FEBS Lett.* **1984**, *176*, 414−420.

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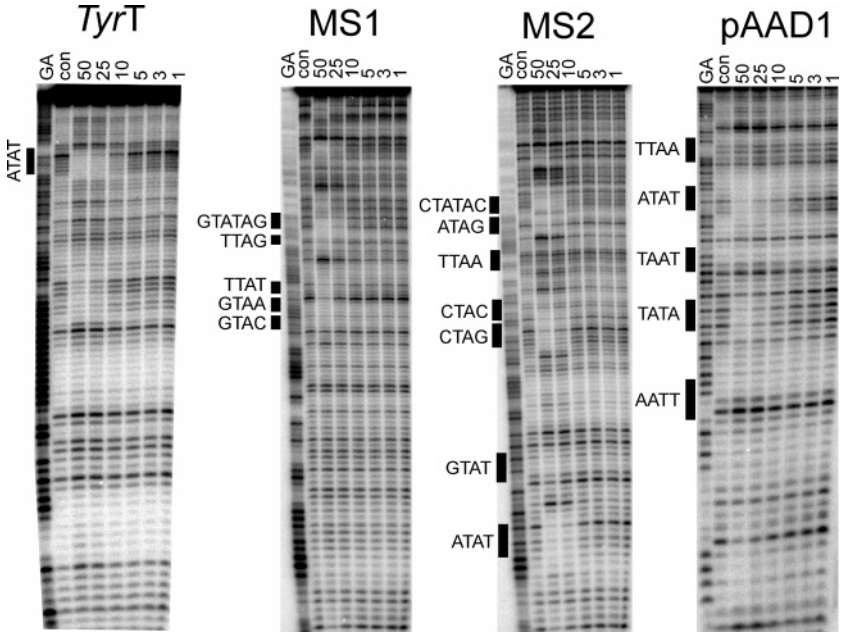

FIGURE 3. DNase I footprinting patterns showing the interaction of TANDEM **4** with four DNA fragments. The concentration of **4** (μM) is shown at the top of each gel lane. Tracks labeled "GA" are Maxam−Gilbert markers specific for purines, while "con" indicates cleavage in the absence of added ligand. The locations of the best binding sites, containing the TpA dinucleotide step, are indicated by the boxes.

thesized TANDEM has the same DNA binding properties, interacting with TpA sites, as previously reported. In contrast, the bis-Acm-protected cyclic depsipeptide **21** and the disulfide bond-containing linear precursor **12** showed no effect on DNase I cleavage at concentrations up to 50 μM (data not shown).

Conclusions

The synthesis of TANDEM on solid phase was achieved by two different routes. Although the first of these routes involved a final, low-yielding peptide cyclization reaction, it still represents a route yielding material in amounts similar to those already published. More gratifyingly, a second route, giving better yields for the macrolactamization prior to disulfide bond formation, is the most efficient route to this DNA-binding cyclic peptide described to date. The new synthesis gave TANDEM with DNA binding characteristics essentially identical to those of the molecule previously synthesized. None of the intermediate synthetic precursors of TANDEM bind to DNA with appreciable affinity.

In this article, we have revisited molecules that have been extensively studied by others but for which structure−activity studies and analogue structures are few. The solid-phase-based protocols give us the opportunity to generate numerous analogues and investigate the ability of these analogues to bind to DNA and inhibit either gene or template function on the duplex. The potential for analogues that may bind to longer sequences is intriguing, and such new entities are currently under investigation in our laboratories and will be described in due course.

Experimental Section

Peptide Synthesis. Peptide synthesis was accomplished manually using a stepwise solid-phase procedure. All peptide couplings were carried out for 30 min in DMF using a 2.5-fold excess (over resin loading) of protected amino acid, activated with an equimolar amount of HBTU and HOBt, in the presence of a 5-fold excess (over resin loading) of iPr$_2$NEt. Each coupling was repeated and completion monitored using the Kaiser test[21] for free amines. Unsuccessful couplings were further repeated until a negative Kaiser test was obtained. N^{α}-Fmoc protection was removed using 20% (v/v) piperidine in DMF (2 × 10 min). Introduction of the quinoxaline-2-carbonyl chromophore was achieved by acylation of the D-serine α-amino group using a 2.5-fold excess of quinoxaline-2-carbonyl chloride in the presence of a 5-fold excess of iPr$_2$NEt (2 × 30 min). D-Serine side-chain O-trityl protection was removed using 2% (v/v) TFA in dry CH_2Cl_2 containing 5% (v/v) iPr$_3$SiH (3 × 5 min; 1 × 20 min). Esterification of the free D-serine β-hydroxyl was performed in DMF using a 10-fold or 5-fold excess of N^{α}-Fmoc-valine and a 5-fold excess of DIC, in the presence of a catalytic amount (0.1 equiv with respect to resin loading) of DMAP (3 × 1 h). Disulfide bond formation (with simultaneous S-Acm deprotection) was performed on the solid phase (if required) using a 10-fold excess of iodine in DMF (1 h), followed by very thorough washing of the resin-bound depsipeptide with copious DMF and CH_2Cl_2.

Peptide Cleavage and Isolation. The resin-bound peptide was washed thoroughly with DMF, CH_2Cl_2, and then 50% (v/v) MeOH in CH_2Cl_2 and dried in vacuo over KOH to constant weight. The peptide was removed from the solid support by acidolysis using TFA containing 2.5% (v/v) water and 2.5% (v/v) iPr$_3$SiH for 2 h at 25 °C. The TFA was removed under reduced pressure. The crude peptide precipitated, was washed with cold anhydrous diethyl ether, and then was extracted into 50% (v/v) acetic acid and lyophilized. The crude product was purified by preparative HPLC (System B). Pure fractions were pooled and lyophilized.

Disulfide Bond-Containing Linear Precursor (12). Disulfide-bridged depsipeptide **12** was synthesized on 0.205 mmol scale (0.50 g of Fmoc-Ala-Wang resin; 0.41 mmol g^{-1} manufacturer's substitution). Synthesis, cleavage, isolation, and HPLC purification (System B) as described earlier afforded

(21) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595.

the desired depsipeptide **12** as a white solid (65 mg; 31% based on manufacturer's resin loading). ES MS ($C_{46}H_{56}N_{12}O_{13}S_2$) 1048.35 *m/z* (%): 1049.4 $[M + H]^+$ (100), 1071.5 $[M + Na]^+$ (60). RP-HPLC (System A): R_t = 14.58 min. ^{1}H NMR (CD_3OH): δ 0.89 (d, 3H, γH Val6), 0.92 (d, 3H, γH Val6), 1.05 (d, 3H, γH Val 2), 1.10 (d, 3H, γH Val 2), 1.41 (d, 3H, βH Ala4), 1.45 (d, 3H, βH Ala8), 2.18 (m, 1H, βH Val6), 2.36 (m, 1H, βH Val2), 3.06 (dd, 1H, βH_a Cys5), 3.10 (dd, 1H, βH_a Cys1), 3.25 (dd, 1H, βH_b Cys5), 3.50 (dd, 1H, βH_b Cys1), 4.31 (dd, 1H, αH Cys1), 4.36 (dd, 1H, αH Ala4), 4.41 (m, 1H, αH Val6), 4.44 (m, 1H, αH Val2), 4.48 (m, 1H, αH Ala8), 4.59 (dd, 1H, βH_a DSer3), 4.65 (d, 2H, βH DSer7), 4.74 (dd, 1H, βH_b DSer3), 4.90 (m, 1H, αH Cys5), 5.03 (m, 1H, αH DSer3), 5.11 (m, 1H, αH DSer7), 7.98−8.03 (m, 4H, Qxc CHAr), 8.04 (d, 1H, NH Val6), 8.20−8.28 (m, 4H, Qxc CHAr), 8.32 (d, 1H, NH Ala4), 8.63 (d, 1H, NH Ala8), 8.67 (d, 1H, NH Val2), 8.70 (d, 1H, NH Cys5), 9.11 (d, 1H, NH DSer3), 9.21 (d, 1H, NH DSer7), 9.56 (s, 1H, Qxc H-3), 9.58 (s, 1H, Qxc H-3).

Bis-Acm-Protected Linear Precursor (18). Bis-Acm-protected linear depsipeptide **18** was synthesized on 0.41 mmol scale (1.00 g of Fmoc-Ala-Wang resin; 0.41 mmol g^{-1} manufacturer's substitution). Synthesis, cleavage, isolation, and purification as described above (omitting the solid-phase disulfide formation step) afforded the desired protected depsipeptide **18** as a white solid (149 mg; 30% based on manufacturer's resin loading). ES MS ($C_{52}H_{68}N_{14}O_{15}S_2$) 1192.44 *m/z* (%): 1193.3 $[M + H]^+$ (100), 1215.5 $[M + Na]^+$ (46). RP-HPLC (System A): R_t = 13.62 min. ^{1}H NMR (CD_3OH): δ 0.83 (d, 3H, γH Val6), 0.85 (d, 3H, γH Val6), 0.93 (d, 3H, γH Val 2), 0.97 (d, 3H, γH Val 2), 1.41 (d, 3H, βH Ala4), 1.45 (d, 3H, βH Ala8), 1.98 (s, 3H, CH_3 Acm5), 2.02 (s, 3H, CH_3 Acm1), 2.12 (m, 1H, βH Val6), 2.26 (m, 1H, βH Val2), 2.76 (dd, 1H, βH_a Cys1), 2.80 (dd, 1H, βH_a Cys5), 3.07 (dd, 1H, βH_b Cys5), 3.17 (dd, 1H, βH_b Cys1), 4.09 (dd, 1H, CH_a Acm1), 4.23−4.27 (m, 2H, CH_a Acm5 and αH Val6), 4.36 (dd, 1H, αH Cys1), 4.40−4.51 (m, 4H, CH_b Acm5, αH Ala8, αH Ala4 and αH Val2), 4.60−4.73 (m, 5H, βH DSer7, βH DSer3 and αH Cys5), 4.77 (dd, 1H, CH_b Acm1), 5.02 (m, 1H, αH DSer3), 5.09 (m, 1H, αH DSer7), 7.92 (d, 1H, NH Val6), 7.95−8.01 (m, 4H, Qxc CHAr), 8.19−8.27 (m, 6H, NH Val2, NH Cys5 and Qxc CHAr), 8.55 (t, 1H, NH Acm5), 8.60 (d, 1H, NH Ala8), 8.68 (d, 1H, NH Ala4), 8.75 (t, 1H, NH Acm1), 9.20 (d, 1H, NH DSer7), 9.28 (d, 1H, NH DSer3), 9.54 (s, 1H, Qxc H-3), 9.54 (s, 1H, Qxc H-3).

Bis-Acm-Protected Cyclic Depsipeptide (21). To a solution of bis-Acm-protected linear precursor **18** (24.2 mg; 20.3 μmol) in dry DMF (1 mL) at 0 °C was added *N*-methylmorpholine (NMM, 2.2 μL, 20.3 μmol, 1 equiv) and *N*-hydroxysuccinimide (HOSu, 9.3 mg, 81.2 μmol, 4 equiv). The reaction mixture was diluted with dry THF (10 mL) and cooled to 0 °C. EDC (12 mg, 60.9 μmol, 3 equiv) was added, and the reaction was stirred at 0 °C under N_2 for 1 h, then at room temperature until completion (4 h, by HPLC). The solvents were removed under reduced pressure (below 40 °C), and the residue was taken up in ca. 60% aqueous MeCN and lyophilized. Preparative HPLC purification (System B) afforded the desired cyclic depsipeptide as a white solid (14.0 mg; 59%). ES MS ($C_{52}H_{66}N_{14}O_{14}S_2$) 1174.43 *m/z* (%): 1175.2 $[M + H]^+$ (47), 1197.1 $[M + Na]^+$ (100). RP-HPLC (System A): R_t = 15.63 min. ^{1}H NMR (CD_3OH): δ 0.93 (d, 12H, γH Val), 1.51 (d, 6H, βH Ala), 1.97 (s, 6H, CH_3 Acm), 2.35 (m, 2H, βH Val), 3.02 (dd, 2H, βH_a Cys), 3.13 (dd, 2H, βH_b Cys), 4.33 (dd, 2H, CH_a Acm), 4.39 (dd, 2H, CH_b Acm), 4.43 (m, 2H, αH Val), 4.49 (quin, 2H, αH Ala), 4.63 (dd, 2H, βH_a DSer), 4.74 (q, 2H, αH Cys), 4.80 (dd, 2H, βH_b DSer), 4.98 (m, 2H, αH DSer), 7.93 (t, 2H, Qxc CHAr), 7.99 (t, 2H, Qxc CHAr), 8.14 (d, 2H, Qxc CHAr), 8.18 (d, 2H, CHAr), 8.19 (d, 2H, NH Val), 8.55 (d, 4H, NH Ala and NH Cys), 8.62 (t, 2H, NH Acm), 9.19 (d, 2H, NH Ser), 9.49 (s, 2H, Qxc H-3).

TANDEM (4). (Method A). To a solution of disulfide-bridged depsipeptide **12** (35 mg, 33 μmol) and HOBt (26 mg, 0.17 mmol) in 40 mL of dry CH_2Cl_2/DMF 7:1 (v/v) was added EDC (32 mg, 0.17 mmol), and the reaction mixture was stirred for 24 h at 25 °C under N_2. The solvents were removed under reduced pressure, and the residue was taken up in chloroform (ca. 30 mL) and washed three times with water (ca. 10 mL) and twice with saturated $NaHCO_3$ solution (ca. 10 mL). The solution was dried ($MgSO_4$), filtered, and evaporated under reduced pressure. The crude product was purified by flash column chromatography on silica gel, using $CHCl_3$/MeOH 19:1 (v/v) as the mobile phase. Pure fractions were pooled and evaporated, and the residue lyophilized from 60% (v/v) MeCN in water to afford TANDEM (**4**) as a white solid (3.9 mg, 11%). MALDI-TOF MS ($C_{46}H_{54}N_{12}O_{12}S_2$) 1030.34 *m/z* (%): 1031 $[M + H]^+$ (100). RP-HPLC (System A): R_t = 17.03 min. HRMS (ES) calcd for $C_{46}H_{55}N_{12}O_{12}S_2$ $[M + H]^+$ 1031.3499, found 1031.3517. ^{1}H NMR: δ 1.11 (d, 6H, γH Val), 1.15 (d, 6H, γH Val), 1.37 (d, 6H, βH Ala), 2.55 (m, 2H, βH Val), 2.92 (d, 4H, βH Cys), 4.51 (quin, 2H, αH Ala), 4.65 (dd, 2H, βH_a DSer), 4.84 (dd, 2H, αH Val), 4.89 (m, 2H, αH DSer), 5.01 (dd, 2H, βH_b DSer), 5.68 (dd, 2H, αH Cys), 6.41 (d, 2H, NH Ala), 7.13 (d, 2H, NH Cys), 7.92 (t, 2H, Qxc H-7), 7.95 (t, 2H, Qxc H-6), 8.12 (d, 2H, Qxc H-5), 8.25 (d, 2H, Qxc H-8), 8.67 (d, 2H, NH Val), 8.84 (d, 2H, NH DSer), 9.66 (s, 2H, Qxc H-3). ^{13}C NMR: δ 17.9 (γC Val), 18.2 (βC Ala), 18.9 (γC Val), 32.2 (βC Val), 46.2 (βC Cys), 49.6 (αC Ala), 53.4 (αC Cys), 54.7 (αC DSer), 57.6 (αC Val), 65.1 (βC DSer), 129.0 (Qxc C-8), 129.7 (Qxc C-5), 131.7 (Qxc C-7), 132.8 (Qxc C-6), 140.6 (Qxc C-8a), 142.4 (Qxc C-2), 142.8 (Qxc C-3), 143.2 (Qxc C-4a), 164.0 (Qxc CO), 167.8 (CO DSer), 170.6 (CO Cys), 171.0 (CO Val), 173.0 (CO Ala).

(Method B). A solution of iodine (35.7 mg, 0.14 mmol, 10 equiv) in absolute MeOH (4.5 mL) was added dropwise over ca. 60 min to a solution of bis-Acm-protected cyclic depsipeptide **21** (16.5 mg, 14.1 μmol) in absolute MeOH (3 mL). The reaction was complete (HPLC) after addition of the iodine. The reaction mixture was cooled to 0 °C, and 1 M aqueous $Na_2S_2O_3$ solution was slowly added dropwise until the solution became colorless. Water was added until no further precipitation was noted (ca. 20 mL), and the precipitate was filtered and washed with water. The precipitate was extracted into 90% aqueous MeCN, and the solution was diluted with a little water and lyophilized to afford TANDEM (**4**) as an HPLC and NMR pure white powder (14.5 mg, quantitative).

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Supporting Information Available: General experimental procedures, synthesis, and analytical details for compounds **13**−**17**, analytical details for compounds **19** and **20**, and footprinting protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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