Synthesis of Cyclopeptidic Analogues of Triostin A with Quinoxalines or Nucleobases as Chromophores

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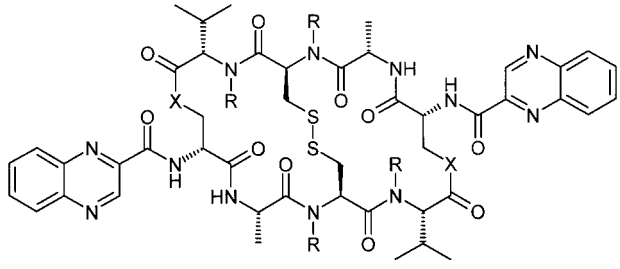
The natural antibiotic triostin A (**1**) is based on a conformationally rigid disulfide-bridged cyclo-octadepsipeptide scaffold. This bicyclic core structure provides a perfect preorganization of two covalently attached quinoxalines resulting in sequence-specific bis(intercalation) of the chromophores in double-stranded DNA. Herein for the first time the corresponding cyclopeptide has been synthesized as a scaffold instead of the cyclodepsipeptide of triostin A by solid-phase peptide synthesis followed by bis(cyclization) in solution. Furthermore, when in contact with DNA the bicyclic peptide provides additional hydrogen-bonding possibilities and greater conformational rigidity in comparison to triostin A. These modifications to the backbone of triostin A might be especially valuable in combination with the use of nucleobases instead of quinoxalines for additional DNA recognition next to bis(intercalation) like major groove binding or detection of abasic DNA damages.

Introduction

Since its first isolation from *Streptomyces* S-2-210,[1] triostin A (**1**)[2,3] is one of the most extensively studied members of the quinoxaline family of antitumor antibiotics.[4,5] As well as the other bicyclic natural products, echinomycin[6,7] and thiocoraline,[8,9] triostin A efficiently blocks both transcription and replication resulting in antibiotic and cytotoxic activity. It is structurally based on a disulfide-bridged cyclooctadepsipeptide composed of two identical tetrapeptides that contain the amino acid sequence D-serine, L-alanine, *N*-methyl-L-cysteine, and *N*-methyl-L-valine (Figure 1). Two quinoxaline chromophores are covalently attached to the serine residues; these two chromophores are at a distance of 10.5 Å which favors the binding of triostin A to double-stranded DNA by bis(intercalation) in the minor groove spanning a dinucleotide.[10,11] Sequence specificity is derived from hydrogen bonding of the depsipeptide scaffold within the DNA minor groove as well as from stacking interactions with nucleobases. Hence, the prevention of hydrogen-bond formation by *N*-methylation of peptide bonds is critical for binding selectivity. Whereas triostin A shows CpG selectivity,[12−14] the demethylated analogue des-*N*-(tetramethyl)triostin (TANDEM, **2**) selectively binds to TpA sequences.[15−17]

As well as the TANDEM derivative **2**, azatriostin (**3**) is another known synthetic modification of triostin A based on a disulfide-bridged cyclopeptide core structure which still contains *N*-methyl groups.[18−20] The substitution of the two ester bonds of the cyclodepsipeptide by amide bonds results in a significant increase in conformational rigidity. In this study, for the first time the bicyclic core structure of des-*N*-(tetramethyl)azatriostin (**4**) was prepared by solid-phase peptide synthesis (SPPS) followed by cyclization reactions. Furthermore, in addition to the modifications of the core structure variations of the quinoxaline chromophores can be addressed.[21−23] In this regard we focused on nucleobases that are covalently linked to the bicyclic scaffold of des-*N*-(tetramethyl)azatriostin instead of quinoxalines as part of our program to develop triostin A analogues with potential new binding modes to DNA double strands.[24] In addition to the binding of the triostin A analogues to



Triostin A (**1**): R = CH_3, X = O
Des-*N*-Tetramethyltriostin (**2**): R = H, X = O
Azatriostin (**3**): R = CH_3, X = NH
Des-*N*-Tetramethylazatriostin (**4**): R = H, X = NH

Figure 1. Structures of triostin A (**1**), des-*N*-(tetramethyl)triostin (TANDEM, **2**), azatriostin (**3**), and des-*N*-(tetramethyl)azatriostin (**4**)

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double-stranded DNA by bis(intercalation) in the minor groove (Figure 2, A) we might also expect major groove binding with specific base-pair recognition at the Hoogsteen site (Figure 2, B). Furthermore, abasic positions known as DNA damages might be specifically detected by our constructs filling in the missing nucleobase and intercalating the second nucleobase (Figure 2, C).[25] In order to generate these different DNA interaction modes it is not entirely predictable whether a depsipeptide or a peptide core structure is more promising. Also the number of *N*-methyl groups and the type of nucleobase are likely to significantly influence the nature of the DNA binding mode. Therefore we have started to synthesize a set of modified cyclopeptide/depsipeptide triostin A compounds with various nucleobases and we report herein the synthesis of des-*N*-(tetramethyl)azatriostin (**4**) as well as its analogues with various pyrimidinyl nucleobases.

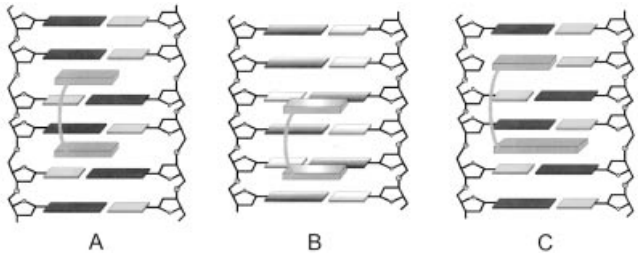


Figure 2. Possible interaction modes of nucleobase-substituted triostin A analogues with double-stranded DNA: (A) bis(intercalation) from the minor groove; (B) Hoogsteen site recognition in the major groove; (C) Watson−Crick recognition of the nucleobase opposed to an abasic site in combination with intercalation of the second nucleobase or chromophore

Results and Discussion

The des-*N*-(tetramethyl)azatriostin backbone is symmetrically composed of two identical tetrapeptides that contain the amino acids D-diaminopropionic acid (Dap), L-alanine, L-cysteine, and L-valine. D-Diaminopropionic acid was chosen instead of the triostin A building block D-serine in order to provide the amide bonds in the cyclopeptide as well as the possibility of linking the quinoxalines or the nucleobases as carboxylic acid derivatives to the α-amino groups.[18] The linear octapeptide **5** (Figure 3) was generated by SPPS and required a protecting group strategy that takes into account the permanent protection of the positions of later disulfide formation and quinoxaline or nucleobase attachment as well as N-terminal Fmoc protection for

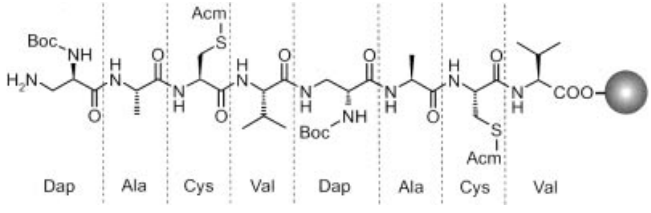


Figure 3. Solid-phase-bound linear octapeptide precursor **5** for the synthesis of des-*N*-(tetramethyl)azatriostin

setting up the peptide chain. Subsequent cyclization reactions were easily performed in solution and the disulfide-bridged cyclopeptide that was obtained was modified at a later stage with quinoxaline or various nucleobases.[26]

Synthesis of the Bicyclic Des-*N*-(tetramethyl)azatriostin Scaffold

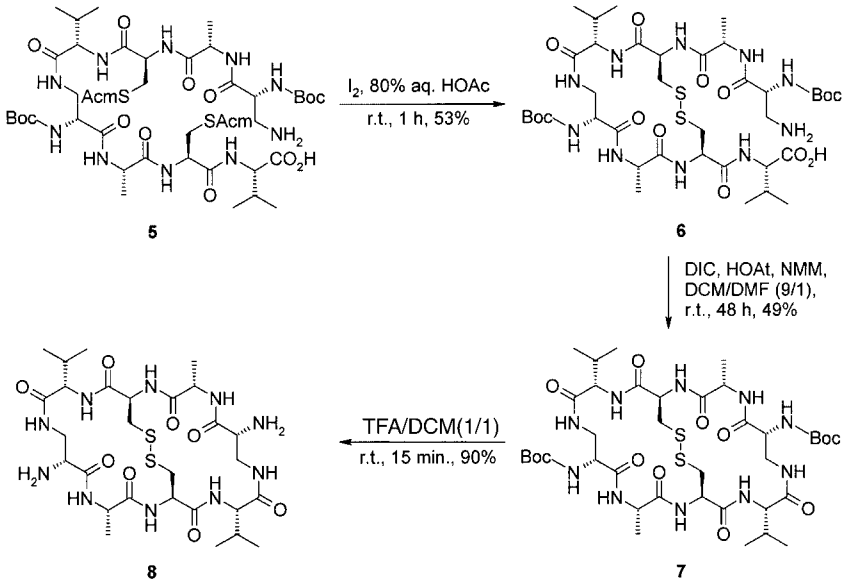
The octapeptide has been prepared previously by manual Fmoc-SPPS protocols[27] without significant formation of by-products by using the amino acids Fmoc-L-Val-OH, Fmoc-L-Cys(Acm)-OH, Fmoc-L-Ala-OH, and Boc-D-Dap(Fmoc)-OH. 2-Chlorotrityl chloride polystyrene has proven to be a suitable resin since the mild cleavage conditions [1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in DCM] tolerate a variety of protecting groups, for example, acetamidomethyl (Acm) and *tert*-butoxycarbonyl (Boc). Valine was loaded onto the resin as the C-terminal amino acid since this was the only way to obtain an amino group bound to a methylene unit for the later macrocyclization step. This cyclization reaction with the Dap side-chain is electronically and sterically beneficial compared with the other possible amide bond formation reactions that would be based on amines bound to higher substituted carbon atoms.

All the amino acids were coupled by activation with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in the presence of Hünig's base with the exception of Fmoc-L-Cys(Acm)−OH which led to considerable epimerization under these conditions.[28−30] Therefore, Fmoc-L-Cys(Acm)−OH was preactivated with *N*,*N*′-diisopropylcarbodiimide (DIC) and Hünig's base and coupled by symmetrical anhydride formation for 1 h. High coupling yields were made possible by the excellent solvating properties of *N*-methylpyrrolidine (NMP) or a NMP/DCM mixture. Cleavage of the peptide from the resin with HFIP provided the linear octapeptide **5** in an overall yield of 87% without significant racemization, as indicated by HPLC purity.

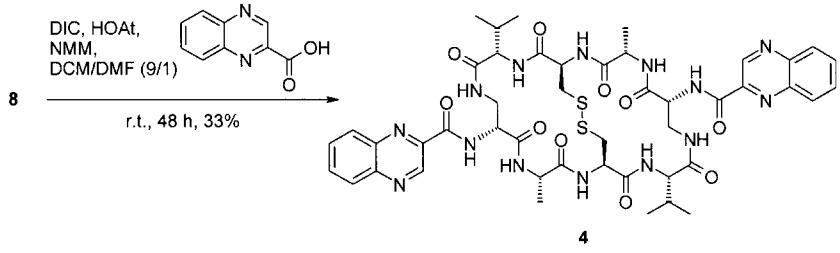
The acetamidomethyl (Acm) protecting group allows iodine-mediated deprotection and formation of the disulfide bond in a single step. Selective intramolecular oxidation to disulfide **6** was performed under high dilution in 80% aqueous acetic acid (Scheme 1).[26,31] The alternative on-resin cyclization in a solution of iodine and DMF turned out to be less efficient than the reaction in solution. The C- and N-terminal ends of the disulfide-bridged peptide **6** were well preorganized to allow the DIC-activated and HOAt-(1-hydroxy-7-azabenzotriazole-)assisted second ring closure to yield the bicyclic peptide **7**. Finally, after cleavage of the Boc side-chain protecting groups of the diaminopropionic acids the des-*N*-(tetramethyl)azatriostin backbone **8** was obtained in 90% yield.

Attachment of Chromophores to the Des-*N*-(tetramethyl)-azatriostin Scaffold 8

Various chromophores can be attached as carboxylic acid derivatives to the des-*N*-(tetramethyl)azatriostin core structure **8** by amide formation. First of all, the respective de-



Scheme 1. Synthesis of the des-*N*-(tetramethyl)azatriostin scaffold **8**



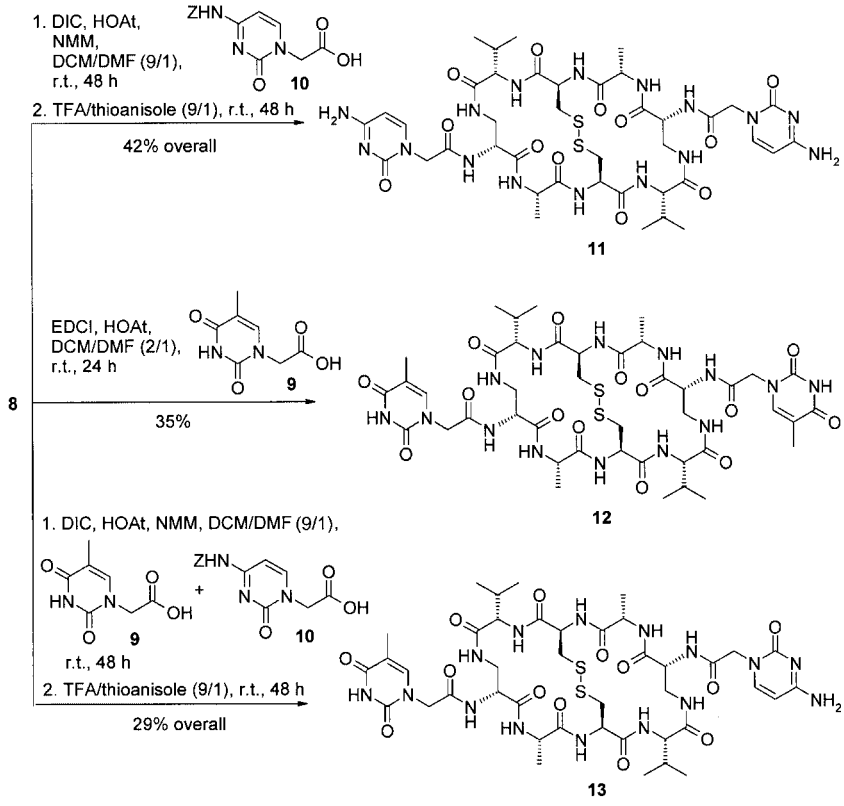
Scheme 2. Synthesis of des-*N*-(tetramethyl)azatriostin **4**

methylated azatriostin analogue **4** was synthesized by attachment of 2-quinoxalinecarboxylic acid to both Dap side-chain amino groups of the peptide scaffold **8** (Scheme 2). The coupling was accomplished by DIC activation of 2-quinoxalinecarboxylic acid in DMF assisted by HOAt to give des-*N*-(tetramethyl)azatriostin **4** in 33% yield.

The first examples of the modification of the bicyclic scaffold **8** with nucleobases were conducted by coupling pyrimidinyl-substituted acetic acids (Scheme 3). Whereas no protecting group was required for the thymine derivative **9**, the exocyclic amino group of cytosine needed to be benzyloxycarbonyl-(Z-)protected which also led to an increase in its solubility. The pyrimidinylacetic acids **9** and **10** were prepared according to the method of Nielsen and co-workers (Scheme 3).[32] For amide coupling of the pyrimidinylacetic acids to the bicyclic scaffold **8**, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI/HOAt) or DIC/HOAt were used as activating reagents both delivering similar yields. Whereas the cytosine-substituted des-*N*-(tetramethyl)azatriostin **11** was isolated in 42% yield after DIC-mediated coupling followed by Z-deprotection under acidic conditions, the thymine-substituted des-*N*-(tetramethyl)azatriostin **12** was synthesized in 35% yield by EDCI activation. For the preparation of des-*N*-(tetramethyl)azatriostin analogues with two different nucleobases, both pyrimidinyl-substituted acetic acids **9** and **10** were used simultaneously in the coupling reaction with the bicyclic scaffold **8** in a combinatorial-like approach. After Z-deprotection the mixed thymine/cytosine-containing des-*N*-(tetramethyl)azatriostin analogue **13** was easily separated from the already known pyrimidinyl derivatives **11** and **12** by preparative HPLC as the main product with a reasonable yield of 29%.

Conclusions

With the synthesis of the bicyclic des-*N*-(tetramethyl)azatriostin scaffold **8** by Fmoc-based SPPS followed by two cyclization steps in solution, a backbone for the preparation of triostin A analogues has been created. Compared with triostin A, this cyclopeptide **8** should provide greater rigidity and a change in the hydrogen-bonding pattern because two ester groups have been replaced by amide bonds. Further scope for hydrogen bonding arises from the missing *N*-methylation. In addition to these structural changes to the bicyclic core structure of triostin A various chromophores were covalently attached to the des-*N*-(tetramethyl)aza-



Scheme 3. Synthesis of nucleobase-substituted des-*N*-(tetramethyl)azatriostins **11**−**13**

triostin scaffold **8**; as well as the original quinoxaline, pyrimidinyl nucleobases were linked to the scaffold. With regard to DNA binding studies, the different binding modes of these compounds are currently being evaluated by cocrystallization, gel electrophoresis, and spectroscopic methods.

Experimental Section

General Remarks: The solvents used in this work were of the highest grade available. Dichloromethane was distilled from calcium hydride prior to use. DMF was purchased dry and stored over molecular sieves (4 Å). Commercially available reagents were of analytical grade and used without further purification. Melting points were obtained with a Büchi 501 Dr. Tottoli apparatus and are uncorrected. Optical rotations were determined with a Perkin−Elmer 241 polarimeter. IR spectra were recorded with a Perkin−Elmer 1600 Series FT-IR spectrometer using KBr pellets. NMR spectra were recorded with a Varian INOVA-600 instrument. Chemical shifts are referenced to the residual solvent peaks of [D_6]DMSO (^{1}H: δ = 2.49 ppm; ^{13}C: δ = 39.5 ppm). ESI-MS data were measured with a LCQ Finnigan spectrometer. HRMS data were determined with a Bruker APEX-Q IV 7T spectrometer. HPLC was performed with a Pharmacia Äkta basic system using YMC J'sphere ODS-H80, RP-C18 columns for both analytical samples (250 × 4.6 mm, 5 μm, 120 Å, 1 mL·min^{-1}) and preparative runs (250 × 20 mm, 5 μm, 120 Å, 10 mL·min^{-1}); eluent A: water/TFA (0.1%); eluent B: acetonitrile/water (10%)/TFA (0.1%). Analytical thin-layer chromatography was performed by using Merck Kieselgel 60 F_{254} precoated aluminium plates and visualized with UV light (254 nm) or by dyeing with ninhydrin (3% in ethanol).

Boc−D-Dap−L-Ala−L-Cys(Acm)−L-Val−β-D-Dap(Boc)−L-Ala−L-Cys(Acm)−L-Val−OH (5): SPPS was performed on a 2-chlorotrityl-TCP resin preloaded with valine (594 mg, 0.85 mmol·g^{-1}, 505 μmol, 1 equiv.). HBTU coupling: Fmoc-amino acid (2.53 mmol, 5 equiv.) and HBTU (958 mg, 5 equiv.) were dissolved in NMP (5 mL). *N*,*N*-Diisopropylethylamine (DIEA) (432 μL, 2.53 mmol, 5 equiv.) was added and the mixture was shaken. After 10 min, the solution was added to the deprotected and NMP-swollen peptidyl resin and shaken for 1 h. After coupling, the resin was washed with NMP (5 × 10 mL). DIC coupling: Fmoc-amino acid (2.53 mmol, 5 equiv.) was dissolved in DCM/NMP (3:1) (5 mL). DIEA (432 μL, 2.53 mmol, 5 equiv.) was added and the mixture was shaken. After 15 min, the solution was added to the deprotected and NMP-swollen peptidyl resin and shaken for 1 h. After coupling, the resin was washed with DCM (5 × 10 mL) and NMP (5 × 10 mL). Deprotection: A solution of DMF/piperidine (4:1) was added to the Fmoc-protected peptidyl resin and shaken for 15 min. After deprotection, the resin was washed with NMP (5 × 10 mL). Cleavage: After Fmoc deprotection the peptidyl resin was shaken with DCM/HFIP (4:1) for 45 min and then washed (2 × 5 min) with the same solution. The combined filtrates were concentrated to dryness and the resulting residue was washed with diethyl ether (3 × 10 mL). The white solid was dissolved in water (100 mL) and lyophilized to give the linear octapeptide **5** (474 mg, 439 μmol, 87%) as a fluffy white solid with a purity of >98% as determined by HPLC (5−60% in 30 min, R_t = 21.2 min). M.p. 200 °C (decomp.). R_F (chloroform/methanol, 7:2) = 0.52. $[\alpha]_D^{25}$ = −14.0 (MeOH, *c* = 0.0125). IR (KBr): $\tilde{\nu}$ = 3430, 2968, 2368, 1636, 1540, 1368, 1251, 1164, 590 cm^{-1}. ^{1}H NMR (600 MHz, [D_6]DMSO, 35 °C): δ = 0.80 (d, ^3J =

7 Hz, 6 H, Val-CH_3), 0.83 (d, ^3J = 5 Hz, 3 H, Val-CH_3), 0.84 (d, ^3J = 5 Hz, 3 H, Val-CH_3), 1.20 (d, ^3J = 6 Hz, 3 H, Ala-CH_3), 1.21 (d, ^3J = 6 Hz, 3 H, Ala-CH_3), 1.36 (s, 9 H, Boc-CH_3), 1.37 (s, 9 H, Boc-CH_3), 1.84 (s, 3 H, Acm-CH_3), 1.85 (s, 3 H, Acm-CH_3), 1.98−2.10 (m, 2 H, Val-Hβ), 2.67−2.76 (m, 2 H, Cys-Hβ), 2.84−2.98 (m, 4 H, Cys-Hβ, Dap-Hβ), 3.31−3.42 (m, 2 H, Dap-Hβ), 4.00 (dd, ^3J = 9, ^3J = 5 Hz, 1 H, Val-Hα), 4.02−4.07 (m, 1 H, Dap-Hα), 4.12−4.19 (m, 4 H, Val-Hα, Dap-Hα, Acm-CH_2), 4.24−4.36 (m, 4 H, Acm-CH_2, Ala-Hα), 4.55−4.60 (m, 1 H, Cys-Hα), 4.60−4.66 (m, 1 H, Cys-Hα), 6.72 (d, ^3J = 7 Hz, 1 H, Dap-NHα), 7.14 (d, ^3J = 7 Hz, 1 H, Dap-NHα), 7.60 (d, ^3J = 7 Hz, 1 H, Val-NH), 7.74−7.82 (m, 2 H, Val-NH, Ala-NH), 7.88−7.93 (m, 1 H, Dap-NHβ), 8.20−8.29 (m, 3 H, Cys-NH, Dap-NH_2), 8.37−8.42 (m, 1 H, Cys-NH), 8.47−8.56 (m, 3 H, Ala-NH, Acm-NH) ppm. ^{13}C NMR (150 MHz, [D$_6$]DMSO, 35 °C): δ = 17.5, 17.9 (Val-CH_3), 18.4, 18.6 (Ala-CH_3), 19.1, 19.2 (Val-CH_3), 22.5 (Acm-CH_3), 28.0, 28.1 (Boc), 30.3, 30.5 (Val-CHβ), 31.8, 32.2 (Cys-CH_2β), 40.0, 40.1 (Acm-CH_2), 40.5, 41.6 (Dap-CH_2β), 48.2 (Ala-CHα), 52.5, 52.6 (Cys-CHα), 54.3, 54.8 (Dap-CHα), 57.5, 58.2 (Val-CHα), 78.5 (Boc), 154.9, 155.3 (Boc-CO), 169.4, 169.5, 169.8, 169.9, 170.0, 171.3, 172.1, 173.7 (Acm-CO, Dap-CO, Cys-CO, Val-CO, Ala-CO) ppm. ESI-MS: *m*/*z* (%) = 1101.5 (100) [M + Na]$^+$. HRMS (ESI): calcd. for $C_{44}H_{78}N_{12}O_{15}S_2$: 1079.5224; found: 1079.5230 [M + H]$^+$.

Boc−D-Dap−L-Ala−L-Cys−L-Val−β-D-Dap(Boc)−L-Ala−L-Cys−L-Val−OH Disulfide (6): Iodine (124 mg, 489 μmol, 10 equiv.) was added in one portion to a solution of octapeptide **5** (52.8 mg, 48.9 μmol) in 80% aqueous acetic acid (25 mL). The suspension was stirred for 1 h at room temperature, before being diluted with water (25 mL). The solution was extracted with tetrachloromethane (6 × 20 mL) until the aqueous phase was colorless. Coevaporation with toluene (5 × 20 mL) gave a residue which was then diluted with methanol, filtered, and purified by HPLC (30−60% in 30 min, R_t = 19.2 min) to afford the disulfide-bridged peptide **6** (24.1 mg, 25.8 μmol, 53%). M.p. 175−180 °C (decomp.). R_F (chloroform/methanol, 6:1) = 0.34. $[\alpha]_D^{25}$ = −10.0 (MeOH, *c* = 0.0075). IR (KBr): $\tilde{\nu}$ = 3447, 2369, 1653, 669 cm^{-1}. ^{1}H NMR (600 MHz, [D$_6$]DMSO, 35 °C): δ = 0.80 (d, ^3J = 7 Hz, 3 H, Val-CH_3), 0.82 (d, ^3J = 7 Hz, 3 H, Val-CH_3), 0.85 (d, ^3J = 6 Hz, 3 H, Val-CH_3), 0.86 (d, ^3J = 6 Hz, 3 H, Val-CH_3), 1.20 (d, ^3J = 7 Hz, 3 H, Ala-CH_3), 1.25 (d, ^3J = 7 Hz, 3 H, Ala-CH_3), 1.38 (s, 9 H, Boc-CH_3), 1.39 (s, 9 H, Boc-CH_3), 2.03−2.14 (m, 2 H, Val-Hβ), 2.89−3.02 (m, 3 H, Cys-Hβ, Dap-Hβ), 3.10−3.16 (m, 2 H, Cys-Hβ, Dap-Hβ), 3.20−3.25 (m, 1 H, Cys-Hβ), 3.30−3.35 (m, 1 H, Dap-Hβ), 3.39−3.46 (m, 1 H, Dap-Hβ), 3.99−4.06 (m, 2 H, Val-Hα, Dap-Hα), 4.14−4.18 (m, 1 H, Val-Hα), 4.18−4.22 (m, 1 H, Ala-Hα), 4.22−4.28 (m, 1 H, Cys-Hα), 4.31−4.37 (m, 1 H, Ala-Hα), 4.62−4.69 (m, 1 H, Dap-Hα), 4.74−4.82 (m, 1 H, Cys-Hα), 6.91−6.96 (m, 1 H, Dap-NHα), 7.08−7.13 (m, 1 H, Cys-NH), 7.58−7.66 (m, 2 H, Ala-NH, Dap-NHβ), 7.82−8.07 (m, 5 H, Val-NH, Ala-NH, DapNH_2), 8.33−8.39 (m, 1 H, Dap-NHα), 8.43−8.48 (m, 1 H, Cys-NHα) ppm. ^{13}C NMR (150 MHz, [D$_6$]DMSO, 35 °C): δ = 17.6 (Val-CH_3), 18.0, 18.5 (Ala-CH_3), 18.9, 19.0 (Val-CH_3), 28.0 (Boc), 29.4, 30.0 (Val-CHβ), 40.0 (Cys-CH_2β), 40.7, 41.6 (Dap-CH_2β), 42.5 (Cys-CH_2β), 48.2, 48.4 (Ala-CHα), 52.1 (2 × Cys-CHα, Dap-CHα), 54.7 (Dap-CHα), 57.0, 58.4 (Val-CHα), 78.7, 79.0 (Boc), 154.6, 155.2, (Boc-CO), 168.4, 169.3, 169.7, 169.8, 171.5, 172.0, 172.5, 172.6 (Dap-CO, Cys-CO, Val-CO, Ala-CO) ppm. ESI-MS: *m*/*z* (%) = 957.4 (100) [M + Na]$^+$. HRMS (ESI): calcd. for $C_{38}H_{66}N_{10}O_{13}S_2$: 935.4325; found: 935.4326 [M + H]$^+$.

Cyclo[β-D-Dap(Boc)−L-Ala−L-Cys−L-Val−β-D-Dap(Boc)−L-Ala−L-Cys−L-Val] Disulfide (7): A solution of the disulfide-bridged peptide **6** (41.7 mg, 44.6 μmol) in dry DCM/DMF (9:1) (25 mL) was cooled to 0 °C and, whilst stirring, treated sequentially with HOAt (6.06 mg, 44.6 μmol, 1 equiv.), NMM (14.7 μL, 134 μmol, 3 equiv.), and DIC (69.8 μL, 446 μmol, 10 equiv.) under argon. After stirring for 1 h, the mixture was allowed to react at room temperature for 48 h, before being concentrated to dryness. The residue was dissolved in methanol, filtered, and purified by HPLC (30−80% in 30 min, R_t = 24.6 min) to give the bicyclic peptide **7** (19.9 mg, 21.7 μmol, 49%) as a white solid. M.p. 210−215 °C. R_F (chloroform/methanol, 10:1) = 0.54. $[\alpha]_D^{25}$ = −43.3 (MeOH, *c* = 0.075). IR (KBr): $\tilde{\nu}$ = 3431, 2364, 1653, 1507, 1166, 669 cm^{-1}. ^{1}H NMR (600 MHz, [D$_6$]DMSO, 35 °C): δ = 0.72 (d, ^3J = 7 Hz, 6 H, Val-CH_3), 0.91 (d, ^3J = 7 Hz, 6 H, Val-CH_3), 1.09−1.15 (m, 6 H, Ala-CH_3), 1.34 (s, 18 H, Boc-CH_3), 2.46−2.53 (m, 2 H, Val-Hβ), 2.92 (dd, ^3J = 14, ^3J = 16 Hz, 2 H, Cys-Hβ), 3.03 (d, ^3J = 12 Hz, 2 H, Dap-Hβ), 3.18 (d, ^3J = 14 Hz, 2 H, Cys-Hβ), 3.81−3.85 (m, 2 H, Dap-Hα), 3.86−3.91 (m, 2 H, Dap-Hβ), 4.32 (d, ^3J = 11 Hz, 2 H, Val-Hα), 4.49−4.57 (m, 2 H, Ala-Hα), 4.84−4.92 (m, 2 H, Cys-Hα), 6.44−6.50 (m, 2 H, Dap-NHα), 6.84 (d, ^3J = 8 Hz, 2 H, Ala-NH), 7.64 (d, ^3J = 10 Hz, 2 H, Val-NH), 7.92−7.97 (m, 2 H, Dap-NHβ), 8.96 (d, ^3J = 9 Hz, 2 H, Cys-NH) ppm. ^{13}C NMR (150 MHz, [D$_6$]DMSO, 35 °C): δ = 15.1, 18.6 (Val-CH_3), 19.5 (Ala-CH_3), 27.8 (Boc), 29.3 (Val-CHβ), 39.4 (Dap-CH_2β), 40.1 (Cys-CH_2β), 46.5 (Ala-CHα), 53.8 (Cys-CHα), 56.6 (Val-CHα), 57.8 (Dap-CHα), 78.9 (Boc), 154.5 (Boc-CO), 170.1, 170.6, 172.9, 173.1 (Dap-CO, Cys-CO, Val-CO, Ala-CO) ppm. ESI-MS: *m*/*z* (%) = 939.4 (100) [M + Na]$^+$. HRMS (ESI): calcd. for $C_{38}H_{64}N_{10}O_{12}S_2$: 917.4219; found: 917.4218 [M + H]$^+$.

Cyclo(β-D-Dap−L-Ala−L-Cys−L-Val−β-D-Dap−L-Ala−L-Cys−L-Val) Disulfide (8): A mixture of DCM/TFA (1:1) (10 mL) was added to the bicyclic peptide **7** (8.10 mg, 8.83 μmol) and stirred at room temperature for 15 min. After concentration to dryness, the residue was dissolved in methanol, filtered, and purified by HPLC (5−40% in 30 min, R_t = 19.1 min) to give the des-*N*-(tetramethyl)azatriostin core structure **8** (5.68 mg, 7.92 μmol, 90%) as a white solid. M.p. 220−225 °C (decomp.). R_F (chloroform/methanol, 8:1) = 0.40. $[\alpha]_D^{25}$ = 12.5 (MeOH, *c* = 0.03). IR (KBr): $\tilde{\nu}$ = 3431, 2344, 1654, 1540, 1203, 1136, 838, 800, 723 cm^{-1}. ^{1}H NMR (600 MHz, [D$_6$]DMSO, 35 °C): δ = 0.82 (d, ^3J = 7 Hz, 12 H, Val-CH_3), 1.38 (d, ^3J = 7 Hz, 6 H, Ala-CH_3), 2.19−2.26 (m, 2 H, Val-Hβ), 2.90 (m, 2 H, Cys-Hβ), 2.95−3.05 (m, 2 H, Cys-Hβ), 3.46 (m, 2 H, Dap-Hβ), 3.74−3.81 (m, 2 H, Dap-Hβ), 3.83−3.87 (m, 2 H, Dap-Hα), 4.15−4.19 (m, 2 H, Val-Hα), 4.22−4.28 (m, 2 H, Ala-Hα), 5.04−5.14 (m, 2 H, Cys-Hα), 7.70−7.78 (m, 2 H, Val-NH), 7.95−8.02 (m, 2 H, Dap-NHβ), 8.11−8.15 (m, 2 H, Ala-NH), 8.15−8.30 (m, 4 H, Dap-NH_2α), 8.80−8.95 (m, 2 H, Cys-NH) ppm. ^{13}C NMR (150 MHz, [D$_6$]DMSO, 35 °C): δ = 16.9 (Val-CH_3), 18.0 (Ala-CH_3), 19.4 (Val-CH_3), 29.6 (Val-CHβ), 39.2 (Dap-CH_2β), 42.2 (Cys-CH_2β), 49.1 (Ala-CHα), 53.2 (Dap-CHα, Cys-CHα), 57.8 (Val-CHα), 166.0, 169.6, 171.8, 172.6 (Dap-CO, Cys-CO, Val-CO, Ala-CO) ppm. ESI-MS: *m*/*z* (%) = 717.3 (100) [M + H]$^+$. HRMS (ESI): calcd. for $C_{28}H_{48}N_{10}O_8S_2$: 717.3171; found: 717.3173 [M + H]$^+$.

Des-*N*-(tetramethyl)azatriostin (4): A solution of cyclopeptide **8** (9.02 mg, 12.6 μmol) in dry DCM/DMF (9:1) (15 mL) was cooled to 0 °C and, whilst stirring, treated sequentially with HOAt (6.85 mg, 50.3 μmol, 4 equiv.), NMM (10.6 μL, 151 μmol, 12 equiv.), DIC (78.4 μL, 503 μmol, 40 equiv.), and 2-quinoxalinecarboxylic acid (8.77 mg, 50.3 μmol, 4 equiv.) under argon. After stirring for 1 h, the mixture was allowed to react at room temperature for 48 h, before being concentrated to dryness. The residue was dissolved in methanol, filtered, and purified by HPLC (30−80% in

30 min, R_t = 18.6 min) to give des-*N*-(tetramethyl)azatriostin (**4**, 4.27 mg, 4.15 μmol, 33%) as a white solid. M.p. 225−230 °C. R_F (chloroform/methanol, 10:1) = 0.42. $[\alpha]_D^{25}$ = −137 (DMSO, *c* = 0.075). IR (KBr): $\tilde{\nu}$ = 3443, 2930, 2363, 1652, 1536 cm^{-1}. ^1H NMR (600 MHz, [D$_6$]DMSO, 35 °C): δ = 0.71 (d, ^3J = 7 Hz, 6 H, Val-CH$_3$), 0.84 (d, ^3J = 7 Hz, 6 H, Val-CH$_3$), 1.14 (d, ^3J = 7 Hz, 6 H, Ala-CH$_3$), 2.56−2.62 (m, 2 H, Val-Hβ), 2.93 (dd, ^3J = 12, ^3J = 15 Hz, 2 H, Cys-Hβ), 3.23 (dd, ^3J = 3, ^3J = 15 Hz, 2 H, Cys-Hβ), 3.32−3.37 (m, 2 H, Dap-Hβ), 4.03−4.08 (m, 2 H, Dap-Hβ), 4.37−4.39 (m, 2 H, Dap-Hα), 4.39−4.41 (m, 2 H, Val-Hα), 4.54−4.61 (m, 2 H, Ala-Hα), 5.01−5.07 (m, 2 H, Cys-Hα), 7.20 (d, ^3J = 8 Hz, 2 H, Ala-NH), 7.77−7.81 (m, 2 H, Dap-NHβ), 7.87 (d, ^3J = 10 Hz, 2 H, Val-NH), 7.94−8.00 (m, 4 H, quinoxaline-H6, quinoxaline-H7), 8.05 (d, ^3J = 8 Hz, 2 H, quinoxaline-H5), 8.19 (d, ^3J = 8 Hz, 2 H, quinoxaline-H8), 8.90 (d, ^3J = 9 Hz, 2 H, Cys-NH), 9.28 (d, ^3J = 6 Hz, 2 H, Dap-NHα), 9.45 (s, 2 H, quinoxaline-H3) ppm. ^{13}C NMR (150 MHz, [D$_6$]DMSO, 35 °C): δ = 16.0 (Val-CH$_3$), 19.0 (Val-CH$_3$), 19.1 (Ala-CH$_3$), 29.7 (Val-CHβ), 40.0 (Dap-CH$_2$β), 41.4 (Cys-CH$_2$β), 47.1 (Ala-CHα), 54.0 (Cys-CHα), 56.5 (Dap-CHα), 56.8 (Val-CHα), 129.0 (quinoxaline-C5), 129.1 (quinoxaline-C8), 131.4 (quinoxaline-C6), 132.0 (quinoxaline-C7), 139.6 (quinoxaline-C2, C4a, C8a), 143.0 (quinoxaline-C2, C4a, C8a), 143.2 (quinoxaline-C3), 143.3, (quinoxaline-C2, C4a, C8a), 163.6, 168.1, 170.1, 172.4, 173.4 (Dap-CO, Cys-CO, Val-CO, Ala-CO, quinoxaline-CO) ppm. ESI-MS: *m*/*z* (%) = 1029.4 (100) [M + H]$^+$. HRMS (ESI): calcd. for $C_{46}H_{56}N_{14}O_{10}S_2$: 1029.3818; found: 1029.3818 [M + H]$^+$.

Cyclo[β-D-Dap(cytosin-1-ylacetyl)−L-Ala−L-Cys−L-Val−β-D-Dap-(cytosin-1-ylacetyl)−L-Ala−L-Cys−L-Val] Disulfide (11): A solution of cyclopeptide **8** (10.0 mg, 13.9 μmol) in dry DCM/DMF (9:1) (15 mL) was cooled to 0 °C and, whilst stirring, treated sequentially with HOAt (7.57 mg, 55.6 μmol, 4 equiv.), NMM (18.4 μL, 167 μmol, 12 equiv.), DIC (87.4 μL, 556 μmol, 40 equiv.), and (N^4-Z-cytosin-1-yl)acetic acid[32] (16.9 mg, 55.6 μmol, 4 equiv.) under argon. After stirring for 1 h, the mixture was allowed to react at room temperature for 48 h, before being concentrated to dryness. TFA/thioanisole (10:1) (5 mL) was added and the mixture was stirred at room temperature for another 48 h and then concentrated in vacuo. The residue was dissolved in methanol, filtered, and purified by HPLC (5−50% in 30 min, R_t = 17.1 min) to give cytosinyl-substituted triostin analogue **11** (6.00 mg, 5.89 μmol, 42%) as a white solid. M.p. 265−270 °C (decomp.). R_F (chloroform/methanol, 2:1) = 0.53. $[\alpha]_D^{25}$ = 0.033 (DMSO, *c* = 32.5). IR (KBr): $\tilde{\nu}$ = 3432, 2367, 1654, 1540, 1458, 1203, 1139, 669 cm^{-1}. ^1H NMR (600 MHz, [D$_6$]DMSO, 35 °C): δ = 0.78 (d, ^3J = 7 Hz, 6 H, Val-CH$_3$), 0.85 (d, ^3J = 7 Hz, 6 H, Val-CH$_3$), 1.21 (d, ^3J = 7 Hz, 6 H, Ala-CH$_3$), 2.30−2.36 (m, 2 H, Val-Hβ), 2.91 (dd, ^3J = 12, ^3J = 15 Hz, 2 H, Cys-Hβ), 3.06 (d, ^3J = 12 Hz, 2 H, Cys-Hβ), 3.31−3.36 (m, 2 H, Dap-Hβ), 3.64−3.69 (m, 2 H, Dap-Hβ), 4.18−4.22 (m, 2 H, Dap-Hα), 4.26 (dd, ^3J = 10, ^3J = 4 Hz, 2 H, Val-Hα), 4.36−4.42 (m, 2 H, Ala-Hα), 4.48−4.52 (m, 4 H, acetyl-CH$_2$), 5.08 (m, 2 H, Cys-Hα), 6.09 (d, ^3J = 7 Hz, 2 H, cytosine-H5), 7.16 (d, ^3J = 6 Hz, 2 H, Ala-NH), 7.51−7.56 (m, 2 H, Dap-NHβ), 7.93 (d, ^3J = 7 Hz, 2 H, cytosine-H6), 7.99 (d, ^3J = 10 Hz, 2 H, Val-NH), 8.34−8.39 (m, 2 H, Dap-NHα), 8.85 (br. s, 2 H, cytosine-NH), 8.89 (d, ^3J = 9 Hz, 2 H, Cys-NH), 9.44 (br. s, 2 H, cytosine-NH) ppm. ^{13}C NMR (150 MHz, [D$_6$]DMSO, 35 °C): δ = 16.5 (Val-CH$_3$), 18.3 (Ala-CH$_3$), 19.3 (Val-CH$_3$), 29.4 (Val-CHβ), 40.6 (Dap-CH$_2$β), 42.2 (Cys-CH$_2$β), 47.7 (Ala-CHα), 51.4 (acetyl-CH$_2$), 53.9 (Cys-CHα), 55.7 (Dap-CHα), 56.9 (Val-CHα), 93.5 (cytosine-C5), 148.6 (cytosine-C4), 150.0 (cytosine-C6), 160.5, 166.5, 168.0, 169.8, 172.5, 173.5 (Dap-CO, Cys-CO, Val-CO, Ala-CO, acetyl-CO, cytosine-C2) ppm. ESI-MS: *m*/*z* (%) = 1019.5 (100) [M + H]$^+$. HRMS (ESI): calcd. for $C_{40}H_{58}N_{16}O_{12}S_2$: 1019.3934; found: 1019.3941 [M + H]$^+$.

Cyclo[β-D-Dap(thymin-1-ylacetyl)−L-Ala−L-Cys−L-Val−β-D-Dap-(thymin-1-ylacetyl)−L-Ala−L-Cys−L-Val] Disulfide (12): A solution of cyclopeptide **8** (22.1 mg, 30.8 μmol) in dry DCM/DMF (2:1) (15 mL) was cooled to 0 °C and, whilst stirring, treated sequentially with HOAt (16.8 mg, 123 μmol, 4 equiv.), EDCI (23.6 mg, 123 μmol, 4 equiv.), and (thymin-1-yl)acetic acid[32] (22.7 mg, 123 μmol, 4 equiv.) under argon. After stirring for 1 h, the mixture was allowed to react at room temperature for 48 h and then concentrated to dryness. The residue was dissolved in methanol, filtered, and purified by HPLC (5−50% in 30 min, R_t = 22.4 min) to give thyminyl-substituted triostin analogue **12** (11.4 mg, 10.9 μmol, 35%) as a white solid. M.p. 255−260 °C (decomp.). R_F (chloroform/methanol, 5:1) = 0.47. $[\alpha]_D^{25}$ = 26.0 (MeOH, *c* = 0.013). IR (KBr): $\tilde{\nu}$ = 3442, 2368, 1654, 1521, 1458, 670 cm^{-1}. ^1H NMR (600 MHz, [D$_6$]DMSO, 35 °C): δ = 0.78 (d, ^3J = 5 Hz, 6 H, Val-CH$_3$), 0.85 (d, ^3J = 5 Hz, 6 H, Val-CH$_3$), 1.21 (d, ^3J = 6 Hz, 6 H, Ala-CH$_3$), 1.76 (s, 6 H, thymine-CH$_3$), 2.30−2.36 (m, 2 H, Val-Hβ), 2.90 (dd, ^3J = 13, ^3J = 16 Hz, 2 H, Cys-Hβ), 3.08 (d, ^3J = 13 Hz, 2 H, Cys-Hβ), 3.32 (m, 2 H, Dap-Hβ), 3.64−3.70 (m, 2 H, Dap-Hβ), 4.16−4.21 (m, 2 H, Dap-Hα), 4.27 (d, ^3J = 9 Hz, 2 H, Val-Hα), 4.32 (m, 4 H, acetyl-CH$_2$), 4.37−4.42 (m, 2H Ala-Hα), 5.06−5.12 (m, 2H Cys-Hα), 7.04−7.10 (m, 2H Ala-NH), 7.47 (s, 2 H, thymine-H6), 7.49−7.54 (m, 2 H, Dap-NHβ), 8.04 (d, ^3J = 9 Hz, 2 H, Val-NH), 8.20−8.24 (m, 2 H, Dap-NHα), 8.87 (m, 2 H, Cys-NH), 11.24 (s, 2 H, thymine-NH) ppm. ^{13}C NMR (150 MHz, [D$_6$]DMSO, 35 °C): δ = 11.9 (thymine-CH$_3$), 16.5 (Val-CH$_3$), 18.4 (Ala-CH$_3$), 19.4 (Val-CH$_3$), 29.3 (Val-CHβ), 40.6 (Dap-CH$_2$β), 42.3 (Cys-CH$_2$β), 47.6 (Ala-CHα), 50.5 (acetyl-CH$_2$), 53.9 (Cys-CHα), 55.9 (Dap-CHα), 56.8 (Val-CHα), 108.5 (thymine-C5), 141.7 (thymine-C6), 151.1 (thymine-C2), 164.3 (thymine-C4), 167.5, 168.1, 169.8, 172.5, 173.5 (Dap-CO, Cys-CO, Val-CO, Ala-CO, acetyl-CO) ppm. ESI-MS: *m*/*z* (%) = 1071.6 (100) [M + Na]$^+$. HRMS (ESI): calcd. for $C_{42}H_{60}N_{14}O_{14}S_2$: 1049.3928; found: 1049.3941 [M + H]$^+$.

Cyclo[β-D-Dap(cytosin-1-ylacetyl)−L-Ala−L-Cys−L-Val−β-D-Dap-(thymin-1-ylacetyl)−L-Ala−L-Cys−L-Val] Disulfide (13): A solution of cyclopeptide **8** (8.12 mg, 11.3 μmol) in dry DCM/DMF (9:1) (10 mL) was cooled to 0 °C and, whilst stirring, treated sequentially with HOAt (6.17 mg, 45.3 μmol, 4 equiv.), NMM (14.9 μL, 136 μmol, 12 equiv.), DIC (70.9 μL, 453 μmol, 40 equiv.), (N^4-Z-cytosin-1-yl)acetic acid[32] (7 mg, 22.7 μmol, 2 equiv.), and (thymin-1-yl)acetic acid[32] (4.17 mg, 22.7 μmol, 2 equiv.) under argon. After stirring for 1 h, the mixture was allowed to react at room temperature for 48 h and was then concentrated to dryness. TFA/thioanisole (10:1) (5 mL) was added and the mixture was stirred at room temperature for another 48 h and then concentrated in vacuo. The residue was dissolved in methanol, filtered, and purified by HPLC (5−50% in 30 min, R_t = 19.4 min) to give the mixed pyrimidinyl-substituted triostin analogue **13** (3.39 mg, 3.28 μmol, 29%) as a white solid. M.p. 236−242 °C (decomp.). R_F (chloroform/methanol, 3:1) = 0.47. $[\alpha]_D^{25}$ = 43.3 (MeOH, *c* = 0.005). IR (KBr): $\tilde{\nu}$ = 3433, 2922, 2372, 1654, 1541, 1385, 1205, 1140, 669 cm^{-1}. ^1H NMR (600 MHz, [D$_6$]DMSO, 35 °C): δ = 0.78 (d, ^3J = 6 Hz, 3 H, Val-CH$_3$), 0.79 (d, ^3J = 6 Hz, 3 H, Val-CH$_3$), 0.85 (d, ^3J = 6 Hz, 3 H, Val-CH$_3$), 0.86 (d, ^3J = 6 Hz, 3 H, Val-CH$_3$), 1.21 (d, ^3J = 3 Hz, 3 H, Ala-CH$_3$), 1.22 (d, ^3J = 3 Hz, 3 H, Ala-CH$_3$), 1.76 (s, 3 H, thymine-CH$_3$), 2.30−2.38 (m, 2 H, Val-Hβ), 2.90 (dd, ^3J = 13, ^3J = 15 Hz, 2 H, Cys-Hβ), 3.07 (dd, ^3J = 10, ^3J = 15 Hz, 2 H, Cys-Hβ), 3.33−3.39 (m, 2 H, Dap-Hβ), 3.64−3.69 (m, 2 H, Dap-Hβ), 4.17−4.21 (m, 2 H, Dap-Hα), 4.26 (m, 2 H, Val-Hα),

4.30−4.47 (m, 6 H, Ala-Hα, acetyl-CH_2), 5.06−5.13 (m, 2 H, Cys-Hα), 5.98 (br. s, cytosine-H5), 7.07 (m, 1 H, Ala-NH), 7.20 (m, 1 H, Ala-NH), 7.47 (s, 1 H, thymine-H6), 7.47−7.53 (m, 2 H, Dap-NHβ), 7.83 (d, ^3J = 7 Hz, 1 H, cytosine-H6), 8.03 (d, ^3J = 10 Hz, 1 H, Val-NH), 8.06 (d, ^3J = 10 Hz, 1 H, Val-NH), 8.23 (d, ^3J = 6 Hz, 1 H, Dap-NHα), 8.29 (d, ^3J = 6 Hz, 1 H, Dap-NHα), 8.85−8.90 (m, 2 H, Cys-NH), 11.24 (s, 1 H, thymine-NH) ppm. ^{13}C NMR (150 MHz, [D_6]DMSO, 35 °C): δ = 11.8 (thymine-CH_3), 16.5, 16.6 (Val-CH_3), 18.2, 18.3 (Ala-CH_3), 19.3, 19.4 (Val-CH_3), 29.3 (Val-CHβ), 40.6 (Dap-CH_2β), 42.3 (Cys-CH_2β), 47.7 (Ala-CHα), 50.5 (acetyl-CH_2), 51.9(acetyl-CH_2), 53.9 (Cys-CHα), 55.7 (Dap-CHα), 56.8 (Val-CHα), 93.6 (cytosine-C5), 108.5 (thymine-C5), 141.7 (thymine-C6), 151.2 (thymine-C2), 164.2, 167.4, 168.0, 168.1, 169.8, 172.4, 172.5, 173.5 ppm. ESI-MS: *m*/*z* (%) = 1056.4 (100) $[M + Na]^+$. HRMS (ESI): calcd. for $C_{41}H_{59}N_{15}O_{13}S_2$: 1034.3931; found: 1034.3933 $[M + H]^+$.

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