Triostin A Derived Cyclopeptide as Architectural Template for the Alignment of Four Recognition Units**

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The DNA bisintercalator triostin A is structurally based on a disulfide-bridged depsipeptide scaffold that provides preorganization of two quinoxaline units in 10.5 Å distance. Triostin A analogues are synthesized with nucleobase recognition units replacing the quinoxalines and containing two additional recognition units in between. Thus, four nucleobase recognition units are organized on a rigid template, well suited for DNA double strand interactions. The new tetra-nucleobase binders are synthesized as aza-TANDEM derivatives lacking the N-methylation of triostin A and based on a cyclopeptide backbone. Synthesis of two tetra-nucleobase aza-TANDEM derivatives is established, DNA interaction analyzed by microscale thermophoresis, cytotoxic activity studied and a nucleobase sequence dependent self-aggregation investigated by mass spectrometry.

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Introduction

Triostin A, derived from Streptomyces triostinicus, is a member of the quinoxaline antibiotics that binds DNA by bisintercalation.[1] It is characterized by a tetra-N-methylated and disulfide-bridged cyclic octadepsipeptide backbone and two covalently side chain-linked quinoxaline chromophores. Potent antitumor activity originates from insertion of the two planar chromophores with a distance of 10.5 Å between the nucleobases into the minor groove of double stranded DNA.[2] The triostin backbone provides a preorganization of the recognition units with respect to DNA binding and a preferential spanning of CpG dinucleotides by bisintercalation. Triostin A derivatives were investigated lacking N-methylation (des-N-tetramethyl, TANDEM)[3] and using cyclopeptides replacing the ester linkages by amides (aza-triostin, aza-TANDEM).[4] TANDEM also binds to DNA double strands, nevertheless, with a TpA preference.[4]

The additional nucleobases were introduced as peptoid building blocks replacing the native valines in the TANDEM backbone by N-alkylated glycine amino acids. Binding studies of tetra-nucleobase aza-TANDEM derivatives 1 (sequence ATTA) and 2 (sequence TTTT) (Figure 2) with DNA double strands performed by microscale thermophoresis indicate a sequence dependent recognition. For the tetra-thymyl derivative 2 a potential for self-association and aggregation was indicated by mass spectrometry.

Results and Discussion

The position for covalent attachment of the additional nucleobases on the aza-TANDEM backbone was chosen based on simple model analysis (Figure 1). All nucleobases were intended to be aligned and oriented in the same direction with a comparable spacing from the cyclopeptide backbone. Furthermore, equidistant stacking of all nucleobases was envisaged. Linkage of the nucleobases to the amide N of the valine amino acids in the TANDEM cyclopeptide by an ethylene
bridge fulfills the geometrical requirements. Since the respective valine peptidoic units turned out to be sterically too demanding, glycines were used for incorporation of the additional nucleobases.

Preparation of the tetra-nucleobase aza-TANDEM derivatives was achieved by solid phase peptide synthesis (SPPS) already incorporating the nucleobases on the resin. In addition, disulfide formation was performed with the peptides still attached to the solid support, whereas macrocyclization was projected under dilute conditions after cleavage from the resin.

Synthesis

The synthesis of aza-TANDEM derivatives containing nucleobases instead of quinoxalines was reported previously based on the diamino propionic acid Fmoc-D-Dap(Trt)-OH (3) (Fmoc = 9-fluorenylmethyloxycarbonyl, Trt = triphenylmethyl), which allows incorporation in the cyclopeptide scaffold as \( \beta \)-amino acid and provides an additional \( \alpha \)-NH\(_2\) residue for attachment of the nucleobases.\(^{[6]}\) Alkylation of the nucleobases furnishing acetic acid derivatives 4 and 5 (Figure 3) allows linkage by amide formation on solid support.

Since SPPS peptide coupling with the secondary N-alkylated glycine turned out to be less effective, the N-alkylated dipeptide Fmoc-L-Cys(Acm)-N-(thymine-1-yl-ethyl)-Gly-OH (9) (Acm = acetamidomethyl) was prepared (Scheme 1) and directly applied in SPPS. 1-(Thymine-1-yl)-2,2-diethoxyethane (6) was obtained by alkylation of thymine with 2-bromo-1,1-diethoxyethane followed by hydrolysis to aldehyde 7 as described by Martinez and Doel.\(^{[10]}\) Reductive amination of 1-(thymine-1-yl)-acetaldehyde pTosOH (7) with H-Gly-OtBu·HCl gave N-(thymine-1-yl-ethyl)-Gly-OtBu (8). Coupling to Fmoc-L-Cys(Acm)-OH turned out to be favorable with N,N-diisopropylcarbodiimide (DIC) activation in absence of a base thereby reducing racemization. After deprotection, the dipeptide Fmoc-L-Cys(Acm)-N-(thymine-1-yl-ethyl)-Gly-OH (9) was obtained for use in SPPS.

In analogy to previous syntheses,\(^{[8]}\) the aza-TANDEM derivatives were prepared by a modified microwave-assisted manual
Fmoc-SPPS (Scheme 2). The orthogonally protected amino acids and dipeptide (9) were coupled on the preloaded Fmoc-Ala-Wang resin using O-(7-azabenzotriazolyl)tetramethyluronium hexafluorophosphate (HATU) and 1-hydroxy-7-aza-benzotriazole (HOAt) for activation. The nucleobase units required for coupling to the diamino propionic acid side chains, (N6-Z-adenine-9-yl)-acetic acid (4) and (thymine-1-yl)-acetic acid (5), were obtained as reported[11] and coupled to the resin-bound, orthogonally protected amino acid Fmoc-d-Dap(Trt)-OH (3) using HOAt, N-methylmorpholine (NMM) and DIC. Still on the resin, the disulfide bridge was formed by adding N-iodosuccinimide (NIS). Deprotection of the terminal Fmoc group was followed by cleavage from the resin providing peptides 10 and 11. Without further purification, the crude disulfide-bridged peptides were cyclized by dropwise addition to a solution of HOAt, NMM and DIC simulating high-dilution conditions. Finally, after deprotection of the Z and side chain protecting groups, the cyclic octa-peptides 1 (ATTA) and 2 (TTTT) carrying four nucleobases each were obtained, purified by HPLC, and characterized by high-resolution mass spectrometry.

**DNA binding studies**

Based on the structural design of tetra-nucleobase aza-TANDEM derivatives, the recognition units have the potential to be well aligned on the peptide backbone in a linear fashion providing a nucleobase stack with distances as known from oligonucleotide double strands. Nucleobases are expected to be linearly lined up similarly to the geometry known from alanyl peptide nucleic acids that are characterized by an intrinsic linear double strand topology originating from base pair recognition.[12] A linear structural arrangement of the four nucleobases on the aza-TANDEM scaffold implies the option for base pair interaction with DNA double strands, for example, by major groove interaction and

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**Scheme 1.** Synthesis of the dipeptide building block 9. *Reagents and conditions:* a) 1. NaH, DMF, 2 h, RT; 2. BrCH₂CH(OEt)₂, 1 4h, 70 °C; 3. 3 h, 180 °C; 35 %. b) pTosOH, H₂O, acetone, reflux, 1 h, 99 %. c) H-Gly-OtBu·HCl, DIEA, DMF, NaBH(OAc)₃, 3 h, RT, 37 %. d) Fmoc-L-Cys(Acm)-OH, DIC, DCM/DMF (1:1, v/v), 2 h, 0 °C to 10 h, RT, 31 %. e) TFA/DCM (1:1, v/v), 2 h, RT, 76 %.

**Scheme 2.** Synthesis of nucleobase-substituted aza-TANDEM derivatives 1 and 2. *Reagents and conditions:* a) 1. NIS, DCM/DMF (1:1, v/v), 2 h, RT; 2. piperidine/DMF (1:5, v/v); 3. TFA/THF/H₂O (95:2.5:2.5, v/v/v), 2 h, RT. b) 1. HOAt, NMM, DIC, DCM/DMF (9:1, v/v), 48 h, RT; 2. TFA/thioanisole (9:1, v/v), 48 h, RT (for 10 only).
hydrogen bonding over the Hoogsteen side. Also, invasion of the aza-TANDEM constructs in the respective DNA double strands might be taken into account. In addition, self-aggregation of two or more tetra-nucleobase aza-TANDEM derivatives by base pair recognition needs to be considered. As experienced by self-aggregation of β-peptide nucleic acids, the pre-organization of nucleobases on a conformational rigid backbone provides base pairing with double strand stabilities that are already sufficiently high at room temperature. The options for DNA recognition or self-aggregation depend on the topology but also on the sequence of nucleobases. Therefore, two tetra-nucleobase aza-TANDEM derivatives were investigated that provide a sequential motif (ATTA) that is not prone to self-aggregation and a TTTT sequence that has a high propensity for aggregation by formation of four T-T base pairs.

First binding studies of the tetra-nucleobase aza-TANDEM derivatives 1 and 2 to DNA were obtained with the DNA double strand of oligomers 5′-AAATTTAATTAAT 3′ (DNA1), offering complementary sequences for recognition of both tetramers 1 and 2. Interaction of aza-TANDEM derivatives with DNA was investigated by microscale thermophoresis (MST). Different 6-carboxyfluorescein (6-FAM)-labeled double stranded DNA sequences consisting of 12 or 14 base pairs were used at a constant concentration of 50 nM for the MST measurements. Aza-TANDEM derivatives were titrated in 1:1 dilutions beginning at 1.34 mM for derivative 1 and 1.38 mM for derivative 2. The samples were incubated for 20 min before loading into capillaries. For aza-TANDEM derivative 1 (ATTA) and DNA1 a K_d value of 26.2±0.7 μM was determined (50 mM phosphate buffer, pH 7.0, 150 mM NaCl, 0.05% Tween-20, 0.05% bovine serum albumin (BSA), 25°C) indicating a weak but significant recognition (Figure 4). In contrast, there is no indication for an interaction between DNA1 and aza-TANDEM derivative 2 (TTTT) (Figure 4B).

To evaluate the option that recognition between aza-TANDEM derivative 1 (ATTA) and DNA1 is due to the ATTA sequence in DNA1, the interaction of 1 and 2 with further DNA oligomers was investigated. The DNA double strands 5′-GGCCGAAAAGCCGG 3′ (DNA2), 5′-GGCCGATTTGCGG 3′ (DNA3), and 5′-GGCCGGTTTTCGGCC 3′ (DNA4) contain a central tetrameric A-T sequence flanked by G-C base pairs. Furthermore, these oligomers differ in the central A-T sequence excluding the ATTA motif. Consequently, for none of the combinations between aza-TANDEM derivatives 1 and 2 and the oligomers DNA2–DNA4 any kind of specific recognition was determined. Overall, it seems reasonable to conclude that the tetra-nucleobase aza-TANDEM derivatives have the potential to specifically interact with DNA double strands when the proper sequence complementarity and topological fitting are provided. Interestingly, aza-TANDEM derivative 2 (TTTT) does not interact with DNA1 or DNA4, although (A-T)_4 tracts are available in the oligomer sequences. This might be due to peptide backbone rigidity that does not allow proper hydrogen bonding with a tetramer of only smaller pyrimidine nucleobases, and therefore, nucleobase hydrogen bonding and stacking have a lower contribution to the DNA binding affinity. Alternatively, the concentration of aza-TANDEM derivative 2 might be reduced by competing self-aggregation of aza-TANDEM derivative 2, which should be beneficial because of the well-organized nucleobases and the hydrogen-bonding complementarity of all-thymine sequences. In order to estimate the aggregation potential of aza-TANDEM derivatives 1 and 2, temperature-dependent UV spectra and mass spectrometry studies were measured.

**Aggregation and recognition potential**

Based on the rigid and well-defined topology offered by the aza-TANDEM backbone, the four nucleobases seem well oriented and preorganized for interaction with a complementary strand. Thus, four base pairs should be sufficient to provide high duplex or aggregate stabilities. Nevertheless, aza-TANDEM derivative 1 (ATTA) lacks a proper self-pairing sequence complementarity, and consequently, no indication for aggregation was indicated by thermal UV denaturation studies (Figure 5). In contrast, the temperature-dependent UV spectrum for aza-TANDEM derivative 2 (TTTT) clearly implies base pair recognition and self-aggregation with a stability higher than 60°C. This sequence-dependent self-aggregation fits nicely to the hy-
Hypothesis of a linear alignment of thymine nucleobases on an organizing and templating aza-TANDEM backbone.

In order to gain further insight in aggregate formation of aza-TANDEM derivative 2 (TTTT), ESI-MS was applied; for comparison aza-TANDEM derivative 1 (ATTA) was probed as well.

Positive ion mode ESI measurements of solutions of TANDEM derivative 2 (c = 10 μM) showed dicaticonic monomers with attached H⁺, NH₄⁺, and/or Na⁺ ions as main species (Figure 6). At higher masses, ions with m/z 1286–1294 were also prominent and included monocations (2⁺NH₄)⁺ and (2⁺Na)⁺. In addition, two multiply charged species were detected, whose isotope patterns showed spacings of 0.5 and 0.33 amu, respectively. On the basis of their exact m/z ratios (see Supporting Information), these species can be assigned to the dicaticonic dimer (2⁺NH₄)₂⁺ and the tricaticonic trimer (2⁺NAH)₃⁺. The dimer is less abundant than the corresponding monomer, but nonetheless makes up a significant fraction of the overall population of 2. In contrast, the signal intensity of the trimer is much lower and did not rise above the noise level in all of the experiments.

Whereas no ions with significant intensity were observed for m/z > 1300, additional ions were found at m/z 858–867 (see Supporting Information). From their exact m/z ratios and their characteristic isotope patterns displaying spacings of 0.33 amu, these ions were identified as dimers of 2, which acquire a triple charge by binding three H⁺, NH₄⁺, and/or Na⁺ ions. This assignment was further supported by collision-induced dissociation (CID) experiments, which showed these dimers to dissociate into singly and doubly charged monomers by Coulomb explosion (e.g., (2⁺3NH₄)³⁺ → (2⁺NH₄)²⁺ + (2⁺Na)⁺, Figure 7).¹⁵

Figure 5. UV melting curves of 1 (ATTA, ———) and 2 (TTTT, ———).

Figure 6. Positive ion mode ESI mass spectrum of an aqueous solution of 2 (NH₄OAc buffer). Inset: Isotope pattern of (2⁺NH₄)⁺ together with the superimposed (2⁺2NH₄)²⁺ and (2⁺3NH₄)³⁺ peaks marked by black and grey arrows, respectively.

Figure 7. Mass spectrum of mass-selected (2⁺3NH₄)³⁺ and its fragment ions produced upon collision-induced dissociation.

Positive ion mode ESI mass spectra measured for solutions of 1 (c = 10 μM) showed ions with m/z 640–670 as the predominant species (Figure 8). These ions correspond to dicaticonic monomers of 1, whose double charge results from the attachment of two H⁺ and/or Na⁺ ions. The latter form an almost ubiquitous low-level contamination of aqueous solutions with

Figure 8. Positive ion mode ESI mass spectrum of 1 in an aqueous (NH₄OAc buffered) solution. Inset: Enlarged section of the same spectrum showing the isotope pattern of (1⁺H)⁺ together with the superimposed (1⁺2H)²⁺ peaks marked by arrows.

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a high affinity to peptides and other biomolecules.\textsuperscript{[14]} The monocation (1 + H)\textsuperscript{+} was also observed, although with much lower signal intensity. Closer inspection reveals that the isotope pattern of the monocation is superimposed by signals of a second species. The 0.5 amu spacings between its different isotopologues as well as the exact m/z ratio suggest that this second species corresponds to the doubly protonated dimer (1\textsuperscript{+} + 2H)\textsuperscript{2+}. Its signal intensity is so low, however, that it does not consistently rise above the noise level in all of the experiments.

**Toxicity of tetra-nucleobase aza-TANDEM derivatives**

Triostin A belongs to a group of antibiotics showing activity against Gram-positive bacteria\textsuperscript{[17]} and a variety of tumor cells;\textsuperscript{[18]} they exert this activity by bisintercalating DNA at nanomolar concentrations.\textsuperscript{[8,4,15,19]} An *Artemia salina* assay was carried out as a simple, rapid and inexpensive bench-top assay. Anhydrous *A. salina* eggs were available from German pet shops. The assay was considered as a useful tool for preliminary assessment of general toxicity\textsuperscript{[20]} of derivative 1 as the only aza-TANDEM analogue with DNA binding affinity in this study. The sample was dissolved in dimethyl sulfoxide (DMSO) and serially diluted starting with 5 \(\mu\)g mL\(^{-1}\). The solutions were added to wells of a microtiter plate containing hatched larvae in artificial seawater. After incubation at permanent light and constant temperature for 24 and 30 h the larvae were still alive. Accordingly, derivative 1 exhibited a median lethal dose (LD\textsubscript{50}) of \(> 5 \mu\)g mL\(^{-1}\), which reflects low cytotoxicity in the *Artemia salina* model.

**Conclusions**

The disulfide-bridged cyclopeptide scaffold of aza-TANDEM derivatives was used as platform for the aligned organization of four nucleobases with the potential to organize with perfect stacking. Derived from the natural product and DNA bisintercalator triostin A, the new derivatives were designed by exchange of intercalating quinoxalines for nucleobases and further peptoid-like attachment of thymines to the cyclopeptide. Aza-TANDEM derivatives with the two sequences ATTA and TTTT were evaluated with respect to DNA binding by microscale thermophoresis (MST) and self-aggregation by temperature-dependent UV spectroscopy and ESI-MS. The aza-TANDEM analogues indicate sequence-dependent DNA binding (ATTA) or base pair-mediated dimer and trimer formation (TTTT) as implied by collision-induced dissociation (CID) experiments. The tetra-nucleobase aza-TANDEM derivatives are especially interesting regarding their potential as architectural building units.

**Experimental Section**

**Materials and Methods**

All chemicals were purchased from NovaBiochem, GL Biochem (Shanghai) Ltd., Sigma–Aldrich, Acros Organics and Carl Roth. HPLC grade CH\(_3\)CN and ultrapure H\(_2\)O (Millipore, Bedford, UK) were used for HPLC chromatography.

**NMR:** \(^1\)H and \(^{13}\)C NMR spectra were recorded with a Varian Unity 300 spectrometer.

**RP-HPLC:** All HPLC analyses were performed on a Pharmacia Äkta basic system (pump type P-900, variable wavelength, detector of type UV-900) using a Nucleodur column, C18 (250 x 10 mm, 5 \(\mu\)m). HPLC runs were performed using a linear gradient of A (0.1% ac trifluoroacetic acid, TFA) and B (80% ac CH\(_3\)CN, 0.1% ac. TFA) within 30 min using a flow rate of 3 mL min\(^{-1}\). UV detection was conducted at 215 nm, 260 nm and 280 nm. The crude samples were dissolved in water and filtered prior to injection.

**MST measurements:** Microscale thermophoresis measurements were performed on a NanoTemper Monolith NT.115 at 60–90 % LED and a MST power of 20–40 %; laser-on time was 30 s, laser-off time s each. The 6-fluorescein amidite (FAM)-labeled DNA was obtained from biomers.net (Ulm, Germany) and was used at concentrations of 50 nm. The derivatives were titrated in 1:1 dilutions starting at the mentioned highest concentrations. A phosphate buffer was used (50 mm supplemented with 150 mm NaCl, 0.05 % Tween-20, 0.05 % bovine serum albumin) at pH 7 and 25 °C. All experiments were performed in standard treated capillaries.

**ESI-MS:** Data were obtained with a Finnigan LCQ instrument and high-resolution mass spectra (HRMS-ESI) with a Bruker Apex-Q IV FT-ICR-MS instrument.

**UV-melting curves:** UV-melting curves were recorded with a Jasco V-550 UV spectrometer (Gross-Ümstadt, Germany) using a Jasco ETC-5055/ETC-50ST Peltier temperature controller while the sample cell was floated with nitrogen. All measurements were carried out in a micro-quartz glass cell of 1 cm path length in a NH\(_4\)OAc buffer solution (10 mm, pH 7.0) at a concentration of 10 \(\mu\)m for each aza-TANDEM analogue. Data were collected at 260 nm with a heating rate of 0.4 °C min\(^{-1}\). The protocol for the melting curve recordings is as follows: 25 °C →85 °C (15 min), 85 °C (2 min), 85 °C →2 °C (25 min) →→ 2 °C (3 min) →85 °C (120 min) →→ 2 °C (120 min) →85 °C (120 min) →→ 2 °C (120 min) →25 °C (10 min). After data collection for the heating cycles, the hyperchromicity (\(H\)) was calculated according to the equation: \(H(%) = 100\% (A(T) - A_\infty) / A_\infty\), where \(A(T)\) is the absorbance given at any temperature and \(A_\infty\) is the minimum absorbance.

**Toxicity assay:** A separation funnel was filled with filtered artificial seawater (1 L) and *A. salina* eggs (200 mg) and kept under aeration and permanent light at a constant temperature of 20 °C until the larvae hatched. Artificial seawater (990 mL) containing 20 or more larvae were pipetted into wells of a 24-well microtiter plate. Dead larvae were recorded under a microscope. The sample was dissolved in dimethyl sulfoxide (DMSO) and serially diluted. From each concentration, 10 \(\mu\)L were added to a well to obtain concentrations of 5, 2.5, 1.25 \(\mu\)L etc. The test was carried out in duplicate. For negative control, 10 \(\mu\)L of DMSO and for positive control, 10 \(\mu\)L of a 1 mg mL\(^{-1}\) solution of actinomycin D in DMSO, were used. After incubation at permanent light and constant temperature, survival of larvae was recorded after 24 h and 30 h. While negative and positive controls displayed 0 % and 100 % lethality, respectively, compound 1 did not affect the survival rates of brine shrimps (LD\textsubscript{50} > 5 \(\mu\)g mL\(^{-1}\) after 24 h and 30 h).

**Analysis of oligomers:** For analysis by ESI-MS, aqueous solutions of 1 and 2 (c = 10–40 \(\mu\)m) containing NH\(_4\)OAc buffer (10 mm, pH 7.0) were prepared and infused into the ESI source of a micro-TOF-Q II instrument (Bruker) at a flow rate of 8 \(\mu\)L min\(^{-1}\). The ESI
source was operated with ESI voltages of −5000 to −3000 V in the positive and +3000 V in the negative ion mode and with N₂ as nebulizer (flow rate of 5 L/min) and drying gas (0.7 bar backing pressure); the temperature of the latter was set to 50 °C to minimize the possibility of thermal decomposition reactions. The ions passed a glass capillary, two ion funnels, a hexapole ion guide and two quadrupole units, the second of which was filled with N₂ and could be used as a collision cell for collision-induced dissociation (CID) experiments. To this end, the ions of interest were mass-selected by the first quadrupole and accelerated by an extra DC voltage applied to the second quadrupole. Sufficiently energetic collisions of the ions with N₂ molecules resulted in the dissociation of the former. The ions were then extracted into a time-of-flight analyzer and finally detected by a multi-channel plate. For calibration, aqueous solutions of sodium formate and solutions of a mixture of trifluoroacetic acid and phosphazenes in H₂O/CH₂CN were employed. As described previously,[11] the instrument’s ion transmission suffers from significant mass discrimination, which strongly depends on two parameters: the amplitude of the RF voltage applied to the second quadrupole unit and the timing of the ion transfer into the time-of-flight analyzer. In the present work, medium settings (RF amplitude of 650 Vpeak-to-peak, transfer time of 77.6 μs) were applied to optimize the detection of ions with m/z ratios in the range of 300–1500. To check for the presence of heavier ions (up to m/z 5000), the parameters were changed accordingly.[11] To ensure good reproducibility of the obtained results, all experiments were repeated with independently prepared sample solutions.

**Synthesis of Fmoc-L-Cys(Acm)-N-(thymine-1-yl-ethyl)-Gly-OH[10]**

(9)

1-(Thymine-1-yl)-2,2-dioethoxyethane (6): Thymine (10.1 g, 80.9 mmol) was suspended in dry N,N-dimethylformamide (DMF, 30 mL) with activated, powdered molecular sieves (4 Å), NaH (60% in mineral oil, 4.65 g, 116 mmol) and was added and the mixture was stirred for 2 h at RT. 2-Bromo-1,1-diethoxyethane (20.1 mL, 10.7 g, 75.5 mmol) in dry CH₂Cl₂/DMF (4:1, 100 mL) was added, and the reaction mixture was stirred for 2 h at reflux. The solution was added to cold H₂O (50 mL) and treated with a diluted NaOH solution (1 M) and allowed to evaporate in vacuo. The resulting white solid was filtered through Celite and washed with DF (50 mL) and treated with a diluted NaOH solution until pH 8 was reached. The solution was extracted with CH₂Cl₂ (3 × 50 mL), the combined organic layers were dried over anhyd MgSO₄, filtered, and the solvent was evaporated in vacuo. The mixture was evaporated in vacuo, dissolved in H₂O (50 mL) and treated with a diluted NaOH solution until pH 8 was reached. The solution was extracted with CH₂Cl₂ (3 × 50 mL), the combined organic layers were dried over anhyd MgSO₄, filtered, and the solvent was evaporated in vacuo. Compound (8) (1.66 g, 5.88 mmol, 37%) was obtained after purification by flash chromatography (EtOAc/MeOH 9:1) as a white solid: HRMS (ESI): [M + H⁺] calcld for C₆H₅N₂O₄: 284.1605, found: 284.1605; [M + H]⁺ NMR (300 MHz, CDCl₃): δ = 9.75 (s, 1H, thymine-N), 7.09 (s, 1H, thymine-C), 3.75 (t, J = 5.9 Hz, 2H, NHCH₂CN), 3.25 (2s, 2H, Gly-CH₂), 2.86 (t, J = 5.9 Hz, 2H, NHCH₂CH₃), 1.84 (d, J = 1.0 Hz, 3H, thymine-CH₃), 1.39 (s, 9H, tBu-CH₃); 13C NMR (125 MHz, CDCl₃); δ = 171.4 (Gly-CH₂), 164.5 (thymine-CH₃), 151.1 (thymine-CHO), 141.0 (thymine-CH₂), 110.0 (thymine-CHO), 81.4 (tBu-C), 51.2 (NHCH₂CH₃), 48.4 (Gly-CH₂), 47.7 (NHCH₂CH₃), 28.0 (tBu-CH₃), 12.2 ppm (thymine-CH₃).

Fmoc-L-Cys(Acm)-N-(thymine-1-yl-ethyl)-Gly-OtBu[8]: Fmoc-L-Cys(Acm)-N-(thymine-1-yl-ethyl)-Gly-OH[9] was dissolved in CH₂Cl₂ (3 mL), TFA (3 mL) was added, and the reaction mixture was stirred for 2 h at RT. The mixture was evaporated in vacuo, dissolved in CH₂O (50 mL) and treated with a diluted NaOH solution until pH 8 was reached. The solution was extracted with CH₂Cl₂ (3 × 50 mL), the combined organic layers were dried over anhyd MgSO₄, filtered, and the solvent was evaporated in vacuo. Compound (8) (1.66 g, 5.88 mmol, 37%) was obtained after purification by flash chromatography (EtOAc/MeOH 9:1) as a white solid: HRMS (ESI): [M + Na⁺] calcld for C₆H₅N₂O₄Na: 702.2568, found: 702.2555; [M + Na]⁺ NMR (300 MHz, CDCl₃): δ = 11.22 (s, 0.5H, thymine-NH), 11.09 (s, 0.5H, thymine-NH), 8.33–8.45 (m, 1H, Acn-N), 7.83–7.91 (m, 2H, Fmoc-CH₂), 7.66–7.80 (m, 3H, CH₂Fmoc), 7.50 (s, 3H, Cys-NH₂), 7.51 (s, 0.5H, thymine-CH), 7.37–7.54 (m, 2H, Fmoc-CH₂), 7.27–7.36 (m, 2H, Fmoc-CH₂), 6.62–7.31 (m, 0.5H, Cys-NH₂), 4.11–4.47 (m, 5.5H, Fmoc-CH₂, Fmoc-CH₃, Acn-CH₃, Cys-CH₃), 3.44–4.10 (m, 5.5H, CH₂, CH₃, CH₂, Gly-CH₂), 2.80–2.94 (m, 1H, Cys-CH₂), 2.61–2.79 (m, 1H, Cys-CH₂), 1.84 (s, 1.5H, Acn-CH₃), 1.83 (s, 1.5H, Cys-CH₃), 1.71 (s, 1.5H, thymine-CH₂), 1.70 (s, 1.5H, thymine-CH₂), 1.40 (s, 4.5H, tBu-CH₃), 1.38 ppm (s, 4.5H, tBu-CH₃).

Fmoc-L-Cys(Acm)-N-(thymine-1-yl-ethyl)-Gly-OH[9]: Fmoc-L-Cys(Acm)-N-(thymine-1-yl-ethyl)-Gly-OtBu[8] (3.02 g, 4.43 mmol) was dissolved in CH₂Cl₂ (3 mL), TFA (3 mL) was added, and the reaction mixture was stirred for 2 h at RT. The solution was added to cold Et₂O. The resulting white solid was washed with cold Et₂O and dried in vacuo. Compound (9) (2.10 g, 3.37 mmol, 76%) was obtained as a yellow solid: HRMS (ESI): [M + Na⁺] calcld for C₆H₅N₂O₄Na: 646.1942, found: 646.1942; [M + Na]⁺ NMR (300 MHz, CDCl₃): δ = 11.22 (s, 0.5H, thymine-NH), 11.10 (s, 0.5H, thymine-NH), 8.33–8.45 (m, 1H, Acn-NH), 7.84–7.91 (m, 2H, Fmoc-CH₂),
SPPS of the nucleobase-substituted aza-TANDEM analogues

Microwave-assisted manual solid phase peptide synthesis (SPPS) was performed on a Fmoc-Ala-preloaded Wang resin (0.48 mmol g⁻¹) with a Discover microwave (MW) reaction cavity (CEM, Matthews, NC, USA). The following reagents, protocols and procedures were used for Fmoc-deprotection (20% piperidine/DMF; 1:30 s, 50 °C, 35 W); HOAt/NMM/DIC/DMF, 2 min, 35 W; HOAt/NMM/DIC/DMF, 2 min, 50 °C, 35 W). The resin was swollen in N-methyl-2-pyrrolidone (NMP) for 4 h. The coupling cycle started with Fmoc-deprotection, followed by washing with CH₂Cl₂ (5 x), NMP (5 x), DMF (5 x). The following amino acid building blocks were used in an excess of three equivalents: Fmoc-Dap-Trt-Oh, Fmoc-L-Cys(Acm)-N-(thymine-1-yl-ethyl)-Gly-Oh, Fmoc-Ala-Oh. The respective building block was dissolved in stock solutions of O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium (HATU) (2.9 equiv) and 1-hydroxy-7-azabenzotriazole (HOAt) (3.0 equiv) in DMF. After 5 min activation, DIEA (10 equiv) was added to the final reaction mixture (0.2 m) before transferring it to the resin. Double couplings were performed for N-(thymine-1-yl)-acetyl acid and N-(W'-Z'-adenine-9-yl)-acetyl acid building blocks (each 4.0 equiv) with the coupling reagents HOAt (4.0 equiv), NMM (4.8 equiv) and DIC (16 equiv) in DMF. The resin was washed with CH₂Cl₂ (5 x), NMP (5 x) and DMF (5 x) after each coupling cycle. Before deprotection of the N-terminal Fmoc group the disulfide bridge was formed. The resin was washed with MeOH (5 x) and CH₃CN (5 x) and swollen in CH₂Cl₂/DMF (1:1). A solution of N-iodosuccinimide (NIS) (2.2 equiv) in DMF/CH₂Cl₂ (1:1) was added to the resin and the mixture was agitated for 2 h. The resin was washed with MeOH (5 x) and CH₂Cl₂ (5 x) and swollen in NMP. After final deprotection, the resin was washed with CH₂Cl₂ (5 x) and MeOH (5 x) and dried in vacuo. Cleavage from the solid support was achieved using the mixture TFA/TEA/H₂O (95:2.5:2.5, v/v/v) for 2 h at RT. The reaction mixture was concentrated under N₂ stream followed by precipitation with cold tert-butyl methyl ether (MTBE). The resulting suspensions were centrifuged at -20 °C. The supernatant was discarded, the peptide pellet was washed with MTBE (3 x) and dried in vacuo.

**Cyclization and deprotection of derivatives 10 and 11**

HOAt (1.0 equiv), NMM (3.0 equiv) and DIC (10 equiv) were dissolved in dry CH₂Cl₂/DMF (9:1, v/v, 0.5 mL mmol⁻¹). A solution of the respective crude peptide disulfide (1.0 equiv) in DMF (0.1 mL mmol⁻¹) was added dropwise to the reaction mixture and stirred for 48 h at RT. After evaporation in vacuo, the residue was dissolved in TFA/thioanisole (9:1) and stirred for 48 h at RT. The resulting solution was evaporated to dryness and the residue purified by HPLC to give a white solid.

Cyclo-[L-Cys-N-(thymine-1-yl-ethyl)-Gly]-β-Dap-N-(N'-Z'-adenine-9-yl-acetyl)-L-Ala-L-Cys-N-(thymine-1-yl-ethyl)-Gly]-β-Dap-N-(N'-Z'-adenine-9-yl-acetyl)-L-Ala-OH]-disulfide (10): HRMS (ESI): m/z [M + H]+ calc'd for C₉₁H₁₇₀N₆₄O₃₈S₂: 1573.5233, found 1573.5234.

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[1] M. J. Waring, L. P. G. Wakelin, Nature 1974, 252, 653 – 657.
[2] K. Katagiri, T. Yoshida, K. Sato, *Antibiotics, Mechanism of Action of Antimicrobial and Antitumor Agents, Vol. 1* (Eds.: J. W. Corcoran, F. E. Hahn), Berlin, Springer, 1975, pp. 234 – 251.
[3] R. K. Olsen, *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, Vol. 7 (Ed.: B. Weinstein), New York, Marcel Dekker, 1983, pp. 1 – 33.
[4] J. A. S. Lee, M. J. Waring, *Biochem. J.* 1978, 173, 115 – 128; b) J. S. Lee, M. J. Waring, *Biochem. J.* 1978, 173, 129 – 144; c) K. R. Fox, R. K. Olsen, M. J. Waring, *Biochim. Biophys. Acta Gene Struct. Expression* 1982, 696, 315 – 322; C. M. Low, R. K. Olsen, M. J. Waring, *FEBS Lett.* 1984, 176, 414 – 420.
[5] a) M. A. Viswanmitra, O. Kennard, W. B. T. Cruse, E. Egert, G. M. Sheldrick, P. G. Jones, M. J. Waring, L. P. G. Wakelin, *Nature* 1981, 289, 817 – 819; b) M. B. Hossain, D. van der Helm, R. K. Olsen, P. G. Jones, G. M. Sheldrick, E. Egert, O. Kennard, M. J. Waring, M. A. Viswanmitra, *J. Am. Chem. Soc.* 1982, 104, 3401 – 3408.
[6] G. M. Sheldrick, J. J. Guy, O. Kennard, R. Rivera, M. J. Waring, *J. Chem. Soc. Perkin Trans. 2* 1984, 1601 – 1605.
[7] a) A. H.-J. Wang, G. Ughetto, G. J. Quigley, T. Hakoshima, G. A. van der Marel, J. H. van Boom, A. Rich, *Science* 1984, 225, 1115 – 1121; b) A. H.-J. Wang, G. Ughetto, G. J. Quigley, A. Rich, *J. Biomol. Struct. Dyn.* 1986, 4, 173.
319 – 342; c) K. J. Addess, J. Feigon, Biochemistry 1994, 33, 12397 – 12404.

[8] a) K. B. Lorenz, U. Diederichsen, J. Org. Chem. 2004, 69, 3917 – 3927; b) B. Dietrich, U. Diederichsen, Eur. J. Org. Chem. 2005, 147 – 153; c) A. Kumar Ray, U. Diederichsen, Eur. J. Org. Chem. 2009, 4801 – 4809; d) E.-F. Sachs, A. Nadler, U. Diederichsen, Amino Acids 2011, 41, 449 – 456.

[9] Maestro, Version 9.1, Schrödinger, LLC, New York, NY, 2010.

[10] a) M. T. Doel, A. S. Jones, N. Taylor, Tetrahedron Lett. 1969, 10, 2285 – 2288; b) W. A. El-Zayat, W. A. El-Sayed, A. A.-H. Abdel-Rahman, Z. Naturforsch. 2009, 64c, 6 – 10; c) A. P. Martinez, W. W. Lee, J. Org. Chem. 1965, 30, 317 – 318.

[11] a) B. E. Watkins, H. Rapoport, J. Org. Chem. 1982, 47, 4471 – 4477; b) B. E. Watkins, J. S. Kiely, H. Rapoport, J. Am. Chem. Soc. 1982, 104, 5702 – 5708; c) K. L. Duhelm, M. Egholm, C. Behrens, L. Christensen, H. F. Hansen, T. Vulpius, K. H. Petersen, R. H. Berg, P. E. Nielsen, O. Buchardt, J. Org. Chem. 1994, 59, 5767 – 5773.

[12] a) U. Diederichsen, Angew. Chem. 1996, 108, 458 – 461; Angew. Chem. Int. Ed. Engl. 1996, 35, 445 – 448; b) U. Diederichsen, D. Weicherdg, N. Diezemann, Org. Biomol. Chem. 2005, 3, 1058 – 1066.

[13] a) A. M. Brückner, P. Chakraborty, S. H. Gellman, U. Diederichsen, Angew. Chem. 2003, 115, 4532 – 4536; Angew. Chem. Int. Ed. 2003, 42, 4395 – 4399; b) P. Chakraborty, U. Diederichsen, Chem. Eur. J. 2005, 11, 3207 – 3216.

[14] M. Jerabek-Willemsen, C. J. Wienken, D. Braun, P. Baaske, S. Duhr, Assay Drug Dev. Technol. 2011, 9, 342 – 353.

[15] N. G. Gotts, A. J. Stace, J. Chem. Phys. 1991, 95, 6175 – 6175.

[16] P. Wang, C. Wesdemiotis, C. Kapota, G. Ohanessian, J. Am. Soc. Mass Spectrom. 2007, 18, 541 – 552.

[17] F. Romero, F. Espliego, J. Perez Baz, T. Gracia de Quesada, D. Gravalos, F. de La Calle, J. J. Fernandez-Puentes, J. Antibiot. 1997, 50, 734 – 737.

[18] a) H. Okada, H. Suzuki, T. Yoshinari, H. Arakawa, A. Okura, H. Suda, A. Yamada, D. Uemura, J. Antibiot. 1994, 47, 129 – 135; b) K. Takahashi, H. Koshino, Y. Esumi, E. Tsuda, K. Kurosawa, J. Antibiot. 2001, 54, 622 – 627.

[19] a) M. S. Searle, J. G. Hall, W. A. Denny, L. P. Wakelin, Biochem. J. 1989, 259, 433 – 441; b) G. J. Quigley, G. Ughetto, G. A. van der Marel, J. H. van Boom, A. H. Wang, A. Rich, Science 1986, 232, 1255 – 1258.

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