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A Convergent Solid-Phase Synthesis of Actinomycin Analogues—Towards Implementation of Double-Combinatorial Chemistry

Glenn Tong and John Nielsen*

The Technical University of Denmark, Department of Organic Chemistry, Building 201, DK-2800, Lyngby, Denmark

Abstract—The actinomycin antibiotics bind to nucleic acids via both intercalation and hydrogen bonding. We found this 'double-action attack' mechanism very attractive in our search for a novel class of nucleic acid binders. A highly convergent, solid-phase synthetic strategy has been developed for a class of peptide–aryl–peptide conjugates modeled upon natural actinomycins. The features of this method include the use of Fmoc solid-phase peptide synthesis, side-chain to side-chain cyclization on the solid phase, a chemoselective cleavage step and segment condensation. The synthetic scheme is consistent with the requirements for combinatorial synthesis and furthermore, the final segment condensation allows, for the first time, double-combinatorial chemistry to be performed where two combinatorial libraries can be reacted with each other. Copyright © 1996 Elsevier Science Ltd

Introduction

The potential targeting of nucleic acids via the use of either antisense[1] or antigene[2] techniques is based on the highly specific and predictable way in which oligonucleotides and their derivatives interact with nucleic acids. This provides a strategy which in principle has the major advantage that it is sequence-based rather than shape/function-based as in traditional drug design. However, the use of oligonucleotides as therapeutic agents is complicated by several factors, such as unfavorable nuclease stability, cell membrane permeability, and oral availability. We believe that there is still great potential in drugs aimed at the gene-expression level, but that molecules other than oligonucleotide analogues should be investigated. In addition, development of synthetic methods which enables us to combine our efforts towards such molecules with the powerful tools of combinatorial synthesis[3] would be attractive. The present report describes an efficient synthetic methodology for the preparation of peptide–aryl–peptide conjugates as potential new DNA/RNA binders. Furthermore, the synthetic scheme described allows, for the first time, the implementation of *double-combinatorial chemistry* (the term *double-combinatorial* arises from the chemical linking of a library consisting of *m* different members with another library of *n* different members, linked by *p* different linkers to form $m \times n \times p$ different library members, Fig. 1).

Actinomycin D (Fig. 2) and triostin are potent antibiotics with characteristics that provided us with some important 'design features' for a new class of nucleic acid binders.[4] Both are conjugates of cyclic peptides with an intercalating moiety. The cyclic peptides are usually very hydrophobic and contain *N*-methyl amino acids which mask the hydrophilicity of the peptide bonds. The specific binding of actinomycin D to DNA has been shown to be enhanced by minimizing water–DNA, water–actinomycin, and *unfavorable* actinomycin–DNA interactions, respectively.[4] Using this natural antibiotic as a model, we set out to develop an efficient synthesis for compound **1** consisting of an aromatic diacid conjugated to two cyclic peptides (Fig. 3). For the validation of our general concept, we selected the same hydrophobic amino acid residues as contained in the cyclic peptide part of Actinomycin D but with no *N*-methylation and likewise, the commercially available naphthoic-2,6-dicarboxylic acid was chosen as our model intercalator. Subsequently, in order to expand the synthetic

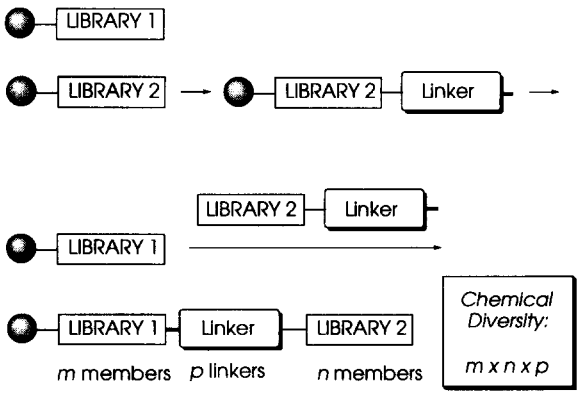


Figure 1. A schematic representation of double-combinatorial chemistry.

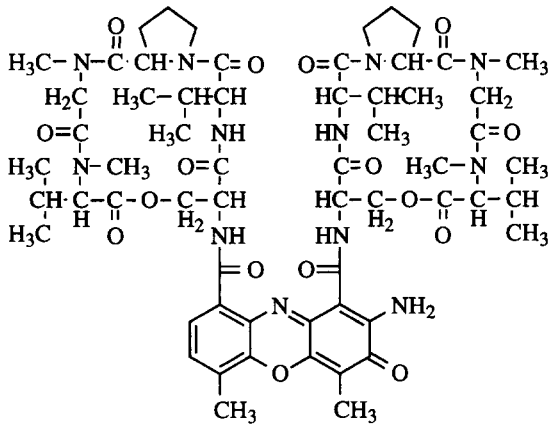


Figure 2. The structure of Actinomycin D.

repertoire, the incorporation of *N*-methyl amino acids was thoroughly investigated.

Results

The availability of monoallyl esters of aromatic dicarboxylic acids is rather limited so a new pathway to these key-monomers has been developed (Scheme 1). The synthesis is based on the alkylation of the mono Cs-salt of the diacid **2** with allyl bromide and resulted in a 30% isolated yield after facile purification by silica and ion-exchange chromatography. The conditions were optimized and the most influential factor being the amount of Cs_2CO_3 (0.5 molar equiv) which had to be added in small portions over an extended time.

The strategy for synthesizing the cyclic peptide-aromatic diacid conjugate **1** (Scheme 2) features the use of Fmoc solid-phase peptide synthesis (Fmoc SPPS),[5] an orthogonal protection scheme and segment condensation. The starting point for the synthesis was the support-bound, hydroxymethyl benzoyl-linked

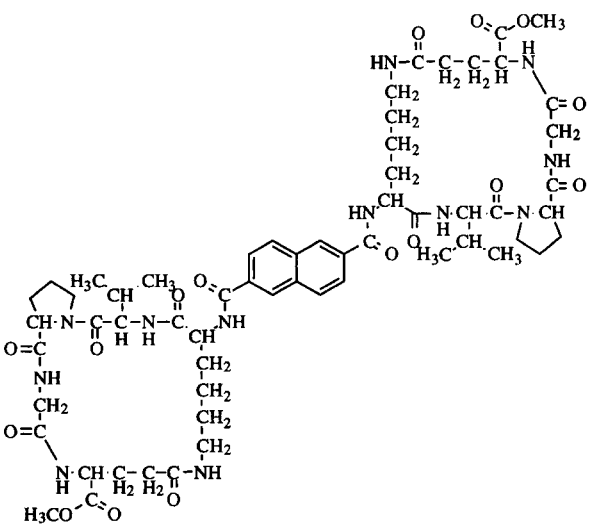
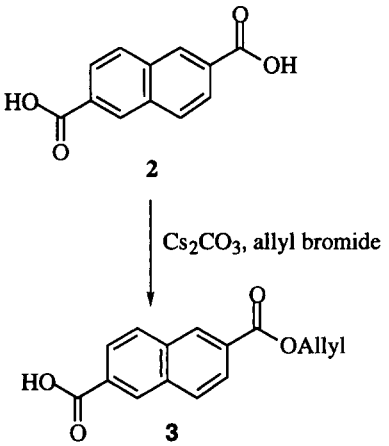


Figure 3. The structure of cyclic peptide–aryl–peptide conjugate **1**.



Scheme 1. Synthesis of monoallylester of 2,6-naphthalenedicarboxylic acid.

(HMB) glutamic acid derivative **4**, which after standard Fmoc SPPS resulted in the linear peptide **5**. The orthogonal protection scheme allowed the allyl-protected γ-carboxyl of the Glu and the Boc-protected ε-amino groups of the Lys to be consecutively and selectively removed using tetrakistriphenylphosphine Pd(0)[6] and TFA, respectively; PyBOP-mediated condensation then gave the cyclic peptide **6**. Subsequently, the resin carrying the support-bound cyclic peptide **6** was split into two portions and one of these was then Fmoc-deprotected and acylated with the monoallyl ester **3** using HATU activation.[7] Removal of the allyl group from the naphthyl carboxyl-group of **7** using Pd(0), followed by a chemoselective methanol–pyridine mediated cleavage of the HMB-linker gave the naphthyl-cyclic peptide segment **8**.[8] An important point to note is that our cyclization conditions gives only the desired cyclic product in good yield with no dimeric side-product or the linear precursor. A second noteworthy observation is that the methanol–pyridine cleavage gives exclusively the C-terminal Glu methyl ester with none of the free acid side product which could potentially be formed from residual moisture in the cleavage mixture being observed. This is very important since the support-bound peptide **7** contains two carboxyl groups and the glutamic carboxyl needs to be selectively blocked so that the naphthyl carboxyl can react with **6**. Finally, the remaining portion of support-bound **6** which was not reacted with the naphthyl derivative **3**, was Fmoc-deprotected and a segment condensation[9] with **8** gave the bis-cyclic peptide–aromatic diacid conjugate **1** after methanol–pyridine mediated release from the HMB-linker. For analytical purposes, small aliquots of solid support were cleaved throughout this sequence of reactions and analyzed by reversed-phase HPLC (RP-HPLC, Fig. 4); products of high purity were observed. The cyclic peptide cleaved from the solid-support carrying the fragment **6** consisted of two very close-running peaks which had identical mass spectra consistent with the proposed structure. Since both the linear peptide precursor cleaved from **5** and the naphthyl-derivatized segment **8** were clearly single sharp peaks, conformational effects are the most likely explanation for these observations.

The HPLC chromatogram of **1** (Fig. 4D) showed complete conversion of the cyclic peptide precursor **6** to one major peak corresponding to the desired conjugate; this in turn corresponds to a very good yield for the segment condensation. A RP-HPLC purified sample of **1** was characterized by LC/ion-spray, PD-MS, UV (data not shown) and ^{1}H NMR spectroscopy (Fig. 5). The LC/ion-spray MS showed two major peaks corresponding to the desired product **1** and a side-product due to the hydrolysis of the C-terminal methyl esters. It was subsequently observed that this hydrolysis only occurred after concentration in aqueous buffer (8 mbar, 40 °C, 3 h). There was no detectable hydrolysis (HPLC, MS) after standing in aqueous buffers at 25 °C (15–20 days), lyophilzation or concentration in low-boiling organic solvents such as methanol and acetonitrile. This suggests that the methyl esters can be either removed from the conjugate by hydrolysis under mild conditions or left intact if the appropriate precautions are adhered to. The PD-MS and UV spectra are consistent with the proposed structure. Distinct resonances characteristic of the symmetrically substituted naphthyl moiety was observed in the ^{1}H NMR spectrum (Fig. 5). The resonances for the cyclic peptides were clean and sharp but unambiguous assignments require multiple dimension NMR experiments; these are now in progress. All characterization data suggest that our reaction scheme provides the desired product in relatively high yield and purity (67% purity of **1** in crude mixture by RP-HPLC; 34% isolated yield).

Since some residues in the natural product are *N*-methylated, we thoroughly investigated the incorporation of *N*-methylated amino acids. The test peptide H-Lys-MeVal-Glu-OH, where the ε-amino group of Lys is protected by the *t*-butyloxycarbonyl group and the γ-carboxyl group of the Glu is protected as an allyl ester, was used to assess the effectiveness of amino acid activation protocols employing PyBrOP,[10] amino acid fluorides,[11] and HATU.[12] PyBrOP gave poor yields while amino fluorides resulted in quantitative coupling albeit with significant racemization (as observed in two very close-running peaks in the RP-HPLC chromatogram which had identical mass spectra from ion-spray MS; data not shown). Optimized conditions for the HATU activation were amino acid (10 equiv), HATU (10 equiv) and DIEA (20 equiv) in anhydrous *N*-methylpyrollidone (NMP, 0.2 M) with two consecutive 17 h couplings. The coupling yield was 89%, and the product was homogeneous by RP-HPLC (data not shown); there was no sign of racemization in this case. In our hands, the conditions described in literature[10-12] gave low coupling yields for the test peptide sequence chosen for this study.

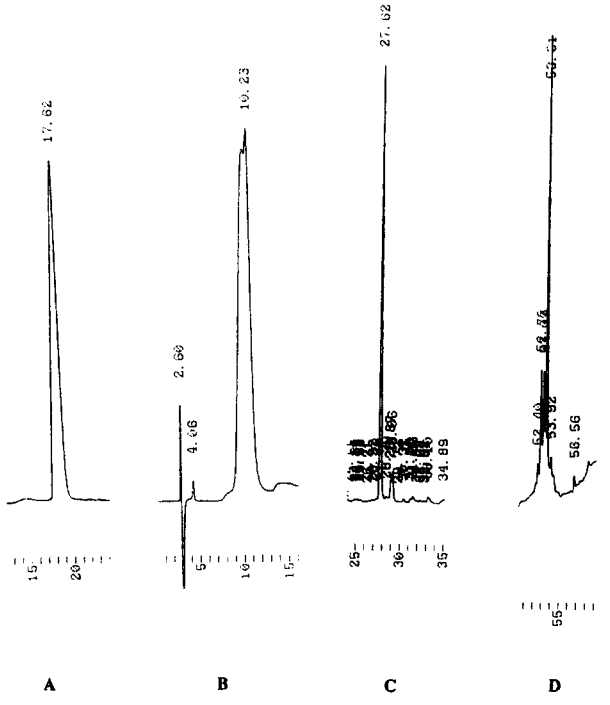
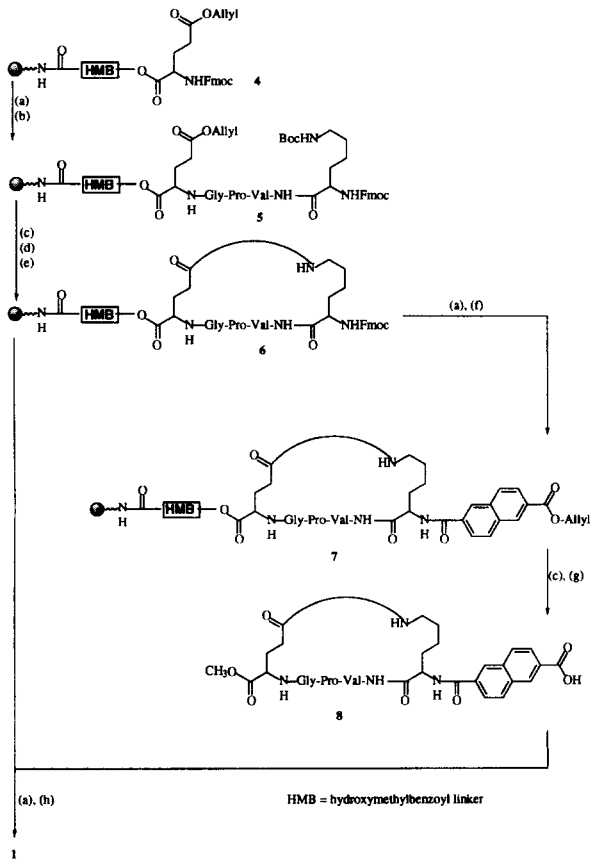


Figure 4. HPLC chromatograms of intermediates in the synthesis of **1**. All peptides were cleaved from the solid support after removal of the Fmoc group as described in ref 16. Column: Merck 50983 LiChrospher 100 RP-18, 5 mm, 250 × 4 mm. Buffer 'Low', 0.1% TFA/H_2O. Buffer 'High', 0.1% TFA, 80% CH_3CN/H_2O. Elution gradient for (A) and (B): 0% 'High' (5 min), 0–20% 'High' over 40 min, 20–100% 'High' over 10 min, then 100% 'High' (5 min). Monitored at l = 220 nm. Elution gradient for (C) and (D): 0% 'High' (5 min), then 0–100% 'High' over 50 min. (A) Linear peptide cleaved from resin **5**. (B) Cyclic peptide cleaved from resin **6**. (C) Naphthoic acid-cyclic peptide segment **8**. (D) Crude conjugate **1**.



Scheme 2. (a) 20% piperidine/DMF, (b) Fmoc amino acid, PyBOP/HOBT/DIEA, (c) tetrakistriphenylphosphine Pd(0), (d) 50% TFA/dichloromethane, (e) PyBOP/HOBT/DIEA, (f) HATU/DIEA, (g) 90% MeOH/pyridine, (h) HATU/DIEA.

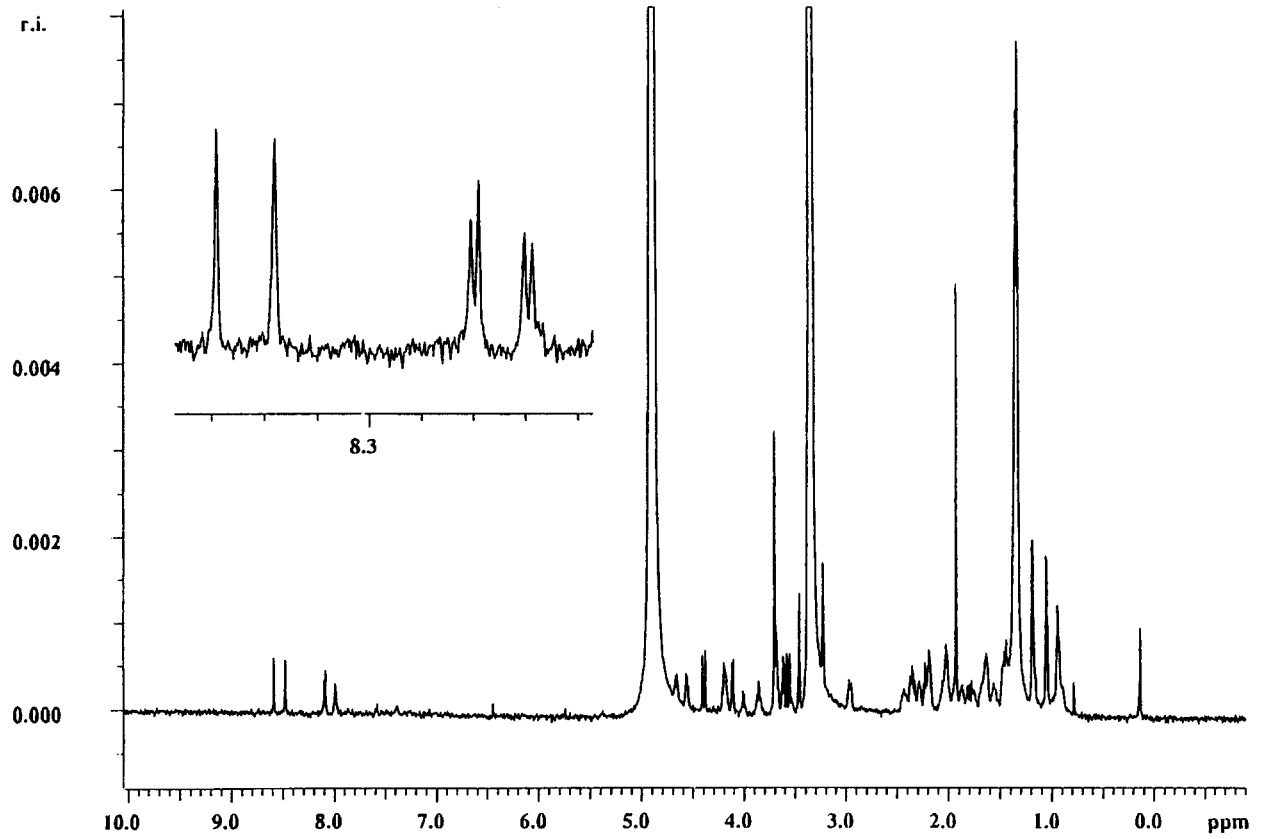


Figure 5. 600 MHz ^{1}H NMR spectrum of the conjugate **1**.

Conclusions

The reported solid-phase synthesis of peptide–aryl–peptide conjugates is convergent and holds the requirements for successful combinatorial synthesis.[3] *Consequently, the present concept can allow for double-combinatorial synthesis of these analogues, with the first level of diversity resulting from the combinatorial synthesis of the linear or cyclic peptides being increased in multiples arising from the consecutive segment condensation.* Currently, our laboratory is focused on exploring the exciting potential of implementing this methodology for the preparation of combinatorial libraries of actinomycin analogues as potential nucleic acid binding molecules and novel antibiotics.

Experimental

General procedures

HATU was a gift from PerSeptive Biosystems Inc. All other reagents (at least AR grade) were purchased and used without further purification. The HPLC experiments were obtained on a Merck Hitachi L7000 system at 40 °C on a LiChrospher 100 RP-18, 5 mm, 250 × 4 mm column. The LC/MS ion-spray was performed on a Perkin-Elmer SciX triple quadrupole instrument. PD-MS was obtained on a Beoen instrument. The NMR spectra for **3** were recorded on a Brucker 250 MHz instrument; a Brucker 600 MHz instrument was used for compound **1**. UV spectra were obtained from a Hewlett Packard 8452A Diode Array spectrophotometer. All melting points are uncorrected.

Naphthoic-2,6-dicarboxylic acid monoallyl ester (3). Cs_2CO_3 (1.60 g, 5.00 mmol) was added in two equal portions over 0.5 h to a solution of **2** (2.16 g, 10.0 mmol) in anhydrous DMF (97 mL). Allyl bromide (17.3 mL, 200 mmol) was then added and the reaction mixture stirred vigorously for 24 h. The same amount of Cs_2CO_3 was then added in two equal portions over 8 h and the reaction allowed to proceed for a further 17 h. The ppt. was filtered and rinsed with DMF (3 × 20 mL). The combined filtrate was then concentrated in vacuo and extracted with hot 50% MeOH–Me_2CO (2 × 100 mL). After evapn of the solvent, residual starting material **2** was removed by silica gel chromatography (Me_2CO:hexane:HOAc, 50:48:2). The resulting fractions which contained a mixture of the monoester **3** and the diester was concentrated, redissolved in 10% MeOH:CH_2Cl_2, and treated with Amberlyst A-26 (hydroxide form) resin (24.0 g, 96 meq) for 17 h. The resin was then filtered and washed with fresh solvent (about 3 L) until no diester was detected in the effluent by TLC ($CHCl_3$:MeOH:HOAc, 94:4:2). The desired compound **3** was then eluted with MeOH:CH_2Cl_2:acetic acid (10:80:10). After solvent removal, recrystallization from acetone gave **3** as a fine colorless solid (0.77 g, 30%); mp 217–218 °C; ^{1}H NMR (250 MHz, DMSO-d_6): δ 4.88 (d, 2H, $C\underline{H}_2$-CH=CH_2, J = 5.1 Hz), 5.30 (m, 1 H, CH_2-CH=C$\underline{H}_2$), 5.45 (m, 1

H, CH_2-CH=C$\underline{H}_2$), 6.10 (m, 1H, CH_2-C$\underline{H}$=CH_2), 8.05 (m, 2H, H-4, H-8), 8.25 (d, 2H, H-1, H-3, J = 8.4 Hz), 8.70 (dd, 2H, H-5, H-7, J = 15.5, 3.9 Hz). Elemental analysis ($C_{15}H_{12}O_4$): C: 69.77% (calcd 70.3%): H: 4.51% (calcd 4.72%). EIMS calculated for $C_{15}H_{12}O_4$ 256, found 256.

HATU-mediated coupling conditions for *N*-methylated amino acids

The test peptide H-Lys-MeVal-Glu-OH, where the ε-amino group of Lys is protected by the *t*-butyloxycarbonyl group and the γ-carboxyl group of the Glu is protected as an allyl ester, was used as the test sequence. Optimized conditions for the HATU activation were amino acid (10 equiv), HATU (10 equiv) and DIEA (20 equiv) in a solution of anhydrous NMP (0.2 M) with two consecutive 17 h couplings. The coupling yield was 89% as assessed by Fmoc Assay.[13]

Peptide synthesis

The basic literature protocol[5] was employed. One synthesis cycle is as follows. Fmoc deprotection with 20% piperidine/DMF (2 × 2.5 min); DMF wash (5 × column vols); coupling with Fmoc amino acid (3 equiv), PyBOP (3 equiv), HOBT (3 equiv) and DIEA (6 equiv); DMF wash (5 × column vols).

Conditions for allyl group removal

A modified version of the literature[6] was used. The resin was treated with tetrakistriphenylphosphine Pd(0) (0.14 M solution in 5% acetic acid and 2.5% *N*-methylmorpholine in chloroform, 3.4 equiv, 2 h, under Ar).

Conditions for removal of the Boc group

The resin was rinsed with 50% TFA–DCM and subsequently incubated with 50% TFA–DCM for two periods (first 2 min, then 18 min). The resin was then rinsed with DCM and 10% DIEA–NMP followed by NMP.

Conditions for peptide cyclization

Conditions are similar to those previously described[6] except the following modifications were included to maximize the yield of cyclic peptide over unwanted side-products: large particle size TentaGel (130 μ) resin with low loading (approximately 160 μmol/g of amino groups compared to the usual 240 μmol/g), PyBOP (4 equiv)/HOBT (4 equiv)/DIEA (8 equiv) in NMP for 1 h. An aliquot was cleaved off the support (90% MeOH–pyridine, 17 h) and analyzed by both LC-ion spray MS and PDMS, which gave the correct molecular weight of 525. No other peaks were observed in either spectra.

Derivatization of support bound cyclic peptide 6 with the monoester 3

After Fmoc deprotection of **6** as described above, the resin was treated with **3** (2 equiv), HATU (2 equiv), and DIEA (4 equiv) in an anhydrous *N*-methylmorpholine solution (0.13 M) for 2 h. After cleavage with 90% MeOH–pyridine followed by solvent removal in vacuo, an isolated yield of 30% was obtained.

Segment condensation

Several modifications were made to the published procedure.[9] After Fmoc deprotection, the resin **6** (0.167 g, 22.9 μmol of amino groups determined by quantitation of the Fmoc group prior to deprotection) was treated with the naphthyl segment **8** (0.026 g, 34.4 μmol), HATU (0.014 g, 34.4 μmol), and DIEA (11.8 mL, 68.8 μmol) in NMP (350 μL) for 17 h. The reaction was shown to be incomplete at this stage by TNBSA assay.[14] More DIEA (50 μL, 291.5 μmol) was added and the reaction allowed to proceed for another 24 h, at which stage the TNBSA assay showed no residual amino groups. The resin was then rinsed with NMP, DCM, Me_2CO, and dried under high vacuum (17 h). Cleavage of the resin with 90% MeOH–pyridine gave the desired conjugate **1** which was one major peak (67%) by HPLC. This corresponded to a yield of 59% for the segment condensation. LC/ion-spray MS for **1**: 1230.8 [M+H] and 1198.4 [M − 2 × CH_3). The UV spectrum (λ_{max} = 204, 242, 286, 296 nm) was consistent with the proposed structure.

Acknowledgments

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