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Solid-Phase Synthesis of Cyclic Peptide–DNA Hybrids

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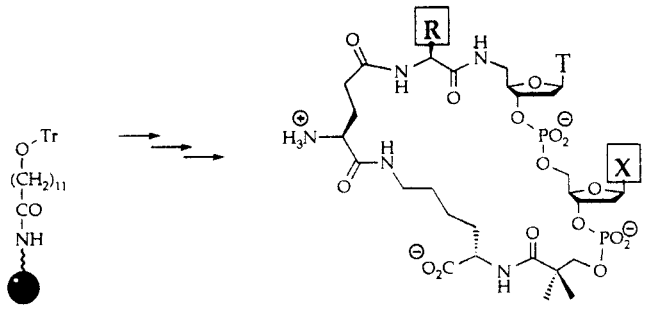
ABSTRACT



A methodology for preparing cyclic peptide–DNA hybrids on controlled pore glass in high yield is reported. This methodology employs Fmoc/Alloc-protected amino acid and nucleoside phosphoramidites on an ω-hydroxylauric acid-derivatized support and is suitable for library synthesis. A cyclic hybrid of the sequence Glu-Leu-T*T-DP-Lys, where Glu and Lys are linked and T* denotes a 5′-amino-5′-deoxynucleotide, exhibited high resistance to exo- and endonucleases.

Cyclic peptides are attractive targets for synthesis as they combine two favorable properties: (i) they adopt fewer conformations than their acyclic counterparts and (ii) they are less susceptible to degradation in biological systems and often possess greater bioavailability than linear peptides.[1] Both make them good leads for drug discovery.[2] Cyclic peptides are, at the same time, more difficult to synthesize than linear oligomers,[3] since the necessary cyclization reaction is often low yielding, unless a particularly stable ring is formed or specific residues or molecular auxiliaries are employed.[4] This seemingly makes the synthesis of diverse libraries of cyclic peptides challenging, particularly so if one was to enrich the repertoire of building blocks with the nucleic acid moieties found in peptide−DNA hybrids.[5,6] In fact, cyclic oligonucleotides, compounds known to be highly specific ligands for DNA, RNA, and proteins, are typically prepared via ligation,[7,8] employing a complementary strand as a template to improve the yield in the cyclization step.[9]

Recent work in these laboratories has focused on creating combinatorial libraries that access the combined structure

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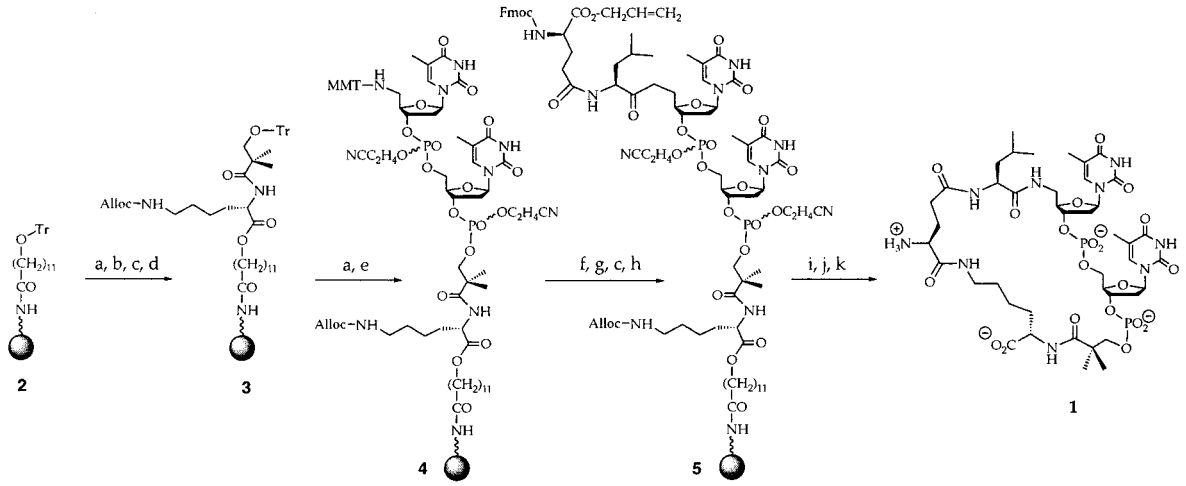
Scheme 1



a) TFA/CH_2Cl_2 (1:1); b) Fmoc-Lys(Alloc)-OH, HOBT, HBTU, DIEA, DMF; c) piperidine/DMF (1:4); d) Tr-DP-OH, HOBT, HBTU, DIEA, DMF; e) DNA synthesis via phosphoramidite protocol; f) TCA/CH_2Cl_2 (3:97); g) Fmoc-Leu-OH, HOBT, HBTU, DIEA, DMF; h) Fmoc-Glu-OCH_2CHCH_2, HOBT, HBTU, DIEA, DMF; i) $Pd(PPh_3)_4$, PPh_3, $[Et_2NH_3]^+HCO_3^-$, CH_2Cl_2; j) HOBT, HBTU, DIEA, DMF; k) NH_4OH.

space of peptides and oligonucleotides.[10−12] Oligonucleotides whose target selectivity and biostability is improved by small acyl and peptidyl substituents have been rapidly identified from these libraries.[13−16] This led us to attempt the synthesis of libraries of cyclic peptide−DNA hybrids, in which we hope to identify compounds that bind specifically to protein surfaces.[17] The successful expansion of cyclic 3′-amino-3′-deoxyadenosine phosphates with peptides by Morr and Wray encouraged us in these attempts.[18,19] Here we report how small libraries of cyclic hybrids can be prepared in high yield.

A methodology for appending peptides to 3′- and 5′-termini of DNA had been established previously.[12] The macrocycle to be prepared here was designed to consist of approximately equal portions of peptide and DNA and to have a ring size between that of FK506 and cyclosporin A. This is the case in hybrids, in which two nucleotides are linked by four peptide residues, including the 3-hydroxy-2,2-dimethylpropionic acid (DP) residue previously optimized as a linker for the 3′-terminus[12] and a glutamic acid residue for the cyclization reaction. The ϵ-amino group of lysine was chosen for the head-to-tail amide formation in the central cyclization step, as it is more sterically accessible than the α-amino group on the same residue. The first synthetic target, compound **1** (Scheme 1), contained two thymidines and leucine as the fourth of the nonnucleic acid residues. The latter is a typical side chain-bearing amino acid and thus a good choice for a model compound.

The synthesis of **1** is shown in Scheme 1. Given the short DNA portion of **1** and the risk of acyl transfer-based chain loss during the cyclization step, a single (rather than three)[12] 12-trityloxylauric acid linker(s) was employed on alkylamine-bearing controlled pore glass to give **2**. After detritylation, the 3′-terminal peptide portion of the hybrid (**3**) was assembled using Fmoc-Lys(Alloc)-OH and the trityl ether of hydroxy dimethylpropionic acid.[12] Detritylation furnished a terminal hydroxyl group, which was used for the assembly of the dinucleotide via automated DNA synthesis, giving **4**. The phosphoramidite of 5′-amino-5′-deoxythymidine[11] as the 5′-terminal residue provided the starting point for construction of the second peptide portion via the Fmoc protocol. Since Fmoc-Glu-OAll but not Fmoc-Glu(All)-OH was commercially available, the second residue was coupled via the side chain carboxylate. Removal of the Alloc and allyl protecting groups liberated amino and carboxy groups on "head" and "tail", respectively (**5**), setting the stage for "on-resin" cyclization. Treatment with HBTU (0.15 M), HOBT (0.167 M), and diisopropyl ethylamine (0.383 M) in DMF for 1 h was used for ring closure. Deprotection and release from support with ammonium hydroxide at room temperature overnight gave **1** in high yield (Figure 1).[20] Structure proof

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(20) A typical hybrid synthesis produced approximately 100 OD_{260} units crude product per gram cpg. HPLC injection yielded **1** as the only MALDI-detectable compound and 65% of the total integrated UV absorbance.

(21) Selected spectroscopic data for **1**: MALDI-TOF MS for $C_{42}H_{64}N_9O_{20}P_2$ $[M-H]^-$ calcd 1076.4, found 1076 ± 2; see Supporting Information for ^1H NMR data.

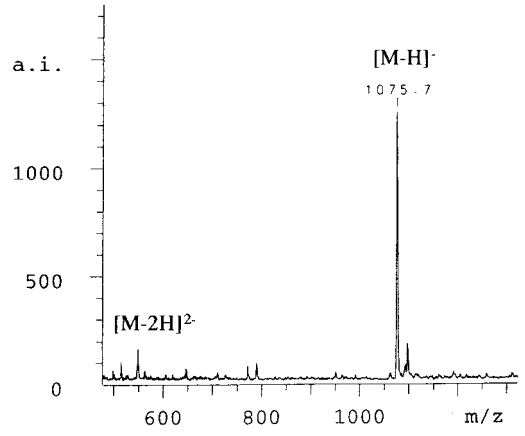

Figure 1. MALDI-TOF MS of crude **1** (calculated *m*/*z* 1076.4 for the $[M-H]^-$ ion and 537.7 for the $[M-2H]^{2-}$ ion).

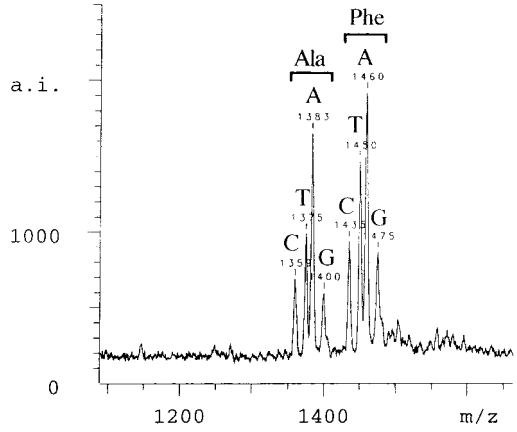

Figure 2. MALDI-TOF mass spectrum of crude **chemset 3**.

for **1** included MALDI-TOF MS, ^{1}H NMR,[21] and nuclease degradation (vide infra).

Exploratory experiments were performed with macrocycles containing four additional nucleotides and five additional amino acid residues, leading to 69-membered rings upon cyclization. The results of these syntheses were judged by MALDI-TOF mass spectra of crudes under conditions previously shown to allow quantitative detection of modified oligonucleotides.[10,14,22] Even on the level of the uncyclized oligomer, the expanded hybrid Glu-$(Gly)_3$-T*GCACG-DP-$(Gly)_3$-Lys gave low yields, but its close relative Glu-$(Gly)_6$-T*GCACG-DP-Lys, where the inserted glycine residues are entirely in the 5′-appendage, gave fewer side products. When comparing the cyclization yield of Glu-$(Gly)_6$-T*GCACG-DP-Lys with that of its derivative Lys-$(Gly)_6$-T*GCACG-DP-Glu, the former was found to give some product, whereas the latter, with its carboxyl group closer to the surface, gave no detectable MALDI peak for the macrocycle, even though these experiments were performed on triester supports.[12] When testing a number of alternative condensation agents, including PyBop/HOBT/DIEA, DCC, DCC/HOBT, DPPA/TEA, CDI, BOP Cl/DIEA, and HONB, none was found to give higher yields than the HBTU/HOBT/DIEA mixture employed thus far. An attempt to cyclize Fmoc-Glu(All)-$(Gly)_6$-T*GCACG-DP-Lys(Alloc)-$(LA)_3$-cpg via metathesis, using the allyl and Alloc protecting groups and bis-(tricyclohexylphosphine)benzylidineruthenium dichloride,[23] also failed to produce MALDI-detectable macrocyclic product. One round of RP-HPLC on a C4-phase gave approximately 50% pure *cyclo*-Glu-$(Gly)_6$-T*GCACG-DP-Lys but gave an approximately 80% pure fraction with the more lipophilic sequence *cyclo*-Glu-$(Ala)_2$-$(Leu)_4$-T*GCACG-DP-Lys (Supporting Information).

The feasibility of library synthesis was tested with derivatives of dinucleotide hybrid **1**. Several libraries or chemsets were prepared via coupling with equimolar mixtures of peptide and/or DNA building blocks, using conditions otherwise unchanged over that for **1**. A mixture of all four phosphoramidites in the first of the two DNA couplings, gave **chemset 1** (Figure 3), whose MALDI-TOF mass spectrum showed as few side products as that of **1**, even though the splitting of the product signal into four peaks makes peaks of impurities more prominent. **Chemset 2** of eight library members was prepared using mixtures of phosphoramidites and the amino acid building blocks Fmoc-Val-OH and Fmoc-Trp-OH. Judged by the MALDI spectrum, the relative reactivity of the two amino acid building blocks was similar to that determined on peptide resins,[24] with the β-branched Fmoc-Val-OH reacting at approximately $^1/_4$ the rate of the Trp building block. Finally, a library of expanded peptide−DNA hybrids containing three additional leucine residues and either alanine or phenylalanine at the 5′-appended position was synthesized (**chemset 3**). The 39-membered macrocycles were again formed in high yield, resulting in a library suitable for monitored selection experiments[10,14] without purification (Figure 2).

As expected,[12] even the largest cyclic peptidyl−DNA hybrids were found to be resistant to nuclease attack from the 3′- and 5′-terminus when treated with snake venom phosphodiesterase (E.C. 3.1.4.1) and calf spleen phosphodiesterase (E.C. 3.1.16.1) (see Supporting Information for **1**). The former nuclease mimics the most important enzymatic activity degrading oligonucleotides in blood.[25] The stability toward nuclease S1 (E.C. 3.1.30.1), an enzyme that hydrolyzes interior phosphodiesters of single-stranded oligonucleotides, was also tested, using the unmodified DNA strand 5′-T_pT_pT-3′ (**6**), which bears the same number of phosphodiesters as **1**, as control. After 5.3 h reaction time, **6** was all but completely hydrolyzed to 5′-T_pT-3′ and monomer products, whereas **1** was largely untouched, with only 20% converted to the open chain form (+18 Da) (Figure 4).

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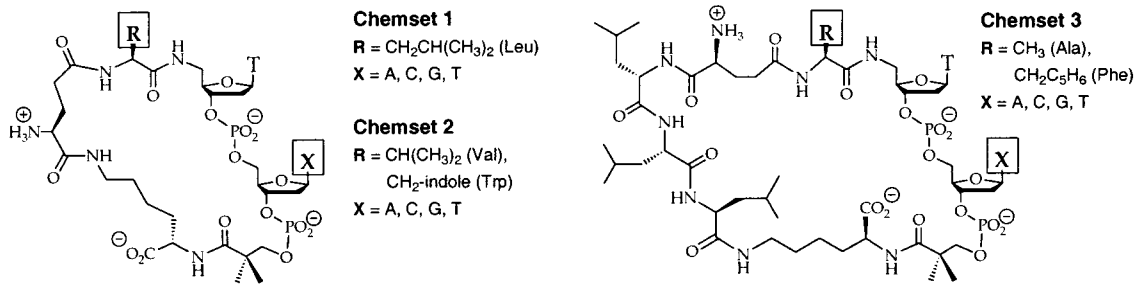

Figure 3.

Dephosphorylation was the only reaction converting the latter, giving a peak at 1015 Da in Figure 4b, indicating that only one of the two phosphodiesters in **1** is a substrate for the enzyme. Nuclease S1 treatment for 24 h showed a more than 10-fold half-life time for **1** compared to **6**. According to exploratory experiments, the linear analogue of **1** has only a 3-fold longer half-life time than **6** under these conditions.

The initial data thus indicates that the hybrids presented here do possess the improved biostability expected for cyclic compounds. The macrocycles also necessarily possess greater conformational rigidity than their acyclic counterparts. The nucleotides with their ribofuranose rings may contribute to this rigidity in a way not unlike that of proline residues in oligopeptides. This is in agreement with the observed tolerance to additional amino acids in hybrids containing a peptide portion with greater contour length than the oligonucleotide (**chemsets 1**, **2**, and **3**). The high yield in which the 39-membered rings of **chemset 3** were formed, together with the much lower yield of the hybrids containing hexanucleotides and octapeptides, corroborates the "proline-like" effect[26] of the nucleosides on the cyclization reaction. A conformational search for **1** converged to closely related three-dimensional structures in which the two nucleobases are in roughly perpendicular orientation to each other in a "DNA loop" and the side chain of Leu2 is the only freely rotating portion of the hybrid (Supporting Information).

The solid-phase methodology presented here is suitable for automation. Hybrid **1** and its derivatives offer a number of sites for further diversification. More diverse libraries may draw from the large number of available amino acids and nucleic acids, including the known phosphoramidites of all four 5′-amino-5′-deoxynucleosides.[27] Their members may be cyclized via shorter side chains by employing aspartic acid, ornithine, or diamino butyric acid. Additional acyl substituents may also be appended to the currently unused amino terminus of the hybrids. Results from the synthetic work on such libraries and the protein binding properties of selected members will be reported in due course.

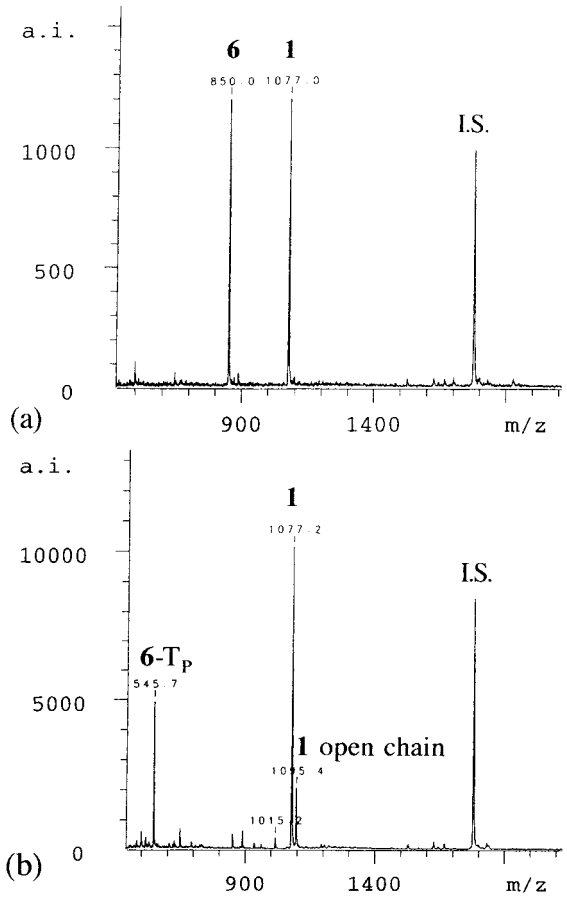

Figure 4. MALDI-TOF mass spectra of a mixture of **1** and DNA control compound 5′-T_PT_PT-3′ (**6**), (a) prior to and (b) after 5.3 h of exposure to nuclease S1 (0.16 u/μL) at 25 °C. I.S. = internal standard.

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Supporting Information Available: MALDI, NMR, and molecular dynamics data for **1**, MALDI data for *cyclo*-Glu-$(Ala)_2$-$(Leu)_4$-T*GCACG-DP-Lys, and MALDI spectra of **1** and **6** prior to and after exposure to snake venom phosphodiesterase. This material is available free of charge via the Internet at http://pubs.acs.org.

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