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Facile Synthesis of Puromycin-Tethered Oligonucleotides at the 3'-End

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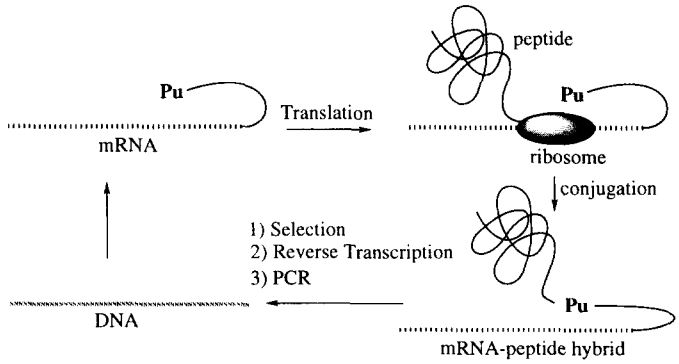
Abstract: A facile method for the synthesis of puromycin-tethered oligonucleotides at the 3'-end is described. The method utilizes a novel CPG support derived from commercially available puromycin. Puromycin-tethered DNA and RNA oligomers were synthesized using a puromycin-tethered CPG support by the usual protocol for automated DNA and RNA synthesis.

Puromycin (Pu) is a member of the 3'-amino-3'-deoxyadenosine family of antibiotics and is known as an extremely powerful inhibitor for protein biosynthesis. Inhibition of protein biosynthesis by Pu is shown to result in the production of a covalent adduct between Pu and the interrupted premature peptide.[2] Recently, using this function of the antibiotic, a specific photocrosslink between 4-thiodTCPu and 23S rRNA to probe the ternary structure of ribosome was investigated.[3] Furthermore, the cross-linking of an elongated peptide and the 3'-end of mRNA was attempted using Pu-tethered mRNA at the 3'-end in order to accomplish a molecular evolution system of proteins as shown in Scheme 1.[4] Although a successful crosslink has recently been demonstrated by the gel electrophoresis technique,[3] the optimization of the linker region between mRNA and Pu has not been examined. Thus, the development of a facile synthetic method of Pu-tethered RNA or DNA is

Scheme 1 A Proposed Molecular Evolution System of Protein using Pu-Tethered RNA



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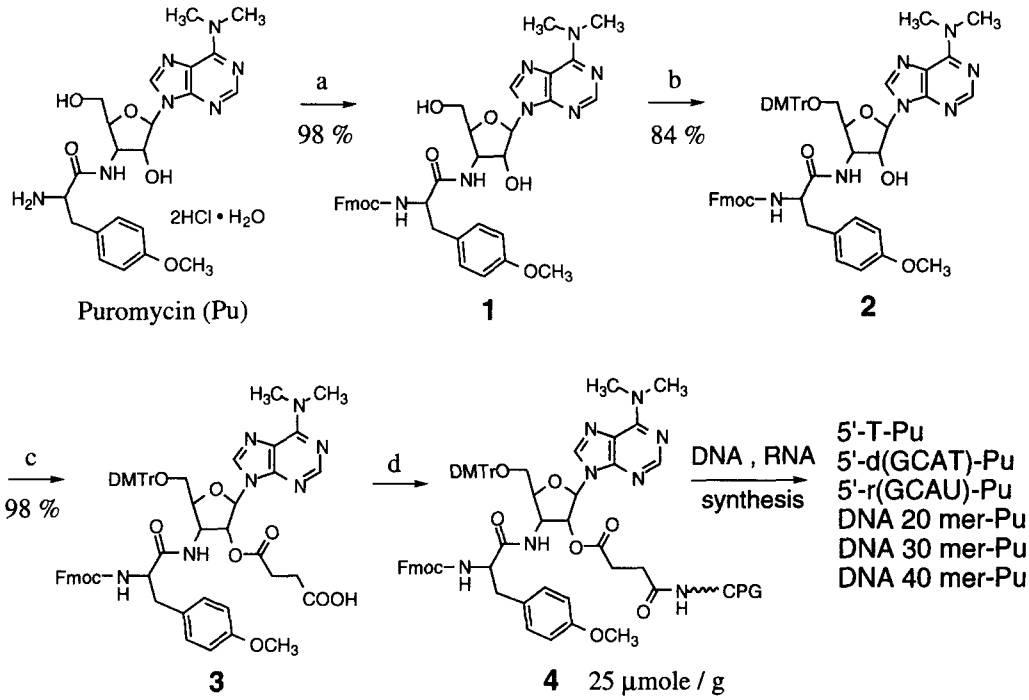
highly desirable. Although the previous syntheses are accomplished by a combination of chemical and enzymatic methods, a large-scale automated synthetic methodology suitable for the screening of peptides had so far not been developed.[5] In this paper we report the synthesis of a novel Pu-attached CPG support. Using this support, various lengths of Pu-tethered oligonucleotides at the 3'-end up to 40 mer were synthesized.

The Pu-attached CPG support **4** was synthesized from commercially available Pu dihydrochloride in four steps (Scheme 2).[6] The amino group and 5'-hydroxyl group of Pu were protected by fluorenylmethoxycarbonyl and dimethoxytrityl groups, respectively. A 2'-Hydroxyl group of **2** was esterified with succinic anhydride.[7] The resulting **3** was attached to an LCAA-CPG support under neutral conditions by the recent Fraser method[8] in order to prevent unwanted release of the Fmoc group.[9] The nucleoside attachment for CPG support **4** was found to be 25 μmole/g by monitoring the released DMTr cation under acidic conditions.[10]

Scheme 2



a. Fmoc-OSu, Et_3N/CH_3CN, b. DMTrCl, Et_3N/pyridine, c. succinic anhydride, DMAP/ pyridine, d. LCAA-CPG, DEC, DMAP, DhbtOH/pyridine

In order to examine the efficacy of the Pu-attached support, we attempted to synthesize DNA and RNA oligomers as shown in Scheme 2 by an automated DNA synthesizer with the standard phosphoramidite method. The averaged coupling efficiency was found to be more than 95 % by monitoring the DMTr cation. After detachment from the support and the subsequent deprotection,[11] the major products were purified by reverse-phase HPLC. The structures of the Pu-tethered oligomers were confirmed by digestion with snake venom

phosphodiesterase (s. v.) and alkaline phosphatase (AP) and by ion spray MS.[12] The structure of T-Pu was further confirmed by ^{1}H NMR.[13] It was revealed that the phosphodiester bond between the normal nucleotide and Pu was completely hydrolyzed under prolonged digestion conditions. Figure 1 shows typical HPLC profiles of (a) the purified sample and (b) enzymatic digest together with (c) ion spray MS of 5'-d(GCAT)-Pu and 5'-r(GCAU)-Pu. Longer DNA-Pu conjugates were also synthesized and identified by ion spray MS.[14] These results clearly show that the CPG support is usable without any modification of the standard phosphoramidite method.

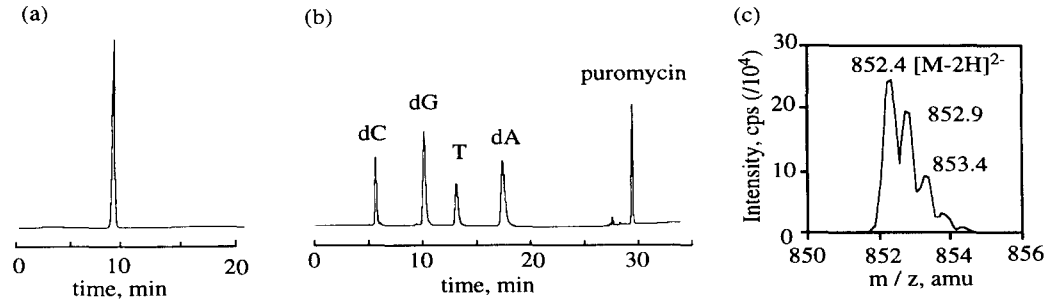


Figure 1. Reverse-phase HPLC profiles of (a) purified 5'-d(GCAT)-Pu, and (b) 5'-d(GCAT)-Pu digested by AP and s. v. PDE (37 °C, 12h). (c) Isotopic distribution of [M-2H]$^{2-}$ by ion spray MS of 5'-d(GCAT)-Pu; calcd 1704.4, found 1704.8.

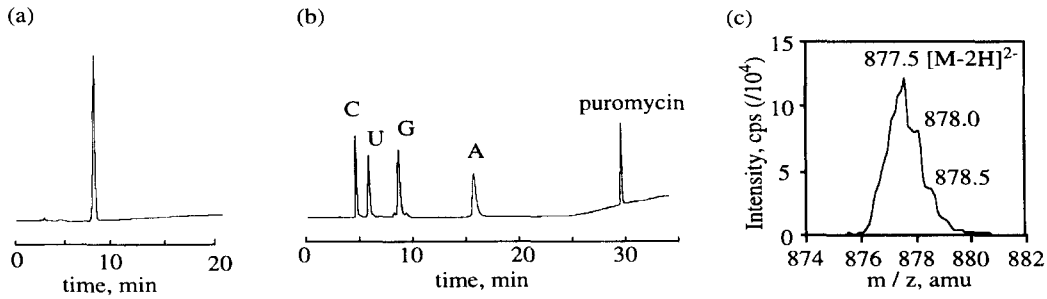


Figure 2. Reverse-phase HPLC profiles of (a) purified 5'-r(GCAU)-Pu, and (b) 5'-r(GCAU)-Pu digested by AP and s. v. PDE (37 °C, 12h). (c) Isotopic distribution of [M-2H]$^{2-}$ by ion spray MS of 5'-r(GCAU)-Pu; calcd 1754.4, found 1755.0.

In summary, we have developed a facile and reliable method to synthesize Pu-tethered oligonucleotides at the 3'-end using a novel support for the standard phosphoramidite method. A preliminary molecular evolution experiment of peptides and the antisense activity of various types of Pu-tethered oligonucleotides at the 3'-end are currently under investigation.

References and Notes

1. Abbreviations: LCAA-CPG (Long Chain Alkyl Amine Controlled Pore Glass), Fmoc-OSu(N(9-Fluorenylmethoxycarbonyloxy)succinimide), DMTrCl(Dimethoxytrityl chloride), DMAP (4-Dimethylaminopyridine), DEC (1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide), DhbtOH (3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine), AP (Alkaline phosphatase), s. v. PDE (snake venom phosphodiesterase).
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5. (a) Fraser, T. H.; Rich, A. *Proc. Nat. Acd. Sci. USA* **1975**, *72*, 8, 3044. (b) Sprizl, M.; Sternbach, H.; von der Haar, F.; Cramer, F. *Eur. J. Biochem.* **1977**, *81*, 579. (c) Uemura, H.; Imai, M.; Ohtsuka, E.; Ikehara, M.; Söll, D. *Nucleic Acids Res.* **1982**, *10*, 20, 6531. (d) Heckler, T. G.; Zama, Y.; Naka, T.; Hecht, S. M. *J. Biol. Chem.* **1983**, *258*, 7, 4492 (e) Palsen, H.; Wintermeyer, W. *Eur. J. Biocem.* **1984**, *138*, 117.
6. Part of the synthesis of Pu-tethered oligonucleotides at the 3' end was presented at the following meetings. (a) Ikeda S,; Sugiyama H.; and Saito I. The 68th Annual Meeting of Chemical Society of Japan, Tokyo, March, **1997.** (b) Ikeda S,; Sugiyama H.; and Saito I. The 214th American Chemical Society National Meeting, Nevada, Sep., **1997.**
7. Compound **1**: ^{1}H NMR (400 MHz, DMSO-d6) δ 2.75 (dd, 1H, J = 10.1, 13.6 Hz, Tyr CH_2), 2.93 (dd, 1H, J = 4.4, 12.9 Hz, Tyr-CH_2), 3.36-3.56 (m, 8H, 5', N-CH_3), 3.67 (s, 3H, Tyr-OCH_3), 3.94 (m, 1H, 4'), 4.10-4.18 (m, 3H, Fmoc-CH, Fmoc-CH_2), 4.32 (ddd, 1H, J = 4.8, 9.0, 9.0 Hz, Tyr-CH), 4.42-4.52 (m, 2H, 2', 3'), 5.13 (t, 1H, J = 5.4 Hz, 5'-OH), 5.99 (d, 1H, J = 2.6 Hz, 1'), 6.06 (br, 1H, 2'-OH), 6.79 (d, 2H, J = 8.4 Hz, Tyr-aromatic), 7.23 (d, 2H, J = 8.6 Hz, Tyr-aromatic), 7.29 (m, 2H, Fmoc-aromatic), 7.39 (m, 2H, Fmoc-aromatic), 7.53 (d, 1H, J = 8.8 Hz, Tyr-NH), 7.63 (d, 1H, J = 7.1 Hz, Fmoc-aromatic), 7.64 (d, 1H, J = 7.3 Hz, Fmoc-aromatic), 7.86 (d, 2H, J = 7.5 Hz, Fmoc-aromatic), 8.06 (d, 1H, J = 7.3 Hz, 3' NH), 8.22 (s, 1H, 2), 8.42 (s, 1H, 8); HRMS (FABMS) calcd for $C_{37}H_{40}N_7O_7$ [(M+H)$^+$] 694.2989, found 694.3012. Compound **2**: ^{1}H NMR (400MHz, $CDCl_3$) δ 2.82 (br, 1H, Tyr CH_2), 3.00 (br, 1H, Tyr-CH_2), 3.30 (dd, 1H, J = 3.8, 10.8 Hz, 5'), 3.43 (dd, 1H, J = 2.0, 10.7 Hz, 5'), 3.20-3.80 (br, 6H, N-CH_3), 3.67 (s, 3H, Tyr-OCH_3), 3.74 (s, 6H, DMTr-OCH_3), 4.13-4.21 (m, 2H, 4', Fmoc-CH), 4.27-4.38 (br, m, 3H, Tyr-CH, Fmoc-CH_2), 4.43 (br, 1H, 3'), 4.62 (br, t, 1H, J = 5.2 Hz, 2'), 5.43 (br, d, 1H, J = 7.0 Hz, Tyr-NH), 5.63 (br, 1H, 1'), 6.42 (br, 1H, 3'-NH), 6.75 (m, 6H, Tyr-aromatic, DMTr-aromatic), 6.98 (m, 2H, Tyr aromatic), 7.13-7.39 (m, 13H, Fmoc-aromatic, DMTr-aromatic), 7.51 (t, 2H, J = 6.8 Hz, Fmoc aromatic), 7.72 (d, 2H, J = 7.5 Hz, Fmoc-aromatic), 7.99 (s, 1H, 2), 8.19 (s, 1H, 8); FABMS calcd for $C_{58}H_{58}N_7O_9$ 996 [(M+H)$^+$] found 996. Compound **3**: ^{1}H NMR (400MHz, $CDCl_3$) δ 2.54-2.75 (m, 6H, Tyr-CH_2, succinic ester-CH_2), 3.30-3.40 (m, 2H, 5'), 3.48 (br, s, 6H, N-CH_3), 3.67 (s, 3H, Tyr-OCH_3), 3.71 (s, 6H, DMTr-OCH_3), 3.89 (br, 1H, 4'), 4.05 (t, 1H, J = 7.0 Hz, Fmoc-CH), 4.12-4.25 (m, 2H, Fmoc-CH_2), 4.73 (m, 1H, Tyr-CH), 5.15 (m, 1H, 3'), 5.77 (d, 1H, J = 5.7 Hz, 2'), 5.82 (d, 1H, J = 8.6 Hz, Tyr-NH), 6.08 (s, 1H, 1'), 6.62-6.70 (m, 3H, 3'-NH, Tyr-aromatic), 6.76 (d, 4H, J = 8.3 Hz, DMTr-aromatic), 6.90 (d, 2H, J = 8.0 Hz, Tyr-aromatic), 7.10-7.50 (m, 15H, Fmoc-aromatic, DMTr-aromatic), 7.70 (d, 2H, J = 7.5 Hz, Fmoc-aromatic), 7.91 (s. 1H, 2), 8.26 (s. 1H, 8); FABMS calcd for $C_{62}H_{62}N_7O_{12}$ 1096 [(M+H)$^+$], found 1096.
8. Walsh, A. J.; Clark, G. C.; Fraser, W. *Tetrahedron Lett.* **1997**, *38*, 1651.
9. Under the previously reported conditions[10] such as triethylamine in pyridine, the Fmoc group of **3** was partially released.
10. Damha, M. J.; Giannaris, P. A.; Zabarylo, S. V. *Nucleic Acids Res.* **1990**, *18*, 3813.
11. The deprotection of RNA-Pu was performed by treatment with 3:1 NH_4OH/MeOH at 55 ℃ for 17 h. Subsequently, 0.4 mL of tetrabutylammonium fluoride solution was added to the residue. The resulting suspension was shaken at room temperature for 6 h and then extracted with ethyl acetate. The aqueous layer was concentrated and subjected to reverse-phase HPLC.
12. Enzymatic digestion of oligomers. To a solution of oligomer in 50 mM sodium cacodylate buffer (pH 7.0) were added s.v. PDE (0.3 unit/ml, final concentration) and calf intestine AP (100 unit/ml, final concentration). The solution was incubated at 37 ℃ for 12 h and was analyzed by reverse-phase HPLC.
13. 5'-T-puromycin: ^{1}H NMR (500 MHz, D_2O) δ 1.62 (ddd, 1H, J = 6.5, 8.5, 14.0 Hz, T2'), 1.67 (s, 3H, T-CH_3), 2.15 (ddd, 1H, J = 2.5, 6.0, 14.0 Hz, T2'), 3.00 (dd, 1H, J = 8.5, 13.5 Hz, Tyr-CH_2), 3.11 (dd, J = 6.5, 14.0 Hz, Tyr-CH_2), 3.17-3.29 (s, 6H, N-CH_3), 3.51 (dd, 1H, J = 4.5, 12.5 Hz, T5'), 3.55 (dd, 1H, J = 3.5, 12.5 Hz, T5'), 3.69-6.75 (m, 4H, Tyr-OCH_3, Pu 4'), 3.85 (m, 1H, Pu 5'), 3.92-3.97 (m, 2H, T4', Pu 5'), 4.13 (dd, 1H, J = 6.5, 8.5 Hz, Tyr CH), 4.51 (m, 1H, T3'), 4.59-4.70 (m, 2H, Pu 2', 3'), 5.72 (dd, 1H, J = 6.0, 8.5 Hz, T1'), 5.84 (d, 1H, J = 3.5 Hz, Pu 1'), 6.92 (d, 2H, J = 8.5 Hz, Tyr aromatic), 7.13 (d, 2H, J = 8.5 Hz, Tyr aromatic), 7.21 (d, 1H, J = 1.5 Hz, T5), 8.02 (s, 1H, Pu 2), 8.18 (s, 1H, Pu 8); ESIMS calcd for 5'-T-Pu 774.3 ([M-H]$^-$) found 774.2.
14. Ion spray MS; 5'-d(GAAGTGAGCGTGAGCGTGAG)-Pu calcd 6820.7; found 6820.5. 5'-d(CAGGATG GCTTGAAGATGTA)-Pu calcd 6754.6; found 6754.7. 5'-d(GAAGTGAGCGTGAGCGTGAG)-T_{10}-Pu calcd 9862.7; found 9863.3. 5'-d(CAGGATGGCTTGAAGATGTA)-T_{10}-Pu calcd 9796.6; found 9797.0. 5'-d(GAAGTGAGCGTGAGCGTGAG)-T_{20}-Pu calcd 12904.6; found 12905.4. 5'-d(CAGGATGGCTTG AAGATGTA)-T_{20}-Pu calcd 12838.6; found 12849.7.