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Synthesis of 2'(3')-*O*-Aminoacyl-pdCpA Carrying a Photofunctional Nonnatural Amino Acid

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A mixed dinucleotide pdCpA, was synthesized and characterized by spectroscopic as well as enzymic analysis. The dinucleotide was acylated with an *N*-protected nonnatural amino acid (Boc-azoAla). The acylation was found to occur at the 2'(3')-*O* position exclusively.

Transfer RNA misacylated with a noncognate amino acid is known to work in protein biosynthetic system and insert the amino acid into a specific site of a protein.[1] Therefore, if one can acylate a tRNA with a nonnatural amino acid, one will obtain an unusual protein in which the nonnatural amino acid is incorporated at the site that should have been occupied by the amino acid cognate to the tRNA. Since direct aminoacylation of tRNA at the 3'-*O* position of the terminal A unit seems intractable, an alternative pathway using enzymic ligation of 3'-*O*-aminoacylated pCpA with a truncated tRNA missing a terminal pCpA unit, has been proposed.[2-7] The semisynthetic tRNA was actually shown to work for the site-specific incorporation of nonnatural amino acids, by Brunner,[5] Schultz,[6] and Chamberlin.[7] Chamberlin and coworkers[7] showed that the C unit of pCpA can be replaced by a deoxy C (dC) unit. Although this markedly simplified the synthesis of the dinucleotide-amino acid conjugate, synthesis of the 2'(3')-*O*-aminoacyl-pdCpA is still a key step in the entire process.

In this communication we describe a synthetic route that is much simpler than earlier,[3-7] to an aminoacyl-pdCpA carrying a nonnatural amino acid, L-*p*-phenylazophenylalanine, i.e., 5'-*O*-phosphoryl-2'-deoxycytidylyl(3'-5')-[2'(3')-*O*-L-phenylazophenylalanyladenosine] = pdCpA-azoAla. The azobenzene units in proteins will photocontrol protein functions.

The synthetic route for pdCpA-azoAla is shown in Scheme 1. As starting materials, a commercially available deoxycytidine derivative, 4-*N*-benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxycytidylyl-3'[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]phosphoramidite **1** (American Bionetics, Inc.) was used as a dC component and 6-*N*,2'-*O*, 3'-*O*-tribenzoyladenosine[8] **2** was employed as an A component. The coupling of the dC component (480 mg) with the A component (277 mg) was carried out in a usual manner.[9] The product was fractionated with a silica gel column [MeOH/dichloromethane(DM)] in 61% yield. The 5'-*O* protecting group of the dinucleotide was removed with 5% trichloroacetic acid in DM. The crude product was

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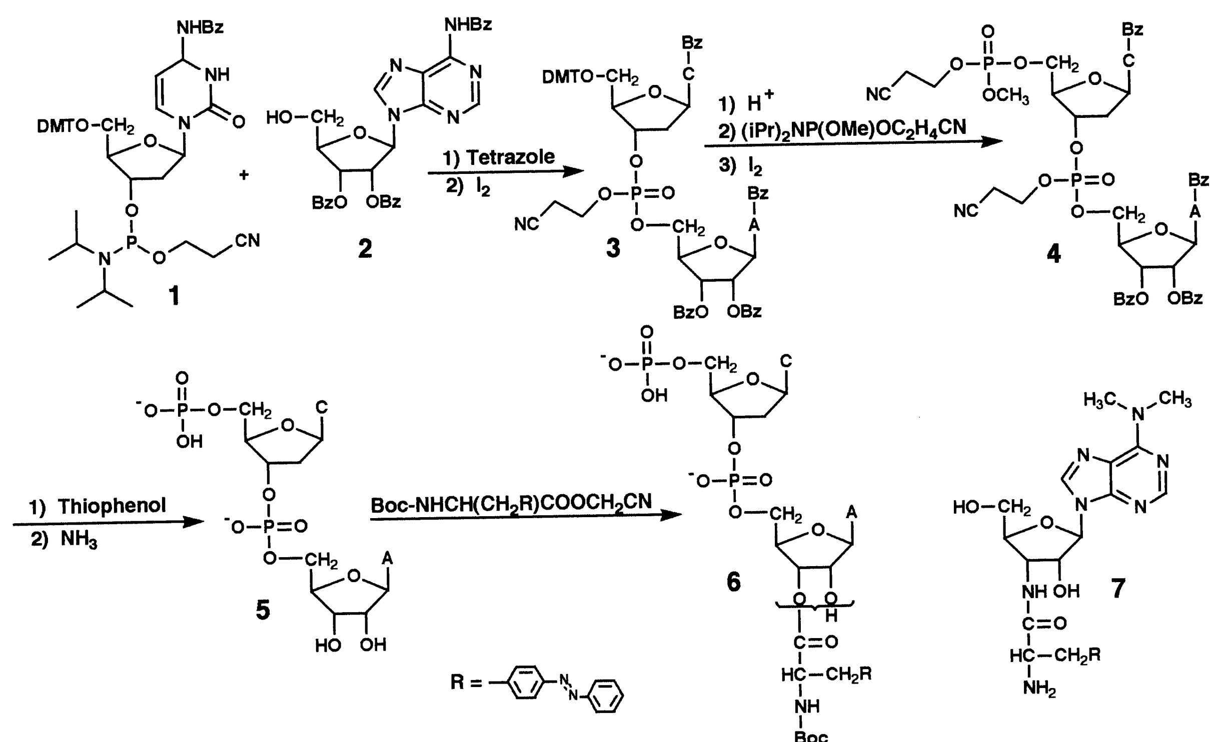

Scheme 1. Synthetic route to pdCpA-azoAla-Boc (**6**) and structure of puromycin analog (**7**).

fractionated with a silica gel column (MeOH/DM) in 86% yield. Phosphorylation of the free 5'-*O* position was achieved with cyanoethyl(diisopropylamino)methoxyphosphine.[9,10] The crude product was fractionated with a silica gel column (MeOH/DM) in 80% yield. ^{31}P NMR showed 4 large peaks that are interpreted by a predominance of two diastereomers among RR, RS, SR, and SS isomers of the two chiral centers on the phosphorus atoms.

The fully protected dinucleotide was deprotected in two steps. The methyl ester at the 5' terminal was removed with thiophenol and the benzoyl group at the nucleic bases and the cyanoethyl esters were deprotected with aqueous ammonia. The product was fractionated with reversed phase HPLC (ODS column, eluent = acetonitrile/0.1 M ammonium acetate, pH 7.0, linear gradient with increasing acetonitrile from 0% to 50% in 50 min). The chromatogram showed three peaks. The first fraction (15%) showed the same ^{1}H NMR spectrum as pdCpA reported by Schultz and coworkers.[6] The third fraction (70%) was assigned to pdCpA with cyanoethyl ester remained at the 5' phosphate. When this fraction was further treated with aqueous ammonia for 24 h, the product was identical to the first fraction. The ^{31}P NMR spectrum of the first fraction showed two equivalent peaks, indicating the absence of chirality on the phosphorus atoms. The assignment of the first fraction to free pdCpA was further supported by enzymic digestion using snake venom phosphodiesterase or nuclease P1. An HPLC analysis of the products of the enzymic reaction indicated an equimolar amount of

5'-dCMP and 5'-AMP, indicating a 1:1 presence of dC and A unit. The total yield of the deprotection was 84%.

Aminoacylationof pdCpA was carried out using an *N*-protected amino acid activated ester in DMF, according to Schultz et al.[6] Free pdCpA ($3x10^{-3}$ mmol) was converted to a pyridinium form using a Dowex 50 W column (pyridinium form, 3 mL, eluted with pyridine/water = 1/1,12 mL). The pyridinium salt was then converted to tetrabutylammonium form using a Dowex 50 W (tetrabutylammonium form, 3 mL, eluted with pyridine/water = 1/1, 12 mL). The eluted solution was evaporated and dried under vacuum. The latter was dissolved in anhydrous DMF (75 μL) containing triethylamine (6 μL) and mixed with 5 equivalents of *t*-butyloxycarbonyl L-*p*-phenylazophenylalanine cyanomethyl ester (Boc-azoAla-OCM) under argon atmosphere. The mixture was stored at room temperature for 4 days and diluted with 50 mM ammonium acetate (pH 4.5)./acetonitrile=1/1 (600 μL). The resultant mixture was subjected to preparative HPLC under the same condition as described above.

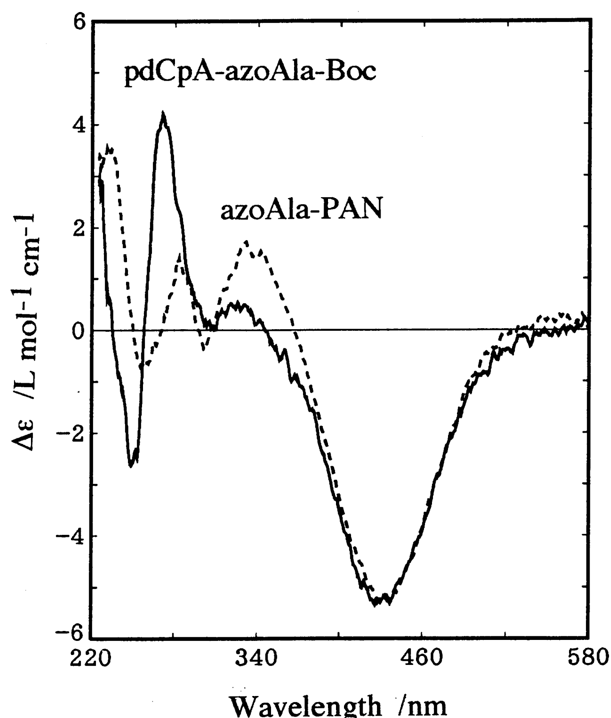

Fig.1. CD spectra of **6** and **7** in 0.1 M ammonium acetate (pH 7).

The HPLC chart showed 6 large peaks. Reasonable absorption spectra were obtained only for two fractions that appeared at 22 min (fraction 1) and at 25 min (fraction 2). The two fractions may be assigned to pdCpA-azoAla-Boc's aminoacylated at 2'-*O* and 3'-*O* position of the adenosine unit. This was confirmed by the interconversion of the two peaks. When fraction 1 was lyophilized and analyzed again with HPLC, the chromatogram showed two peaks with the same intensity ratio (1:4) as before. The same phenomenon was observed for the fraction 2. Similar interconversion has been reported on 2'(3')-*O*-phenylalanyladenosine with a preference of the 3'-*O* derivative than the 2'-*O* derivative.[11] Therefore, the fraction 1 may be the 2'-*O* derivative and the fraction 2 may be the 3'-*O* isomer. The combined yield of the two isomers was 69%. Both fractions showed trans/cis photoisomerization when they were irradiated with a 350 nm light (trans to cis) or with a 450 nm light (cis to trans), indicating the presence of azobenzene unit. pdCpA-azoAla-Boc showed a characteristic CD pattern at the azobenzene absorption band (Fig.1). The CD pattern is similar to that of a puromycin analog *N*-acylated with azoAla (**7**).[12] The resemblance strongly supports that the aminoacylation is occurring at the 3'-*O* position. The *N*-protecting group (Boc) of pdCpA-azoAla-Boc could be removed by an acid treatment. The lyophilized substance was dissolved

in trifluoroacetic acid at 0 °C for 30 min, and the acid was purged off by a stream of nitrogen. The residue was washed with ether and dried under vacuum. HPLC analysis showed complete removal of the Boc group. The pdCpA-azoAla could be stored for several weeks at -20 °C.

In conclusion, pdCpA aminoacylated with a nonnatural amino acid at the 2'(3')-*O* position was synthesized from commercially available intermediates. The pdCpA-amino acid will be used to incorporate the nonnatural amino acid into proteins at any specific sites.

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10) β-Cyanoethanol (2 g) was dissolved in DM (50 mL) at 0 °C. Diisopropylaminomethoxychlorophosphine (5 g) and *N,N*-diisopropylethylamine (26.5 g) were mixed and stirred at 25 °C for 30 min and the precipitate was removed. The filtrate was diluted with ethyl acetate, washed with water, dried over Na_2SO_4, and evaporated. Yield 3.9 g.
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