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Synthesis of the Ribosomal P-Site Substrate CCA-pcb

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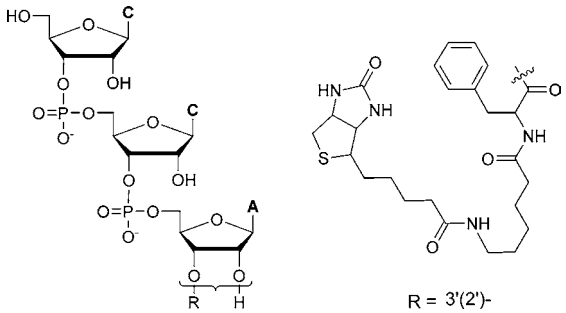
ABSTRACT



CCA-pcb (cytidylyl-(3′5′)-cytidylyl-(3′5′)-3′(2′)-*O*-(*N*-(6-D-(+)-biotinoylaminohexanoyl)-L-phenylalanyl)adenosine), a ribosomal P-site substrate, was synthesized by phosphoramidite chemistry in 26 steps with an overall yield of 18%, starting from biotin. The synthesis relies on the judicious selection of orthogonal silyl protecting groups for the 5′-hydroxyls and acid-labile protecting groups (DMTr, AcE, and MeE) at other reactive sites to ensure the intactness of the labile ester. Both 3′-esterification and nucleotide coupling were accomplished by in situ activation with imidazolium ions.

The catalytic mechanism of peptide bond formation by the ribosome is an area of ongoing research. Proposed catalytic strategies include general acid−base catalysis,[1] substrate-assisted catalysis,[2] and catalysis derived solely from substrate alignment.[3] It is also possible that the reaction may follow a pathway different from the stepwise mechanism involving a tetrahedral intermediate that is observed for aminolysis reactions in aqueous solution.[4] Improved chemical and enzymatic tools are needed to differentiate between these mechanistic possibilities.

In addition to the reaction with full-sized tRNAs, the ribosome can also catalyze peptide bond formation between minimal A-site and P-site substrates. We previously reported a modified fragment reaction that used the A-site substrate, C-puromycin, and a P-site substrate with an extended peptidyl-like chain, CCA-pcb.[5] These substrates have played a valuable role in the structural and biochemical characterization of the peptidyl transfer (PT) reaction.[5,6] Studies have included the use of N^{15}-labeled C-puromycin for kinetic isotope effect (KIE) analysis,[6e] a method that can be used to characterize the transition-state structure of a chemical reaction. Our goal is to determine intrinsic KIEs at several

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atomic positions in both the A-site and P-site substrates in order to explore the transition state of the PT reaction. This necessitates that isotopic substitutions be introduced into CCA-pcb, but an efficient chemical synthesis of CCA-pcb has not been reported.

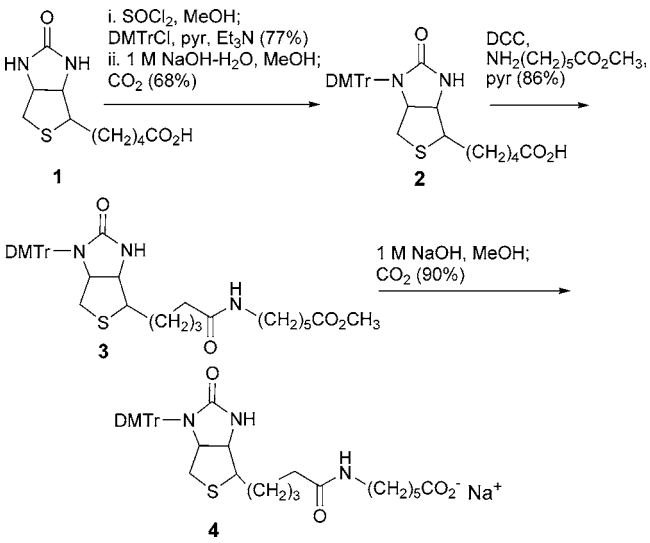
The synthesis of CCA-pcb offers a synthetic challenge because the ester linkage of aminoacylribonucleotide derivatives is an activated, high energy bond with a free energy of hydrolysis comparable to that of ATP.[7] Fast and reversible migration of the amino acyl occurs between the 2′- and 3′-hydroxyl of the ribofuranose. This results in a mixture of 2′- and 3′-*O*-aminoacylated isomers. Most recent syntheses of 2′/3′-*O*-aminoacyloligonucleotides utilized the cyanomethyl ester of protected amino acids.[8] The only synthetic procedure reported for CCA-pcb was by direct coupling between CpCpA and the cyanomethyl ester of pcb, which is expected to provide low yields due to a mixture of mono-, di-, and multiple aminoacylated byproducts.[6a] Here we report an alternative route that provides an efficient synthesis of CCA-pcb.

It was observed that the absence of a neighboring hydroxyl stabilizes the ester bond of 2′/3′-aminoacylnucleotides against hydrolysis and that the hydrolysis rate decreases dramatically at low pH.[9] A half-life of 250 h at pH 2.5 was observed for a 2′/3′-*O*-L-phenylalanyladenosine methyl phosphate derivative.[10] Therefore, we reasoned that the 2′-OH and the amino groups should be protected by acid-labile protecting groups such as ketal or ortho ester and trityl group, respectively. In our synthesis, CCA-pcb was prepared in two different schemes by phosphoramidite chemistry using acid-labile 2′-bisacetoxyethoxymethyl (AcE), 2′-bismethoxyethoxymethyl (MeE), and 4/6-(4,4′-dimethoxytrityl) (DMTr) as protecting groups.

Synthesis of A-pcb Fragment 19. Biotin **1** is insoluble in most organic solvents. Its solubility was enhanced by 1-*N*-tritylation. Esterification of biotin by treatment with $SOCl_2$ in methanol and tritylation with 4,4′-dimethoxytrityl chloride (DMTrCl) in pyridine in the presence of Et_3N,[11] followed by hydrolysis of methyl ester in 1 M NaOH/MeOH, gave the tritylated biotin **2** (52%). This biotin derivative is highly soluble even in DCM. DCC-mediated coupling between compound **2** and methyl 6-aminohexanoate in pyridine and hydrolysis of the methyl ester in 1 M NaOH/MeOH produced compound **4** (77%) (Scheme 1). Significant loss of **2** during purification was observed, and a crude separation followed by amide formation gave **3** in much higher yield (83%).

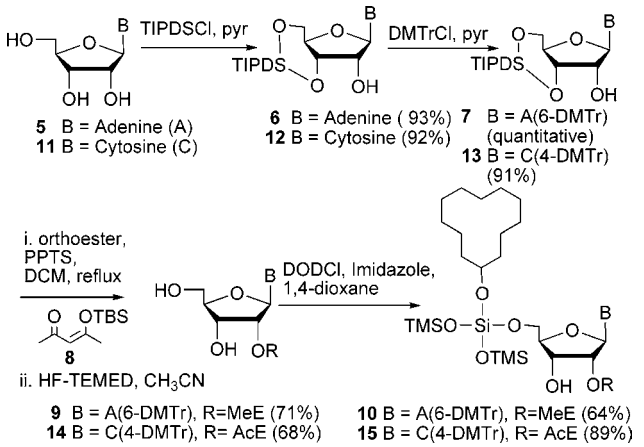
Adenosine **5** was converted into 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)adenosine **6** (93%) via reaction with 1,3-dichloro-1,1,3,3-tetraisopropyl-disiloxane in pyridine. The

Scheme 1. Synthesis of 6-(1-*N*-(4,4′-Dimethoxytrityl)-D-(+)-biotinoyl)aminohexanoic Acid



6-amino group of adenosine was protected with DMTr by reaction with DMTrCl to give quantitatively compound **7**.[12] MeE was introduced into the 2′-position by reflux of compound **7**, with trismethoxyethoxy orthoformate, 4-*tert*-butyldimethyl-siloxy-3-penten-2-one, and PPTS in DCM. The derived syrup after chromatography was treated with HF−TEMED/CH_3CN to provide compound **9** (71%).[13] Compound **9** was further protected by a sterically hindered silyl group at the 5′-position by treatment with bis(trimethylsiloxy)cyclododecyloxylsilyl (DOD) chloride and imidazole in cold THF to give **10** (64%) (Scheme 2). Compound **10** is

Scheme 2. Synthesis of A and C Monomers



unstable, and significant migration of the 2′-*O*-MeE to the 3′-position occurred during storage; thus, it was freshly prepared and purified prior to the subsequent acylation reaction.

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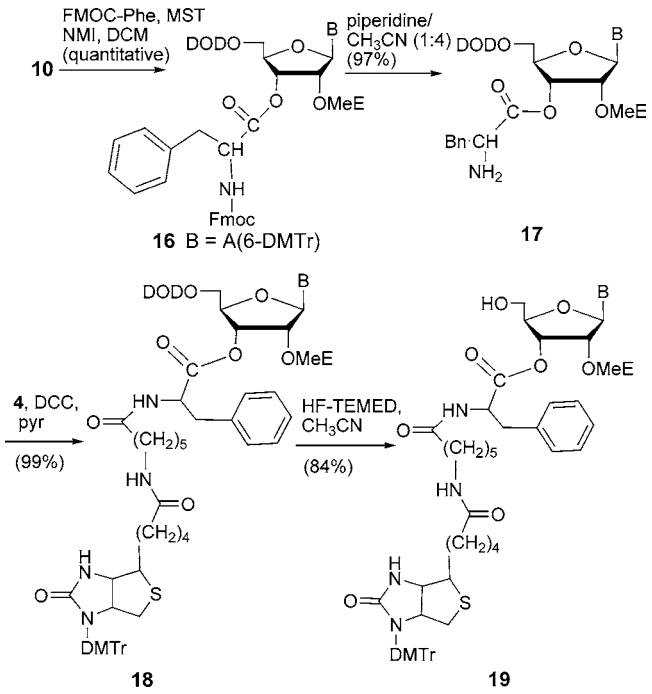
Aminoacylation of compound **10** was first attempted via either the cyanomethyl ester[8] or the acyl fluoride[14] of N-protected L-phenylalanine. Both gave low to moderate yields probably due to significant steric hindrance caused by DOD and MeE. Aminoacylation was then performed with a stronger promoter. Compound **10** was treated with *N*-FMOC-L-phenylalanine in the presence of mesitylenesulfonyl tetrazole (MST)[15,16] and excess *N*-methylimidazole in DCM at ambient temperature to produce quantitatively the fully protected derivative **16**. The reaction involves the imidazolium cation, as shown by ^{1}H NMR, which was nucleophilically displaced as a neutral leaving group by the 3′-hydroxyl of **10**. Removal of FMOC by treatment of **16** with 20% piperidine in CH_3CN resulted in **17** (97%). DCC mediated amide formation with **4** (99%), followed by desilylation by treatment with HF−TEMED in CH_3CN, gave **19** in a yield of 84% (Schemes 2 and 3).

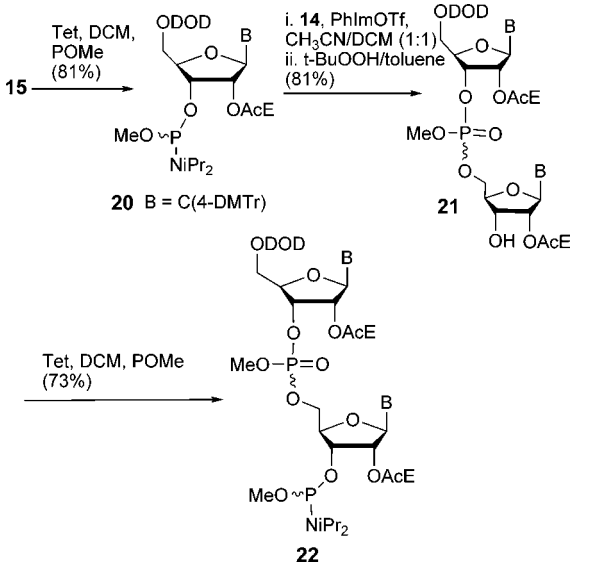
Scheme 3. Synthesis of A-pcb Derivative



Synthesis of C(3′5′)C Phosphoramidite. Cytidine was treated with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane in pyridine to afford 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-cytidine (92%). Compound **12** was selectively tritylated at the N4 to produce **13** (91%), which was further protected at the 2′-position by reflux with a mixture of trisacetoxyethoxy orthoformate, 4-*tert*-butyldimethylsiloxy-3-penten-2-one, and PPTS in DCM. The syrup that resulted after chromatography was treated with HF−TEMED in CH_3CN to provide compound **14** (68%), which was further protected by DOD (89%) (Scheme 2). The cytidylyl phosphoramidite **20** was obtained by treatment of compound **15** with methyl tetraisopropylphosphorodiamidite (POMe) in the presence of tetrazole (Tet) in DCM overnight (81%). Compound **20** was a mixture of two diastereomers due to the chirality of the phosphoramidite as shown by ^{1}H NMR and ^{31}P NMR.

Compound **20** was directly coupled to compound **14** in the presence of PhImOTf[17] and molecular sieves in CH_3CN/DCM (1:1), followed by oxidation with *t*-BuOOH in toluene. The reaction selectively gave **21** in good yield (81%) after chromatography. The diribonucleotide was transformed into its phosphoramidite **22** as before (73%) (Scheme 4).

Scheme 4. Synthesis of C(3′5′)C Phosphoramidite



Compound **22** is a mixture of four diastereomers due to the chirality of phosphorus triester and phosphoramidite. Synthesis of the dinucleotide with tetrazole and S-Et-tetrazole only gave moderate yields, which might be caused by the bulky silyl protecting group.

Synthesis of Protected CCA-pcb and Deprotection. Diribonucleotide phosphoramidite **22** was coupled to 1 molar equiv of compound **19** using PhImOTf and molecular sieves as the promotor in CH_3CN/DCM (1:1) to form the fully protected CCA-pcb **23** in good yield (72%). Reactions with tetrazole and S-Et-tetrazole gave only traces of products. The success of the coupling reaction involves imidazolium cation, which was displaced by the 5′-OH to release a neutral leaving group.[17] Desilylation of compound **23** by treatment with HF−TEMED in CH_3CN (73%) and cleavage of methyl phosphate ester with S_2Na_2 (88%), followed by complete removal of DMTr and orthoformate ester in 0.5 M HCOOH in MeOH−

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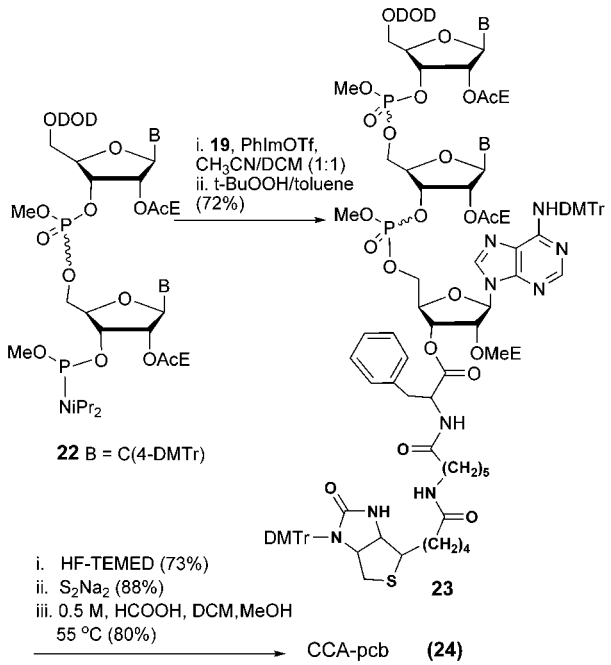
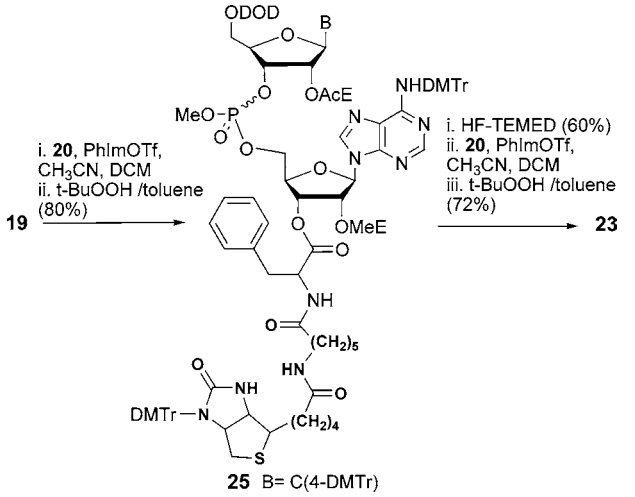
Scheme 5. Synthesis of Fully Protected CCA-pcb and Its Deprotection



Scheme 6. Synthesis of CCA-pcb by Sequential Addition



DCM (1:1) at 55 °C for 5 h gave compound **24** (80%) as a mixture of two regiosiomers (2′/3′-ester) (Scheme 5). The overall yield from biotin was 18%.

Compound **23** was also synthesized by sequential addition of cytidylyl phosphoramidite **20** to A-pcb derivative **19** (Scheme 6). Coupling of **19** with **20** in the presence of PhImOTf and molecular sieves gave fully protected compound CpA-pcb, **25** (80%). Compound **25** was then desilylated (60%) and coupled to **20** a second time to afford a white foam (72%), which was deprotected as described above to produce a compound spectroscopically identical to compound **24** in an overall yield of 9%.

In conclusion, we have accomplished the first efficient synthesis of CCA-pcb, which relies on the judicious selection of orthogonal silyl protecting groups for 5′-hydroxyls and acid-labile protecting groups (DMTr, ACE, and MeE) at other reactive sites to ensure the intactness of the labile ester. Both 3′-esterification and nucleotide coupling were accomplished by in-situ activation with imidazolium ions. This efficient synthesis will make it possible to prepare a series of isotopically substituted P-site substrates for KIE analysis of the PT reaction.

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Supporting Information Available: Experimental procedures and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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