# Positional assignment of differentially substituted bisaminoacylated pdCpAs†

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The synthesis and NMR analysis of a 2′-*O*-alanyl, 3′-*O*-[1-$^{13}$C]valyl-pdCpA derivative has permitted the definitive assignment of the positions of acylation of tandemly activated pdCpAs, and the bisaminoacylated transfer RNAs derived therefrom.

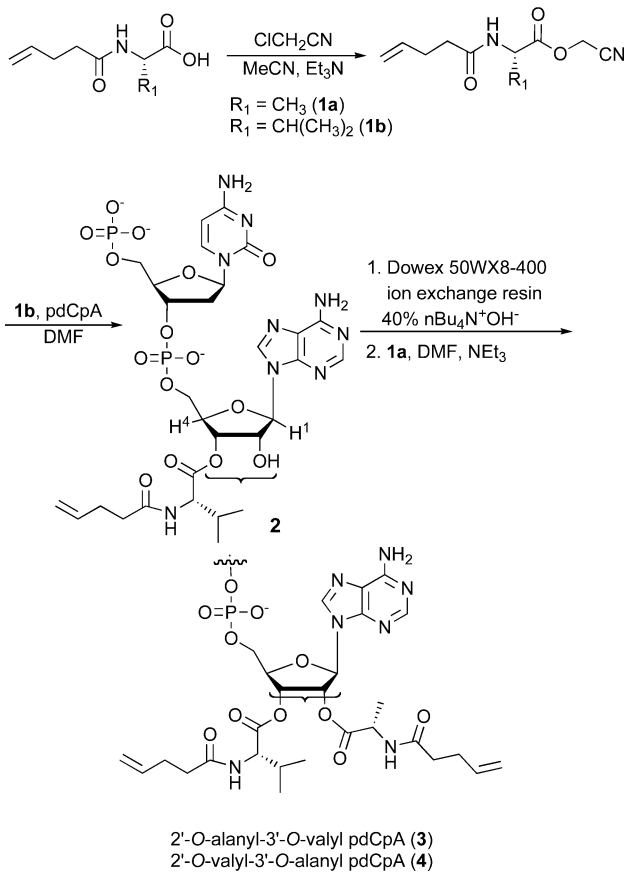
## Introduction

A number of strategies for the preparation of transfer RNAs bearing noncognate amino acids have been developed during the last two decades.[1,2a] One of the most versatile involves enzyme-mediated condensation of an N-protected, chemically synthesized aminoacyl-pdCpA derivative with a tRNA lacking the 3′-terminal dinucleotide, pCpA. The misacylated tRNAs produced in this fashion have been useful in protein synthesizing systems for the elaboration of numerous proteins containing unnatural amino acids at predetermined positions.[2]

Recently, our laboratory has demonstrated that tRNAs tandemly activated with amino acids on both the 2′- and 3′-OH groups of the 3′-terminal adenosine moiety participate normally in protein synthesis.[3] While this was the first report describing a biochemical function of bisaminoacyl-tRNAs, bisphenylalanyl-tRNA has been reported to occur naturally in *Thermus thermophilus*.[4] While most of the bisaminoacylated pdCpA derivatives described to date have had the same amino acids on the 2′-OH and 3′-OH positions of the adenosine ribose moiety,[5] the synthesis of analogues differentially substituted at these positions was necessary for mechanistic studies of peptide bond formation.[3] The synthesis of bisaminoacylated dinucleotides parallels that of the monoacylated pdCpA derivatives, with only a second acylation being necessary. However, the chemical synthesis of the dinucleotides containing two different amino acids at the 2′- and 3′-OH groups of the terminal adenosine moiety in a regiochemically defined fashion is more complex, owing to the rapid migration of the aminoacyl residue of the intermediate monoacylated pdCpAs between the vicinal *cis*-hydroxyl groups of adenosine.[6] This phenomenon results in an equilibration between the 2′- and 3′-aminoacyl isomers of pdCpA complicating product analysis (*vide infra*). Presently, we describe the preparation and positional assignment of bisaminoacyl-pdCpA derivatives having different amino acids on the 2′- and 3′-OH groups of the adenosine moiety.

## Results and discussion

The syntheses of bisaminoacylated pdCpA derivatives **3** and **4**, which are differentially substituted with valine and alanine, was accomplished by acylation of a monoacylated pdCpA derivative, as shown in Scheme 1. The monoacylated valyl-pdCpA was prepared as described previously.[7a] (*S*)-Alanine and (*S*)-valine were N$^{\alpha}$-protected using succinimidyl 4-pentenoate to afford the respective *N*-pentenoyl amides. Treatment with chloroacetonitrile in the presence of $NEt_3$ gave the respective cyanomethyl esters **1a** and **1b**.[7] Treatment of activated ester **1b** with the tris(tetrabutylammonium)salt of pdCpA in DMF provided the *N*-(4-pentenoyl)valyl-pdCpA (**2**) as a mixture of 2′- and 3′-*O*-valyl




**Scheme 1** Synthesis of bisaminoacylated pdCpA derivatives **3** and **4**.

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† Electronic supplementary information (ESI) available: gDQCOSY spectrum of [$^{13}$C]labeled bisaminoacylated pdCpA and HPLC separation of regioisomeric 2′,3′-*O*-valyl-pdCpAs

esters. It has been demonstrated previously that related species having an unprotected amino group undergo rapid equilibration between the 2′- and 3′-OH groups of ribose [$t_{1/2} = 1$–$11\ s^{-1}$, pH 7.3, 37 °C],[6] but it seemed possible that acylation of the α-amino group would slow the equilibration to a manageable rate. However, while the two isomers could be cleanly separated by HPLC, immediate reinjection of the ostensibly pure 2′- or 3′-isomers resulted in two peaks with the same retention times as the original mixture (ESI†). Further, the relative intensity of these peaks was comparable to that in the original mixture of positional isomers, indicating that despite $N^{\alpha}$-protection, rapid equilibration between the 2′- and 3′-aminoacyl isomers of pdCpA had taken place.[8] It is likely that the mechanism of equilibration involves the intermediacy of an orthoacid (Fig. 1).

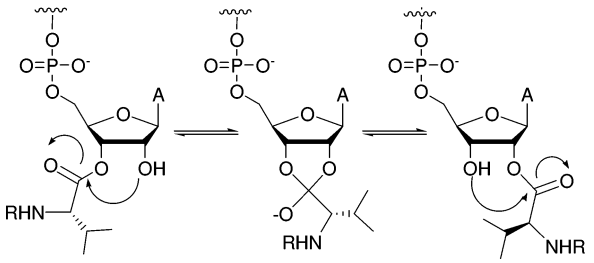

**Fig. 1** Putative mechanism for equilibration between the 2′-OH and 3′-OH groups.

As the individual monoacylated pdCpA derivatives equilibrate rapidly, they could not be separated for regiocontrolled introduction of the second requisite aminoacyl group. Therefore, the mixture of the 2′- and 3′-isomers was treated with an ion exchange resin to form the tris(tetrabutylammonium) salt of the regioisomeric valyl-pdCpAs. The resulting salt was treated with a large excess of *N*-pentenoylalanine cyanomethyl ester (**1a**), affording the isomeric substituted 2′,3′-bisaminoacylated pdCpA derivatives in about a 3 : 2 ratio. Because the equilibrium abundance of 3′- and 2′-isomers of monoacylated nucleosides typically falls within the same range as the approximate 3 : 2 ratio of products noted for the regioisomers of pdCpA acylated with both valine and alanine (**3** and **4**), it was tempting to assign the major product as regioisomer **3**, *i.e.* the product resulting from acylation of the major positional isomer of **2** with *N*-pentenoylalanine (Fig. 2). However, given the observed rapid equilibration between positional isomers of precursor **2** (Fig. S1, ESI†) and significant difference in $pK_a$s of the 2′- and 3′-OH groups of adenosine,[8] we could not exclude the possibility that the major bisacylated regioisomer of pdCpA actually arose from acylation of the 2′-*O*-valyl-pdCpA (**2**) with **1a** (Fig. 2).[9] In any case, these products could be separated by $C_{18}$ reversed phase HPLC using a gradient of 1–50% $CH_3CN$ in 50 mM $NH_4OAc$, pH 4.5, over a period of 75 min (Fig. 3).

To resolve the ambiguity concerning the position of acylation, the synthesis of **3** and **4** shown in Scheme 1 was repeated using [1-$^{13}$C]valine. The major bisaminoacylated product was isolated by HPLC for NMR analysis. Initially, we sought to define the chemical shifts of the various protons on the ribose moiety in the $^1$H NMR spectrum. Analysis of $^1$H-$^1$H COSY and TOCSY NMR spectral data clearly revealed the chemical shifts of these protons: $\delta_H$ 6.31 (H-1′), $\delta_H$ 5.99 (H-2′), $\delta_H$ 5.78 (H-3′) and $\delta_H$ 4.58 (H-4′) (Fig. S2). Additionally, these NMR experiments allowed

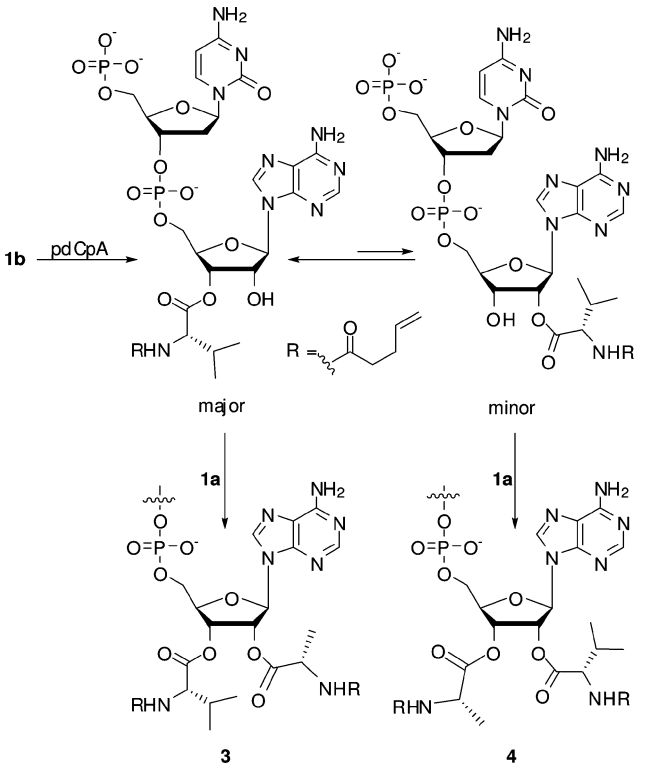


**Fig. 2** Proposed mechanism for the formation of the isomeric bisaminoacylated pdCpAs.

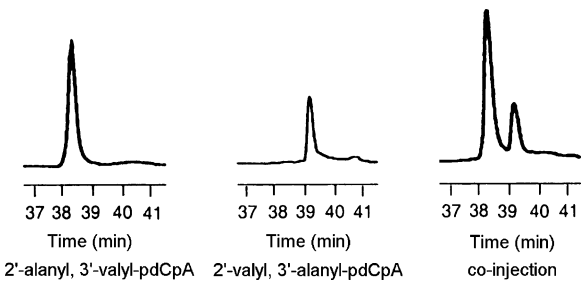


**Fig. 3** $C_{18}$ reversed phase HPLC analysis of two isomeric pdCpA derivatives (also indicating the putative regiochemical assignments). The retention times of **3** and **4** were 38.3 min and 39.2 min, respectively.

for the assignment of the chemical shifts of both $H_\alpha$ and $H_\beta$ of the $^{13}$C-labeled valine ester (Fig. 4). With this information, the analysis of the $^1$H-$^{13}$C HMBC NMR spectral data was possible. Importantly, this technique is typically limited to two- and three-bond couplings. Thus one would only expect the following correlations for the putative 2′-*O*-alanyl-3′-*O*-$^{13}$C-valyl-pdCpA product: ($^{13}$C-H-3′), ($^{13}$C-$H_\alpha$) and ($^{13}$C-$H_\beta$). Notably, the unlabeled carbonyl carbon of alanine would not be seen in the NMR spectrum due to the low natural abundance of $^{13}$C and the limited quantity used for spectral analysis. As shown in Fig. 4, intense cross peaks were observed between the valine carbonyl carbon and H-3′, $H_\beta$ and $H_\alpha$, *i.e.* exactly what would be expected for 2′-*O*-alanyl-3′-*O*-$^{13}$C-valyl-pdCpA. Had the $^{13}$C-valine been on the 2′-OH group, an intense cross peak with H-2′ (at 5.99 ppm) would have been present; this was not observed. These results indicate that the major bisacylation product is 2′-*O*-alanyl-3′-*O*-valyl-pdCpA (**3**). The ratio of this product to the isomeric

 

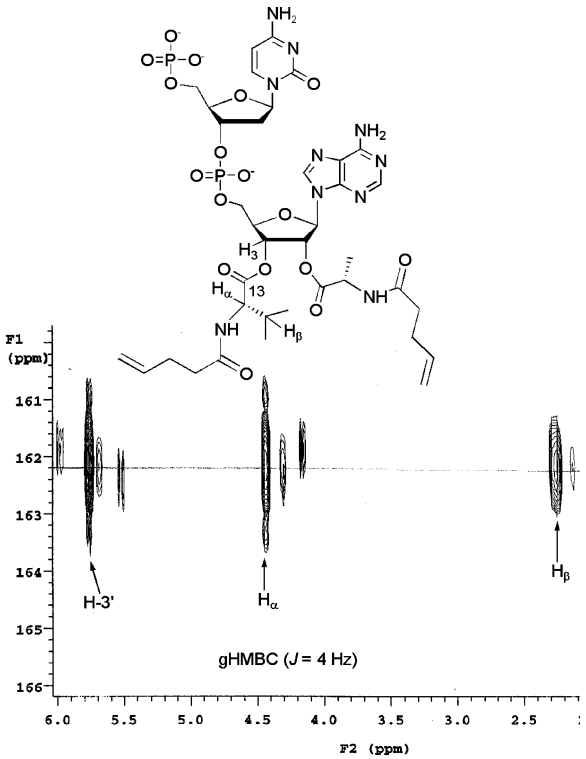


**Fig. 4** $^{1}$H-$^{13}$C Heteronuclear correlation (HMBC) of 2′-*O*-alanyl-3′-*O*-[$^{13}$C]valyl-pdCpA.

3′-*O*-alanyl-2′-*O*-valyl-pdCpA (**4**) was comparable to the ratio of the intermediate monovalyl-pdCpA derivatives (**2**) (Scheme 1 and Fig. 2), arguing that the product ratio is likely defined by the ratio of the (equilibrating) monovalyl-pdCpA intermediates.

## Conclusion

This paper describes the synthesis and positional assignment of a bisaminoacyl-pdCpA derivative. It provides unambiguous assignment of the position of acylation through the use of 2D NMR techniques. The acylation of pdCpA with an activated valine gave predominantly the 3′-*O*-valyl derivative, with the second acylation occurring at the 2′-position. Further, this paper provides a strategy for unambiguously assigning the positions of substitution of other unsymmetrical bisaminoacylated pdCpA derivatives.

## Experimental

### General methods

Anhydrous grade THF, $CH_2Cl_2$ and acetonitrile were purchased from VWR. All reactions involving air or moisture-sensitive reagents or intermediates were performed under a nitrogen or argon atmosphere. Flash chromatography was performed using Silicycle 40–60 mesh silica gel. Analytical TLC was performed using 0.25 mm EM silica gel 60 $F_{250}$ plates that were visualized by irradiation (254 nm) or by staining with ninhydrin. $^{1}$H and $^{13}$C NMR spectra were obtained using 300 MHz and 500 MHz Varian NMR instruments. Chemical shifts are reported in parts per million (ppm, $\delta$) referenced to the residual $^{1}$H resonance of the solvent ($CDCl_3$: 7.26 ppm; DMSO-$d_6$: 2.49 ppm). $^{13}$C spectra were referenced to the residual $^{13}$C resonance of the solvent ($CDCl_3$: 77.3 ppm; DMSO-$d_6$: 39.5 ppm). Splitting patterns are designed as follows: s, singlet; br, broad; d, doublet; m, multiplet. High resolution mass spectra were obtained at the Michigan State University-NIH Mass Spectrometry Facility.

**2′-*O*-[*N*-(4-Pentenoyl)-(*S*)-alanyl]-3′-*O*-[*N*-(4-pentenoyl)-(*S*)-valyl]-pdCpA ester (3).** To a conical vial containing 120 mg (0.57 mmol) of *N*-(4-pentenoyl)-(*S*)-alanine cyanomethyl ester (**1a**) was added a solution of the tetrabutylammonium salt of 2.0 mg (2.45 μmol) of *N*-(4-pentenoyl)-(*S*)-valyl-pdCpA[7a] (**2**) in 50 μL of freshly distilled DMF, followed by 5 μL of triethylamine. The reaction mixture was stirred at 25 °C and monitored by HPLC. A 5 μL aliquot of the mixture was diluted with 45 μL of 1 : 2 $CH_3CN$–50 mM $NH_4OAc$, pH 4.5. Ten microlitres of the diluted aliquot was analyzed by HPLC on a $C_{18}$ reversed phase column (250 × 10 mm). The column was washed with 1 → 63% $CH_3CN$ in 50 mM $NH_4OAc$, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL min$^{-1}$ (monitoring at 260 nm). After 4 days, the reaction mixture was diluted to a total volume of 400 μL with 1 : 1 $CH_3CN$–50 mM $NH_4OAc$, pH 4.5, and purified using the same semi-prep $C_{18}$ reversed phase column (retention time 22.3 min). After lyophilization of the appropriate fractions, 2′-*O*-[*N*-(4-pentenoyl)-(*S*)-alanyl]-3′-*O*-[*N*-(4-pentenoyl)-(*S*)-valyl]-pdCpA ester (**3**) was obtained as a colorless solid: yield 0.6 mg (25%); mass spectrum (electrospray ionization), *m*/*z* 969.4 (M − H)$^+$, theoretical *m*/*z* 969.3 (M − H)$^+$. A smaller amount of isomeric **4** was also isolated.

**2′-*O*-[*N*-(4-Pentenoyl)-(*S*)-valyl]-3′-*O*-[*N*-(4-pentenoyl)-(*S*)-alanyl]-pdCpA ester (4).** Prepared as described for the synthesis of **3**; however, **1b** was treated with *N*-(4-pentenoyl)-(*S*)-alanyl-pdCpA.[7b] Compound **4** was obtained as a colorless solid: 0.4 mg (21%); mass spectrum (electrospray ionization) *m*/*z* 969.5 (M − H)$^+$, theoretical *m*/*z* 969.3 (M − H)$^+$. A smaller amount of isomeric **3** was also isolated.

***N*-(4-Pentenoyl)-(*S*)-[1-$^{13}$C]valine.** Prepared as described for the unlabeled derivative in ref. 7. Colorless solid: 0.39 g (93%); $^{1}$H NMR ($CDCl_3$) $\delta$ 0.94 (d, 3H, *J* = 6.9 Hz), 0.98 (d, 3H, *J* = 6.9 Hz), 2.20–2.27 (m, 1H), 2.35–2.42 (m, 4H), 4.95–5.17 (m, 2H), 5.79–5.95 (m, 1H), 6.15 (d, 1H, *J* = 8.1 Hz) and 6.16–6.50 (br s, 1H); $^{13}$C NMR ($CDCl_3$) $\delta$ 17.9, 19.3, 29.8, 31.2, 35.9, 57.4, 116.2, 136.9, 173.6 and 177.3; mass spectrum (electrospray ionization), *m*/*z* 201 (M + H)$^+$, theoretical *m*/*z* 201 (M + H)$^+$; mass spectrum (FAB) *m*/*z* 201.1319 (M + H)$^+$ ($C_9H_{18}O_3N^{13}C$ requires 201.1321).

***N*-(4-Pentenoyl)-(*S*)-[1-$^{13}$C]valine cyanomethyl ester.** Prepared as described for the unlabeled derivative in ref. 7. Colorless solid: yield 0.30 g (73%); $^{1}$H NMR ($CDCl_3$) $\delta$ 0.91 (d, 3H, *J* = 6.9 Hz), 0.94 (d, 3H, *J* = 6.9 Hz), 2.00–2.20 (m, 1H), 2.31–2.40 (m, 4H), 4.51–4.60 (m, 1H), 4.64–4.85 (m, 2H), 4.97–5.00 (m, 2H), 5.65–5.85 (m, 1H) and 6.15 (m, 1H); $^{13}$C NMR ($CDCl_3$) $\delta$ 18.0, 19.1, 29.6, 31.1, 35.6, 48.9, 56.0, 57.5, 116.0, 137.0, 171.1 and 172.8; mass spectrum (electrospray ionization), *m*/*z* 240 (M + H)$^+$, 262 (M + Na)$^+$, theoretical *m*/*z* 240 (M + H)$^+$; mass spectrum (FAB) *m*/*z* 240.1430 ($C_{11}H_{19}O_3N_2{}^{13}C$ requires 240.1430).

***N*-(4-Pentenoyl)-(*S*)-[1-$^{13}$C]valyl-pdCpA.** Prepared as described for the synthesis of the unlabeled derivative in ref. 7. Colorless solid: 1.9 mg (49%); mass spectrum (electrospray

ionization) $m/z$ 819.2 $(M + H)^+$, theoretical $m/z$ 819 $(M + H)^+$; mass spectrum (FAB), $m/z$ 819.2305 $(M + H)^+$ ($C_{29}H_{42}O_{15}N_9P_2{}^{13}C$ requires 819.2310).

**2′-*O*-[*N*-(4-Pentenoyl)-(*S*)-alanyl]-3′-*O*-[*N*-(4-pentenoyl)-(*S*)-[1-$^{13}$C]valyl-pdCpA ester.** Prepared as described for the synthesis of unlabeled **3**. Colorless solid: yield 2.8 mg (28%); mass spectrum (electrospray ionization) $m/z$ 972 $(M + H)^+$, 995 $(M + H + Na)^+$, theoretical $m/z$ 972 $(M + H)^+$; mass spectrum (FAB) $m/z$ 972.3103 ($C_{36}H_{53}O_{17}N_{10}P_2{}^{13}C$ requires 972.3099).

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8 See ESI for $C_{18}$ reversed phase HPLC traces of the monoaminoacylated pdCpA derivatives.† The 3′-*O*-aminoacylated species is the predominant species, due to the greater acidity of the 2′-OH group.

9 As well established by the Curtin–Hammett principle, D. Y. Curtin, *Rec. Chem. Prog.*, 1954, **15**, 111; J. I. Seeman, *Chem. Rev.*, 1983, **83**, 83, the less abundant of two equilibrating intermediates can afford the major product if it does so by a pathway that is energetically favored overall.

 