T4 RNA ligase mediated preparation of novel "chemically misacylated" tRNAPheS

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T4 RNA Ligase Mediated Preparation of Novel "Chemically Misacylated" $tRNA^{Phe}$s†

Thomas G. Heckler, Li-Ho Chang, Yoshiyuki Zama, Takehiko Naka, Mukund S. Chorghade, and Sidney M. Hecht*

ABSTRACT: T4 RNA ligase was employed for the condensation of *Escherichia coli* $tRNA^{Phe}$ missing cytidine-75 and adenosine-76 ($tRNA^{Phe}$-C_{OH}; the acceptor "oligomer") with each of several chemically acylated derivatives of pCpA (the donor "oligomer"). The resulting "chemically misacylated" $tRNA^{Phe}$s were obtained in 20–65% yields following chromatographic workup on DEAE-cellulose and benzoylated DEAE-cellulose. Characterization of the chemically misacylated tRNAs was accomplished by (i) enzymatic reaminoacylation of chemically misacylated $tRNA^{Phe}$ with phenylalanine by *E. coli* phenylalanyl-tRNA synthetase following chemical deacylation of the "incorrect" amino acid, (ii) comparison of the hydrolytic effects of Cu^{2+} solutions on chemically and enzymatically prepared samples of *N*-acetyl-L-phenylalanyl-$tRNA^{Phe}$s, and (iii) measurement of the chromatographic behavior of the tRNA species derived from chemical misacylation.

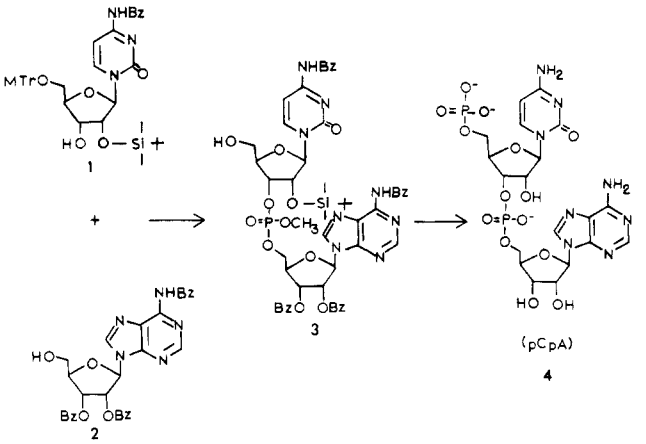
The considerable potential of T4 RNA ligase for the construction of specific oligonucleotides has been realized in recent years (Gumport et al., 1980; Ohtsuka et al., 1981; Bruce & Uhlenbeck, 1982). Concurrently, the use of RNA ligase for the modification of the 3′-terminus of tRNA has been equally successful. For example, T4 RNA ligase has been utilized for the specific radiolabeling of the CCA acceptor stem (England et al., 1980) and for the construction of tRNAs having extended 3′-termini (Bruce & Uhlenbeck, 1978). Also described has been a potentially general approach for the preparation of "chemically misacylated" tRNAs (Hecht et al., 1978), wherein T4 RNA ligase was employed to effect the transfer of aminoacylated adenosine moieties from suitably protected aminoacylated P^1,P^2-bis(5′-adenosyl) diphosphates onto the 3′-end of abbreviated *Escherichia coli* tRNA-CC_{OH}'s.[1] However, the large (>200-fold) excess of the aminoacylated P^1,P^2-bis(5′-adenosyl) diphosphates required to effect successful ligation (as well as the accompanying solubility and technical problems[2] associated with this excess) has prompted the development of a more effective alternative (Heckler et al., 1983).

Presently, we describe the preparation of several chemically misacylated $tRNA^{Phe}$s by the T4 RNA ligase mediated coupling of 2′(3′)-O-acylated pCpA derivatives (using a more modest 20-fold excess of these enzyme substrates) with $tRNA^{Phe}$-C_{OH}. Several misaminoacylated $tRNA^{Phe}$s were isolated in satisfactory yields, as were a number of acylated $tRNA^{Phe}$s containing carboxylic acids other than naturally occurring amino acids. Also described is the characterization of the chemically misacylated tRNAs.

Materials and Methods

Materials. DEAE-cellulose was purchased from Whatman and BD-cellulose from Boehringer Mannheim. T4 RNA ligase (sp act. 2000 units/mg) was obtained from P-L Biochemicals; one unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol of phosphatase-resistant ^{32}P from 5′-^{32}P-labeled oligo(rA)$_n$ in 30 min at 37 °C. RNase T_2 was purchased from Sigma, and [^{3}H]phenylalanine (22.5 Ci/mmol) was obtained from New England Nuclear. *E. coli* $tRNA^{Phe}$ (sp act. 1200 pmol/A_{260} unit) was purchased from Boehringer Mannheim; *E. coli* phenylalanyl-tRNA synthetase was purified as described (Pezzuto & Hecht, 1980).

Scheme I



General Procedures. Thin-layer chromatography was performed on silica gel F_{254} (Merck) and cellulose F_{254} plates (Analtech). Solvent systems used were as follows: (solvent A) dichloromethane–methanol (9:1); (solvent B) 2-propanol–concentrated ammonium hydroxide–water (7:1:2); (solvent C) 1-butanol–glacial acetic acid–water (5:2:3); (solvent D) 2-propanol–concentrated ammonium hydroxide–water (55:10:35); (solvent E) acetone–ethanol–water (4:4:2). Nucleotides and their derivatives were detected by UV visualization.

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[1] Abbreviations: tRNA-CC_{OH}, tRNA missing the 3′-terminal adenosine moiety; tRNA-C_{OH}, tRNA missing the 3′-terminal cytidine and adenosine moieties; BD-cellulose, benzoylated diethylaminoethylcellulose; Na^+-Hepes, sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; Tris, tris(hydroxymethyl)aminomethane; Na^+-Pipes, sodium 1,4-piperazinediethanesulfonate; Cbz, carbobenzyloxy; THF, tetrahydrofuran; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

[2] Limited chemical deacylation of aminoacylated P^1,P^2-bis(5′-adenosyl) diphosphates, at the molar ratios (200 equiv of aminoacylated nucleotide/equiv of tRNA-CC_{OH}) required for successful ligation, can yield amounts of $AppA_{OH}$ that are significant relative to the amounts of tRNA-CC_{OH} present in the incubation mixture. As $AppA_{OH}$ is a better substrate for T4 RNA ligase than many aminoacylated dinucleoside diphosphates, nonproductive formation of tRNA-CCA_{OH} can result.

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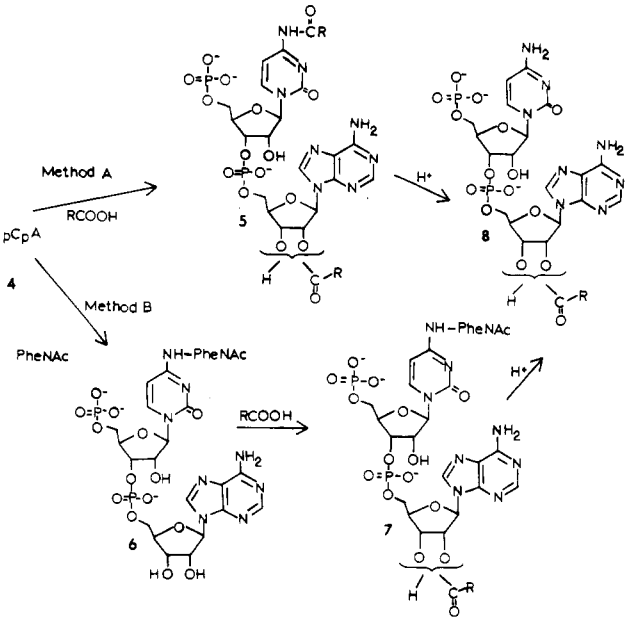
Synthesis of pCpA (**4**). N^4-Benzoyl-2′-*O*-(*tert*-butyldimethylsiyl)-5′-*O*-(monomethoxytrityl)cytidine (**1**) (Scheme I) (Ogilvie et al., 1979) and $N,O^{2'},O^{3'}$-tribenzoyladenosine (**2**) (Ikehara et al., 1966) were condensed in a 1:0.8 ratio with 1.3 equiv of methyl phosphorodichloridite in 9:1 tetrahydrofuran–collidine at −78 °C, according to the procedure of Ogilvie et al. (1980). Detritylation of the fully protected dinucleoside monophosphate with 1% benzenesulfonic acid afforded compound **3** in 45% yield: R_f 0.42 (silica gel, solvent A); $\lambda_{max}^{CH_3OH}$ 318 (sh), 261, and 230 nm; $\lambda_{min}^{CH_3OH}$ 248 and 217 nm; partial ^{1}H NMR [Me_2SO-d_6-$(CH_3)_4Si$] δ 0.78 (s, 9), 5.91 (d, 1), 6.80 (d, 1), 8.42 (d, 1), 8.76 (s, 1), and 8.80 (s, 1).

Compound **3** (112 mg, 0.1 mmol) in tetrahydrofuran (2.0 mL) was added dropwise to a cold (−78 °C) 2.0-mL solution of collidine–THF (1:3) containing $POCl_3$ (47 μL, 0.5 mmol). After 30 min, water (0.5 mL) was added; the solution was allowed to stand at 0 °C for 30 min and then evaporated to dryness. Chloroform (30 mL) was added to dissolve the residue and the solution washed with water. The chloroform layer was evaporated, the syrup was dissolved in 0.5 mL of thiophenol reagent (thiophenol–triethylamine–dioxane, 1:2:2) (Daub & van Tamelen, 1977), and the resulting solution was stirred at room temperature for 1 h. The solution was then evaporated, and the residue was partitioned in benzene–water (1:1). The aqueous layer was concentrated to afford a powder that was treated with concentrated NH_4OH (40 mL) for 17 h. The NH_4OH was removed by repeated concentration of portions of water, and the residue was dissolved in a minimal amount of water and applied to a preparative silica gel TLC plate. Following development with solvent B, 2′-*O*-(*tert*-butyldimethylsilyl)-pCpA was extracted with 7:3 2-propanol–water, concentrated to dryness (735 A_{260} units; 35% yield), and treated with 1 M tetrabutylammonium fluoride in tetrahydrofuran (7 mL) at 25 °C. After 12 h, the reaction mixture was diluted with water (100 mL) and applied to a DEAE-cellulose column (1.5 × 10 cm, HCO_3^- form). The column was washed with water (100 mL) and then with 0.5 M ammonium bicarbonate (100 mL). The appropriate A_{260}-absorbing fractions were combined and lyophilized, yielding 650 A_{260} units (88%) of pCpA (**4**) as a white powder: R_f 0.11 (cellulose TLC, solvent C), R_f 0.29 (silica gel TLC, solvent D); λ_{max} (pH 2) 265 nm, λ_{min} (pH 2) 235, λ_{max} (pH 7) 261, λ_{min} (pH 7) 232; partial ^{1}H NMR (D_2O) δ 5.72 (d, 1), 5.97 (d, 1), 6.03 (d, 1), 7.87 (d, 1), 8.17 (s, 1), and 8.40 (s, 1). Digestion of pCpA (**4**) with RNase T_2 yielded pCp and A in a molar ratio of 1:1.

Synthesis of Acylated pCpA's. Method A (*Scheme II*). *N*-Acylamino acid (0.1 mmol) and 1,1′-carbonyldiimidazole (16.2 mg, 0.1 mmol) in dry Me_2SO (0.2 mL) was stirred over molecular sieves (4 Å) for 30 min under N_2. To this solution was added pCpA (180 A_{260} units, 9 μmol). Stirring was continued under N_2 at 25 °C for 60–70 h. An additional amount of 1,1′-carbonyldiimidazole (5.5 mg, 0.03 mmol) was added, and the mixture was stirred for 26–72 h.

The reaction mixture was added dropwise to 6 mL of *n*-hexane–ether–acetone (1:1:1). The precipitate (or syrup) was collected by centrifugation, dissolved in 0.01 N acetic acid, and applied to a preparative cellulose TLC plate (10 × 20 cm). Development with solvent C yielded a UV-active band (R_f 0.7–0.85), which was extracted from the plate with 0.01 N acetic acid. The solution was concentrated to a small volume and lyophilized to yield a white powder. The powder was suspended in 0.01 N acetic acid, and the solution was adjusted to pH 2 with 0.01 N HCl. The solution was stirred at 25 °C for 26 h and then neutralized with Dowex 50W (Na^+ form). The solution was then concentrated to a small volume (1 mL) and applied to a preparative cellulose TLC plate. Development with butanol saturated with 10% acetic acid, followed by solvent C, gave a major band (R_f 0.40–0.53) that was extracted with 0.01 N acetic acid (2 × 5 mL). The combined extract was concentrated to a small volume (1 mL) and lyophilized. The desired *N*-acylaminoacyl dinucleotides (**8a–e**, **8g**, and **8j**) were obtained in typical yields of 20–35%.

Scheme II



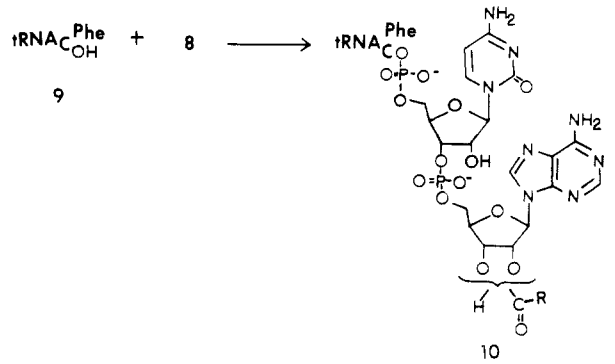
Method B. *N*-Acetyl-DL-phenylalanine (8.3 mg, 0.04 mmol) and 1,1′-carbonyldiimidazole (6.5 mg, 0.04 mmol) in dry Me_2SO (0.3 mL) were stirred at room temperature for 20 min. To this solution was added pCpA (**4**) (180 A_{260} units, 9 μmol), and stirring was continued under N_2 for 20 h. The reaction mixture was then added dropwise to 2 mL of dry acetone, and the precipitate was collected by centrifugation. The precipitate was dissolved in water (0.5 mL), applied to a preparative cellulose plate (10 × 20 cm), and developed with solvent E. The major band was extracted with water (2 × 30 mL); the extract was concentrated to a small volume and lyophilized to afford 99 A_{260} units (55%) of compound **6**: R_f 0.52 (cellulose TLC, solvent E); λ_{max} (pH 7) 302 and 252 nm, λ_{min} (pH 7) 284 and 288 nm; A_{302}/A_{252} 0.32; partial ^{1}H NMR (D_2O) δ 1.86 (s, 3), 5.74 (d, 1), 6.10 (d, 1), and 7.20 (m, 5).

A solution of the appropriate carboxylic acid (0.05 mmol) and 1,1′-carbonyldiimidazole (8.1 mg, 0.05 mmol) in dry dimethyl sulfoxide (0.2 mL) was stirred at room temperature for 20 min. To this solution was added dinucleotide **6** (100 A_{260} units, 5 μmol); stirring was continued under N_2 for 70 h. The reaction mixture was then worked up as described under method A. Acylated dinucleotides **8f**, **8h**, and **8i** were obtained in 10–25% yields.

N-Acetylaminoacyl dinucleotides **8k–n** were prepared from the respective *N*-carboxybenzyloxyaminoacyl dinucleotides **8a–d** by catalytic hydrogenolysis over palladium on carbon, followed by *N*-acetylation with *N*-acetoxysuccinimide, essentially as described by Quiggle et al. (1981).

tRNAs. E. coli $tRNA^{Phe}$-C_{OH} (**9**) (Scheme III) was prepared from $tRNA^{Phe}$-CC_{OH} (Alford et al., 1977) by successive treatments with periodate, lysine (pH 8.0), and alkaline phosphatase (Neu & Heppel, 1964). L-Phenylalanyl-$tRNA^{Phe}$ was prepared under standard aminoacylation conditions as described by Pezzuto & Hecht (1980). *N*-Acetyl-L-phenyl-

Scheme III



alanyl-tRNA^{Phe} was prepared essentially as described by Rappoport & Lapidot (1974) and purified by chromatography on BD-cellulose (see legend to Figure 1, panel B).

Results

Synthesis of Acylated pCpA's. The synthesis of pCpA (**4**) was accomplished by employing the phosphorochloridite procedure described by Ogilvie et al. (1980) to effect the coupling of N^4-benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(monomethoxytrityl)cytidine (**1**) (Ogilvie et al., 1979) and $N,O^{2'},O^{3'}$-tribenzoyladenosine (**2**) (Ikehara et al., 1966) to afford dinucleoside monophosphate **3** in 45% yield after detritylation. Subsequent phosphorylation of **3** with phosphorus oxychloride and suitable deprotection afforded pCpA (**4**) in an overall yield of ~15%. Degradation of pCpA (**4**) with RNase T_2 yielded equimolar quantities of pCp and adenosine.

The synthetic routes employed for the preparation of compounds **8a–e**, **8g**, and **8j** (method A) and compounds **8f**, **8h**, and **8i** (method B) are illustrated in Scheme II. In the preparation of compounds **8a–e**, **8g**, and **8j** protection of the N^4-amino group of cytosine was found to be unnecessary as treatment of the initially formed diaminoacylated species (**5**) with 0.01 N HCl hydrolyzed the N^4-aminoacyl group. In contrast, preparation of compounds **8f**, **8h**, and **8i** (method B) required prior protection of the cytidine amino group, so as to avoid the more troublesome deblocking of these acyl groups from N^4. *N*-Acetyl-DL-phenylalanine was found to be a convenient protecting group for N^4 of cytidine, and this group greatly simplified the subsequent deprotection chemistry. Thus, treatment of **4** with 1,1′-carbonyldiimidazole and *N*-acetyl-DL-phenylalanine for a limited period of time (20 h), followed by suitable chromatographic workup, afforded the protected species (**6**) in 55% yield. Condensation of dinucleotide **6** with *N*-acetyl-DL-β-phenylalanine, 3-phenylpropionic acid, and *trans*-cinnamic acid then afforded respective dinucleotides **7**, which were formally analogous to **5**. Accordingly, treatment of dinucleotides **7** with 0.01 N HCl afforded pCpA derivatives **8f**, **8h**, and **8i**.

N-Acetyl-L-aminoacyl-pCpA derivatives **8k–n** were prepared from the respective *N*-carbobenzyloxy species **8a–d** by catalytic hydrogenolysis over palladium on carbon, followed by acetylation with *N*-acetoxysuccinimide. Characterization of acylated dinucleotides **8a–n** was effected by alkaline hydrolysis to the parent dinucleotide (**4**), characterization of chromatographic mobilities, and UV spectra of the dinucleotides (Table I), as well as analysis by mass spectrometry.

T4 RNA Ligase Mediated Preparation of Chemically Misacylated tRNA^{Phe}s. *E. coli* tRNA^{Phe}-C_{OH} (**9**) was prepared from abbreviated tRNA^{Phe}-CC_{OH} (Alford et al., 1977) by sequential treatment with periodate, amine-catalyzed β-elimination of the resulting oxidized ribose moiety, and subsequent removal of the resulting terminal 3′-phosphate with alkaline phosphatase (Neu & Heppel, 1964). Incubation of tRNA^{Phe}-C_{OH} (8.6 A_{260} units/mL) and acylated dinucleotides **8a–n** (4.6 A_{260} units/mL) in the presence of T4 RNA ligase (340 units/mL) for 16 h at 4 °C was found to effect ligation optimally. Chromatography of the incubation mixture on DEAE-cellulose, to effect separation of tRNAs **9** and **10** from RNA ligase and other reaction components, and subsequent chromatography of the tRNA-containing fractions on BD-cellulose permitted isolation of the desired tRNA species (**10a–n**). The isolated yields of "chemically aminoacylated" tRNAs ranged from 20 to 65% (Table II).

Characterization of Chemically Aminoacylated tRNA. A sample of chemically aminoacylated DL-β-phenylalanyl-tRNA^{Phe} (**10f**), prepared and isolated as described in the legend to Figure 1, was deacylated by treatment with 50 mM Tris-HCl (pH 9.5) buffer and again subjected to chromatography on BD-cellulose (Figure 1, panel C). Enzymatic

Table I: 2′(3′)-O-Acylated Cytidylyl(3′→5′)adenosine 5′-Phosphates (**8**)

acylated dinucleotides	method	UV max (nm) pH 7	UV max (nm) pH 2	R_f[a]
pCpA-L-Phe^{Cbz} (**8a**)	A	262	264	0.53
pCpA-D-Phe^{Cbz} (**8b**)	A	262	263	0.53
pCpA-L-Tyr^{Cbz} (**8c**)	A	262	266	0.48
pCpA-D-Tyr^{Cbz} (**8d**)	A	262	266	0.48
pCpA-Gly^{Cbz} (**8e**)	A	262	266	0.46
pCpA-DL-β-Phe^{Ac} (**8f**)	B	262	263	0.40
pCpA-DL-PhGly^{Ac}[c] (**8g**)	A	262	266	0.45
pCpA-3-phenylpropionate (**8h**)	B	262	266	0.46
pCpA-*trans*-cinnamate (**8i**)	B	262	268	0.44
pCp(2′dA)-DL-Phe^{Ac} (**8j**)	A	262	266	0.55
pCpA-L-Phe^{Ac} (**8k**)[b]		262	266	0.47
pCpA-D-Phe^{Ac} (**8l**)[b]		262	266	0.47
pCpA-L-Tyr^{Ac} (**8m**)[b]		262	266	0.47
pCpA-D-Tyr^{Ac} (**8n**)[b]		262	266	0.47
pCpA (**4**)		262	266	0.11

[a] Cellulose TLC, solvent C. [b] Prepared from the respective *N*-carboxybenzyloxy-pCpA derivatives **8** by catalytic hydrogenolysis and subsequent chemical acetylation as described under Materials and Methods. [c] PhGly is phenylglycine.

Table II: Preparation of Chemically Misacylated tRNA^{Phe}s[a]

acylated pCpA derivative	isolated yield of ligated acyl-tRNA^{Phe} (%)
L-Phe^{Ac} (**8k**)	45 (**10k**)
D-Phe^{Ac} (**8l**)	47 (**10l**)
L-Phe^{Cbz} (**8a**)	20 (**10a**)
D-Phe^{Cbz} (**8b**)	25 (**10b**)
L-Tyr^{Ac} (**8m**)	65 (**10m**)
D-Tyr^{Ac} (**8n**)	20 (**10n**)
L-Tyr^{Cbz} (**8c**)	32 (**10c**)
D-Tyr^{Cbz} (**8d**)	50 (**10d**)
Gly^{Cbz} (**8e**)	47 (**10e**)
DL-β-Phe^{Ac} (**8f**)	57 (**10f**)
DL-PhGly^{Ac}[b] (**8g**)	29 (**10g**)
3-phenylpropionate (**8h**)	41 (**10h**)
trans-cinnamate (**8i**)	21 (**10i**)
pCp(2′dA)-L-Phe^{Ac} (**8j**)	35 (**10j**)

[a] RNA ligase mediated preparation of chemically misacylated tRNA^{Phe}s (**10**). *E. coli* tRNA^{Phe}-C_{OH} (**9**) (0.75 nmol) was incubated in the presence of 2′(3′)-O-acylated cytidylyl(3′→5′)-adenosine 5′-diphosphates (**8**) (~15 nmol) and 24 units of T4 RNA ligase in 55 mM Na^+-Hepes buffer, pH 7.5, containing 15 mM $MgCl_2$, 250 μM ATP, 20 μg/mL bovine serum albumin, and 10% dimethyl sulfoxide. The ligated chemically misacylated tRNA^{Phe}s (**10**) were isolated by successive chromatographies on DEAE- and BD-cellulose, as described in legend to Figure 1. [b] PhGly is phenylglycine.

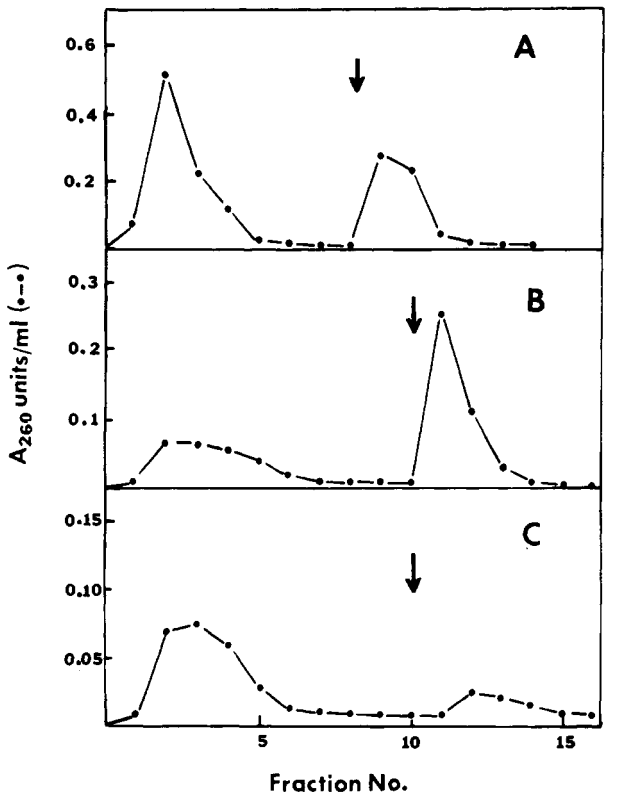


FIGURE 1: Chromatography of chemically misacylated *E. coli* *N*-acetyl-DL-β-phenylalanyl-tRNA^{Phe} on DEAE-cellulose (panel A), on BD-cellulose (panel B), and on BD-cellulose after alkali treatment (panel C). *E. coli* tRNA^{Phe}-C_{OH} (**9**; 0.6 A_{260} unit) was incubated with pCpA-DL-β-phenylalanyl-tRNA^{Phe} (**8f**; 0.3 A_{260} unit) in a reaction volume (70 μL) containing 55 mM Na^+-Hepes, pH 7.5, 15 mM $MgCl_2$, 250 μM ATP, 20 μg/mL bovine serum albumin, 10% dimethyl sulfoxide, and 24 units of T4 RNA ligase. The reaction mixture was incubated at 4 °C for 16 h and then diluted to 0.5 mL with 50 mM NaOAc, pH 4.5, containing 10 mM $MgCl_2$. The sample was applied to a 1.0-mL DEAE-cellulose column (4 °C) that had been equilibrated in the same buffer, and the column was washed with (eight 1.0-mL fractions of) 50 mM NaOAc, pH 4.5, containing 10 mM $MgCl_2$ and 0.25 M NaCl to remove RNA ligase and other non-tRNA reaction components. Elution of tRNAs **9** and **10** was then effected with five 1.0-mL fractions of the same buffer containing 1.0 M NaCl (panel A). The tRNA-containing fractions were pooled and applied to a 1.0-mL BD-cellulose column (4 °C) that had been equilibrated with 50 mM NaOAc, pH 4.5, containing 10 mM $MgCl_2$ and 1.0 M NaCl. Unreacted tRNA^{Phe}-C_{OH} (**9**) was removed by washing the column with 10 mL of the same buffer; the column was then eluted with the same buffer containing 25% ethanol to effect elution of *N*-acetyl-DL-β-phenylalanyl-tRNA^{Phe} (panel B). Fractions 11 and 12 were pooled, dialyzed against 1 mM NaOAc, pH 4.5, and concentrated to a small volume. The isolated yield of *N*-acetyl-DL-β-phenylalanyl-tRNA^{Phe} was 0.34 A_{260} unit (57%). *N*-Acetyl-DL-β-phenylalanyl-tRNA^{Phe} (0.25 A_{260} unit) was treated with 0.5 mL of 50 mM Tris-HCl, pH 9.5, containing 10 mM $MgCl_2$ at 4 °C for 16 h, and then diluted with 1 mL of 50 mM NaOAc, pH 4.5, containing 10 mM $MgCl_2$ and 1.0 M NaCl, and again subjected to chromatography on BD-cellulose (panel C). The elution conditions were identical with those described for panel B.

reaminoacylation of a portion of this deacylated sample, in comparison with untreated *E. coli* tRNA^{Phe} and abbreviated tRNA^{Phe}-C_{OH}, was carried out by utilizing [^{3}H]phenylalanine and an *E. coli* phenylalanyl-tRNA synthetase preparation. As illustrated in Figure 2, the deacylated species could be reaminoacylated to approximately the same extent (940 pmol/A_{260}), and at a similar rate, as untreated *E. coli* tRNA^{Phe} (1200 pmol/A_{260}). Abbreviated tRNA^{Phe}-C_{OH}, however, was not a substrate for phenylalanyl-tRNA synthetase under identical incubation conditions.

Chemically aminoacylated tRNA was additionally characterized by comparison of the behavior of *N*-acetyl-L-phenylalanyl-tRNA^{Phe} (**10k**) with "authentic" samples of L-phenylalanyl-tRNA^{Phe} and *N*-acetyl-L-phenylalanyl-tRNA^{Phe} in the presence of aqueous Cu^{2+}. As shown by analysis of chromatographic behavior on BD-cellulose (Figure 3), treatment with 20 mM $CuSO_4$ in 50 mM sodium acetate buffer (pH 5.5) effected complete deacylation of L-phenylalanyl-tRNA^{Phe} to tRNA^{Phe}, whereas both the chemically aminoacylated and authentic *N*-acetyl-L-phenylalanyl-tRNA^{Phe}s were stable, as would be predicted.

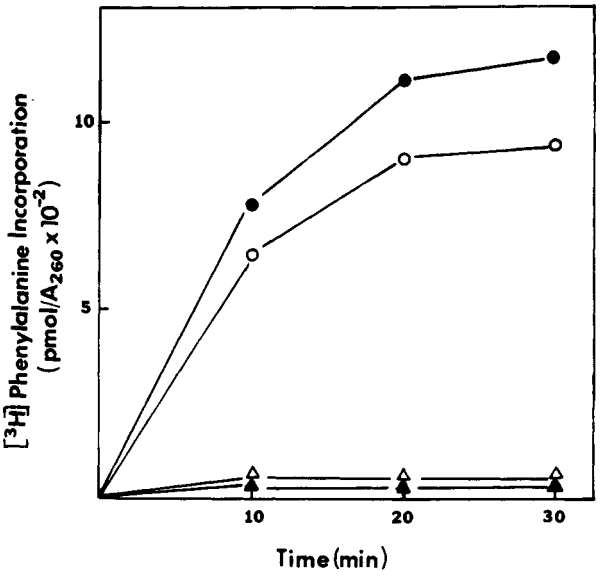


FIGURE 2: Reaminoacylation of deacylated *N*-acetyl-DL-β-phenylalanyl-tRNA^{Phe}. Dialyzed and concentrated deacylated *N*-acetyl-DL-β-phenylalanyl-tRNA^{Phe} (○) (Figure 1, panel C) was aminoacylated in comparison with untreated *E. coli* tRNA^{Phe} (●), abbreviated *E. coli* tRNA^{Phe}-C_{OH} (**9**; △), and a control lacking tRNA (▲). Assays were performed in 75 μL of 90 mM Na^+-Pipes buffer, pH 7.0, containing 100 mM KCl, 15 mM $MgCl_2$, 0.5 mM EDTA, 2.5 mM ATP, 10 μM [^{3}H]phenylalanine (1450 cpm/pmol), 0.1 A_{260} unit of tRNA, and 3 μL of *E. coli* phenylalanyl-tRNA synthetase (Pezzuto & Hecht, 1980). Aliquots (20 μL) were removed at the indicated time intervals and spotted onto glass-fiber disks that had been presoaked with 0.5 M cetyltrimethylammonium bromide solution in 1% acetic acid. The disks were washed thoroughly with 1% acetic acid, dried, and used for determination of radioactivity.

Discussion

The first preparation of a misacylated tRNA was reported by Chapeville et al. (1962), who prepared alanyl-tRNA^{Cys} by chemical desulfurization of cysteinyl-tRNA^{Cys}. The ability of this misacylated tRNA to effect the substitution of alanine for cysteine in response to hemoglobin mRNA (von Ehrenstein et al., 1963), as predicted by the adaptor hypothesis (Crick, 1958; Hoagland, 1959), and more recent observations utilizing a purified in vitro protein biosynthesizing system (Hecht, 1980; Pezzuto & Hecht, 1980) suggest the potential utility of misacylated tRNAs for effecting amino acid substitutions at predetermined sites in polypeptides. The availability of a general technique for the preparation of misacylated tRNAs would also facilitate studies of the mechanism of tRNA activation and proofreading (Eldred & Schimmel, 1972; Hopfield, 1974; von der Haar & Cramer, 1976), as well as investigations of the partial reactions of protein biosynthesis and the mechanism(s) employed to maintain the overall fidelity of that process [Loftfield, 1963; Thompson & Karin (1982) and references cited therein]. In this paper we describe the preparation and characterization of a number of misacylated tRNAs by a potentially general method for the elaboration of such species.

The scheme envisioned for the preparation of the mis-

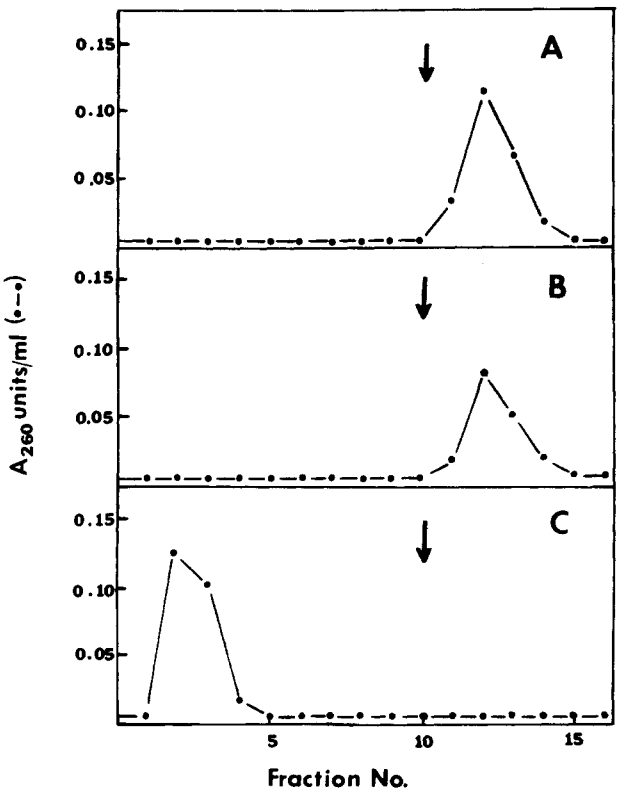


FIGURE 3: BD-cellulose chromatography of Cu^{2+}-treated chemically aminoacylated *N*-acetyl-L-phenylalanyl-tRNA^{Phe} (**10k**), authentic *N*-acetyl-L-phenylalanyl-tRNA^{Phe}, and L-phenylalanyl-tRNA^{Phe}. Authentic *N*-acetyl-L-phenylalanyl-tRNA^{Phe} (panel A; 0.30 A_{260} unit), chemically aminoacylated *N*-acetyl-L-phenylalanyl-tRNA^{Phe} (panel B; 0.22 A_{260} unit; prepared analogously to the sample described in the legend to Figure 1), and L-phenylalanyl-tRNA^{Phe} (panel C; 0.30 A_{260} unit) were each dissolved in 0.5 mL of 50 mM NaOAc buffer, pH 5.5, containing 10 mM $MgCl_2$ and 20 mM $CuSO_4$. After 30 min at 25 °C, the samples were diluted with 0.5 mL of 50 mM NaOAc buffer, pH 4.5, containing 10 mM $MgCl_2$ and 1.0 M NaCl and subjected to chromatography on BD-cellulose as described in the legend to Figure 1 for panel B.

acylated tRNAs took advantage of the fact that all tRNAs have the common sequence CCA at the 3′-terminus. As shown in Table II, following conversion of tRNA^{Phe}-CCA$_{OH}$ to tRNA^{Phe}-C$_{OH}$, several misacylated tRNA^{Phe}s were prepared in 20–65% yields by the T4 RNA ligase mediated coupling of 2′(3′)-O-acylated pCpA derivatives with tRNA^{Phe}-C$_{OH}$. The requisite dinucleotides themselves were prepared by acylation of pCpA, as described above. Salient features of the present scheme relative to procedures described previously for the synthesis of similar dinucleotides (Bhuta et al., 1981; Quiggle et al., 1981) included the use of pCpA as a common intermediate for the indirect elaboration of all of the O-acylated derivatives, the novel use of an acylated aminoacyl group for the simultaneous protection of N^4 in cytidine and $O^{2'}(O^{3'})$-derivatization of the adenosine moiety in pCpA, and the development of techniques that permitted isolation of the final aminoacylated pCpA derivatives in reasonable yield and a good state of purity.

Special precautions were taken for the preparation of *N*-acetyl-L-aminoacyl dinucleotides **8k–n**. These derivatives were necessarily prepared from the respective *N*-carbobenzyloxy analogues **8a–d** by catalytic hydrogenolysis and subsequent N^{α}-acetylation to ensure retention of optical activity, since significant racemization of *N*-acetyl-L-amino acids is known to occur upon activation with 1,1′-carbonyldiimidazole (Quiggle et al., 1981). That configurational integrity was maintained may be appreciated from the very different activities of the derived D- and L-phenylalanyl-tRNA^{Phe}s and D- and L-tyrosyl-tRNA^{Phe}s in the peptidyltransferase reaction (Heckler et al., 1983).

Chemically misacylated tRNA^{Phe}s prepared by this technique were characterized by (i) their chromatographic properties on DEAE- and BD-cellulose (Figure 1), (ii) the ability of misacylated tRNA^{Phe} to act as a substrate for phenylalanyl-tRNA synthetase following chemical deacylation (Figure 2), and (iii) the insensitivity of *N*-acetyl-L-phenylalanyl-tRNA^{Phe} to Cu^{2+}-mediated hydrolysis (Figure 3). The last of these criteria derives from the observation that Cu^{2+} mediated the rapid hydrolysis of aminoacyl-tRNA, but not of N-acylated aminoacyl-tRNAs (Schofield & Zamecnik, 1968).

Registry No. **1**, 72409-30-4; **2**, 14985-43-4; **3**, 88867-00-9; **4**, 15648-73-4; **6**, 88854-01-7; **8a**, 88854-03-9; **8b**, 88854-04-0; **8c**, 88854-05-1; **8d**, 88854-06-2; **8e**, 88854-07-3; **8f**, 88854-08-4; **8g**, 88854-09-5; **8h**, 88854-10-8; **8i**, 88854-11-9; **8j**, 88854-02-8; **8k**, 88854-12-0; **8l**, 88854-13-1; **8m**, 88854-14-2; **8n**, 88854-15-3; RNA ligase, 37353-39-2.

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Isolation and Identification of 1α,25-Dihydroxy-24-oxovitamin D_3, 1α,25-Dihydroxyvitamin D_3 26,23-Lactone, and 1α,24(*S*),25-Trihydroxyvitamin D_3: In Vivo Metabolites of 1α,25-Dihydroxyvitamin D_3†

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ABSTRACT: Three new in vivo metabolites of 1α,25-dihydroxyvitamin D_3 were isolated from the serum of dogs given large doses (two doses of 1.5 mg/dog) of 1α,25-dihydroxyvitamin D_3. The metabolites were isolated and purified by methanol–chloroform extraction and a series of chromatographic procedures. By cochromatography on a high-performance liquid chromatograph, ultraviolet absorption spectrophotometry, mass spectrometry, Fourier-transform infrared spectrophotometry, and specific chemical reactions, the metabolites were identified as 1α,25-dihydroxy-24-oxovitamin D_3, 1α,25-dihydroxyvitamin D_3 26,23-lactone, and 1α,24(*S*),25-trihydroxyvitamin D_3. According to these procedures, the total amounts of the isolated metabolites were as follows: 1α,25-dihydroxyvitamin D_3, 23.6 μg; 1α,25-dihydroxy-24-oxovitamin D_3, 1.8 μg; 1α,25-dihydroxyvitamin D_3 26,23-lactone, 9.2 μg; 1α,24(*R*),25-trihydroxyvitamin D_3, 15.4 μg; 1α,24(*S*),25-trihydroxyvitamin D_3, 1.0 μg. With recovery corrections, the serum levels of each metabolite were approximately 49 ng/mL for 1α,25-dihydroxyvitamin D_3, 3.7 ng/mL for 1α,25-dihydroxy-24-oxovitamin D_3, 19 ng/mL for 1α,25-dihydroxyvitamin D_3 26,23-lactone, 32 ng/mL for 1α,24(*R*),25-trihydroxyvitamin D_3, and 2.1 ng/mL for 1α,24(*S*),25-trihydroxyvitamin D_3.

It has been well established that 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$][1] is a metabolite of vitamin D_3 active in intestinal calcium absorption and bone mineral mobilization (Norman, 1979). In recent studies, 1α,25$(OH)_2D_3$ may undergo further metabolism to several additional secosteroids, including (i) 1α,24,25-trihydroxyvitamin D_3 [1α,24,25$(OH)_3D_3$] (Kleiner-Bossaler & DeLuca, 1974; Tanaka et al., 1977; Ribovich & DeLuca, 1978; Reinhardt et al., 1982), (ii) side-chain oxidation and cleavage to yield CO_2 (Kumar et al., (1976) and a shortened side chain, C-23-COOH-containing secosteroid designated calcitroic acid (Esvelt et al., 1979), (iii) 1α,25,26-trihydroxyvitamin D_3 [1α,25,26$(OH)_3D_3$] (Reinhardt et al., 1981; Tanaka et al., 1981b), and (iv) 1α,25-dihydroxyvitamin D_3 26,23-lactone [1α,25$(OH)_2D_3$-26,23-lactone] (Ohnuma et al., 1980; Ishizuka et al., 1981). The stereochemical configurations of the biosynthesized 1α,24,25$(OH)_3D_3$ and 1α,25,26$(OH)_3D_3$ were determined to be 24(*R*) and 25(*S*), respectively (Tanaka et al., 1977; Reinhardt et al., 1981). Furthermore, the stereochemical configuration at the C-23 and C-25 positions of the 1α,25-$(OH)_2D_3$-26,23-lactone was determined to be (23*S*,25*R*)-1α,25$(OH)_2D_3$-26,23-lactone (Ishizuka et al., 1981).

During the course of the investigation of the further metabolism of 1α,25$(OH)_2D_3$, we became aware of three new metabolites of 1α,25$(OH)_2D_3$ that appeared in the serum of dogs given large doses of 1α,25$(OH)_2D_3$. It is the purpose of this paper to describe the isolation and to establish the structures of these new metabolites as 1α,25-dihydroxy-24-oxovitamin D_3 [1α,25$(OH)_2$-24-oxo-D_3], 1α,25$(OH)_2D_3$-26,23-lactone, and 1α,24(*S*),25-trihydroxyvitamin D_3 [1α,24(*S*),25$(OH)_3D_3$].

Materials and Methods

Compounds. The syntheses of 1α,25$(OH)_2D_3$ and the 24-isomers of 1α,24,25$(OH)_3D_3$ were carried out in our laboratory

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[1] Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 23(*S*),25-$(OH)_2D_3$, 23(*S*),25-dihydroxyvitamin D_3; 24(*R*),25-$(OH)_2D_3$, 24(*R*),25-dihydroxyvitamin D_3; 25(*S*),26$(OH)_2D_3$, 25(*S*),26-dihydroxyvitamin D_3; 25-OH-D_3-26,23-peroxylactone, 25-hydroxyvitamin D_3 26,23-(peroxylactone); 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1α,25$(OH)_2D_3$-26,23-lactone, 1α,25-dihydroxyvitamin D_3 26,23-lactone; 1α,25$(OH)_2D_3$-26,23-peroxylactone, 1α,25-dihydroxyvitamin D_3 26,23-(peroxylactone); 23(*S*),25(*R*),26$(OH)_3D_3$, 23(*S*),25(*R*),26-trihydroxyvitamin D_3; 1α,24(*R*),25$(OH)_3D_3$, 1α,24(*R*),25-trihydroxyvitamin D_3; 1α,24(*S*),25$(OH)_3D_3$, 1α,24(*S*),25-trihydroxyvitamin D_3; 1α,25(*S*),26$(OH)_3D_3$, 1α,25(*S*),26-trihydroxyvitamin D_3; 25-OH-24-oxo-D_3, 25-hydroxy-24-oxovitamin D_3; 1α-OH-25,26,27-trinor-24-CHO-D_3, 1α-hydroxy-25,26,27-trinor-24-oxovitamin D_3; 1α,23(*S*),25$(OH)_3D_3$, 1α,23(*S*),25(*R*)-trihydroxyvitamin D_3; 1α,23(*S*),25(*R*),26$(OH)_4D_3$, 1α,23(*S*),25(*R*),26-tetrahydroxyvitamin D_3; HPLC, high-performance liquid chromatography.

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