Sobell, H. M. (1973) *Prog. Nucleic Acid Res. Mol. Biol. 13*, 153–190.
Sobell, H. M., & Jain, S. C. (1972) *J. Mol. Biol. 68*, 21–34.
Stec, W. J., Zon, G., Egan, W., Byrd, R. A., Phillips, L. R., & Gallo, K. A. (1985) *J. Org. Chem. 50*, 3908–3913.
Walker, G. T., Stone, M. D., & Krugh, T. R. (1985) *Biochemistry 24*, 7471–7479.
Wang, A. H., Ughetto, G., Quigley, G. J., Hakoshima, T., van der Marel, G. A., van Boom, J. H., & Rich, A. (1984) *Science (Washington D.C.) 255*, 1115–1121.
Waring, M., (1981) in *The Molecular Basis of Antibiotic Action* (Gale, F., et al., Eds.) pp 314–333, Wiley, London.
Wells, R. D., & Larson, J. E. (1970) *J. Mol. Biol. 49*, 319–342.
Wilson, W. D. (1987) *Prog. Drug Res. 31*, 193–221.
Wilson, W. D., Jones, R. L., Zon, G., Banville, D. L., & Marzilli, L. G. (1986a) *Biopolymers 25*, 1997–2015.
Wilson, W. D., Jones, R. L., Zon, G., Scott, E. V., Banville, D. L., & Marzilli, L. G. (1986b) *J. Am. Chem. Soc. 108*, 7113–7119.
Wüthrich, K. (1986) in *NMR of Proteins and Nucleic Acids*, Wiley, New York.

Mischarging *Escherichia coli* tRNA^{Phe} with L-4′-[3-(Trifluoromethyl)-3*H*-diazirin-3-yl]phenylalanine, a Photoactivatable Analogue of Phenylalanine†

Giulia Baldini,‡ Bruno Martoglio, Andreas Schachenmann, Carmen Zugliani, and Josef Brunner*
Laboratorium für Biochemie der Eidgenössischen Technischen, Hochschule, ETH-Zentrum, CH-8092 Zürich, Switzerland
Received March 25, 1988; Revised Manuscript Received June 14, 1988

ABSTRACT: The Boc-protected derivative of a photoactivatable, carbene-generating analogue of phenylalanine, L-4′-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenylalanine [(Tmd)Phe], was used to acylate 5′-*O*-phosphorylcytidylyl(3′–5′)adenosine (pCpA). A diacyl species was isolated which upon successive treatments with trifluoroacetic acid and 0.01 M HCl yielded a 1:1 mixture of 2′(3′)-*O*-(Tmd)phenylalanyl-pCpA and of its 2′–5′-phosphodiester isomeric form. Adapting a procedure introduced by Hecht's group [Heckler, T. G., Chang, L. H., Zama, Y., Naka, T., Chorghade, M. S., & Hecht, S. M. (1984) *Biochemistry 23*, 1468–1473], brief incubation of a 15 molar excess of this material with *Escherichia coli* tRNA^{Phe}, missing at the acceptor stem the last two nucleotides (pCpA), in the presence of T4 RNA ligase and ATP afforded "chemically misaminoacylated" tRNA^{Phe} in approximately 50% yield. Following chromatographic purification on DEAE-Sephadex A-25, benzoylated DEAE-cellulose, and Bio-Gel P-6, the misaminoacylated tRNA^{Phe} was characterized by (i) urea–polyacrylamide gel electrophoresis, (ii) enzymatic reaminoacylation under homologous conditions following chemical deacylation, and (iii) its ability to stimulate protein synthesis in an in vitro translation system which, through the addition of the phenylalanyl-tRNA synthetase inhibitor phenylalaninyl-AMP, was unable to charge its endogenous tRNA^{Phe}. The data demonstrate that we have prepared a biologically active misaminoacylated tRNA^{Phe}.

T4 RNA ligase mediated coupling of 2′(3′)-O-acylated pCpA derivatives with tRNAs missing the 3′-terminal cytidine and adenosine moieties (tRNA-C_{OH})[1] has in the last years been utilized by Hecht's group to prepare a number of novel "chemically misacylated" tRNAs (Hecht et al., 1978; Heckler et al., 1983, 1984a,b; Roesser et al., 1986). Prebound to the P site of *Escherichia coli* ribosomes, several of these tRNAs were shown to mediate dipeptide formation upon admixture of phenylalanyl-tRNA^{Phe} to the A site (Heckler et al., 1983; Roesser et al., 1986). Since the current methodology for chemical misacylation can be applied only to acyl moieties that do not contain a free amino group (e.g., an N-protected amino acid), these misacylated tRNAs do not function in the ribosomal A site and, hence, cannot be used to incorporate biosynthetically nonnatural analogues of amino acids into polypeptides. In view of the considerable potential such application would have, it was very tempting to search for procedures by which it would be possible to prepare also the "free", N-deprotected aminoacyl-tRNAs.

An approach that could lead to such tRNAs would be to include in Hecht's general scheme a step that generates the free amino group from its protected form. Such deprotection may either follow directly acylation of pCpA (prior to ligation) or, alternatively, be carried out after ligation on the misacylated tRNA itself. In the present paper we describe the successful misaminoacylation of *E. coli* tRNA^{Phe} with an analogue of phenylalanine, (Tmd)Phe. For transient protection of the amino function, the butoxycarbonyl (Boc) group was used and deprotection was done immediately after acylation of pCpA. Evidence is also presented that the resulting

† This work was supported by a research grant from the Swiss National Science Foundation, Bern (3.228-0.85 to J.B.). Dedicated to Prof. G. Semenza on the occasion of his sixtieth birthday.
* Address correspondence to this author.
‡ Permanent address: Dipartimento di Biochimica Biofisica e Chimica delle Macromolecole, Universita degli Studi di Trieste, I-34127 Trieste, Italy.

[1] Abbreviations: (Tmd)Phe, L-4′-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenylalanine; Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; tRNA-CC_{OH}, tRNA missing the 3′-terminal adenosine moiety; tRNA-C_{OH}, tRNA missing the 3′-terminal cytidine and adenosine moieties; tRNA^{Phe}, phenylalanyl-specific tRNA; Na^+-Hepes sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/88/0427-7951$01.50/0

mischarged tRNA retained biological activity.

(Tmd)Phe is a photoactivatable, carbene-generating amino acid based on 3-(trifluoromethyl)-3-phenyldiazirine (Richards & Brunner, 1980; Brunner et al., 1980) and was first synthesized by Nassal (1984) and Shih and Bayley (1985). There is growing experimental evidence suggesting that the highly reactive carbene generated from this diazirine is capable of inserting into all types of amino acid side chains including aliphatic ones. Incorporated into proteins, the (Tmd)Phe residues can, therefore, be expected to undergo efficient cross-linking to the nearest amino acid residue, thus providing a basis for studying spatial relationships in proteins. The potential of this approach may be illustrated by some recent cross-linking studies following biosynthetic incorporation into nascent polypeptides of chemically or photochemically reactive derivatives of lysine that had been prepared by specific modification of the corresponding (charged) lysyl-tRNA^{Lys} (Johnson et al., 1978; Johnson & Cantor, 1980; Johnson & Slobin, 1980; Kurzchalia et al., 1986; Krieg et al., 1986; Wiedmann et al., 1987a,b).

Experimental Procedures

Materials. All chemicals and solvents were commercial grades of highest purity. *E. coli* tRNA and *E. coli* tRNA^{Phe} were from Boehringer (Mannheim). Tritiated water (1500 Ci/mL) was from Amersham and [^{14}C]Phe (512 mCi/mmol) from NEN. Phenylalanyl-tRNA synthetase was isolated from bakers' yeast lysate by ammonium sulfate (80%) precipitation as described by Gillam et al. (1967) and partially purified by DEAE-cellulose and phosphocellulose ion-exchange chromatography as described by Roe et al. (1973). With yeast tRNA^{Phe} (Sigma) the specific activity was 34 EU/mg under standard homologous conditions (Roe et al., 1973). Phosphodiesterase (25 units/mg) and T4 RNA ligase (RNase free; sp act. 1500 units/mg of protein) were from Pharmacia; ribonuclease T_2 and crude *E. coli* aminoacyl-tRNA synthetase were from Sigma. CTP(ATP):tRNA nucleotidyltransferase (EC 2.7.7.25) was isolated from bakers' yeast according to Alford et al. (1979). Phenylalaninyl-AMP has been prepared essentially as described by Cassio et al. (1967). Its purity was assessed by TLC, descending paper chromatography, and paper electrophoresis. Rabbit reticulocyte lysate was a generous gift from Dr. Hans Wacker.

General Procedures. UV/vis spectra were recorded on a Cary 219 or Zeiss PMQII spectrophotometer. ^{1}H NMR and ^{31}P NMR spectra were recorded on a 300-MHz Bruker instrument. $CDCl_3$ or DMSO-d_6 was used a solvent, and $(CH_3)_4Si$ (δ 0.00) was used as an internal standard. Thin-layer chromatography was performed on precoated silica gel 60 F_{254} plates (Merck, Darmstadt, FRG). Spots were visualized by fluorescence quenching under a 254-nm UV light source or with ninhydrin. Phosphate was determined according to the procedure of Chen et al. (1956). Paper chromatography was performed by the descending technique using Whatman 3MM paper. Nucleotides and related compounds were detected by viewing under an UV lamp; amino acid derivatives were detected by spraying the chromatograms with ninhydrin (Merck). Paper electrophoresis was performed in a pherograph type Mini 65 (Hormuth-Vetter, Heidelberg, FRG). The medium normally used was 10% acetic acid. The average potential was 20 V/cm. 3′-End nucleoside and integrity of the various *E. coli* tRNA species were analyzed by determining their capacity to accept [^{14}C]Phe; abbreviated tRNAs were first treated with CTP(ATP):tRNA nucleotidyltransferase in the presence of either ATP or CTP or both nucleotides. The incubation mixture (0.1 mL) contained 200 mM sodium cacodylate, pH 7.5, 4 mM ATP, 20 mM KCl, 20 mM $MgCl_2$, 100 μM [^{14}C]Phe (50 mCi/mmol), 0.050 A_{260} unit of *E. coli* tRNA^{Phe}, and 10 μg of aminoacyl-tRNA synthetase from *E. coli*. After incubation for 15 min at 37 °C, the tRNA was precipitated with 5% TCA, transferred on a GF/C filter (Whatman), and washed five times with 5-mL portions of 5% TCA and twice with ethanol and then counted.

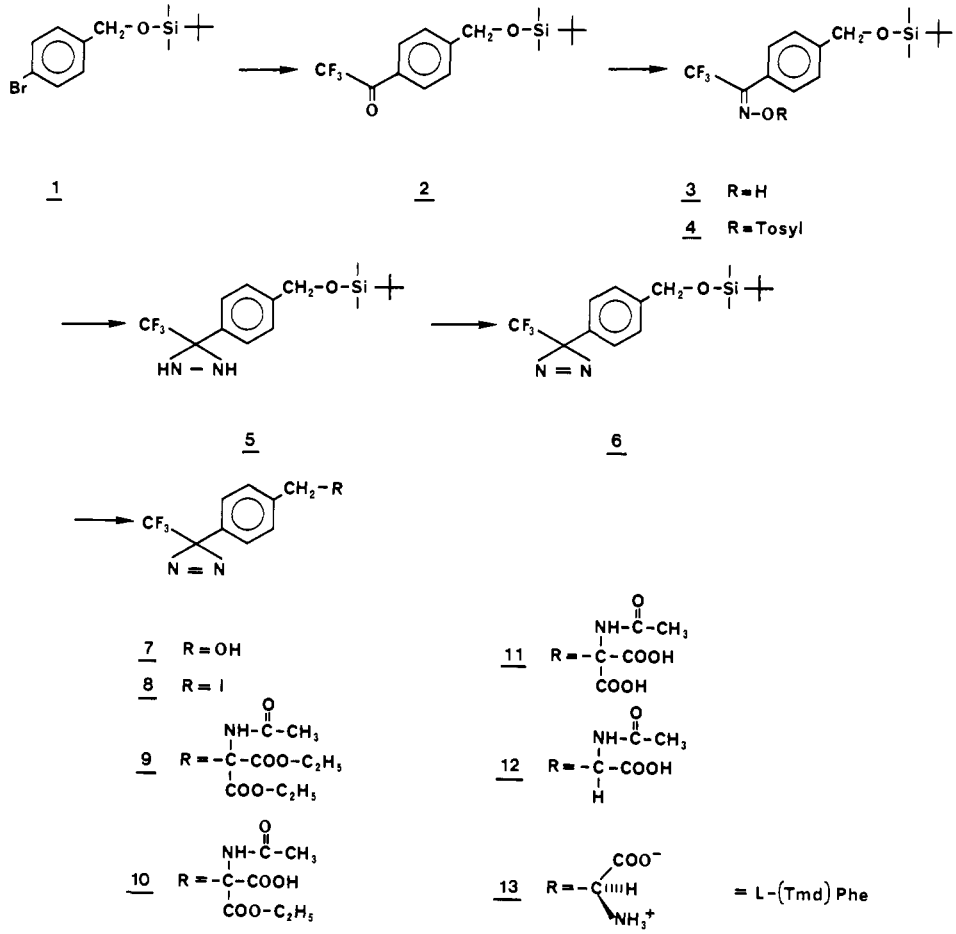
Synthesis of (Tmd)Phe and [^{3}H](Tmd)Phe (Scheme I). (*A*) *4′-[[(tert-Butyldimethylsilyl)oxy]methyl]-2,2,2-trifluoroacetophenone* (**2**). A solution of 69.4 g (0.23 mol) of [(*p*-bromobenzyl)oxy]-*tert*-butyldimethylsilane (**1**) [Shih & Bayley, 1985; bp 148 °C (14 mmHg)] in 1150 mL of ether was cooled to −30 °C and treated over a period of 45 min with 158 mL of a 1.6 M solution of butyllithium in hexane and then allowed to warm up within 2 h to 0 °C. After cooling to −50 °C, a solution of 41.7 g of (trifluoroacetyl)piperidine (Nassal, 1984) in 245 mL of ether was added and the mixture reacted for 3 h at −50 °C. After the reaction was quenched with a saturated solution (300 mL) of ammonium chloride, the organic phase was washed with water and dried and the solvent evaporated. Distillation (140–142 °C/13 mmHg) afforded 61.3 g (84%) of **2** as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 0.154 (s, 6), 0.97 (s, 9), 4.97 (s, 2), 7.6–8.1 (m, 4).

(*B*) *4′-[3-(Trifluoromethyl)-3H-diazirin-3-yl]benzyl Iodide* (**8**). Compound **2** was converted via oxime **3** and *O*-tosyl oxime **4** to the diazirine **5** as described by Shih and Bayley (1985). For oxidation of **5** we followed the procedure of Church and Weiss (1970): 10 g (30 mmol) of the diaziridine **5** were dissolved in 20 mL of methanol and 7 mL of triethylamine. Then I_2 (a total of 6.3 g) was added in small portions. The brown solution (excess iodine) was neutralized with 10% citric acid, treated with a few drops of a 5% $NaHSO_3$ (to reduce excess I_2), and extracted with 300 mL of ether. Following evaporation of the solvent, the residue was chromatographed on silica gel with CH_2Cl_2/hexane (2:1 v/v), yield 7.3 g (73%) of a colorless oil. The subsequent steps [removal of the silyl group and conversion of the alcohol **7** (colorless oil) to the iodide **8** (yellow needles, mp 39 °C)] were carried out again as described by Shih and Bayley (1985). As reported, nearly quantitative yields were obtained.

(*C*) *4′-[3-(Trifluoromethyl)-3H-diazirin-3-yl]phenylalanine* [*(Tmd)Phe*] (**13**). In order to prepare the title compound from the intermediate **8**, we essentially followed the scheme of Nassal (1984), which involved intermediates **9**–**12** (Scheme I). For deacetylation of the racemic **12**, a resin-bound acylase was used: 1.9 g (6 mmol) of compound **12** was dissolved in a minimal amount of 1 M NaOH. To this solution, 150 mL of sodium phosphate buffer (20 mM, pH 6.6) containing 0.5 mM $CoCl_2$ and 5.4 g of washed, immobilized acylase (Plexazym AC, Fluka) was added and the dispersion treated at 37 °C. Cleavage of the amide was essentially complete after 10 h as concluded from measurements of free amine groups in aliquots of the supernatant using the TNBS assay (Fields, 1972). Following removal of the resin, the filtrate was processed further as described (Nassal, 1984).

(*D*) *[^{3}H]-4′-[3-(Trifluoromethyl)-3H-diazirin-3-yl]-phenylalanine* [[^{3}*H*](*Tmd*)*Phe*] (**13**). Tritium labeling of (Tmd)Phe was done via the oxazolone following essentially the protocol of Shih and Bayley (1985). A mixture of *N*-acetyl-(Tmd)Phe (33 mg, 0.106 mmol), 38 μL (0.40 mmol) of acetic anhydride, and tritiated water (4 μL, 5 Ci) was treated at 50 °C for 22 h. Following hydrolysis of the reaction mixture, volatile radioactivity was condensed into a cold trap (liquid nitrogen) and the residue was treated several times with 50-μL portions of water (after each treatment the water was

Scheme I



distilled into the cold trap). The amorphous residue containing DL-[^{3}H]-*N*-acetyl-(Tmd)Phe was then dissolved in phosphate buffer (see above), and the L form was cleaved specifically by acylase. The free amino acid was converted to the Boc derivative following the protocol of Nassal (1985). L-[^{3}H]-Boc-(Tmd)Phe was then purified by column chromatography (silica gel 60) using CH_2Cl_2/methanol/acetic acid (17:2:1), and final purification was achieved by HPLC using a Nucleosil C18-120-5 (Macherey Nagel) reverse-phase column (4.6 × 250 mm). A linear gradient formed from 50% aqueous ethanol containing 2% HCOOH (solvent A) and 2% HCOOH in absolute ethanol (solvent B) was used. The flow rate was 0.5 mL/min. L-[^{3}H]Boc-(Tmd)Phe eluted as a sharp peak at 65% ethanol; its specific radioactivity was 3.5×10^6 cpm/nmol. Yield was approximately 50 mCi.

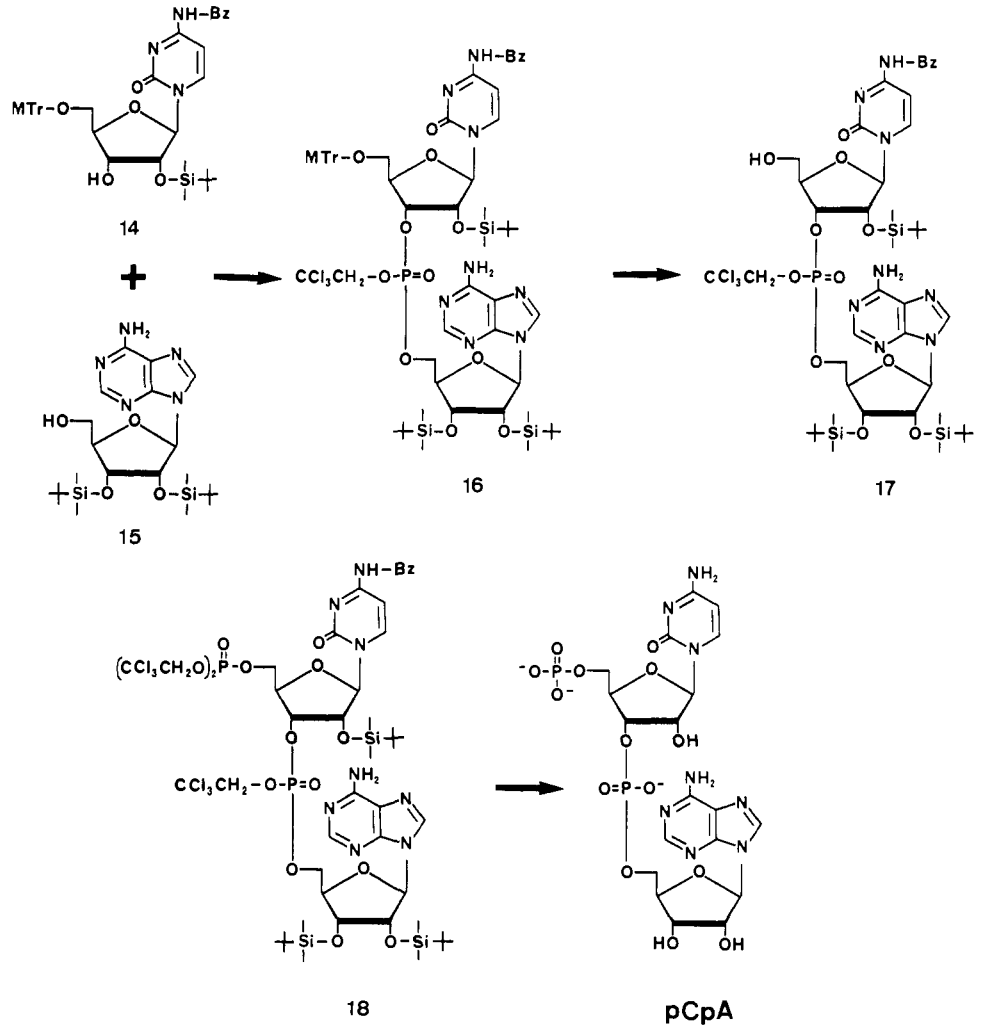
Synthesis of pCpA (*Scheme II*). (*A*) *N*^{4}*-Benzoyl-2′-O-(tert-butyldimethylsilyl)-5′-O-(monomethoxytrityl)cytidine* (**14**). This compound was prepared according to Ogilvie et al. (1979). Purification by column chromatography using CH_2Cl_2/methanol (98:2) yielded a white amorphous material which crystallized upon treatment with ether (mp 159–162 °C): partial ^{1}H NMR (DMSO-d_6) δ 0.09 (s, 3), 0.13 (s, 3), 0.89 (s, 9), 3.3–3.5 (m, 3), 3.76 (s, 3), 4.05–4.15 (m, 3), 5.15 (s, 1), 5.78 (s, 1), 6.9–8.4 (m, 21), 11.25 (s, 1).

(*B*) *2′,3′-Bis-O-(tert-butyldimethylsilyl)adenosine* (**15**). This derivative was obtained from 2′,3′,5′-tris-*O*-(*tert*-butyldimethylsilyl)adenosine by treatment with 80% HOAc at 95 °C for 3 h according to Ogilvie et al. (1978). ^{1}H NMR (DMSO-d_6) δ −0.47 (s, 3), −0.15 (s, 3), 0.11 (s, 3), 0.12 (s, 3), 0.69 (s, 9), 0.92 (s, 9), 3.5–3.8 (m, 2), 3.99 (s, 1), 4.3 (d, 1), 4.9 (m, 1), 5.7–5.8 (m, 1), 5.9 (d, 1), 7.4 (s, 2), 8.14 (s, 1), 8.4 (s, 1).

(*C*) *2,2,2-Trichloroethyl N*^{4}*-Benzoyl-2′-O-(tert-butyldimethylsilyl)-5′-O-(monomethoxytrityl)cytidylyl(3′–5′)-[2′,3′-bis-O-(tert-butyldimethylsilyl)adenosine*] (**16**). A solution of 2,4,6-collidin (2.9 mL), THF (7.5 mL), and 2,2,2-trichloroethyl phosphorochloridite (Aldrich) was cooled to −78 °C and treated dropwise with a solution of the protected cytidine derivative **14** (3.65 g, 4.9 mmol) in THF (12 mL). After 15 min at −78 °C, a solution of the adenosine derivative **2** (2.06 g, 4.15 mmol) in THF (16 mL) was added dropwise and reacted for an additional 10 min. The mixture was warmed up and treated with a solution of I_2 (2.4 g, 9 mmol) in 31 mL of THF/water (2:1) containing 0.5 mL of pyridine. The mixture was concentrated to dryness under reduced pressure, and the residue was dissolved in 300 mL of methylene chloride. This solution was then washed with a solution of $NaHSO_3$ and water, dried over $MgSO_4$, and concentrated. Column chromatography on silica gel (CH_2Cl_2/methanol, 20:1 v/v) provided the pure dinucleoside phosphate **16** as white crystals (mp 148–150 °C), yield 5.19 g (88% of theory): partial ^{1}H NMR (DMSO-d_6) δ −0.42 to +0.13 (m, 18), 0.70–0.95 (m, 27), 3.72 (s, 3), 6.8–8.4 (m, 25), 11.33 (s, 1); ^{31}P NMR (DMSO-d_6) δ −3.26 and −3.67 (two signals with equal intensities); phosphate content, 1 P per formula weight of 1423.

(*D*) *2,2,2-Trichloroethyl N*^{4}*-Benzoyl-2′-O-(tert-butyldimethylsilyl)cytidylyl(3′–5′)[2′,3′-bis-O-(tert-butyldimethylsilyl)adenosine*] (**17**). **16** (5.09 g, 3.6 mmol) was dissolved in 350 mL of CH_2Cl_2/methanol (2:1 v/v). Benzenesulfonic acid (4.25 g) was added, and the mixture was allowed to react for 4 h at room temperature. Following addition of 200 mL of CH_2Cl_2 and extraction with a solution of $NaHCO_3$ and with water, the organic phase was dried and the residue subjected to column chromatography (silica gel 60) using CH_2Cl_2/

Scheme II



methanol (100:6 v/v), yield 3.35 g (82%) of a colorless solid: partial ^{1}H NMR (DMSO-d_6) δ −0.5 to +0.20 (m, 18), 0.70–0.95 (m, 27), 6.8–8.5 (m, 11), 11.3 (s, 1).

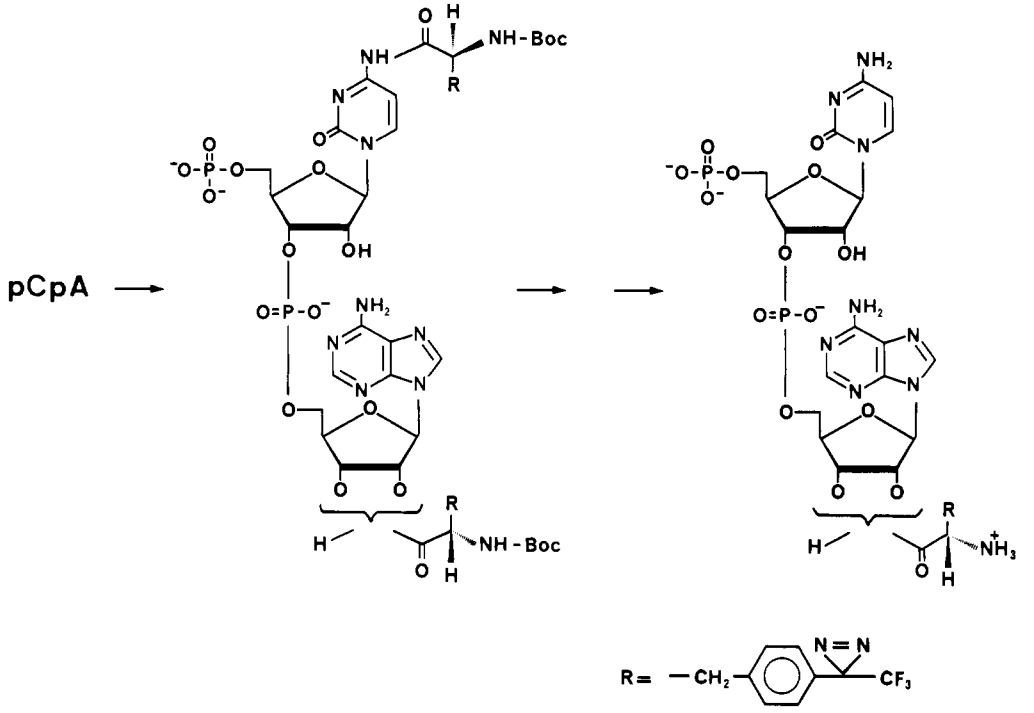
(*E*) *2,2,2-Trichloroethyl N^4-Benzoyl-2′-O-(tert-butyldimethylsilyl)-5′-O-[[bis(2,2,2-trichloroethyl)oxy]-phosphoryl]cytidylyl(3′–5′)[2′,3′-bis-O-(tert-butyldimethylsilyl)adenosine]* (**18**). **17** (3.13 g, 2.7 mmol) (dried in vacuo at 50 °C for 10 h) was dissolved in 50 mL of anhydrous THF. Then, 18 g of pulverized molecular sieves (3 Å, dried at 110 °C for 4 h under reduced pressure) was added and the dispersion cooled to −78 °C. Bis(2,2,2-trichloroethyl) phosphorochloridite [prepared according to Imai and Torrence (1981), bp 86–89 °C (0.08 mmHg)] was added while the suspension was vigorously stirred at −78 °C. After 30 min, the reaction mixture was warmed to 0 °C, 1 mL of water was added, and the molecular sieves were removed by filtration and washed with a total of 250 mL of ether. To the combined filtrate was added a solution of $NaHCO_3$ (125 mL, 0.5 M) followed by an ether solution of iodine (2 g in 125 mL), which was added dropwise. When the oxidation was complete, the organic layer was separated, extracted with aqueous Na_2SO_3, washed with saturated NaCl solution, dried over $MgSO_4$, and concentrated in vacuo. Chromatography on silica gel (200 g) with CH_2Cl_2/methanol (100:4 v/v) afforded **18** as a white solid with mp 151–153 °C, yield 2.95 g (74%): ^{1}H NMR (DMSO-d_6) δ −0.40 to +0.14 (m, 18), 0.71–0.91 (m, 27), 3.25–3.35 (m, 2), 4.1–5.2 (m, 15), 5.8–6.0 (m, 2), 7.25–8.4 (m, 11), 11.35 (s, 1). The ^{31}P NMR showed two signals at δ −3.27 and −4.12. Phosphate analysis gave 2 P per formula weight of 1493.

(*F*) *5′-O-Phosphorylcytidylyl(3′–5′)adenosine* (*pCpA*). Deblocking of compound **18** was carried out by treatment of **18** (1.78 g, 1.2 mmol) with a zinc–copper couple (3.9 g, mmol) and 3.9 g of acetylacetone in 24 mL of DMF for 2 h at 55 °C and removal of metal ions with Chelex resin according to Imai and Torrence (1981). Following evaporation of the solvent, the yellow oily residue was treated with half-saturated methanolic ammonia for 20 h at 25 °C, and upon concentration to dryness the residue was dissolved in 100 mL of 0.7 M tetrabutylammonium fluoride in THF and maintained at room temperature overnight.[2] The mixture was then diluted with 1000 mL of water and applied to a (100-mL, HCO_3^- form) DEAE-Sephadex A-25 column that had been equilibrated with 50 mM $NH_4^+HCO_3^-$ and washed with the same buffer. Elution of pCpA was effected with a linear gradient (from 50 mM to 0.8 M) of $NH_4^+HCO_3^-$. The appropriate fractions containing pure pCpA as judged by TLC were pooled and lyophilized, yield approximately 350 mg (41%): partial ^{1}H NMR (DMSO-d_6) δ 5.73 (d, 1), 5.89–5.91 (m, 2), 7.72 (d, 1), 8.14 (s, 1), 8.81 (s, 1). The ^{31}P NMR showed two signals at δ 0.59 and 0.93.

Synthesis of 2′(3′)-O-[[^{3}H](Tmd)phenylalanyl]pCpA (*Scheme III*). A solution containing 188 mg (0.50 mmol)

[2] Anhydrous tetrabutylammonium fluoride was less effective than the commercially available (Fluka) trihydrate.

Scheme III



of L-[^{3}H]Boc-(Tmd)Phe (20 mCi/mmol) and 81 mg (0.50 mmol) of 1,1′-carbonyldiimidazole in 1.0 mL of DMSO was stirred at room temperature for 10 min and then reacted with 32.5 mg (0.05 mmol) of pCpA (**8**). Stirring was continued at 25 °C for 3 h, and then the reaction was quenched by the addition of 1 mL of methanol. The clear yellow solution was subjected to gel filtration on a column (3 × 15 cm) of Sephadex LH-20 equilibrated and eluted with methanol. Fractions (2 mL) containing diacyl-pCpA were pooled, concentrated, and dried at 0.01 mmHg. This residue was treated with anhydrous TFA (0.3 mL) for 2 min at room temperature. TFA was then evaporated by means of a stream of dry nitrogen and the residue dissolved in ethanol (2 mL) and dried again by rotevaporation (temperature 25 °C). This step was repeated three more times, and then the residue was dissolved in 0.01 M HCl (pH 2). After 24 h at room temperature, the sample was lyophilized to afford a pale powder that was dissolved in a small volume of 10% aqueous acetic acid. The solution was applied to Whatman 3MM paper and chromatographed in *n*-butanol/acetic acid/water (5:2:3 v/v). The product-containing band (R_f 0.25; identified by fluorescence quenching and red ninhydrin color) was eluted with 0.02 M acetic acid. Lyophilization provided a white material that was further purified by paper electrophoresis (Whatman 3MM) in 10% acetic acid. Elution with 0.02 M acetic acid followed by lyophilization provided approximately 3 μmol (6%) of the desired compound. It was stored at −20 °C.

Preparation of E. coli tRNA^{Phe}-C_{OH}. tRNA^{Phe} was shortened at the terminal adenylic acid residue according to the procedure described by Alford (1979). The resulting tRNA^{Phe}-CC_{OH} was purified by DEAE-Sephadex A-25 ion-exchange chromatography. The 3′-terminal cytidylic acid residue was then removed according to the procedure of Sprinzl and Sternbach (1979).

Preparation of "Chemically Misaminoacylated" (Tmd)-phenylalanyl-tRNA^{Phe}. The procedure used was, in essence, that described by Heckler et al. (1984b) except that the T4 RNA ligase mediated reaction was done at 37 °C. The resulting (Tmd)phenylalanyl-tRNA^{Phe} was purified as described for *E. coli N*-acetyl-DL-β-phenylalanyl-tRNA^{Phe} by Heckler et al. (1984b) except that DEAE-Sephadex A-25 was used in place of DEAE-cellulose. The biochemical integrity of the purified material was assessed by enzymatic aminoacylation following chemical deacylation at pH 9.0 for 1 h at 37 °C.

Preparation and Purification of Phenylalanyl-tRNA^{Phe}. The aminoacylation mixture (0.6 mL) contained 50 mM Tris-HCl, pH 7.6, 20 mM $MgCl_2$, 0.5 mM EDTA, 10 mM ATP, 50 μM Phe, 6 A_{260} units of *E. coli* tRNA^{Phe}, and 0.3 mg of yeast phenylalanyl-tRNA synthetase. After 10 min at 37 °C, the reaction was stopped by the addition of 150 μL of 0.2 M NaAc, pH 4.5. The charged tRNA^{Phe} was purified by extraction with phenol (450 μL), precipitation with 2.5 volumes of ethanol, and passing through a column (1.5 × 10 cm) of Bio-Gel P-6 using 2 mM NaOAc, and 1 mM $MgCl_2$, pH 5.2.

Results and Discussion

Synthesis of (Tmd)Phe and [^{3}H](Tmd)Phe. The synthetic route used in the present study to prepare the title compounds is depicted in Scheme I. It is the result of an effort to further improve the preparation of this compound by combining those steps in the two original syntheses (Nassal, 1984; Shih & Bayley, 1985) that proved to both be simple and result in relatively high yields.

Synthesis of pCpA (Scheme II). Chemical misacylation of tRNAs involves as a key intermediate pCpA, the synthesis of which has been described by Heckler et al. (1984a,b). Difficulties (e.g., in preparing the poorly characterized tribenzoyladenosine) prompted us to examine an alternative synthesis.

The present route involved condensation of N^4-benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(monomethexytrityl)cytidine (**14**) and $O^{2'}$,$O^{3'}$-bis(*tert*-butyldimethylsilyl)adenosine (**15**) with 2,2,2-trichloroethyl phosphorodichloridite as described by Ogilvie et al. (1978). The protected dinucleoside monophosphate (**16**) was obtained in 88% yield after chromatographic purification. Detritylation with benzenesulfonic acid followed by chromatography afforded compound **17** (82% yield). A significant improvement of the present procedure

Table I: Physical Properties of Compounds Involved in the Synthesis and Characterization of 2′(3′)-*O*-(Tmd)phenylalanyl-pCpA

compd	R_f value: silica gel F_{254}	R_f value: Whatman 3MM	electrophoretic mobility[a]	mp (°C)
14	0.64[b]			159–162[e]
15	0.24[b]			249–252
16	0.33[b]			148–150
17	0.14[b]			
18	0.31[b]			151–153
pCpA	0.05[c] 0.15[d]	0.10[c]	−0.07	
(Tmd)Phe		0.70[c]	0.38	
N^4-[Boc-(Tmd)Phe]pCpA	0.42[c]			
[Boc-(Tmd)Phe]$_2$pCpA	0.47 and 0.50[c]			
2′(3′)-*O*-(Tmd)phenylalanyl-pCpA	0.25[c]	0.43		
pCp[f]		0.08[c]	−0.67	
2′(3′)-*O*-[(Tmd)phenylalanyl]adenosine[g]			1.31	

[a] The medium was 10% acetic acid. Values recorded are relative to the mobility of adenosine (=1.0). [b] The solvent used was CH_2Cl_2/methanol (95:5 v/v). [c] The solvent used was 1-butanol/HOAc/water (5:2:3 v/v). [d] Solvent: 2-propanol/concentrated aqueous ammonia/water (55:10:35 v/v). [e] This melting point differed markedly from that (112–117 °C) reported by Ogilvie et al. (1979). [f] Obtained by digestion of pCpA of 2′(3′)-*O*-(Tmd)Phe-pCpA with RNase T_2. [g] This compound was synthesized as described for 2′(3′)-*O*-phenylalanyladenosine (Gottikh et al., 1970); the same compound also resulted from digestion of 2′(3′)-*O*-(Tmd)phenylalanyl-pCpA upon digestion with RNase T_2.

appears to be the use of bis(2,2,2-trichloroethyl) phosphorochloridite for phosphorylation of the 5′-OH group of **17**. Unlike phosphorylation by $POCl_3$, the resultant triester is relatively unpolar and can be easily purified on silica gel. Moreover, the use of this reagent does not require blocking of the amino group in the adenosine moiety. After chromatographic purification, compound **18** was obtained in 74% yield. Deblocking was effected by sequential treatments of **18** with (i) zinc–copper couple/2,4-pentanedione/DMF as described by Imai and Torrence (1981), (ii) half-saturated ammonia in methanol, and (iii) 0.7 M tetrabutylammonium fluoride in THF. Progress of each step could be followed by TLC, but the intermediates obtained were not isolated and characterized. The final product was characterized by UV, ^1H and ^{31}P NMR spectroscopy, and quantitative RNAse T_2 mediated hydrolysis. In addition, on TLC, pCpA was indistinguishable from a reference sample obtained by Dr. Hecht.

Synthesis of 2′(3′)-O-(Tmd)phenylalanyl-pCpA. The overall strategy for the chemical aminoacylation of tRNA is determined to a great extent by the nature of the amino protecting group used along with the corresponding chemistry for its removal. Another important aspect that had to be considered is the pronounced susceptibility of the ester bond in aminoacyl-pCpA to hydrolysis even under neutral conditions. Therefore, a synthetic scheme involving deprotection prior to ligation suffers from the disadvantage that some of the aminoacyl-pCpA (as well as some of the formed aminoacyl-tRNA) may be cleaved in the course of the ligation reaction, resulting in formation of free pCpA (and free intact tRNA) which also represents a donor (acceptor) for T4 RNA ligase and, hence, would lead to side products. These risks would be reduced if deprotection were carried out following the ligation reaction. Our search for a protecting group compatible with such a scheme led us to examine the dithiasuccinoyl group introduced by Barany and Merrifield (1977) for "orthogonal" peptide synthesis. This group can be removed with a number of reducing agents [e.g., alcoholic sodium borohydride, triphenyl(tri-*n*-butyl)phosphine, thiols (Barany & Merrifield, 1979)]. Unfortunately, however, under the conditions employed for the condensation of the Dts-amino acid with pCpA (in anhydrous DMSO as solvent), this group proved not stable enough (data not shown).

In view of its acid lability we then focused upon the Boc protecting group. Implicit in this decision was that deprotection had to be carried out prior to the ligation reaction (this is because tRNA would hardly survive the TFA treatment utilized to effect deblocking). This group was previously used by Mercer and Symons (1972) for the synthesis of *N*-acetylaminocyl di- and trinucleotides. Starting from Boc-(Tmd)Phe and pCpA, we were able to prepare the desired (Tmd)phenylalanyl-pCpA although the yield was low (6% based on radioactivity). As expected, the intermediate initially formed were due to acylation of the N^4 amino group of the cytosine moiety. With increasing reaction time, two new products were formed with nearly identical R_f values on TLC (0.47 and 0.50, respectively). Both were found to contain 2 (Tmd)Phe residues per pCpA and are assumed to be due to additional acylation at either the 2′- or 3′-hydroxyl group of the adenosine ribose moiety as suggested by the finding that they underwent relatively rapid interconversion on silica gel TLC plates. Treatment of these putative *N,O*-diacyl dinucleotides with TFA and 0.01 M HCl afforded 2′(3′)-*O*-(Tmd)phenylalanyl-pCpA, which was purified by descending paper chromatography and high-voltage paper electrophoresis. It was stable at −20 °C when stored as a lyophilized powder.

Several criteria were used to characterize this product. In addition to demonstrating that it contained one aminoacyl residue per pCpA and that it was rapidly hydrolyzed under slightly alkaline conditions, yielding pCpA and (Tmd)Phe, we also examined the material by RNase T_2 digestion. Only 50% could be degraded, whereby two new products were formed which were identified as pCp and 2′(3′)-*O*-[(Tmd)phenylalanyl]adenosine, respectively (Table I). From this result we concluded that the purified material we had isolated was of a 1:1 mixture of the 2′–5′- and 3′–5′-phosphodiester isomers (Figure 1). We have not investigated whether this putative isomerization had occurred during acylation (via an acylphosphodiester intermediate) or during the TFA treatment used to effect removal of the Boc group. Interestingly, it was reported previously (Mercer & Symons, 1972) that treatment of aminoacyl di- and trinucleotides with anhydrous TFA for 2 min at 20 °C caused considerable degradation but that isomerization of the phosphodiester group did not occur to a significant extent. Since only the 3′–5′- but not the 2′–5′-phosphodiester can serve as a donor in the subsequent T4 RNA ligase catalyzed reaction (Kikuchi et al., 1978), no further attempt has been made to separate the isomers or to avoid their formation.

Preparation of E. coli tRNA^{Phe}-C$_{OH}$. The main results from the abbreviation of *E. coli* tRNA^{Phe} are summarized in Table II. It demonstrates that we lost approximately 30% (from 1300 to 960 pmol/A_{260} unit of tRNA) of the phenylalanine-

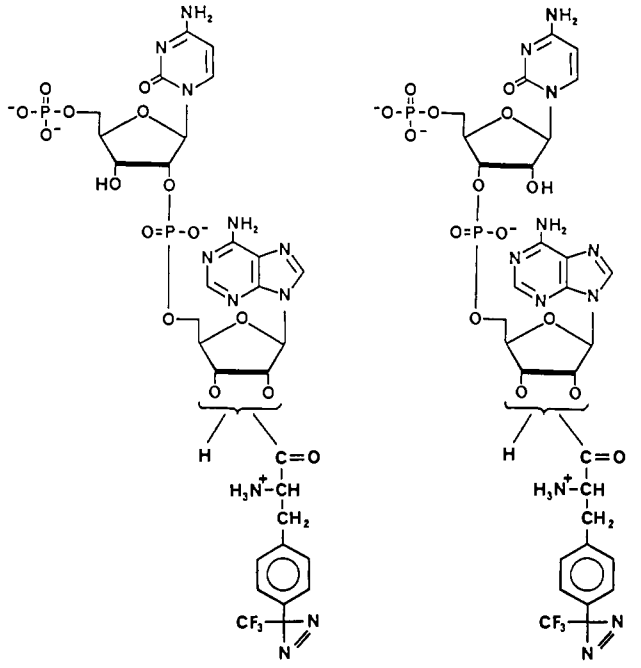


FIGURE 1: Structures of isomeric 2′(3′)-*O*-(Tmd)Phe-pCpA's. The 2′–5′-phosphodiester isomer (left) is not degraded by RNAse T_2.

Table II: Incorporation of [^{14}C]Phenylalanine into Various tRNA Species Catalyzed by *E. coli* Aminoacyl-tRNA Synthetase (pmol/A_{260} unit of tRNA)[a]

tRNA species examined	no pretreatment	incubation[b] in the presence of ATP	incubation[b] in the presence of CTP + ATP
$tRNA^{Phe}$[c]	1350		
$tRNA^{Phe}$-CC_{OH} and $tRNA^{Phe}$-C_{OH}[d]	0	260	1210
$tRNA^{Phe}$-CC_{OH}[e]		1300	1365
$tRNA^{Phe}$-C_{OH}[f]			960
$tRNA^{Phe}$	780		

[a] Phenylalanine acceptancy was measured as described under Experimental Procedures. [b] Prior to aminoacylation, the tRNAs were incubated with ATP(CTP):tRNA nucleotidyltransferase. Incubation mixtures (20 μL) contained 0.05 A_{260} unit of tRNA, 25 ng [sp act. 1150 nmol/(mg·min)] of transferase, 0.63 mM CTP (1 mM ATP) in 120 nM Tris-HCl, 40 mM KCl, and 8 mM $MgCl_2$, pH 9.0. After incubation for 20 min at 32 °C, the tRNAs were subjected to aminoacylations. [c] Commercial *E. coli* $tRNA^{Phe}$ (Boehringer). [d] Obtained by venom exonuclease treatment of *E. coli* $tRNA^{Phe}$. [e] Obtained upon incubation of exonuclease digested *E. coli* $tRNA^{Phe}$ with ATP(CTP):tRNA nucleotidyltransferase. [f] "Abbreviated" *E. coli* $tRNA^{Phe}$ resulting from successive treatments of $tRNA^{Phe}$ with (i) exonuclease, (ii) ATP(CTP):tRNA nucleotidyltransferase in the presence of excess CTP, and (iii) periodate/lysine followed by alkaline phosphatase. [g] Obtained by deacylation of the chemically misaminoacylated (Tmd)-phenylalanyl-$tRNA^{Phe}$.

accepting capacity of the tRNA during the periodate/lysine/alkaline phosphatase treatment.

T4 RNA Ligase Mediated Preparation of (Tmd)phenylalanyl-$tRNA^{Phe}$. Figure 2 demonstrates the time course of the T4 RNA ligase mediated coupling of 2′(3′)-*O*-[^{3}H]-(Tmd)phenylalanyl-pCpA to $tRNA^{Phe}$-C_{OH}. Although maximal radioactivity incorporation was not reached within less than 10 min, for preparative purposes the reaction was stopped after 6 min by adjusting the pH to 5.2 and cooling to 0 °C. The rationale was to reduce the extent of side reactions due to hydrolysis of 2′(3′)-*O*-(Tmd)phenylalanyl-pCpA or (Tmd)phenylalanyl-$tRNA^{Phe}$.

For purification of the putative (Tmd)phenylalanyl-$tRNA^{Phe}$, we used essentially the procedure of Heckler et al. (1984b) involving ion-exchange chromatography on DEAE-

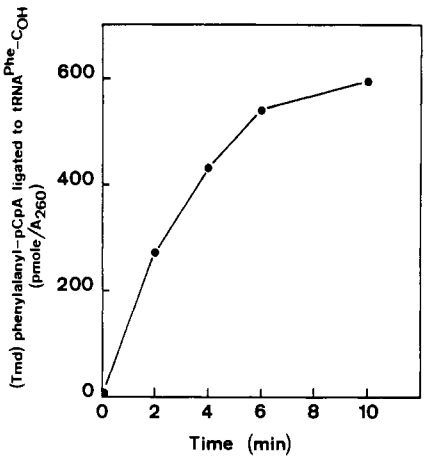


FIGURE 2: Time course of the T4 RNA ligase mediated coupling of 2′(3′)-*O*-[^{3}H](Tmd)phenylalanyl-pCpA to $tRNA^{Phe}$-C_{OH}. $tRNA^{Phe}$-C_{OH} (1.14 A_{260} unit) was incubated at 37 °C with 0.6 A_{260} unit (30 nmol; 1.85 × 10^4 cpm/nmol) of 2′(3′)-*O*-[^{3}H](Tmd)-phenylalanyl-pCpA in the presence of 30 units of T4 RNA ligase. The mixture (total volume 114 μL) contained Na^+-Hepes (55 mM), pH 7.5, 15 mM $MgCl_2$, 250 μM ATP, 20 μg/mL bovine serum albumin, and 10% DMSO. At the time points indicated, 20-μL aliquots were taken and immediately mixed with 80 μL of cold 50 mM sodium acetate buffer, 1 M NaCl, and 10 mM $MgCl_2$, pH 5.2, containing 10 A_{260} units of *E. coli* tRNA. Following extraction with phenol (60 μL), the tRNA was precipitated by the addition of 2.5 volumes of ethanol and the tRNA collected by centrifugation. Upon removal of the supernatant, the tRNA was dissolved in 100 μL of the pH 5.2 buffer and reprecipitated. This last procedure was repeated three more times, and the tRNA was then dissolved in 50 μL of buffer. Therefrom 20-μL aliquots were taken for scintillation and A_{260} measurements.

Sephadex A-25 and BD-cellulose. In addition, the material was passed through a column of Bio-Gel P-6 which had been equilibrated and eluted with 2 mM NaOAc and 1 mM $MgCl_2$, pH 5.2. By means of this gel filtration ethanol and traces of UV-absorbing compounds leaching out from the BD-cellulose column could be removed. The specific radioactivity of 2′-(3′)-*O*-[^{3}H](Tmd)phenylalanyl-pCpA was 18.5 × 10^3 cpm/nmol, and that of the purified [^{3}H](Tmd)phenylalanyl-$tRNA^{Phe}$ corresponded to 18.7 × 10^3 cpm/A_{260} unit or, assuming 960 pmol/A_{260} unit of tRNA (Table II), 19.5 × 10^3 cpm/nmol. This demonstrates that there was 1 (Tmd)Phe bound per $tRNA^{Phe}$.

Characterization of (Tmd)phenylalanyl-$tRNA^{Phe}$. (i) Samples of *E. coli* $tRNA^{Phe}$, $tRNA^{Phe}$-CC_{OH}, $tRNA^{Phe}$-C_{OH}, and the purified (Tmd)phenylalanyl-$tRNA^{Phe}$ were subjected to urea–PAGE [preparation of the samples is assumed to cause complete deacylation of (Tmd)phenylalanyl-$tRNA^{Phe}$]. As illustrated in Figure 3, all tRNA species showed a characteristic "doublet" of bands indicating heterogeneity already in the starting $tRNA^{Phe}$ (lanes 1 and 5). The two truncated species (lanes 3 and 4) showed the expected incremental increase in mobility when compared with the intact $tRNA^{Phe}$. The tRNA obtained from the T4 RNA ligase mediated reaction (lane 2) was indistinguishable from starting $tRNA^{Phe}$, a result that rules out the possibility that limited hydrolysis of 2′(3′)-*O*-(Tmd)phenylalanyl-pCpA during the ligation reaction yielded amounts of pCpA that lead to significant formation of uncharged $tRNA^{Phe}$, which in turn then functioned as an acceptor for (an) additional pCpA unit(s).

(ii) A sample of the putative (Tmd)phenylalanyl-$tRNA^{Phe}$ was deacylated by brief exposure to 50 mM Tris-HCl (pH 9.0) and reaminoacylated by utilizing [^{14}C]phenylalanine and an *E. coli* aminoacyl-tRNA synthetase preparation. As shown in Table II, the deacylated $tRNA^{Phe}$ obtained from the ligase

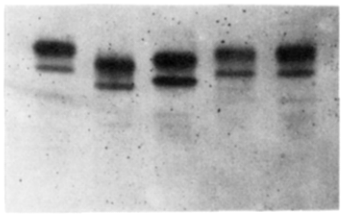


FIGURE 3: Electrophoretic analysis of various tRNA species. Samples containing approximately 0.6 pmol of tRNA were subjected to urea–polyacrylamide gel electrophoresis using essentially the method described by Igloi (1983). tRNA was visualized by the silver staining technique according to Sammons et al. (1981). Lanes: (1 and 5) *E. coli* $tRNA^{Phe}$ from Boehringer; (2) $tRNA^{Phe}$-C_{OH}; (3) $tRNA^{Phe}$-CC_{OH}; (4) $tRNA^{Phe}$ as obtained from T4 RNA ligase mediated condensation of $tRNA^{Phe}$-C_{OH} with 2′(3′)-*O*-(Tmd)-phenylalanyl-pCpA. The figure shows only that sector of the gel which contained the tRNA.

reaction could be reaminoacylated to nearly the same extent (780 pmol/A_{260} unit of tRNA) as $tRNA^{Phe}$ regenerated from $tRNA^{Phe}$-C_{OH} upon incubation with CTP(ATP):tRNA nucleotidyltransferase in the presence of ATP and CTP (960 pmol/A_{260} unit). Abbreviated $tRNA^{Phe}$-C_{OH}, however, was not a substrate for aminoacyl-tRNA synthetase (Table II).

(iii) The biological activity of chemically aminoacylated (Tmd)phenylalanyl-$tRNA^{Phe}$ was further assessed by comparison of the ability of this species with that of enzymatically charged phenylalanyl-$tRNA^{Phe}$ to stimulate protein synthesis in a reticulocyte lysate. Protein synthesis was followed by measuring incorporation of [^{3}H]leucine into TCA-precipitable material. As depicted in Figure 4, the presence of 0.03 mM phenylalaninyl-AMP, a potent inhibitor of phenylalanyl-tRNA synthetase (Cassio et al. 1967), completely abolished protein synthesis. However, supplementation of the system with either the enzymatically charged phenylalanyl-$tRNA^{Phe}$ (60 pmol) or chemically misaminoacylated (Tmd)phenylalanyl-$tRNA^{Phe}$ (35 pmol) showed a clear stimulation in protein biosynthesis whereas with 60 pmol of uncharged $tRNA^{Phe}$ (control) no effect was seen. Assuming that only globin was synthesized [implying the incorporation of 18 Leu per 7 Phe [or (Tmd)-Phe]] and on the basis of the known specific radioactivity of the [^{3}H]Leu added, we calculated that in both cases about 40% of the expected incorporation of radioactivity was obtained. In this calculation we considered neither the endogenous Leu nor any deacylation of the aminoacyl-$tRNA^{Phe}$s during the translation experiments. We think, therefore, that these results do strongly support the view that the (Tmd)phenylalanyl-$tRNA^{Phe}$ is biologically active.

CONCLUSIONS

By extending the general scheme introduced by Hecht and colleagues for the chemical misacylation of tRNA, we have in this study developed a procedure for the misaminoacylation of *E. coli* $tRNA^{Phe}$ with the photoactivatable, carbene-generating analogue of phenylalanine, (Tmd)Phe. Central in this study were the elaboration of procedures that allowed the preparation of 2′(3′)-*O*-(Tmd)phenylalanyl-pCpA and the ligation of this key intermediate to $tRNA^{Phe}$-C_{OH}. The methodology used here should, in principle, be applicable to any other amino acid or tRNA.

We have obtained evidence that the mischarged $tRNA^{Phe}$ is active in an in vitro protein-biosynthesizing system. This implies that proteins can be made biosynthetically with different types of amino acid substitutions: (i) replacement of a single type (e.g. Phe) by (Tmd)Phe, (ii) "random" substitution of amino acids by using (Tmd)Phe mischarged to unfractionated tRNA (this would represent a "simple" way of generating a very large population of distinctly different photoactivatable polypeptides which might be interesting for affinity labeling), and (iii) replacement of a single residue, e.g., by chemically mischarging suppressor tRNA and introducing the amino acid specifically at amber mutation sites as already suggested by Shih and Bayley (1985).

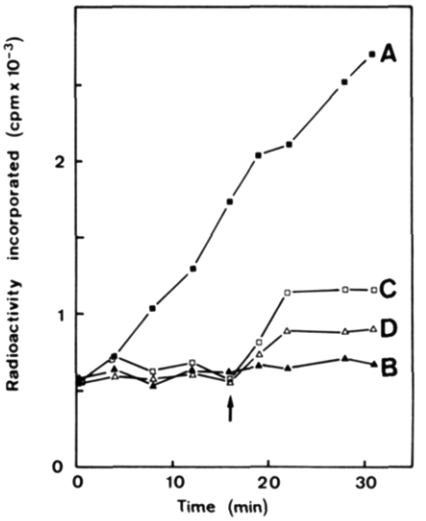


FIGURE 4: Stimulation of protein biosynthesis by *E. coli* phenylalanyl-$tRNA^{Phe}$ and chemically misaminoacylated *E. coli* (Tmd)-phenylalanyl-$tRNA^{Phe}$. To reticulocyte lysates were added (final concentration) creatine kinase (67 μg/mL), hemin (26 μM), dithiothreitol (2.2 mM), creatine phosphate (10 mM), KCl (100 mM), and a mixture of amino acids that includes all except Phe and contained [^{3}H]Leu (sp radioact. 1.3 Ci/mmol) (100 μM). In experiments B–D the translation mixture was further supplemented with the phenylalanyl-tRNA synthetase inhibitor phenylalaninyl-AMP (30 μM). In each experiment (A–D) after 16 min (arrow), 12 μL of a solution (in 2 mM NaOAc and 1 mM $MgCl_2$, pH 5.2) containing the following component was added to each 61 μL of the lysate: (A) buffer alone (control), (B) uncharged $tRNA^{Phe}$ (60 pmol), (C) enzymatically charged phenylalanyl-$tRNA^{Phe}$ (60 pmol), or (D) chemically mischarged (Tmd)phenylalanyl-$tRNA^{Phe}$ (35 pmol). Translation was done at 32 °C. At time points indicated, aliquots (2 μL) were withdrawn and incorporation of [^{3}H]Leu into TCA-precipitable material was determined by the filter paper disk method.

ACKNOWLEDGMENTS

We thank Drs. S. Hecht and F. Cramer for valuable discussions, Dr. H. Wacker for a gift of reticulocyte lysate, and Dr. N. Mantei for critical comments on the manuscript. We are especially obliged to Dr. G. Semenza in whose laboratory this work was carried out.

REFERENCES

Alford, B. L., Chinault, A. C., Jolly, S. O., & Hecht, S. M. (1979) *Methods Enzymol. 59*, 121–134.

Barany, G., & Merrifield, R. B. (1977) *J. Am. Chem. Soc. 99*, 7363–7365.

Barany, G., & Merrifield, R. B. (1979) *Anal. Biochem. 95*, 160–170.

Brunner, J., Senn, H., & Richards, F. M. (1980) *J. Biol. Chem. 255*, 3313–3318.

Cassio, D., Lemoine, F., Waller, J.-P., Sandrin, E., & Boissonas, R. A. (1967) *Biochemistry 6*, 827–836.

Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.

Church, R. F. R., & Weiss, M. J. (1970) *J. Org. Chem. 35*, 2465–2471.

Fields, R. (1972) *Methods Enzymol. 25*, 464–468.

Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E., & Tener, G. M. (1967) *Biochemistry 6*, 3043–3056.
Gottikh, B. P., Krayevsky, A. A., Tarussova, N. B., Purygin, P. P., & Tsilevich, T. L. (1970) *Tetrahedron 26*, 4419–4433.
Hecht, S. M. (1977a) *Acc. Chem. Res. 10*, 239–245.
Hecht, S. M. (1977b) *Tetrahedron 33*, 1671–1696.
Hecht, S. M., Alford, B. L., Kuroda, Y., & Kitano, S. (1978) *J. Biol. Chem. 253*, 4517–4520.
Heckler, T. G., Zama, Y., Naka, T., & Hecht, S. M. (1983) *J. Biol. Chem. 258*, 4492–4495.
Heckler, T. G., Chang, L. H., Zama, Y., Naka, T., & Hecht, S. M. (1984a) *Tetrahedron 40*, 87–94.
Heckler, T. G., Chang, L. H., Zama, Y., Naka, T., Chorghade, M. S., & Hecht, S. M. (1984b) *Biochemistry 23*, 1468–1473.
Igloi, G. L. (1983) *Anal. Biochem. 134*, 184–188.
Imai, J., & Torrence, P. F. (1981) *J. Org. Chem. 46*, 4015–4021.
Johnson, A. E., & Cantor, C. R. (1980) *J. Mol. Biol. 138*, 273–297.
Johnson, A. E., & Slobin, L. I. (1980) *Nucleic Acids Res. 8*. 4185–4200.
Johnson, A. E., Miller, D. L., & Cantor, C. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3075–3079.
Kikuchi, Y., Hishinuma, F., & Sakaguchi, K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1270–1273.
Krieg, U. C., Walter, P., & Johnson, A. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8604–8608.
Kurzchalia, T. V., Wiedmann, M., Girshovich, A. S., Bochkareva, E. S., Bielka, H., & Rapoport, T. A. (1986) *Nature (London) 320*, 634–636.
Mercer, J. F. B., & Symons, R. H. (1972) *Eur. J. Biochem. 28*, 38–45.
Nassal, M. (1984) *J. Am. Chem. Soc. 106*, 7540–7545.
Ogilvie, K. K., Beaucage, S. L., Schifman, A. L., Theriault, N. Y., & Sadana, K. L. (1978) *Can. J. Chem. 56*, 2768–2780.
Ogilvie, K. K., Schifman, A. L., & Penney, C. L. (1979) *Can. J. Chem. 57*, 2230–2238.
Richards, F. M., & Brunner, J. (1980) *Ann. N.Y. Acad. Sci. 346*, 144–164.
Roe, B., Sirover, M., & Dudock, B. (1973) *Biochemistry 12*, 4146–4154.
Roesser, J. R., Chorghade, M. S., & Hecht, S. M. (1986) *Biochemistry 25*, 6361–6365.
Sammons, D. W., Adams, L. D., & Nishizawa, E. E. (1981) *Electrophoresis 2*, 17–23.
Shih, L. B., & Bayley, H. (1985) *Anal. Biochem. 144*, 132–141.
Sprinzl, M., & Sternbach, H. (1979) *Methods Enzymol. 59*, 182–190.
Wiedmann, M., Kurzchalia, T. V., Bielka, H., & Rapoport, T. A. (1987a) *J. Cell Biol. 104*, 201–208.
Wiedmann, M., Kurzchalia, T. V., Hartmann, E., & Rapoport, T. A. (1987b) *Nature (London) 328*, 830–833.