# Donor Site of Ribosomal Peptidyltransferase: Investigation of Substrate Specificity Using 2′(3′)-*O*-(*N*-Acylaminoacyl)dinucleoside Phosphates as Models of the 3′ Terminus of *N*-Acylaminoacyl Transfer Ribonucleic Acid†

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ABSTRACT: The unambiguous chemical synthesis of C-A-(fMet) (**4a**), C-A(f-D-Met) (**4b**), C-A(AcMet) (**4e**), C-A-(AcLeu) (**4f**), C-2′-dA(AcLeu) (**4c**), C-3′-dA(AcLeu) (**4d**), C-BrA(AcMet) (**4g**) (BrA = 8-bromoadenosine), and C-N-(AcMet) (**4h**) (N = nebularine) is described. These compounds were investigated as peptidyltransferase donor substrates (*Escherichia coli* 70S ribosomes) by using Phe-tRNA as the acceptor. C-A(fMet) (**4a**), C-A(AcMet) (**4e**), and C-A(AcLeu) (**4f**) were found to be active as donor substrates, whereas C-A(f-D-Met) (**4b**), C-2′-dA(AcLeu) (**4c**), and C-3′-dA(AcLeu) (**4d**) had no donor activity. However, compounds **4b**, **4c**, and **4d** could inhibit the peptidyltransferase reaction and, thus, are presumably bound to the P site. The 3′-*O*-(*N*-acylaminoacyl) derivative **4c** appears to be a stronger inhibitor than the parent 2′ ester **4d**. C-BrA(AcMet) (**4g**), with the 3′ nucleoside in the syn conformation, has donor activity similar to that of unmodified C-A(AcMet) (**4e**), whereas C-N(AcMet) (**4h**), with a 3′ nucleoside unable to form a Watson–Crick pair, is completely inactive. These results show that the peptidyltransferase P site (1) requires the L configuration of the aminoacyl portion of a substrate, (2) requires the 2′-OH group, probably of the 3′-*O*-(*N*-acylaminoacyl) derivative, for the reaction, (3) interacts with the aminoacyl portion of the donor substrate in a way similar to interactions in proteolytic enzymes, (4) can accept a donor substrate with the 3′ nucleoside in syn conformation, and (5) cannot accept a donor substrate in which the 3′ nucleoside cannot form two hydrogen bonds.

The substrate specificity of the peptidyltransferase acceptor and donor sites (A and P sites) could be conveniently investigated by using relatively simple substrate analogues based on the charged C-C-A termini of either AA- or peptidyl-tRNA.[1] Use of these analogues, 2′(3′)-*O*-(aminoacyl)nucleosides or -oligonucleotides, free of the other portions of tRNA, makes it possible to resolve the peptidyltransferase reaction from the other reactions of protein biosynthesis with which peptidyl transfer is normally linked. Although relatively exhaustive studies have been carried out on the peptidyltransferase A site, resulting in the description in considerable detail of the pertinent interactions of the 3′ terminus of AA-tRNA with that site, much less is known about the specificity of the peptidyltransferase P site [for review, see Sprinzl & Cramer (1979) and Chládek (1980)]. Monro and co-workers (Monro & Marcker, 1967; Monro et al., 1968) established that 2′(3′)-*O*-(*N*-acylaminoacyl) derivatives of C-C-A (as analogues of the 3′ terminus of AcAA-tRNA) react as peptide (*N*-acylamino acid) donors on the ribosome with puromycin as an acceptor substrate. This fragment reaction catalyzed by peptidyltransferase is dependent upon the presence of monovalent cations, divalent cations, and alcohol. The alcohol is apparently indispensable; it may be that it promotes an interaction of the donor substrate with the ribosome, since C-C-A(AcMet) and similar compounds do not bind to ribosomes under normal conditions (Monro & Marcker, 1967). Later it was discovered that even more simple compounds, such as 2′(3′)-*O*-(*N*-acylaminoacyl) derivatives of adenosine 5′-phosphate [e.g., pA(fMet)], possess donor activity in the peptidyltransferase reaction, provided that they are used at very high concentrations (above 1 mM) (Černá et al., 1973). In spite of the apparent insensitivity of this system, the donor properties of several 2′(3′)-*O*-(*N*-formylmethionyl)nucleoside 5′-phosphates were investigated [for review see Krayevsky & Kukhanova (1979)], although it was extremely difficult to detect the lower donor activity of the modified compounds. Further, the synthetic method used yielded compounds of uncertain optical purity leaving many previous biochemical results open to criticism.

We decided that further studies of the peptidyltransferase P site were warranted once optically pure 2′(3′)-*O*-(*N*-acylaminoacyl) derivatives of C-A and C-C-A became available. Since the C-C-A sequence of peptidyl-tRNA interacts with the P site of peptidyltransferase, the *N*-acylaminoacyl derivatives of C-A or C-C-A should bind more strongly than the pA derivatives (Monro et al., 1968; Quiggle & Chládek, 1980), and substrate specificity studies should give clearer results.

It is well-known that both AA-tRNA and AcAA-tRNA exist under physiological conditions as mixtures of 2′ and 3′ isomers due to rapid transacylation of the aminoacyl residue within the cis diol grouping of the 3′-terminal adenosine residue

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[1] Abbreviations used: AA-tRNA, aminoacyl transfer ribonucleic acid; AcAA-tRNA, *N*-acylaminoacyl transfer ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane; AcLeu, *N*-acetyl-L-leucine; fMet, *N*-formyl-L-methionine; similar abbreviations are used for other aminoacyl derivatives; Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; NPS, *p*-nitrophenylsulfonyl; DCC, dicyclohexylcarbodiimide; $Me_4Si$, tetramethylsilane; DSS, 4,4-dimethyl-4-silapentane-5-sulfonate; DMF, *N*,*N*-dimethylformamide; tRNA-C-C-3′-dA, tRNA with 3′-deoxyadenosine incorporated at the 3′ end; tRNA-C-C-2′-dA, tRNA with 2′-deoxyadenosine incorporated at the 3′ end; BrA, 8-bromoadenosine; N, nebularine, purine riboside; A-Leu, 2′(3′)-*O*-L-leucyladenosine; A(AcMet), 2′(3′)-*O*-(*N*-acetyl-L-methionyl)adenosine; 3′-dA(AcLeu), 2′-*O*-(*N*-acetyl-L-leucyl)-3′-deoxyadenosine; similar abbreviations are used for dinucleotide derivatives and other aminoacyl derivatives.

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(Griffin et al., 1966). The use of nonisomerizable models, in which the transacylation is prevented by chemical modification of the 3′-terminal ribose residue, is the only method available for investigating the role of AA-tRNA isomers in protein biosynthesis [for review, see Sprinzl & Cramer (1979) and Chládek (1980)]. We felt that some of these studies could be conveniently performed with simple nonisomerizable analogues of the 3′ termini of AcAA-tRNA, namely, modified derivatives of C-A or C-C-A.

In this paper we report the specific chemical synthesis of several such dinucleotide derivatives and discuss their behavior as donor substrates in the peptidyltransferase reaction on *Escherichia coli* 70S ribosomes. Attention is also paid to the problem of stereospecificity and isomer specificity at the donor site of peptidyltransferase. A portion of this material has been presented at meetings (Quiggle et al., 1979; Chládek, 1980).

## Materials and Methods

*General Methods.* General methods for chemical synthesis were the same as described in the previous papers of this series (Chládek et al., 1974; Ryu et al., 1977). Thin-layer chromatography (TLC) was performed on silica gel coated aluminum foils (silica gel 60 F 254, E. Merck, Darmstadt, West Germany) by using solvent systems $S_1$, $CH_2Cl_2$–$CH_3OH$ (95:5), and $S_2$, $CH_2Cl_2$–$CH_3OH$ (9:1), and on cellulose (13254 cellulose with fluorescent indicator, Eastman chromatogram sheet, Eastman-Kodak Co., Rochester, NY) in systems $S_3$, 2-propanol–concentrated ammonium hydroxide–water (7:1:2), $S_4$, 1-butanol–acetic acid–water (5:2:3), and $S_5$, 1-butanol saturated with 10% acetic acid. Preparative TLC was performed by using Uniplate silica gel GF (2000 μm) and Uniplate Avicel F (1000 μm) (both from Analtech, Inc., Newark, DE) in the same solvent systems. Column chromatography was performed over short columns (Hunt & Rigby, 1967) of silica gel 60H (for TLC) (EM Reagent, E. Merck, Darmstadt, West Germany) by using a $CH_2Cl_2$–$CH_3OH$ gradient as the eluent. Paper electrophoresis was conducted on a Savant Flat plate by using 1 M acetic acid as a buffer on Whatman No. 1 (analytical) or Whatman 3MM (preparative) paper at 40 V/cm for 2–4 h. NMR spectra were recorded on a JEOL FX100 instrument by using solvents and standards as specified in Table I. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter. Starting materials were made as described before (Chládek et al., 1974; Žemlicka et al., 1975; Chládek & Žemlička, 1969). Z, Boc, and NPS amino acids were commercial products (Sigma Chemical Co., St. Louis, MO).

*Racemization of N-Formyl-L-methionine and N-Acetyl-L-methionine by Carboxyl-Activating Reagents.* A solution of *N*-acylamino acid (0.1 mmol) was treated either with carbonyldiimidazole (2 equiv) in DMF (0.15 mL) or with mesitylenesulfonyltriazolide (2 equiv) or DCC (2 equiv) in pyridine (0.15 mL). After the appropriate time, water (0.1 mL) was added, the sample was made up with pyridine to a 1-mL volume, and the specific rotation was determined. The changes in the specific rotation indicated that *N*-acetylmethionine was completely racemized by carbonylimidazole, mesitylenesulfonyltriazolide, or DCC in 24 h. Similarly, *N*-formylmethionine was 97.5% racemized by mesitylenesulfonyltriazolide in 24 h.

*2(3′)-O-(N-Acylaminoacyl)dinucleoside Phosphates. Route A.* 2′(3′)-*O*-(Aminoacyl)dinucleoside phosphate (**5a–c**, 1 equiv) was dissolved in dry DMF (0.2 mL/mol), acetylating reagent was added (*N*-acetoxysuccinimide or 8-acetoxy-5-chloroquinoline, 10 equiv), and the reaction mixture was stirred at room temperature for 48 h. Following evaporation of the solution in vacuo, the residue was purified by cellulose TLC in system $S_5$ and by preparative electrophoresis in 1 M $CH_3COOH$. The products **4e–g** were isolated by elution of the appropriate bands by 80% acetic acid and subsequent lyophilization of the eluate. Yields were determined spectrophotometrically (Chládek et al., 1974) and were in the 40–60% range. The final compounds were kept at −70 °C as a powder obtained by freeze-drying of the solution in 80% acetic acid. The properties and characterization of the final products are reported in Table II.

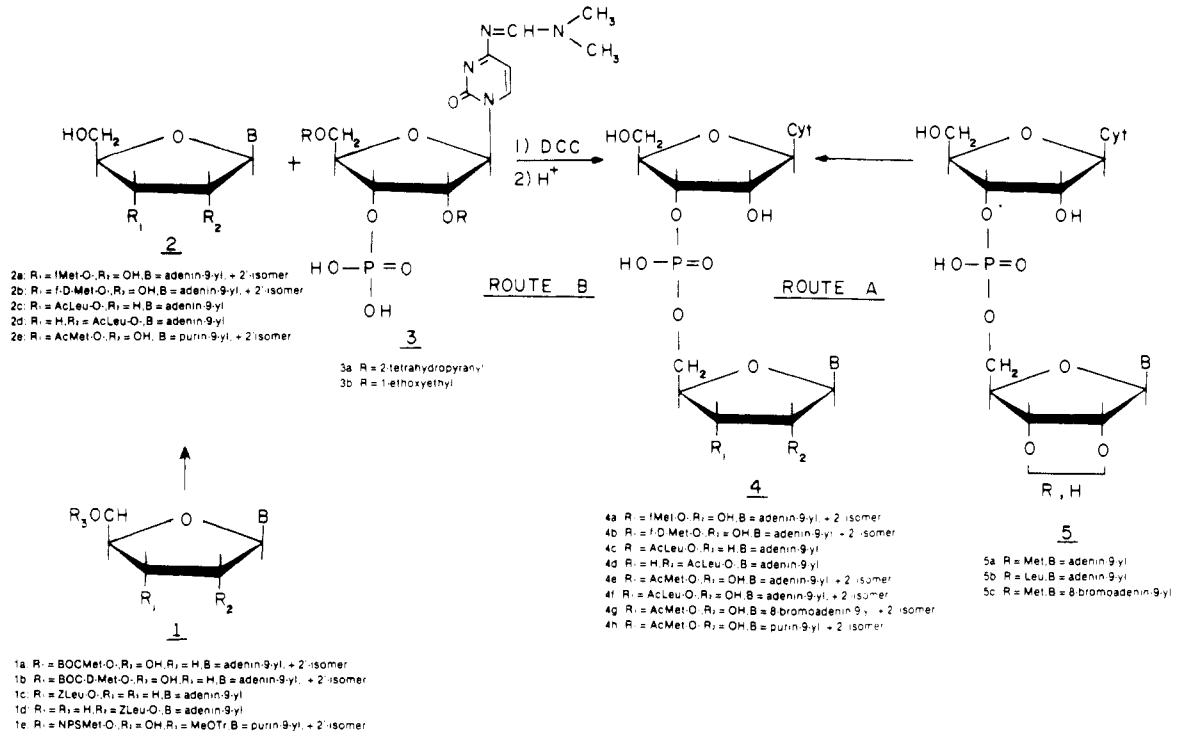
*Route B.* Compounds **4a–d** and **4h** were prepared via DCC-mediated condensation of 2′(3′)-*O*-(*N*-acylaminoacyl)-nucleosides **2a–e** with nucleotide components **3a** and **3b** as previously described (Ryu et al., 1977). The component **3b** was used for the condensation with nucleoside component **2c** containing deoxyadenosine. The products **4a–d** and **4h** were obtained after deblocking of the crude reaction products by successive treatment with chromatographic system $S_2$ and 0.01 N HCl in dioxane (Chládek et al., 1974) (when component **3a** was used) or by treatment with $S_2$ and 80% acetic acid (when component **3b** was employed). Isolation and characterization of the products was performed in the same manner as described in route A.

*2′(3′)-O-(N-Acylaminoacyl)nucleosides* **2a–e**. The protected 2′(3′)-*O*-(aminoacyl)nucleosides **1a–e** (containing either Z, Boc, or NPS groups) were used as starting materials for syntheses of the title compounds. Removal of Z or Boc groups was accomplished, as described previously, by either hydrogenolysis or by treatment with trifluoroacetic acid (Chládek et al., 1970). The NPS group in compound **1e** was removed (together with 5′-monomethoxytrityl group) by overnight treatment with 80% acetic acid. The solution was freeze-dried, and the residue was washed with petroleum ether. The purity of the 2′(3′)-*O*-(aminoacyl)nucleoside intermediates was checked by electrophoresis in 1 M acetic acid, and these intermediates were extensively dried in vacuo to remove the last traces of acid. The 2′(3′)-*O*-(aminoacyl)nucleosides were dissolved in dry DMF (3 mL/0.1 mmol), acylating reagent (10 equiv) was added, and the mixture was stirred at room temperature for 48 h or until the TLC in system $S_2$ showed almost quantitative conversion to a faster moving product. The reaction mixture was evaporated to dryness in vacuo, and the residue was purified by using short-column chromatography on silica gel (linear gradient of $CH_2Cl_2$–$CH_2Cl_2$ with 10% $CH_3OH$). The products **2a–e** were obtained by evaporation of the appropriate pooled fractions and characterized as shown in Table I. The yields were in the 50–60% range.

*Preparation of Biochemical Materials.* Ribosomes (3 times $NH_4Cl$ washed) were prepared from *E. coli* MRE-600 (RNase $1^-$) cells as described previously (Chládek et al., 1974). *E. coli* B tRNA (Gibco) was charged with [$^{14}$C]phenylalanine (~440 Ci/mol) as described previously (Chládek et al., 1974) to give 0.36 nmol of Phe/mg of tRNA.

*Assay of Peptidyltransferase Activity.* The incubation mixture for the donor assay of aminoacyldinucleotides contained 50 mM Tris-HCl, pH 7.4, 400 mM KCl, 20 mM $MgCl_2$, 3 $OD_{260}$ units of ribosomes, 10 nmol of [$^{14}$C]Phe-tRNA (~7000 cpm), the donor at the desired concentration, and 16 μL of MeOH in a final volume of 50 μL (Monro et al., 1968). The mixture, minus methanol, was preincubated at 37 °C for 10 min and equilibrated at 0 °C before initiation of the reaction by the addition of cold methanol. After 60 min at 0 °C, the reaction was terminated by the addition of 50 μL of 3 N NaOH. The mixture was incubated 30 min at 37 °C to hydrolyze the dipeptide product from the tRNA. This

Scheme I




incubation mixture was then acidified with 400 μL of 5 N HCl, and the dipeptide was extracted with 1.5 mL of ethyl acetate (Leder & Bursztyn, 1966). The radioactive product in 1 mL of the ethyl acetate extract was then counted by using 10 mL of RPI 3a701b scintillation fluid (Research Products International, Elk Grove Village, IL).

*Analysis of Peptidyltransferase Reaction Products.* fMet-[$^{14}$C]Phe was prepared from $8.4 \times 10^{-4}$ M C-A(fMet) and $1.2 \times 10^{-6}$ M [$^{14}$C]Phe-tRNA by using the normal peptidyltransferase assay procedure. The ethyl acetate extract containing the product was electrophoresed, as described by Bhuta et al. (1981). The product fMet-[$^{14}$C]Phe was identified by radioactive counting and comparison to standards (results not shown).

*Inhibition of Peptidyltransferase Reaction.* Inhibition of f-L-Met-[$^{14}$C]Phe-tRNA formation by various inhibitors was carried out by the normal assay procedure, as indicated in the legend to Figure 2.

## Results and Discussion

*Synthesis.* In this study, 2′(3′)-*O*-(*N*-acylaminoacyl)dinucleoside phosphate derivatives of methionine and leucine were used, since derivatives of these amino acids were expected to give the highest intrinsic donor activity (Monro et al., 1968). The routes used for the syntheses of 2′(3′)-*O*-(*N*-acylaminoacyl)dinucleoside phosphates, **4**, are shown in Scheme I. 2′(3′)-*O*-(Aminoacyl)dinucleoside phosphates, **5**, prepared by using previously described methods (Ryu et al., 1977; Bhuta et al., 1981), were selectively acetylated at the amino group of the aminoacyl moiety by using *N*-acetoxysuccinimide or 8-acetoxy-5-chloroquinoline (Chládek, 1972) as the acetylating reagent (route A). It is known that this reaction does not produce undesirable isomerization of the 3′→5′ phosphodiester linkage and that acetylation of the reactive cytosine residue is insignificant (Chládek & Žemlička, 1970). Since the starting 2′(3′)-*O*-(aminoacyl) derivative of dinucleotide **5** has to be prepared by deblocking of the corresponding Boc derivative, this route is relatively inconvenient. Therefore, another synthetic route was sought, in which 2′(3′)-*O*-(*N*-acylaminoacyl)nucleosides, **2**, were employed as nucleoside components for the condensation with appropirately protected derivatives of cytidine 3′-phosphate, **3** (route B).

The Boc and Z protecting groups, used for route A, were removed at the dinucleotide stage to produce C-BrA-Met (**5c**) analogously to C-A-Met (**5a**) (Bhuta et al., 1981) and C-A-Leu (**5b**) (Ryu et al., 1977). In route B, the Boc group was also removed from nucleosides **1a** and **1b** by treatment with trifluoroacetic acid, the Z group from compounds **1c** and **1d** by hydrogenolysis on a palladium catalyst, and the NPS group in compound **1e** by treatment with acetic acid (concomitantly with the 5′-monomethoxytrityl group). The resulting 2′-(3′)-*O*-(aminoacyl)nucleosides were then selectively acetylated or formylated on the amino group of the aminoacyl moiety by reaction with *p*-nitrophenyl acetate or *p*-nitrophenyl formate (Chládek & Žemlička, 1968). The resulting 2′(3′)-*O*-(*N*-acylaminoacyl)nucleosides **2a–e** (Table I) were then used as nucleoside components for DCC-mediated coupling with protected cytidine 3′-phosphate, **3** (Chládek & Žemlička, 1974; Chládek et al., 1974). The use of component **3b** is preferable for the coupling with nucleoside components containing 2′-deoxyadenosine (such as **2c**) since the cleavage of 1-ethoxyethyl groups in the latter stage of synthesis can be achieved by short treatment with dilute acetic acid, which does not cleave the acid-sensitive 2′-deoxyadenosine glycosidic bond (Ryu et al., 1977). After the appropriate deblocking of the dinucleotide intermediates the final products, **4**, were isolated by preparative TLC on cellulose and characterized by the usual procedures (Chládek et al., 1974). This isolation step was employed in both routes A and B. The negligible amount of material resistant to pancreatic ribonuclease, or snake venom diesterase in compounds **4**, proves that the final products contained almost exclusively 3′→5′ phosphodiester linkages (Table II).

Other synthetic routes have recently been reported involving direct aminoacylation of protected (Alexandrova & Smrt, 1977; Smrt & Jonák, 1979) or even unprotected (Tarussova et al., 1975; Krayevsky et al., 1976) dinucleoside phosphates with *N*-acylamino acids activated on the carboxyl group with

Table I: 2′(3′)-*O*-(*N*-Acylaminoacyl)nucleosides and Protected 2′(3′)-*O*-(Aminoacyl)nucleosides

| compound[a] | UV (95% EtOH) $\lambda_{max}$ ($\epsilon \times 10^{-3}$) | $^1$H NMR spectra ($\delta$)[b] H-8 (S-1H) | H-2 (S-1H) | H-1 (1 H, $J_{1',2'}$ in Hz) | amino acid side chain | *N*-acyl group |
|---|---|---|---|---|---|---|
| A(fMet) (**2a**) | 260 (13.84) | 8.41 | 8.2 | 5.95 (90%) (d, 7.5), 6.15 (10%) (d, 7.5) | 2.09 (s), 2.54 (m) | 7.31 (s) |
| A(f-D-Met) (2b) | 260 (13.07) | 8.39 | 8.17 | 5.9 (83.4%) (d, 7.32), 6.1 (16.6%) (d, 7.3) | 2.09 (s), 2.54 (m) | 7.39 (s) |
| 2′-dA(AcLeu) (**2c**) | 260 (15.2) | 8.36 | 8.16 | 6.37 (q) | 0.93 (2d), 2.5 (m) | 1.9 (s) |
| 3′-dA(AcLeu) (**2d**) | 260 (14.48) | 8.36 | 8.16 | 6.1 (d, 1.7) | 0.92 (2d), 2.58 (m) | 1.89 (s) |
| N(AcMet) (**2e**) | 262 (6.99) | 9.22[c] | 8.98 | 6.05 (75%) (d, 7.5), 6.25 (25%) (d, 7.5) | 2.09 (s), 2.5 (m) | 2.07 (s) |
| A(Boc-D-Met)[e] (**1b**) | 258 (12.57) | 8.39 | 8.16 | 5.91 (82%) (d, 7.4) 6.11 (18%) (d, 7.4) | 2.09 (s), 2.54 (m) | 1.36 (m) |
| MeOTrN(NPS-Met) (**1e**) | 264[f] (7.29), 232 (14.59) | 9.1[c,d] | 8.97 | 6.05 (d, 7.5) | 2.03 (s), 2.18 (m) | |

[a] Satisfactory elemental analyses were obtained for these compounds. [b] $Me_2SO$-$d_6$; DSS as internal standard. [c] H-6, 8.9 (s); OMe, 3.75 (s); phenyl, 7.3 (m). [d] $CDCl_3$; $Me_4Si$ as internal standard. [e] Prepared analogously to A(BocMet) (1a) according to Bhuta et al. (1981). [f] Shoulder.

Table II: 2′(3′)-*O*-(*N*-Acylaminoacyl)dinucleoside Phosphates

| compound | route | 2′,5′[a] (%) | Cp/N[a] | 3′,3′[b] or 3′,2′ (%) |
|---|---|---|---|---|
| C-A(fMet)[c] (**4a**) | B | 0 | 0.95 | 0 |
| C-A(f-D-Met)[c] (**4b**) | B | 0 | 0.97 | 0 |
| C-2′-dA(AcLeu)[c] (**4c**) | B | 3.9 | 1.02 | |
| C-3′-dA(AcLeu)[c] (**4d**) | B | 5.0 | 1.03 | |
| C-A(AcMet)[c] (**4e**) | A | 8.0 | 0.99 | 0 |
| C-A(AcLeu)[c] (**4f**) | A | 0 | 0.84 | 0 |
| C-BrA(AcMet)[d] (**4g**) | A | 4.9 | 0.8 | 0 |
| C-N(AcMet)[e] (**4h**) | B | 2.7 | 1.15 | |

[a] Determined by degradation with pancreatic ribonuclease. [b] Determined by degradation with snake venom phosphodiesterase. [c] UV spectra (0.01 in HCl) analogous to that of C-A. [d] UV spectra (0.01 in HCl): $\lambda_{max}$ = 265 nm; 250/260 = 0.66; 280/260 = 0.33. [e] UV spectra (0.01 in HCl): $\lambda_{max}$ = 275 nm; 250/260 = 0.62; 280/260 = 1.13; 290/260 = 0.86.

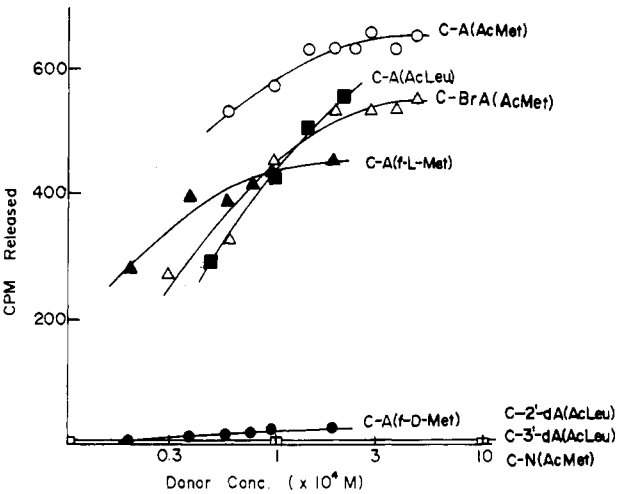



FIGURE 1: Donor activities of (*N*-acylaminoacyl)dinucleotide models **4a–h** in peptidyltransferase reaction with [$^{14}$C]Phe-tRNA as acceptor, assayed by release of [$^{14}$C]Phe from the tRNA, as described under Materials and Methods. The donors are as follows: C-A(AcLeu) (■); C-A(f-L-Met) (▲); C-A(f-D-Met) (●); C-2′-dA(AcLeu) or C-3′-dA(Leu) (□); C-A(AcMet) (○); C-BrA(AcMet) (BrA = bromoadenosine) (△); C-N(AcMet) (N = nebularine) (□).

various reagents. However, it is well-known from the literature that the carboxyl activation of *N*-acylamino acids or peptides usually leads to significant racemization of the aminoacyl residue through the formation of an azlactone intermediate [see, e.g., Bondansky et al. (1976)]. As our results show, the reaction of both AcMet and fMet with various reagents, which could be used for carboxyl activation, leads to significant racemization of the amino acid. These clear-cut and expected results are difficult to reconcile with a report of Alexandrova & Smrt (1977), who did not observe racemization of fMet under the same conditions while using, e.g., *p*-toluenesulfonyltriazolide as the activating reagent. In addition to this problem, Tarussova et al. (1975) and Krayevsky et al. (1976) have aminoacylated unprotected C-A with carboxyl-activated fMet to produce C-A(fMet).

It is, thus, quite clear that such reactions must inevitably lead to significant racemization and in the latter case also to a complex mixture of products and, thus, the purity of the final products is debatable. On the other hand, the synthetic approaches described in this report guarantee the purity of the products and are, therefore, preferable.

*Biochemical Results.* 2′(3′)-*O*-(*N*-Acylaminoacyl)dinucleoside phosphates, **4**, were investigated as potential donors of the *N*-acylaminoacyl residue in the nontemplate system (fragment reaction; Monro & Marcker, 1967) containing 70S ribosomes and Phe-tRNA as an acceptor. By analogy with successful studies of the peptidyltransferase A site (Bhuta et al., 1981), we attempted to investigate the role of the 2′ and 3′ isomers of AcAA-tRNA as donors in the peptidyltransferase reaction by using the nonisomerizable models C-2′-dA(AcLeu) (**4c**) and C-3′-dA(AcLeu) (**4d**).

As can be seen from Figure 1, neither of the two nonisomerizable models **4c** and **4d** could function as a donor in the peptidyltransferase reaction, although the unmodified parent derivative C-A(AcLeu) (**4f**) has shown reasonably high activity. The failure of the models **4c** and **4d** to react is most likely due to the absence of an activating hydroxyl group adjacent to the *N*-acylaminoacyl residue (Griffin & Reese, 1964). Analogous behavior has been observed by other authors with similarly modified derivatives of intact AcAA-tRNA

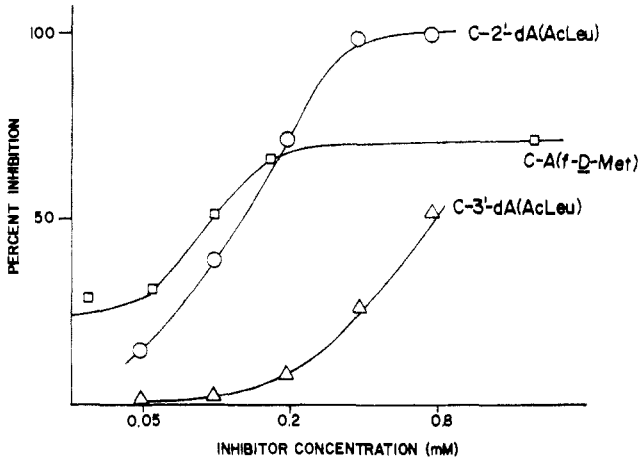



FIGURE 2: Inhibition of fMet-[$^{14}$C]Phe-tRNA formation from C-A-(f-L-Met) ($5.35 \times 10^{-5}$ M) and [$^{14}$C]Phe-tRNA, using the normal assay procedure as described under Materials and Methods, except for the addition of inhibitors **4b–d** at the indicated concentrations. The inhibitors are as follows: C-A(f-D-Met) (□); C-2'-dA(AcLeu) (○); C-3'-dA(AcLeu) (△).

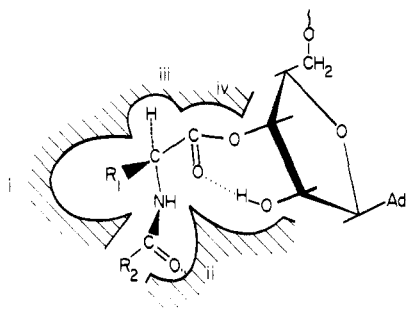



FIGURE 3: Schematic drawing of peptidyltransferase P site showing interactions with L-aminoacyl moiety of donor substrate (presumed to be the 3' isomer). The four indicated enzyme subsites are as follows: (i) the site which binds the side chain of the amino acid ($R_1$) and is presumed to bind especially strongly to side chains of the N-acylated methionine or leucine (Monro et al., 1968); (ii) the acylamide site (e.g., $R_2$ = H, $CH_3$) where the carbonyl may be bound by a hydrogen bond; (iii) the putative $\alpha$-hydrogen site [in analogy to the site on chymotrypsin (Schultz et al., 1977) or the A site of peptidyltransferase (Bhuta et al., 1981)]; (iv) the nucleophilic site into which the scissible bond of the substrate is placed (with the carboxyl presumably activated by a hydrogen bond to the 2'-OH) (Griffin & Reese, 1964).

(Chinali et al., 1974; Hecht et al., 1974) and with other *N*-acylaminoacyl nucleotide models (Krayevsky & Kukhanova, 1979) in the *E. coli* systems. On the other hand, de Groot et al. (1979) have observed the donor activity of both 2' and 3' nonisomerizable derivatives of AcTyr-tRNA (AcTyr-tRNA-C-C-2'-dA and AcTyr-tRNA-C-C-3'-dA) in the rat liver ribosomal system. The 3' isomer, AcTyr-tRNA-C-C-2'-dA, was the more effective donor. Therefore, the requirement for the neighboring hydroxyl group in the donor models may not be absolute and may depend upon the ribosomal system used. Thus, investigations of fragments similar to **4c** and **4d**, or analogous trinucleotide derivatives in other ribosomal systems, should be of considerable interest. Both nonisomerizable models **4c** and **4d** inhibit the transfer of an fMet residue from C-A(fMet) (**4a**) to Phe-tRNA (Figure 2), which shows that these two compounds can bind to the peptidyltransferase P site in spite of the absence of a hydroxyl group adjacent to the aminoacyl residue. A comparison of the inhibitory activities of C-2'-dA(AcLeu) (**4c**) and C-3'-dA(AcLeu) (**4d**) shows that the former is a stronger inhibitor in accordance with other indications from the *E. coli* system that 3'-AcAA-tRNA is probably the prefered donor substrate (Sprinzl et al., 1975). Also seen in Figure 1 is that C-A(f-L-Met) (**4a**) is clearly the prefered donor substrate, as compared to the nearly inactive C-A(f-D-Met) (**4b**). This finding also provides an indirect proof that our synthetic routes to the donor models **4** are practically racemization free, since even a minute amount of the highly active C-A(f-L-Met) (**4a**) would be detected in the assay.[2] On the other hand, C-A(f-D-Met) (**4b**) can also bind to the P site of peptidyltransferase,[3] since it inhibits the peptidyltransferase-catalyzed transfer of the fMet residue from C-A(fMet) (**4a**) to Phe-tRNA (Figure 2).

Therefore, it appears that stereochemical control of the peptidyltransferase reaction occurs at both sites of peptidyltransferase (Bhuta et al., 1981) and that control at the donor site is achieved in a way very similar to that of the active sites of proteolytic enzymes, e.g., chymotrypsin (Schultz et al., 1977). Moreover, the productive association of the *N*-acylaminoacyl portion of a donor substrate would include the interaction of the amino acid side chain, the acylamido bond, and the scissile carbonyl bond with appropriate regions (loci) of the enzyme.[4] The proposed association of the *N*-acylaminoacyl portion of a donor substrate with the peptidyltransferase P site is illustrated in schematic drawing (Figure 3). As with the A site of peptidyltransferase, the aminoacyl residue in the D configuration of the donor substrate, C-A-(f-D-Met) (**4b**), would interact nonproductively (Bhuta et al., 1981). This is the case with chymotrypsin as well (Bosshard, 1974). Therefore, the model of the donor site interactions is quite similar to that of the acceptor site, with a notable difference in the requirement for an acylated amino group of the donor vs. a free amino group of the acceptor.

The role of the 3'-terminal moiety of the donor substrate in binding to the peptidyltransferase P site was investigated by employing compounds **4g** and **4h**. These compounds have the "natural" adenosine of the 3' terminus replaced by 8-bromoadenosine (**4g**) or by nebularin (**4h**). As results in Figure 1 show, the 3'-terminal adenosine residue in C-A-(AcMet) (**4e**) can be replaced by 8-bromoadenosine (existing in syn conformation; Tavale & Sobell, 1970) with practically no loss of donor activity of compound **4g**, as compared to **4e**. Popovkina et al. (1978) also observed some donor activity of p-BrA(fMet) only at high substrate concentration (~1 mM), and this activity was considerably lower than that of unmodified pA(fMet). On the other hand, Žemlička et al. (1975) observed that BrA-Phe has only very low activity in the peptidyltransferase reaction as an acceptor. Replacement of the 3'-terminal adenosine in C-A(AcMet) (**4e**) by nebularin to form the compound C-N(AcMet) (**4h**) leads to complete loss

[2] For further discussion pertinent to the racemization problem, see also Bhuta et al. (1981). The apparent inactivity of C-A(f-D-Met) (**4b**) as a donor substrate is difficult to correlate with results of Kharshan et al. (1977), who investigated the donor activities of pA(fMet-Leu) containing L or D (or combinations of both) amino acids and found considerable donor activity with, e.g., pA(f-L-Leu-D-Leu). Since it is apparent (vide supra) that in the synthesis by these authors, racemization of the aminoacyl moieties must have taken place (via activation of the carboxyl group of the *N*-formyl dipeptide), their results are ambiguous.

[3] As with compounds **4c** and **4d**, also in this case, it is assumed that the inhibitor competes with donor substrate C-A(AcMet) (**4e**) and not with the A site bound Phe-tRNA. It has already been shown that the 2'(3')-*O*-(*N*-acylaminoacyl)nucleosides or -oligonucleotides have a very low affinity for the peptidyltransferase A site (Celma et al., 1979; Pestka et al., 1970; Rychlik et al., 1970).

[4] Although the influence of the nature of the amino acid side chain on the donor activity of the substrate was not investigated in this report, the existence of a binding site for the amino acid side chain within the peptidyltransferase P site can be inferred from the work of Monro et al. (1968).

of the donor activity. It is important to mention that N-Phe is an excellent acceptor in the peptidyltransferase reaction when compared to the natural A-Phe (Žemlička et al., 1975). Thus, the results obtained with C-BrA(AcMet) (**4g**) and C-N(AcMet) (**4h**) appear to indicate the differences in the interactions of the 3′-terminal base of acceptor and donor substrates with the respective sites of peptidyltransferase. However, it should be emphasized that in the virtual absence of suitable donor models with fixed conformation (for comparison, see models for the A site; Žemlička & Bhuta, 1979), the question of conformation preference at the P site cannot be answered unambiguously. On the other hand, it is of considerable interest that an 8-bromoadenosine residue in the syn conformation is presumably capable of forming an asymetrical complementary pair (by using two hydrogen bonds) with a base residue in the anti conformation (Topal & Fresco, 1976) whereas nebularin can, in principle, form only one hydrogen bond. Since 23S RNA was found in the neighborhood of the peptidyltransferase P site by using affinity labeling approaches [for review, see Cooperman (1978)], the models suggesting base pairing between the 3′ terminus of charged tRNA on the P site and the corresponding sequence of 23S RNA should be seriously considered.

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