L-Phenylalanine Esters of Open-Chain Analog of Adenosine as Substrates for Ribosomal Peptidyl Transferase†,‡,§

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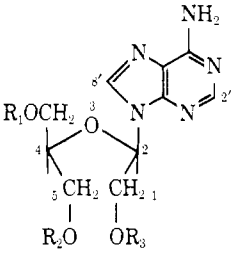
ABSTRACT: The chemical synthesis of the open-chain analogs of 2′- and 3′-*O*-(L-phenylalanyl) and 2′,3′-bis-*O*-(L-phenylalanyl)-adenosine (compounds Ih–j) is described. Compounds Ih and Ij were active in the release of *N*-Ac-Phe-tRNA catalyzed by ribosomes: at 0.1 mM, compound Ih released 8% and Ij, 12% and at 1 mM, 40 and 50%, respectively, of the amount of Ac-Phe released by A-Phe. The results indicate that peptidyl transferase requires the 3′-aminoacyl derivative and that an intact furanose ring is of importance for the peptide transfer reaction.

The structural requirements of ribosome-catalyzed peptide bond formation have been of interest since the mechanism of puromycin inhibition of protein biosynthesis was first elucidated (Nathans and Neidle, 1963). It has been shown that the terminal aminoacyladenosine of the aminoacyl-tRNA is the smallest unit capable of accepting the peptide chain from peptidyl tRNA, with phenylalanyladenosine (A-Phe)[1] exhibiting the greatest activity (Rychlík *et al.*, 1969). One series of compounds that has been under investigation in our laboratory is the open-chain analogs of A-Phe that contain a cleaved C(2′)–C(3′) bond in the ribose moiety (see formulas Ih–j). The study of these derivatives can contribute to the analysis of the roles of the ribofuranose ring and the position (2′ or 3′) of the aminoacyl group in peptide bond formation catalyzed by ribosomal peptidyl transferase. Ofengand and Chen (1972) suggested[2] that enzymatically aminoacylated tRNA containing an open terminal adenosine ribose is inactive in the peptidyl transferase catalyzed peptide bond formation because aminoacylation occurs specifically at the 2′-hydroxy group. Due to cleavage of the C(2′)–C(3′) bond, the subsequent isomerization (Wolfenden *et al.*, 1964) to the 3′ isomer, which is the substrate for peptidyl transferase, is unlikely. While our investigations were in progress, Hussain and Ofengand (1973) reported a study of a mixture of Ih and Ii (ratio of isomers not given) in peptidyl transferase systems, for which they proposed that only isomer Ih exhibits acceptor activity.[3] The present communication describes the chemical synthesis and acceptor activity of the pure isomers Ih and Ii and the bis-Phe derivative Ij.



Ia, R_1 = MeOTr; R_2 = R_3 = H
b, R_1 = MeOTr; R_2 = ZPhe; R_3 = H
c, R_1 = MeOTr; R_2 = H; R_3 = ZPhe
d, R_1 = MeOTr; R_2 = R_3 = ZPhe
e, R_1 = R_3 = H; R_2 = ZPhe
f, R_1 = R_2 = H; R_3 = ZPhe
g, R_1 = H; R_2 = R_3 = ZPhe
h, R_1 = R_3 = H; R_2 = Phe
i, R_1 = R_2 = H; R_3 = Phe
j, R_1 = H; R_2 = R_3 = Phe

Z = $C_6H_5CH_2OCO-$, MeOTr = *p*-methoxytrityl

Materials and Methods

General Methods. Thin-layer chromatography (tlc) was performed on silica gel coated aluminum foils (E. Merck, Darmstadt, Germany). Preparative tlc was carried out on 4-mm thick (37 × 12 cm) nonadhering (loose) layers of silica gel (70–325 mesh, ASTM, E. Merck, Darmstadt, Germany) containing 1% of fluorescent indicator (Leuchtpigment ZS Super, Riedel DeHaën, Hannover, Germany) or 2-mm thick layers (20 × 20 cm) of Stahl's silica gel (GF 254, E. Merck, Darmstadt, Germany) in solvents S_1 ($CHCl_3$–CH_3OH, 95:5) and S_2 ($CHCl_3$–CH_3OH, 9:1). Paper electrophoresis was performed using a Savant flat plate. Ultraviolet (uv) spectra were

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‡ This paper is No. XVII in the series, "Aminoacyl Derivatives of Nucleosides, Nucleotides, and Polynucleotides." For a preceding report of this series (Paper XVI), see Žemlička and Chládek (1971). The synthesis of the title compounds was reported at the 162nd National Meeting of the American Chemical Society, Washington, D. C., Sept 1971, Abstract BIOL-104.

§ The correct nomenclature of the parent compound is 2-(adenin-9-yl)-4′-methylol-3-oxapentane-1,5-diol (see formula I, R_1 = R_2 = R_3 = H).

[1] Abbreviations used are: *N*-Ac-Phe-tRNA, *N*-acetyl-L-phenylalanyl transfer ribonucleic acid; Ac-Phe, *N*-acetyl-L-phenylalanine; A-Phe, 2′(3′)-*O*-(L-phenylalanyl)adenosine; tlc, thin-layer chromatography; Z-Phe, *N*-benzyloxycarbonyl-L-phenylalanine; RNase, ribonuclease; A-(Phe)$_2$, 2′,3′-*O*-bis(L-phenylalanyl)adenosine; A-(Ala)$_2$, 2′,3′-*O*-bis(L-alanyl)adenosine; A-(Leu)$_2$, 2′,3′-*O*-bis(L-leucyl)adenosine; Z, benzyloxycarbonyl; MeOTr, *p*-methoxytrityl.

[2] The possibility of the preferential enzymatic formation of one isomer of aminoacyl-tRNA, its nonenzymatic isomerization, and the use of the other isomer in the subsequent steps of protein biosynthesis were first invoked by Griffin *et al.* (1966).

[3] The term "acceptor activity" refers to the capacity of a given derivative to accept a peptide residue from peptidyl-tRNA in a process catalyzed by ribosomal peptidyl transferase.

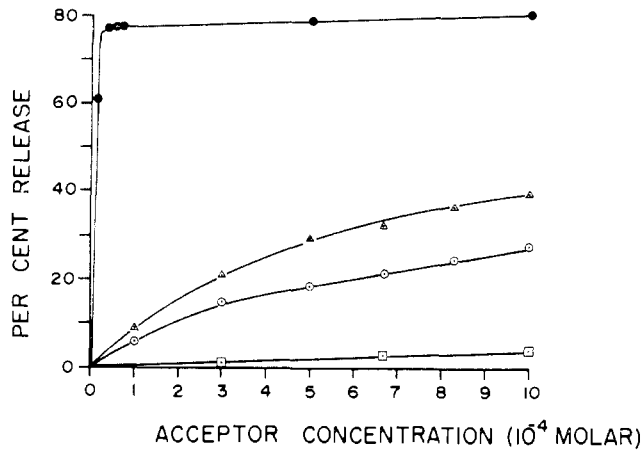

FIGURE 1: Extent of Ac-Phe release from *N*-Ac-Phe-tRNA in the peptidyl transferase reaction with A-Phe and A-Phe analogs. Each reaction mixture contained in 0.15 ml: 50 mM Tris (pH 7.4), 100 mM NH_4Cl, 10 mM $MgCl_2$, 3.9 A_{260} of high salt washed ribosomes, 10 μg of poly(U), and 0.15 A_{260} of *N*-Ac-[^{14}C]Phe-tRNA (1300 cpm). The reaction was initiated by the addition of the acceptor at the concentrations shown in the figure. Following an incubation of 30 min at 37° the reactions were stopped by the addition of 2.0 ml of 2.5% CCl_3COOH at 4°. The stopped reactions were kept at 4° for 15 min and then filtered through HAWP-Millipore membranes followed with three 2.0-ml 2.5% CCl_3COOH washings. After the membranes were dried, the radioactivity was counted in a PPO–POPOP–toluene scintillation mixture. Per cent of Ac-Phe release was obtained from the acceptor dependent decrease in CCl_3COOH precipitated counts trapped by the membranes. Electrophoresis of the released radioactivity in the CCl_3COOH wash confirmed that it was in the form of the peptidyl product A-(AcPhePhe) or its open-chain analog, data not shown: (●) A-Phe, (△) Ij, (○) Ih, and (□) Ii.

obtained by using a Cary recording spectrophotometer (Model 11) or a Beckman grating spectrophotometer, Model DB-GT, and optical rotations were measured on a Perkin-Elmer Model 141 polarimeter in CH_3CN or $CHCl_3$. Nuclear magnetic resonance (nmr) spectra were determined with a Varian A-60A spectrometer.

Ribosome Assay. Bacterial ribosomes were prepared from late-log-phase *Escherichia coli* MRE 600 (RNase 1$^-$) cells (General Biochemicals, Inc., Chargin Falls, Ohio) (Belitsina and Spirin, 1970), and were washed three times by ultracentrifugation in 0.5 M NH_4Cl (Ravel and Shorey, 1971). Such high salt-washed ribosomes showed no requirement for preactivation (Miskin *et al.*, 1968) to give maximum activity in assay systems.

N-Ac-[^{14}C]Phe-tRNA (220,000 cpm/mg of tRNA, *i.e.*, 0.4 mμmol of [^{14}C]phenylalanine/mg of tRNA) was prepared as described previously (Rychlík *et al.*, 1969). Detailed assay conditions are specified in the text (Figure 1). The quantity of *N*-acetylaminoacyl residue transferred from donor to acceptor substrate by the peptidyl transferase catalyzed reaction was determined as the difference between radioactivity released from the donor during incubation with and without acceptor and expressed as percentage radioactivity of the donor used in the assay. This indirect method of product analysis has been demonstrated in this laboratory to give results complementary to those obtained using the ethyl acetate method (Leder and Bursztyn, 1966) for the direct measurement of peptidyl transferase products.

Synthesis. 2-(Adenin-9-yl)-4-O-(p-methoxytrityl[4]*)-4'-methylol-pentane-1,5-diol (Ia).* The described method (Lerner, 1970) was modified as follows: to the solution of 5'-*O*-(*p*-methoxytrityl)adenosine (Aldrich Chemical Co., Milwaukee, Wis., 3.0 g, 5.55 mmol) in a mixture of dioxane (70 ml), ethanol (25 ml), and water (30 ml), under ice cooling and stirring, a solution of $NaIO_4$ (1.2 g, 5.6 mmol) in water (25 ml) was added. Oxidation was shown to be quantitative by tlc in solvent S_1 in 2 hr. The solution was then added, in the dark, to the $NaBH_4$ (1.2 g, 32 mmol) in dioxane–water (1:1, 50 ml) at room temperature. After 2 hr of stirring, the reaction mixture was evaporated almost to dryness and extracted with $CHCl_3$ (500 ml). The $CHCl_3$ extract was washed once with 5% $NaHSO_3$ (100 ml) and twice with water, then dried with $MgSO_4$, and evaporated *in vacuo* and the residue triturated with dry ether to give amorphous Ia, tlc homogeneous (solvent S_2). For the yield, analysis, and characterization, see Table I.

*N-Benzyloxycarbonyl-*L*-phenylalanyl Derivatives Ie, Ij, and Ig.* The solution of compound Ia (1.1 g, 2 mmol) and *N*-benzyloxycarbonyl-L-phenylalanine (Z-Phe) in pyridine (12 ml) was cooled to 0°. Dicyclohexylcarbodiimide (0.52 g, 2.5 mmol) in pyridine (6 ml) was added and the reaction mixture kept for 1 hr at 5° and 21 hr at room temperature (Chládek *et al.*, 1970). Ice was then added, the mixture was extracted with petroleum ether, and the solids were filtered off. The filtrate was evaporated and the residue chromatographed on eight loose layers of silica gel in solvent S_1. Three uv-absorbing zones were eluted with the solvent: pure starting material Ia, band A containing compounds Ib and Ic,[5] and band B containing compound Id. Band A was rechromatographed on four plates of Stahl's silica gel and pure compounds Id and Ic were eluted with the solvent S_2. Products Ib–d were dissolved in 80% acetic acid (each in 15 ml); the solution was kept for 17 hr at room temperature and then lyophilized. The residues were dissolved in $CHCl_3$ and precipitated with petroleum ether and the resultant crude products Ie–g chromatographed (each on two plates) on loose layers of silica gel in solvent S_2. Elution with the solvent and drying at 10^{-2} mm afforded amorphous compounds Ie–g homogeneous on tlc (S_1, S_2). For yields, analyses, and characterization, see Table I.

L-Phenylalanyl Derivatives Ih, Ii, and Ij. PdO–$BaSO_4$ (5%, 50 mg) was added to compounds Ie, If, or Ig (0.1 mmol) dissolved in cold 80% acetic acid (3 ml) and the suspension was stirred at 0° for 1.5 hr in the presence of bubbling hydrogen (Chládek *et al.*, 1970). The catalyst was filtered off through a Celite pad and the filtrate diluted to 10 ml with 80% acetic acid. Samples were taken for uv spectra and paper electrophoresis. The stock solutions were stored at −20° for several weeks without any decomposition of compounds Ih–j. For biochemical studies, aliquots containing 1 μmol were lyophilized, stored at −20°, and dissolved in water to suitable concentrations just before use in assays.

Results and Discussion

Synthesis. The synthesis of compounds Ih and Ii followed the general procedure for the preparation of 2'(3')-*O*-L-phenylalanyl nucleosides (Chládek *et al.*, 1970). The mixture of products Ib–d and unreacted Ia was separated by preparative

[4] A correct name for *p*-methoxytrityl is diphenyl(*p*-methoxy)phenylmethyl.

[5] The nmr spectrum of this mixture showed the following composition as judged from the heights of the H_2 (middle peak) signals: 70% of Ic and 30% of Ib.

TABLE I: Characterization of the Reaction Products and Intermediates.

Compd	Yield (%)	Calculated / Found % C	% H	% N	Uv Spectra λ_{max} (nm)	λ_{min} (nm)	Optical Rotations $[\alpha]_D^{24-25}$ (deg)	Nmr Spectra[a] $H_{3'}$[b]	$H_{2'}$[b]	H_2[c]	Electrophoretic Mobility[d]	R_F[e]
Ia	77	65.43 65.26	5.86[f] 6.00		234[g] 261	229 247	+9.8[h]	8.29	8.24	6.10		
Ie	5	57.95 57.81	5.58 5.69	15.02[f] 14.69	259[g]	228	+29[i]	8.34	8.23	6.12		0.95
If	12	57.03 57.23	5.67 5.48	14.78[j] 14.66	259[g]	227	+3.6[i]	8.38	8.25	6.33		0.95
Ig	6	63.53 63.33	5.45 5.52	11.79 11.71	259[g]	229	+2.6[i]	8.38	8.28	6.34		0.95
Ih	77[k]				260[l]	230					4.7	0.74
Ii	85[k]				260[l]	228					4.7	0.74
Ij	83[k]				258[l]	233					5.3	0.78

[a] In CD_3COCD_3 containing D_2O using Me_4Si as an internal standard. Values of chemical shifts are expressed in ppm (δ units). [b] A singlet corresponding to 1 proton. [c] A symmetrical triplet ($J_{2,1}$ = 5 Hz) corresponding to 1 proton. [d] Whatman No. 1 paper in 1 M acetic acid at 30 V/cm for 1.5 hr. Mobility of Phe = 1.00. [e] 1-Butanol–acetic acid–water (5:2:3), Whatman No. 1 paper, descending development. Spots of Ih–j gave violet coloration after spraying with 0.1% ninhydrin in ethanol. In 2-propanol–ammonium hydroxide–water (7:1:2) products Ih–j partially decompose to parent compound I (R_1 = R_2 = R_5 = H) and Phe. [f] Calculated for compound containing $0.5H_2O$. [g] In 95% C_2H_5OH. [h] $CHCl_3$ (0.5%). [i] In CH_3CN (0.5%). [j] Calculated for compound containing 1 H_2O. [k] Determined spectrophotometrically at λ_{max}. [l] 0.01 N HCl.

tlc on silica gel. Compounds Ib–d were detritylated with 80% acetic acid to give the *N*-benzyloxycarbonyl derivatives Ie–g. The structural assignment of the 1- and 2-Z-Phe isomers was performed by nmr spectroscopy. In analogy to the corresponding ribonucleoside derivatives (Griffin *et al.*, 1966; Sonnenbichler *et al.*, 1964), the structure of the 1 isomers Ic and If was assigned to the products having the more deshielded H_2 proton. According to tlc, the 1 isomer If was more polar (slower moving) than the 5 isomer Ie. The final products Ih–j were obtained by hydrogenolysis of the corresponding *N*-benzyloxycarbonyl-L-phenylalanyl derivatives Ie–g (Chládek *et al.*, 1970). (For characterization of products Ih–j and intermediates, see Table I.) It is of interest to note that the reaction of Ia with Z-Phe and dicyclohexylcarbodiimide produced the 1 and 5 isomers in the ratio of 70:30 as determined by nmr spectra. On the other hand, in the corresponding ribonucleoside derivatives, the 3′ isomers usually prevail. Apparently, in both cases the 2′-OH in ribonucleosides and the 1-OH in Ia are attacked first, but owing to a rapid 2′ ⇆ 3′ acyl migration (Wolfenden *et al.*, 1964) the composition of the isolated products in the case of ribonucleosides reflects thermodynamic (with the 3′ isomer prevailing) rather than kinetic control. In the case of derivatives Ib and Ic, such an isomerization is more difficult, if not impossible, and the isomeric composition of the products is controlled kinetically.

Biochemical Results. As can be seen from Figure 1, the 5 isomer Ih and bis-Phe derivative Ij were active in release of *N*-acetyl-L-phenylalanine from *N*-Ac-Phe-tRNA catalyzed by ribosomes. The 1-*O* isomer Ii which corresponds to 2′-*O*-Phe-adenosine was virtually inactive in the concentration range tested. The activity of Ih and Ij at 0.1 mM was about 8 and 12% of that of A-Phe. At 1 mM the activity of both compounds corresponded to 40% (Ih) and 50% (Ij) of A-Phe. The results for Ih are in agreement with those of Hussain and Ofengand (1973) who reported *ca.* 16% activity of A-Phe for the mixture of Ih and Ii (per cent composition of the mixture was not given) at 0.1 mM and *ca.* 50% at 1 mM. The fact that the bis-Phe derivative Ij was slightly more active than compound Ih is in accord with our previous observations (Černá *et al.*, 1970) on the activity of A-$(Phe)_2$, A-$(Ala)_2$, and A-$(Leu)_2$ in the release of Ac-Leu from Ac-Leu pentanucleotide under the conditions of fragment reaction. Our results show the necessity of the 3′-aminoacyl group as an absolute requirement for the peptide chain release activity and thus for ribosomal peptidyl transferase. The moderate activity of Ih and Ij indicates that an intact ribofuranose ring is not an absolute requirement for release. However, the substantial decrease of activity observed for Ih and Ij in comparison with A-Phe (particularly at lower concentrations) shows the important role played by a relatively rigid ribofuranose ring in the effectiveness of the peptide chain transfer. Thus differences in the activities of Ih (and Ij) and A-Phe may be the result of A-Phe's more favorable entropic factors. It is of interest to note that another open-chain analog of puromycin, 6-dimethylamino-9-[2-hydroxy-3-(*p*-methoxyphenyl-L-alanylamino)propyl]purine, also exhibited a moderate inhibitory activity in the poly(UC)-directed polyphenylalanine synthesis (21% inhibition at 1 mM) (Vince and Isakson, 1973). The latter compound lacks a considerable portion of the ribofuranose system (unlike Ih), but it contains an intact covalent bond between "C-2‴" and "C-3′." Unfortunately, owing to considerable differences in biological assay systems, it is impossible to correlate the above results, even in a qualitative fashion, with those described in this paper or those reported by Hussain and Ofengand (1973).

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Polynucleotides Containing 2′-Amino-2′-deoxyribose and 2′-Azido-2′-deoxyribose†

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ABSTRACT: 2′-Azido-2′-deoxycytidine 5′-diphosphate, 2′-amino-2′-deoxycytidine 5′-diphosphate, and 2′-amino-2′-deoxyuridine 5′-diphosphate are substrates for polynucleotide phosphorylase from *Micrococcus luteus*. Poly(2′-azido-2′-deoxycytidylic acid) [poly(Cz)], poly(2′-amino-2′-deoxycytidylic acid) [poly(Ca)], and poly(2′-amino-2′-deoxyuridylic acid) [poly(Ua)] are obtained with *s* values of 8.6, 4.8, and 3.1, respectively. All these polymers are stable to alkali and pancreatic ribonuclease. They are degraded by snake venom phosphodiesterase and micrococcal nuclease at rates considerably slower than for poly(rC) and poly(rU).

The need to understand the way in which a substituent at the 2′ position of the ribose ring influences the structure and function of the corresponding nucleosides, nucleotides, and polynucleotides has led to the synthesis and study of a number of compounds of this type, some of which have not been found in nature. Thus, polynucleotides have been reported which contain 2′-*O*-methyl (Zmudzka and Shugar, 1970; Zmudzka *et al.*, 1969) and 2′-*O*-ethyl substituents (Kusmierek *et al.*, 1973; Khurshid *et al.*, 1972), 2′-fluoro (Janik *et al.*, 1972), and 2′-chloro (Hobbs *et al.*, 1972b) substituents, and also the 2′-azido (Hobbs *et al.*, 1972a; Torrence *et al.*, 1972) substituent. A further spur to the investigation of such compounds has been the wish to determine the structure–function relationships of potential interferon inducers analogous to the homopolynucleotide duplex, poly(rI·rC). The data available at present indicate that the presence of a 2′-hydroxyl group in the polynucleotide is an absolute requirement for interferon inducers (Black *et al.*, 1972; DeClercq *et al.*, 1972). However, the compounds which have been tested have lacked the ability of the 2′-hydroxyl group to act as an electron donor, while simultaneously possessing a hydrogen atom suitable for the formation of hydrogen bonds. Both these properties are likely to be important in determining the structure of the polynucleotides and their complexes. The amino group would thus be a substituent of interest, since it possesses these properties. We wish to report the syntheses of poly(2′-azido-2′-deoxycytidylic acid) [poly(Cz)][1] and poly(2′-amino-2′-deoxycytidylic acid) [poly(Ca)], and to describe some of their properties, along with some of those of the previously reported poly(2′-azido-2′-deoxyuridylic acid) [poly(Uz)] (Hobbs *et al.*, 1972a; Torrence *et al.*, 1972) and poly(2′-amino-2′-deoxyuridylic acid) [poly(Ua)].

Experimental Section

Materials and Methods. Synthesis of Nucleosides. 2′-Azido-2′-deoxyuridine was synthesised by the method of Verheyden *et al.* (1971). It was converted to 2′-azido-2′-deoxy-3′,5′-diacetyluridine as described by the same authors, and 2′-azido-2′-deoxycytidine was prepared from this latter compound as described below. 2′-Amino-2′-deoxyuridine and 2′-amino-2′-deoxycytidine were prepared from the corre-

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[1] Abbreviations used are: poly(Cz), poly(2′-azido-2′-deoxycytidylic acid); poly(Ua), poly(2′-amino-2′-deoxyuridylic acid); poly(Ca), poly(2′-amino-2′-deoxycytidylic acid); poly(Ucl), poly(2′-chloro-2′-deoxyuridylic acid); poly(Ccl), poly(2′-chloro-2′-deoxycytidylic acid); poly(Uz), poly(2′-azido-2′-deoxyuridylic acid).