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Substrate Specificity of Ribosomal Peptidyltransferase. Effect of Modification in the Heterocyclic, Carbohydrate and Amino Acid Moiety of 2′(3′)-*O*-L-Phenylalanyladenosine†

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ABSTRACT: The chemical synthesis of 2′(3′)-*O*-L-phenylalanyl derivatives of nebularine (Id), 6-methoxynebularine (Ih), N^6,N^6-dimethyladenosine (Ik), 6-methylthionebularine (Io), 8-bromoadenosine (Ir), tubercidin (Iu), and 3′-*O*-L-phenylalanyl derivatives of 1-(β-D-arabinofuranosyl)cytosine (IIIg) and 9-(β-D-arabinofuranosyl)adenine (IIIl) is described. 2′(3′)-*O*-(3-Phenyl)propionyladenosine (Iv) was obtained by reaction of adenosine with ethyl 3-phenylorthopropionate and subsequent hydrolysis of the orthoester intermediate IV with formic acid. Compounds Id, Ih, Ik, Io, and Iu were active in the release of Ac-Phe from *N*-Ac-Phe-tRNA·poly(U)·70S ribosome complex: at 0.01 m*M* the release of Ac-Phe was 50–100% of that of A-Phe. At 1 m*M*, compounds Ir and IIIg released 30 and 25% of Ac-Phe relative to A-Phe whereas derivatives Iv and IIIl were virtually inactive. The results indicate the following conclusions regarding ribosomal peptidyltransferase activity of 2′(3′)-*O*-aminoacyl nucleosides: (a) the presence of the 2′-hydroxy group in the ribo configuration is more important for a highly active substrate (A-Phe) than for one of moderate activity (C-Phe); (b) the heterocyclic (purine) residue is in the anti conformation although this requirement is not absolute; (c) the presence of the amino group of the aminoacyl moiety is required; (d) acceptor activity is dependent upon the substituent in the position 6 of the purine moiety.

2′(3′)-*O*-Aminoacyladenosine is the smallest unit of aminoacyl-tRNA[1] capable of accepting the peptide chain from peptidyl-tRNA (Rychlík et al., 1969, and references cited therein) in a reaction catalyzed by ribosomal peptidyltransferase. Thus, it is possible to study the structural requirements of ribosomal peptidyltransferase using modified 2′(3′)-*O*-aminoacyl nucleosides. Previous work has shown that changes in the heterocyclic, ribose, and amino acid moieties of such substrates can greatly influence the acceptor activity of a given 2′(3′)-*O*-aminoacyl nucleoside in the peptide transfer reaction. Studies with A-Phe, I-Phe, C-Phe, G-Phe, and U-Phe have indicated that acceptor activity sharply decreases from adenosine to cytidine (G-Phe and U-Phe were inactive; Rychlík et al., 1969). Less information is available on the role of the ribose (carbohydrate) portion. The consequence of the methylation of the 2′-hydroxy group of A-Phe was only decreased acceptor activity (Pozdnyakov et al., 1972), whereas dA-Phe has almost no acceptor activity (Rychlík et al., 1969). On the other hand, the cleavage of the C(2′)–C(3′) bond in A-Phe resulted in substantially decreased but not yet abolished acceptor activity (Chládek et al., 1973). It has also been shown that the nature of the amino acid residue affects the activity of a given 2′(3′)-*O*-aminoacyl nucleoside, with A-Phe being the most active compound whereas the corresponding glycyl analogue, A-Gly, does not exhibit any activity (Rychlík et al., 1970).

Studies of 2′(3′)-*O*-aminoacyl nucleosides have been restricted to compounds derived either from the C-C-A termi-

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[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-phenylalanyladenosine and similarly for other 2′(3′)-*O*-aminoacyl nucleosides; dA-Phe, 2′-deoxy-3′-*O*-L-phenylalanyladenosine; Z-Phe, *N*-benzyloxycarbonyl-L-phenylalanine; Boc-Phe, *N*-*tert*-butoxycarbonyl-L-phenylalanine; araA, 9-(β-D-arabinofuranosyl)adenine; araC, 1-(β-D-arabinofuranosyl)cytosine; DCC, dicyclohexylcarbodiimide; CPK models, Corey-Pauling-Koltun models; MeOTr, 4-methoxytrityl(*p*-anisyldiphenylmethyl); C-C-A, cytidylyl-(3′ → 5′)-cytidylyl-(3′ → 5′)-adenosine; TCEC, 2,2,2-trichloroethoxycarbonyl; Ms, methylsulfonyl.

nus of tRNA or from naturally occurring nucleosides. It was anticipated that further investigations of other modified (not naturally occurring) substrates may provide valuable information on the substrate specificity of ribosomal peptidyltransferase.

As proposed earlier (Rychlík et al., 1969), the electron density in the purine (pyrimidine) moiety may influence the acceptor activity of the substrate. We therefore examined a series of 2′(3′)-*O*-L-phenylalanyl derivatives of 6-substituted purine ribonucleosides Id, Ih, Ik, and Io bearing substituents which cover a relatively wide range of resonance and/or inductive effects (H, MeO, Me_2N, and MeS). To shed more light on the importance of the heterocyclic (ring) nitrogen atoms, we also prepared 2′(3′)-*O*-L-phenylalanyltubercidin (Iu), an analogue of A-Phe having the N-7 replaced by a CH group. Finally, 8-bromo-2′(3′)-*O*-L-phenylalanyladenosine (Ir) was considered to be a good model of a compound with a syn conformation of the base residue (Tavale and Sobell, 1970). Because the base conformation of tRNA nucleoside units is probably anti (Cramer and Saenger, 1971), it seemed particularly intriguing to look into the problem of anti-syn conformation of the base in relation to the acceptor activity.

We were also interested in the problem of the configuration of the 2′-hydroxy group and its possible influence on the acceptor activity of the 2′(3′)-*O*-aminoacyl nucleoside.[2] Therefore, arabino analogues of substrates of high (A-Phe) and moderate (C-Phe) activity were chosen as model compounds IIIg and IIIl.

Finally, 2′(3′)-*O*-(3-phenyl)propionyladenosine (Iv) was prepared to study the influence of removal of the amino group in the aminoacyl residue when other structural factors important for the acceptor activity of A-Phe (aromatic moiety, adenine portion, and 2′-hydroxy group) are present.

The chemical synthesis and biochemical investigation of the above model compounds in the *Escherichia coli* ribosomal system are the subject of this communication.

Materials and Methods

General Methods. (See Chládek et al., 1973.) Unless stated otherwise, all evaporations were carried out on a Büchi rotary evaporator at temperatures below 35°. Thin-layer chromatography (TLC) was carried out as described earlier (Chládek et al., 1973) in solvents S_1 ($CHCl_3$ or CH_2Cl_2–MeOH, 95:5), S_2 ($CHCl_3$ or CH_2Cl_2–MeOH, 9:1), and S_3 ($CHCl_3$ or CH_2Cl_2–MeOH, 4:1). Paper chromatography was performed on Whatman No. 1 paper in S_4 (1-butanol–acetic acid–water, 5:2:3) and S_5 (2-propanol–concentrated NH_4OH–water, 7:1:2). Paper electrophoresis was performed as described previously (Chládek et al., 1973) on Whatman No. 1 paper in 1 *M* acetic acid at 30 V/cm for 1.5 hr. The spots were visualized by uv light (Mineralight) and ninhydrin-positive substances were detected by spraying with 0.1% ninhydrin in ethanol. Nuclear magnetic resonance (NMR) spectra were determined with a Varian A-60A spectrometer using Me_4Si as an internal standard with CD_3COCD_3 and CD_3COOD and 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard with CD_3SOCD_3. Melting points were determined on a Thomas-Hoover apparatus and are uncorrected.

Starting Materials. 9-(β-D-Ribofuranosyl)purine (nebularine), 6-methoxy-9-(β-D-ribofuranosyl)purine, 6-methylthio-9-(β-D-ribofuranosyl)purine, 8-bromoadenosine, and Z-Phe were products of Sigma Chemical Co., St. Louis, Mo. Boc-Phe was purchased from Aldrich Chemical Co., Milwaukee, Wis. Tubercidin was a generous gift of Dr. L. B. Townsend, Department of Chemistry, University of Utah, Salt Lake City, Utah. AraA, araC, and the corresponding N^4-(2,2,2-trichloroethoxycarbonyl)-5′-*O*-trityl derivative IIIa (Gish et al., 1971) were kindly furnished by Dr. W. J. Wechter, The Upjohn Co., Kalamazoo, Mich. 2′(3′)-*O*-L-Phenylalanyladenosine (A-Phe) was prepared as previously described (Chládek et al., 1970).

N^6,N^6-Dimethyladenosine. The described procedure (Žemlička and Šorm, 1965) was modified as follows. The mixture of 6-chloro-9-(β-D-ribofuranosyl)purine (2 g, 7 mmol), dimethylamine hydrochloride (1.14 g, 14 mmol), triethylamine (5 ml, 35 mmol), and dimethylformamide (15 ml) was stirred for 21 hr at room temperature. The precipitated triethylamine hydrochloride was filtered off and washed with dimethylformamide and the filtrate was evaporated at 0.04 mm and room temperature. The solid residue was washed with acetone and then crystallized from methanol (35 ml). White crystalline N^6,N^6-dimethyladenosine was filtered off and dried at 0.04 mm and room temperature (1.04 g, 50%): mp 182–185°; uv max (95% ethanol) 276 nm (ε 18750) and min 235 (ε 1900) (literature (Žemlička and Šorm, 1965) gives mp 183–184°, uv max (ethanol) 275 nm, min 235 nm); NMR (CD_3SOCD_3, external Me_4Si) δ 8.67 (s, 1, H_8), 8.53 (s, 1, H_2), 6.30 (d, $J_{1',2'}$ = 6 Hz, 1, $H_{1'}$), 3.78 (s, 6, NMe_2). Mother liquors on cooling to 0° afforded another portion of the same product (0.2 g, 9%).

9-(β-D-Arabinofuranosyl)-N-dimethylaminomethyleneadenine was prepared according to the procedure described for the synthesis of *N*-dimethylaminomethylene 2′-deoxynucleosides (Žemlička and Holý, 1967): yield 100%; mp 225–226°; uv max (95% ethanol) 310 and 230 nm (ε 30000, 12400); NMR (CD_3SOCD_3) δ 8.95 (s, 1, CH of Me_2NCH=), 8.44 (s, 1, H_8), 8.33 (s, 1, H_2), 6.37 (d, $J_{1',2'}$ = 4 Hz, 1, $H_{1'}$), 3.02 (d, 6, Me_2N). Literature (Hanessian, 1973) gives mp 214–215° and uv max (ethanol) 310 and 232 nm.

8-Bromo-N-dimethylaminomethyleneadenosine was prepared as described for the synthesis of *N*-dimethylaminomethylene ribonucleosides (Žemlička and Holý, 1967): yield 95%; mp 144–149°; uv max (95% ethanol) 318, 237 nm (ε 27600, 11400), min 256 (ε 2600). Anal. Calcd for $C_{13}H_{17}N_6O_4Br \cdot H_2O$: C, 37.24; H, 4.56; N, 20.04. Found: C, 36.97; H, 4.42; N, 19.86.

N-Dimethylaminomethylenetubercidin was prepared as above. Yield 79%; mp 164–168°; uv max (95% ethanol) 315, 262, 224 nm (ε 14800, 7200, 11200), min 278, 248 (ε 4300, 5200); NMR (CD_3SOCD_3 + D_2O) δ 8.75 (s, 1, CH of Me_2NCH=), 8.40 (s, 1, H_2);[3] 7.50 (d, 1, H_8), 6.85 (d, 1, H_7), 6.15 (d, 1, $H_{1'}$), 3.18 (s, 6, Me_2N). Anal. Calcd for $C_{14}H_{19}N_5O_4 \cdot \frac{1}{2}H_2O$: C, 50.80; H, 6.10; N, 21.20. Found: C, 51.27; H, 5.90; N, 21.32.

Ethyl 3-Phenylorthopropionate. This compound was prepared according to the literature (McElvain and McShane, 1952) from ethyl hydrocinnamimidate hydrochloride (9.06 g, 0.043 mol). The crude product (5.36 g) was, however, not fractionated by distillation. According to

[2] It has been shown that 3′-aminoacyl nucleosides are acceptors in peptidyltransferase-catalyzed reactions whereas 2′ derivatives are inactive (Chládek et al., 1973).

[3] For the sake of proton numbering, tubercidin is considered to be 7-deazaadenosine.

Table I: 5′-*O*-(4-Methoxy)trityl Nucleosides.

Compd	Yield[a] (%)	Calcd/Found % C	% H	% N	Max (nm)[b] (ε × 10⁻³)	Min (nm) (ε × 10⁻³)	NMR Constants (CD_3COCD_3) Chemical Shifts, δ (No. of Protons, Multiplicity[c]) H_8	H_2	Aromatic Protons[d]	$H_{1'}$	$J_{1',2'}$ (Hz)	MeO
Ia	75	68.69 68.34	5.38 5.71		264,232	252	9.05	8.84[e]	7.32, 6.74	6.22	4.5	3.77
Ie	80	67.13 66.95	5.45 5.51	10.10 9.99	256,[f]236 (9.4,14.9)	224 (12.0)	8.42[g]	8.12	7.22, 6.70	6.01	5.5	3.71[h]
Ii	100	67.71 67.69	5.86 6.31		275,232 (16.3,13.4)	249 (7.1)	8.18	8.13	7.27, 6.84	6.11	4.0	3.77[i]
Il	97	64.23[j] 64.51	5.39 5.89		291,284,228 (15.4,16.7,19.0)	255 (4.4)	8.48	8.28	7.25, 6.78	6.12	4.5	3.76[k]
Ip	52			11.32 11.33	266,230	247		8.50	7.25, 6.80	6.05	4.5	3.72
Is	76	68.78 68.38	5.94 6.18		313,260 (16.3,8.3)	276,252 (6.5,7.4)	*l*	8.28	7.25, 6.80	6.27	4.5	3.73
IIIh	64			12.17[m] 12.15	259,234 (13.6,14.5)	245,226 (10.9,13.5)	8.25	8.20	7.34, 6.80	6.47	4.0	3.80

[a] TLC (S_1 or S_2) homogeneous. [b] 95% ethanol. [c] In all cases, the H_8 and H_2 signals were one-proton singlets, those of $H_{1'}$ were one-proton doublets, and those of MeO were three-proton singlets. [d] The signal of C_6H_5 (m) is overlapped with the low-field half of the A_2B_2 system (d) belonging to *p*-$MeOC_6H_4$, total number of aromatic protons was 14 in each case. [e] 8.54 (H_6, s, 1). [f] Shoulder. [g] $CDCl_3$. [h] 4.13 (s, 1, MeO of 6-methoxypurine). [i] 3.48 (s, 6, Me_2N). [j] Hemihydrate. [k] 2.68 (s, 3, MeS). [l] Overlapped with the C_6H_5 signal. Other signals: 8.81 (s, 1, CH of $Me_2NCH{=}$), 6.55 (d, 1, $J_{7,8}$ = 4 Hz, H_7), 3.17, 3.12 (two s, 6, Me_2N). For proton numbering, see footnote 3. [m] Dihydrate.

the ir (CCl_4) 1738 cm^{-1}, CO ester, 1065, strong, C–O–C) and NMR (CCl_4) the product was a mixture of orthoester and ester containing as estimated from the ratio of methylene (ethoxy) proton signals 58% of orthoester. This product was used in the preparation of compound IV.

Adenosine Cyclic 2′,3′-(Ethyl 3-phenylorthopropionate) (IV) and 2′(3′)-O-(3-Phenylpropionyl)adenosine (Iv). Adenosine (0.27 g, 1 mmol), a mixture of orthoester and ester from the previous experiment (1.3 g, ca. 3 mmol of ethyl 3-phenylorthopropionate), dimethylformamide (10 ml), and CF_3COOH (0.1 ml) were stirred for 17 hr at room temperature. Triethylamine (0.5 ml, 5 mmol) was then added and the solution was evaporated at 0.1 mm. The residue was partitioned between CH_2Cl_2 (20 ml) and a saturated solution of $NaHCO_3$ (10 ml). The aqueous phase was extracted with additional CH_2Cl_2 (20 ml) and the combined organic layers were dried ($MgSO_4$). Evaporation in vacuo followed by precipitation of the resultant sirup from chloroform with petroleum ether gave a foam which was dissolved in CH_2Cl_2 (4 ml) and applied on two 2-mm thick loose layers (25 × 10 cm) of silica gel. Development in solvent S_2 containing 0.2% of triethylamine afforded a major band which was rechromatographed in the same solvent (without triethylamine). The main band (compound IV) obtained after elution with the same solvent was TLC (S_2) homogeneous, NMR (CD_3COCD_3) δ 8.25 (s, 1, H_8), 8.23 (s, 1, H_2), 7.26 (s, 5, phenyl), 6.43 (d, $H_{1'}$ of endo[4] diastereoisomer, $J_{1',2'}$ = 3 Hz), 6.23 (d, $H_{1'}$ of exo[4] diastereoisomer, $J_{1',2'}$ = 3 Hz) both signals integrate for 1 proton, 4.45 (m, $H_{2'}$ + $H_{3'}$, 2), 4.47 (m, 1, $H_{4'}$), 3.82 (d, 2, $H_{5'}$) partially overlapped with methylene proton signals of the ethoxy group, 3.62 (q, 2, CH_2 of C_2H_5, partially overlapped with $H_{5'}$), 2.87 and 2.32 (two m, 2 protons each, CH_2 of phenylethyl group), 1.13 (t, 3, CH_3). A solution of product IV in 88% HCOOH (10 ml) was kept at room temperature for 20 min and then lyophilized. The residue was lyophilized again from water, the resultant solid was dissolved in acetone (4 ml) and applied to a 2-mm thick loose layer of silica gel (15 × 25 cm) which was developed in CH_2Cl_2–MeOH (85:15). The major band of compound Iv was eluted with the same solvent; the eluate was evaporated and the residue precipitated from chloroform by the addition of petroleum ether to give 0.125 g (31%) of Iv: TLC (S_2) homogeneous, mp 147–149°; uv max (95% ethanol) 260 nm (ε 12500), min 227 (ε 1800); $[\alpha]^{24}D$ −70° (*c* 0.5, acetone); NMR (CD_3SOCD_3 + D_2O) δ 8.24 (s, 2, H_8 + H_2), 7.25 (s, 5, phenyl), 6.00 (1, d, $H_{1'}$, $J_{1',2'}$ = 6.5 Hz), the ribose signals were not well resolved. Anal. Calcd for $C_{19}H_{21}N_5O_5 \cdot \frac{1}{4}H_2O$: C, 56.50; H, 5.37; N, 17.34. Found: C, 56.30; H, 5.27; N, 17.38.

5′-O-(4-Methoxy)trityl Nucleosides (General Procedure). A solution of a nucleoside or its *N*-dimethylaminomethylene derivative (2 mmol, dried by evaporation with pyridine at 0.01 mm and room temperature) and 4-methoxytrityl chloride (0.74 g, 2.4 mmol) in pyridine (10 ml) was kept for 24 hr at room temperature. The reaction mixture was poured onto ice (ca. 200 g) and extracted with $CHCl_3$ or CH_2Cl_2 (3 × 50 ml), and the organic layer was dried ($MgSO_4$) and evaporated. After the sirupy residue was evaporated with ethanol (100 ml), it was dissolved in $CHCl_3$ or CH_2Cl_2 and petroleum ether or cyclohexane (200 ml) was added. The partially sirupy product gradually solidified to give the 5′-*O*-(4-methoxy)trityl derivative which was used as such (compound Ia). In the case of compound Ip the crude residue was dissolved in a mixture of ethanol (15 ml), concentrated NH_4OH (15 ml), and dioxane (10 ml); the resultant solution was kept for 40 hr at room temperature and then evaporated. In the preparation of IIIh the residue was dissolved in methanol saturated with NH_3 at 0° (50 ml) and the solution kept for 24 hr at room temperature before evaporation. In most cases, the products were purified either by column chromatography (compound Ip) or preparative TLC (compounds Ie, Ii, Il, Is, and IIIh) on silica gel (70–325 mesh). They were obtained after precipitation from $CHCl_3$ or CH_2Cl_2 with petroleum ether or cyclohexane as an amorphous powder. Yields, analyses, uv, and NMR spectra are listed in Table I. N^4-(2,2,2-Trichlo-

[4] Exo and endo assignments are tentative.

Table II: 2′(3′)-*O*-(*N*-Protected)-L-phenylalanyl Purine Nucleosides.

Compd	Yield[a] (%)	Calcd/Found % C	% H	% N	Max (nm)[a] ($\epsilon \times 10^{-3}$)	Min (nm) ($\epsilon \times 10^{-3}$)	NMR Constants (CD_3COCD_3) Chemical Shifts, δ (Multiplicity, No. of Protons[a]) H_8	H_2	Aromatic Protons[b]	MeO	3′ Isomer[c] $H_{1'}$	$J_{1',2'}$ (Hz)	%	2′ Isomer[c] $H_{1'}$	$J_{1',2'}$ (Hz)	%
Jb							9.15	8.96[d]	7.30,6.75	3.78	6.18	6	83.2	6.33	4.5	16.8
Ic	23	58.79[e]	4.93	12.69	263	228	9.14	8.90[f]	7.33,7.18		6.11	7	76.4	6.38	6	23.6
		59.42	5.09	12.63	(6.8)	(2.1)										
If							8.37[g]	8.27[h]	7.27,6.83	3.74[i]	6.08	5.5	83.5	6.27	4.3	16.5
Ig	28.5	59.67	5.19	12.43	250–254[j]	224	8.09	8.01	7.28,7.17	*i*	5.98	6.5	79.7	6.28	6	20.3
		59.65	5.47	12.48	(8.2)	(2.1)										
Ij	18	59.48[k]	6.03		276	236	8.17	8.08	7.47,7.17		5.87	7.5	~90	6.19	7	~10[l]
		59.41	5.68		(18.5)	(1.9)										
Im							8.53[m]	8.32	7.37,6.78	3.70[n]	6.09	6	68.5	6.33[o]	4.5	31.5
In	26	54.14[k]	5.82	12.63	290,284	242[p]	8.61	8.42[q]	7.44,7.17	*r*	6.03	6.5	75.3	6.31[o]	6.5	24.7
		53.96	5.66	12.64	(20.2,20.7)	(2.0)										
Iq	26	61.14	5.23	9.70[s]				8.13[t]	7.30,6.70	3.77	6.05	3.5	60.5	6.20	3.5	39.5
		61.30	5.26	9.64												
It	31			12.79	270	239	*u*	8.15	7.30,7.17		6.00	6.5	73.6	6.32	6	26.4
				12.49	(9.3)	(1.5)										
IIIi											6.42	3.5	100			
IIIj	19	57.24[e]	5.34	14.84	258	228	8.50[v]	8.33	7.27		6.35	3.5	100[w]			
		57.83	5.76	14.72	(11.8)	(1.9)										

[a] See footnotes *a–c* in Table I. [b] See Table I, footnote *d*. In the case of 5′-*O*-(4-methoxy)trityl derivatives, the total number of aromatic protons was 24 whereas in detritylated compounds the total number amounted to 10. [c] Total integration of the $H_{1'}$ signals of both the 2′ and 3′ isomers gave one proton. [d] 8.80 (s, 1, H_6). [e] Monohydrate. [f] 8.60 (s, 1, H_6). [g] Poorly resolved singlets of both 2′ and 3′ isomers. [h] Partially overlapped with that of 2′ isomer (s, 8.26). Total integration of both signals gave one proton. [i] 4.12 (s, 3, MeO of 6-methoxypurine). [j] Shoulder at 262 (ϵ 5000). [k] Hemihydrate. [l] $[\alpha]^{25}D$ −51.5° (*c* 0.517, $CHCl_3$). [m] 2′ isomer. Signal is partially overlapped with that of the 3′ isomer (8.50, s). Both integrated for one proton. [n] 2.64 (s, 3, MeS). [o] 1.37, 1.32 (2s, 9, *t*-Bu). [p] Shoulder at 227 (ϵ 11900). [q] 3′ isomer. The 2′ isomer: 8.38 (s), total integration 1 proton. [r] 2.68 (s, 3, MeS). [s] Calcd: Br, 9.22. Found: 9.21. [t] 2′ isomer (CD_3COCD_3 + D_2O). The 3′ isomer: 8.07 (s), total integration 1 proton. [u] Overlapped with phenyl protons. For proton numbering, see footnote 3. [v] CD_3COOD. [w] $[\alpha]^{25}D$ −23° (*c* 0.5, CH_3SOCH_3).

roethoxy)carbonyl-1-β-D-(5-*O*-tritylarabinofuranosyl)cytosine (IIIa, Gish et al., 1971) had uv max (95% ethanol) 298, 232 nm (ϵ 8100, 16500), min 267 (ϵ 2900), and NMR (CD_3COCD_3) δ 8.02 (d, 1, H_6, $J_{6,5}$ = 7.5 Hz), 7.34 (m, 15, trityl), 6.94 (d, 1, H_5, $J_{5,6}$ = 7.5 Hz), 6.22 (d, 1, $H_{1'}$, $J_{1',2'}$ = 4 Hz), 4.91 (s, 2, CH_2 of TCEC), ribose protons not well resolved. The sample was a solvate with ethyl acetate (1 mol). The latter was removed by drying at 100° and 10^{-3} mm. For aminoacylation, both solvate and dried material can be used.

Aminoacylation of 5′-O-(4-Methoxy)trityl Nucleosides. The general procedure (Chládek et al., 1970, 1973) was followed. The trityl nucleosides listed in Table I were used as starting materials. In most instances, the residues obtained after evaporation of the preparative TLC fractions were dissolved in 80% acetic acid; the solution was kept for 20 hr at room temperature and then lyophilized. The residue was worked up as described previously (Chládek et al., 1970, 1973) by preparative TLC in solvent S_2. The same method was used for the preparation of araA derivatives IIIj and IIIk. For yields, analyses, uv, and NMR spectra see Tables II and III.

The analogous tubercidin derivative It was prepared in 80% yield by dissolving the 5′-*O*-trityl derivative in 80% CH_3COOH; the solution was kept for 15 hr at room temperature and then diluted with water (10 ml) and 1-butanol (20 ml). It was kept for 24 hr at room temperature to remove the *N*-dimethylaminomethylene group (Žemlička and Holý, 1967), evaporated at 0.1 mm, and then worked up as above.

1-β-D-[3-O-(N-Benzyloxycarbonyl)-L-phenylalanyl-5-O-tritylarabinofuranosyl]-N⁴-(2,2,2-trichloroethoxycarbonyl)cytosine (IIIb) and 1-β-D-[2,3-O-bis(N-benzyloxycarbonyl)-L-phenylalanyl-5-O-tritylarabinofuranosyl]-N⁴-(2,2,2-trichloroethoxycarbonyl)cytosine (IIIc). DCC (0.412 g, 4 mmol) in pyridine (2 ml) was added to a solution of the trityl derivative IIIa (ethyl acetate solvate, 0.749 g, 1 mmol) and Z-Phe (1.2 g, 4 mmol) in pyridine (12 ml) with stirring at 0°. The stirring was continued for 1 hr at 0° and then 20 hr at room temperature, dicyclohexylurea was filtered off and washed with pyridine, and the filtrate was evaporated at 0.1 mm. The residue was partitioned between $CHCl_3$ (50 ml) and saturated aqueous $NaHCO_3$ (20 ml); the chloroform layer was washed with water (20 ml) and dried ($MgSO_4$). After evaporation in vacuo the resultant sirup was coevaporated with water to give an amorphous powder (1.03 g). This material (0.1 g) was chromatographed on one Stahl silica gel GF 254 (2-mm thick layer, one 20 × 20 cm plate) in $CHCl_3$ containing 3% of MeOH. In addition to the starting material IIIa, two distinct bands containing IIIb and IIIc were obtained and eluted with solvent S_2 and the eluates were evaporated to afford both compounds as a colorless glass. Compound IIIb was dissolved in chloroform (5 ml) and petroleum ether (100 ml) was added. The white amorphous precipitate was collected by filtration and washed with petroleum ether (35 mg, 37%): mp 95–100° (transition point), TLC (S_1) homogeneous; NMR (CD_3COCD_3) δ 7.92 (d, 1, H_6, $J_{6,5}$ = 7.5 Hz), 7.32 (m, 25, phenyl), 6.10 (d, 1, $H_{1'}$, $J_{1',2'}$ = 3.5 Hz), 5.00 (d, overlapped with some ribose protons, H_5, $J_{5,6}$ = 7.5 Hz). Anal. Calcd for $C_{48}H_{43}Cl_3N_4O_{10}$: C, 61.18; H, 4.60; N, 5.95. Found: C, 61.04; H, 4.60; N, 6.19.

In the same manner, amorphous IIIc was prepared from the glassy material: yield 10 mg (8%); mp 110–115° (tran-

Table III: 2',3'-*O*-Bis(*N*-protected)-*L*-phenylalanyl Ribonucleosides.

Compd	Yield[a] (%)	Calcd/Found % C	% H	% N	Max (nm)[a] ($\epsilon \times 10^{-3}$)	Min (nm) ($\epsilon \times 10^{-3}$)	NMR Constants (CD_3COCD_3) Chemical Shifts, δ (Multiplicity, No. of Protons[a]) H_8	H_2	Aromatic Protons[b]	$H_{1'}$	$J_{1',2'}$ (Hz)	Mp (°C)	$[\alpha]^{25}D$[c] (deg)
IIa	6			10.31 9.94	264	236	9.13	8.92[d]	7.26			*e*	−40.8
IIb	7	63.97 63.99	5.25 5.17	9.95 9.69	248–253[f] (9.1)	236 (7.6)	8.48	8.22	7.20	*g*		142–145	−91.2
IIc	3	64.40 64.62	5.52 5.92	11.42 10.92	276 (16.4)	236 (2.1)						*e*	−64
IIIk	6[h]	62.32 62.35	5.34 5.22	11.56[i] 11.80	258 (12.4)	230 (2.4)	8.47[j]	8.28	7.22, 7.05	6.55	4.0	*e*	−23.8[k]

[a] See footnotes *a–c*, Table I. [b] The total number of aromatic protons was 20. [c] *c* 0.5, $CHCl_3$. [d] 8.55 (s, 1, H_6). [e] Amorphous compound. [f] Shoulder at 262 nm (ϵ 5600). [g] 4.13 (s, 3, MeO). [h] When the molar ratio ZPhe/IIIh = 3 was used, the isolated yield of compounds IIIj and IIIk was 16 and 21%, respectively. [i] Monohydrate. [j] CD_3COOD. [k] *c* 0.5, CH_3SOCH_3 at 24°.

sition point), NMR (CD_3COCD_3) δ 7.92 (d, 1, H_6, $J_{6,5}$ = 7.5 Hz), 7.45 (m, 35, phenyl), 6.24 (d, 1, $H_{1'}$, $J_{1',2'}$ = 4.5 Hz), 5.00 (d, overlapped with some ribose protons, H_5, $J_{5,6}$ = 7.5 Hz). Anal. Calcd for $C_{65}H_{58}Cl_3N_5O_{13}\cdot\frac{1}{2}H_2O$: C, 63.33; H, 4.83; N, 5.68. Found: C, 63.23; H, 4.81; N, 5.87.

From another experiment using the trityl derivative IIIa dried at 10^{-3} mm and 100° (reaction time was 17 hr at room temperature), 38% of the 3' derivative (IIIb) and 33% of the bis derivative (IIIc) were obtained.

1-β-D-[3-O-(N-Benzyloxycarbonyl)-L-phenylalanyl-2-O-methylsulfonyl-5-O-tritylarabinofuranosyl]-N⁴-(2,2,2-trichloroethoxycarbonyl)cytosine (IIId). A solution of IIIb (0.35 g, 0.362 mmol) in pyridine (5 ml) was cooled to −20°, methylsulfonyl chloride (0.056 ml, 0.724 mmol) was added, and the mixture was kept at −20° for 24 hr. The reaction was incomplete as shown by TLC (S_2), additional methylsulfonyl chloride (0.11 ml, 1.45 mmol) was added, and the solution was kept at −20° overnight and at 0° for 10 days. Ethanol (2 ml) was added and the mixture evaporated. The residue was partitioned between CH_2Cl_2 (20 ml) and saturated aqueous $NaHCO_3$ (10 ml). The organic layer was washed with water (10 ml), dried ($MgSO_4$), and evaporated. The residue was evaporated with dioxane (20 ml), dissolved in CH_2Cl_2 (1 ml), and applied to one 3 mm thick 10 × 25 cm loose layer of silica gel which was developed in solvent S_1. The major band was eluted and rechromatographed in solvent S_2. Elution of the corresponding band and precipitation of the product from $CHCl_3$ with petroleum ether gave a fluffy powder (IIId): TLC (S_1) homogeneous; yield (determined spectrophotometrically, using ϵ_{295} 8100) 0.12 mmol (33%); uv max (95% ethanol) 295, 234 (sh) nm, min 269; NMR (CD_3COCD_3) δ 8.03 (d, 1, H_6, $J_{6,5}$ = 7.5 Hz), 7.33 (m, 25, phenyl), 6.31 (d, 1, $H_{1'}$, $J_{1',2'}$ = 4 Hz), 5.04 (d, overlapped with ribose protons, H_5, $J_{5,6}$ = 7.5 Hz), 3.07 (s, 3, CH_3SO_2).

1-β-D-[3-O-(N-Benzyloxycarbonyl)-L-phenylalanylarabinofuranosyl]cytosine (IIIe). Trityl derivative IIIb (0.3 g, 0.318 mmol) was dissolved in 95% CH_3COOH (30 ml), zinc powder (90%, 0.3 g) was added, and the reaction mixture was stirred for 6 hr at room temperature. The insoluble material was filtered off and the filtrate was lyophilized. The uv spectrum of the filtrate in 95% ethanol (max 272, min 250 nm) showed the absence of the TCEC group. The residue was dissolved in CF_3COOH (5 ml), and the solution was kept for 10 min at room temperature and then lyophilized. The residue was dissolved in acetone (4 ml), applied on a 3-mm thick loose layer of silica gel (15 × 37 cm), and developed in solvent S_3. The major band was eluted with the S_3 and the eluate was evaporated. The resultant sirup solidified on prolonged drying at 0.1 mm and room temperature to give 85 mg of IIIe, presumably as the trifluoroacetate salt. The product was dissolved in dioxane–water (1:1, 10 ml) and the solution stirred with Dowex 1-X2, 200–400 mesh (acetate form, 2 ml), for 30 min at room temperature. The resin was filtered off and washed with dioxane and the eluate was lyophilized. The resultant white fluffy powder was precipitated from chloroform with petroleum ether as above: 65 mg (51%) of IIIe, TLC (S_2 and S_3) homogeneous; mp 100–110° (transition point); uv max (95% ethanol) 274 nm (ϵ 8800), min 250 (ϵ 6300), $[\alpha]^{24}D$ +29.4° (*c* 0.5, acetone); NMR (CD_3COCD_3 + D_2O) δ 7.85 (d, 1, H_6, $J_{6,5}$ = 7.5 Hz), 7.32 (s, 10, phenyl), 6.06 (d, 1, partially overlapped with H_5, $H_{1'}$, $J_{1',2'}$ = 3.5 Hz), 5.97 (d, 1, partially overlapped with $H_{1'}$, H_5, $J_{5,6}$ = 7.5 Hz). Anal. Calcd for $C_{26}H_{28}N_4O_8\cdot\frac{1}{2}H_2O$: C, 58.53; H, 5.48; N, 10.50. Found: C, 58.15; H, 5.41; N, 10.20.

1-β-D-[2,3-O-Bis(N-benzyloxycarbonyl)-L-phenylalanylarabinofuranosyl]cytosine (IIIf). Trityl derivative IIIc (0.5 g, 0.41 mmol) was stirred with zinc in CH_3COOH for 2.5 hr, followed by treatment with CF_3COOH in the same fashion as given for compound IIIe. The sirupy residue obtained after lyophilization was dissolved in $CHCl_3$ (25 ml) and the solution was washed with aqueous saturated $NaHCO_3$ (10 ml). A white insoluble material was filtered off, the layers were separated, and the aqueous portion was extracted with additional $CHCl_3$ (25 ml). The combined $CHCl_3$ extracts were dried ($MgSO_4$) and evaporated. The sirupy residue was dissolved in $CHCl_3$ (5 ml) and petroleum ether (100 ml) was added. The precipitated amorphous product (0.28 g, 85%) was contaminated with a faster moving component shown by TLC (S_2). Pure compound IIIf was obtained after chromatography of the above material in solvent S_2 and precipitation of the main band with petroleum ether in the usual fashion: 0.1 g (30%) of IIIf; TLC (S_2) homogeneous; uv max (95% ethanol) 272, 242 nm (ϵ 6900, 6500); $[\alpha]^{23}D$ +18.9° (*c* 0.45, acetone); NMR (CD_3COCD_3) δ 7.91 (d, 1, H_6, $J_{6,5}$ = 7.5 Hz), 7.25 (d, 20, phenyl), 6.28 (d, 1, $H_{1'}$, $J_{1',2'}$ = 4 Hz), 5.92 (d, 1, H_5, $J_{5,6}$ = 7.5 Hz). Anal. Calcd for $C_{42}H_{43}N_5O_{11}$: C, 64.09; H, 5.38; N, 8.69. Found: C, 64.01; H, 5.32; N, 8.68.

Ammonolysis of Compounds IIIe and IIIf. Compounds IIIe and IIIf (2 mg) were dissolved in methanolic ammonia

Table IV: 2′(3′)-*O*-L-Phenylalanyl Nucleosides.

Compd	Yield[a] (%)	Max[b] (nm)	A_{250}/A_{260}	A_{280}/A_{260}	A_{290}/A_{260}	Electrophoresis[c]	R_f (S_4)[d]
Id	91 (5900)[e]	263	0.71	0.19	0.08	2.7	
Ih	73 (11200)[f]	246, 250	1.46	[g]	[g]	1.9[h]	
Ik	89 (18400)[i]	267	0.60	0.88	0.42	4.0[h]	0.83
Io	100[j]	290,[j] 284	0.95	3.94	7.81	1.8[h]	0.72
Ir	72 (19000)[k]	263	0.67	0.53	0.16	3.4	0.65
Iu	75 (12200)[l]	273	0.51	1.19	0.77	4.2	0.47
IIIg	85 (13400)[m]	278	0.47	2.00	1.42	3.5[h]	
IIIl	84 (14600)[n]	258	0.79	0.22	0.02	3.9	0.50

[a] Determined spectrophotometrically after dilution of a corresponding aliquot of the stock solution (see Materials and Methods) with 0.01 *N* HCl unless stated otherwise. The values in parentheses indicate ϵ_{max} which were used for calculation of the yield. [b] 0.01 *N* HCl. [c] See Materials and Methods. Mobilities in cm toward the cathode are based on the mobility of Phe = 1.00. All products were ninhydrin positive. In all cases, authentic samples of Phe and the corresponding parent nucleosides were run on the same paper to ascertain the absence of the hydrolysis products. [d] In solvent S_5 the compounds were hydrolyzed during chromatography (ca. 16 hr at room temperature) to the parent nucleoside and Phe. [e] ϵ_{262}, pH 1 (Sober, 1970d). [f] ϵ_{250}, pH 1 (Johnson et al., 1958). [g] No absorption at 280 and 290 nm. [h] 40 V/cm, 1 hr. [i] ϵ_{268}, pH 1 (Sober, 1970b). [j] 95% ethanol. See ϵ_{290} for compound In, Table II. [k] ϵ_{262}, pH 1 (Holmes and Robins, 1964). [l] ϵ_{272}, pH 2 (Suhadolnik, 1970). [m] The value for cytidine (ϵ_{280}, pH 2), was used (Sober, 1970c). [n] The value for adenosine (ϵ_{257}, pH 2) was used (Sober, 1970a).

saturated at 0° and the solution was kept for 16 hr at room temperature. Paper electrophoresis (0.02 *M* $Na_2B_4O_7$) showed only araC.

2′(3′)-O-L-Phenylalanyl Nucleosides. The general procedure (Chládek et al., 1970, 1973) was followed. Aliquots from the filtered reaction mixtures were taken for uv spectra, paper electrophoresis, chromatography, and alkaline hydrolysis in solvent S_5. AraC and araA derivatives IIIg and IIIl were hydrolyzed in concentrated NH_4OH for 16 hr at room temperature prior to chromatography in S_5. All compounds were homogeneous as established by paper chromatography and electrophoresis. For yields and other characterization, see Table IV. For acceptor activity assays, 1-μmol aliquots were lyophilized and dissolved in water just before use.

Compound Io was prepared by dissolving In in 90% CF_3COOH (ca. 40 μmol/0.2 ml). After 5 min at room temperature the solution was lyophilized and the resultant white powder was characterized as above (Table IV). Compound Ir was prepared similarly: the solution of trityl derivative Iq in 90% CF_3COOH (ca. 40 μmol/ml) was kept for 30 min at room temperature and then lyophilized. The solid residue was triturated with dioxane–petroleum ether, then filtered, and washed with petroleum ether. For characterization, see Table IV.

Ribosome Assay

Assay of Peptidyltransferase Activity. The ability of the various acceptor compounds to participate in the peptidyltransferase reaction was measured as previously described (Chládek et al., 1973). A typical reaction mixture contained in 0.1 ml: 0.05 *M* Tris-HCl (pH 7.4), 0.10 *M* NH_4Cl, 0.01 *M* $MgCl_2$, 3.5 A_{260} units of NH_4Cl-washed 70S ribosomes from *E. coli* MRE-600 cells, 10 μg of poly(U), and 0.14 A_{260} units of *N*-acetyl[^{14}C]phenylalanyl-tRNA (2000 cpm). Reactions were initiated by the addition of acceptor compounds at concentrations indicated in the text and allowed to incubate for 30 min at 37° before termination of the reaction by the addition of cold aqueous 2.5% CCl_3COOH. The amount of unreacted Ac[^{14}C]Phe-tRNA was determined by measurement of CCl_3COOH precipitated counts trapped by Millipore membranes as described before (Ringer et al., 1975). The amount of Ac[^{14}C]Phe residue transferred from Ac[^{14}C]Phe-tRNA to the acceptor was determined as the difference between radioactivity retained on the filter following reaction in the absence of acceptor compound and that remaining on the filter after reaction in the presence of acceptor. It is expressed as the percentage of the radioactivity of Ac[^{14}C]Phe-tRNA added to the incubation mixture (Figure 1 and Table V).

Results and Discussion

Synthesis. The synthesis of 6-substituted 2′(3′)-*O*-L-phenylalanyl purine ribonucleosides Id, Ih, Ik, and Io followed the general procedure described earlier (Chládek et al., 1970). The 5′-*O*-(4-methoxy)trityl nucleosides Ia, Ie, Ii, and Il (Table I) were condensed with Z-Phe using DCC in pyridine to give intermediates Ib, If, and Im which were separated from unreacted starting materials and the corresponding diaminoacyl derivatives by preparative TLC (Tables II and III). In the case of Ii, the separation of mono- and diaminoacyl derivatives was incomplete. Therefore, the separation was performed after the detritylation step. Detritylation in 80% CH_3COOH (20 hr at room temperature) afforded the corresponding mono- (Ic, Ig, Ij, and In, Table II) and diaminoacyl derivatives IIa, IIb, and IIc (Table III). Essentially the same approach was used for the synthesis of tubercidin derivative Iu. In this case, however, the parent nucleoside was converted to the corresponding *N*-dimethylaminomethylene derivative prior to tritylation to prevent the formation of the *N*-tritylated product (Žemlička and Holý, 1967). The reaction of *N*-dimethylaminomethylene 5′-*O*-(4-methoxy)tritylubercidin (Is) with Z-Phe was carried out as above. After the detritylation in 80% acetic acid, the *N*-dimethylaminomethylene group was removed in solvent S_4 (Žemlička and Holý, 1967) to give

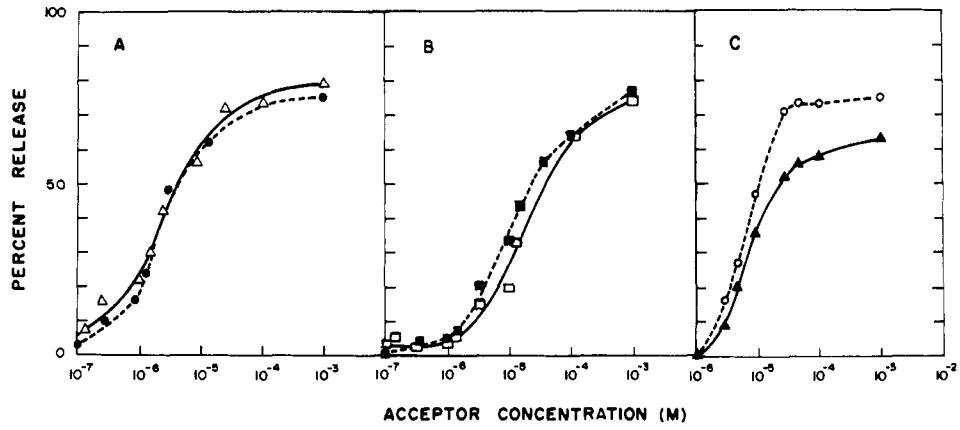
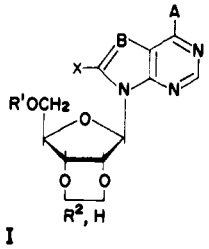


FIGURE 1: Extent of Ac-Phe release from *N*-Ac-Phe-tRNA in the peptidyltransferase-catalyzed reaction with 2′(3′)-*O*-L-phenylalanyl ribonucleosides as acceptors. For reaction conditions, see Materials and Methods. (A) A-Phe (●), 2′(3′)-*O*-L-phenylalanyltubercidin (Iu, △); (B) 6-methoxy-2′(3′)-*O*-L-phenylalanylnebularine (Ih, ■); 2′(3′)-*O*-L-phenylalanylnebularine (Id, □); (C) N^6,N^6-dimethyl-2′(3′)-*O*-L-phenylalanyladenosine (Ik, ○); 6-methylthio-2′(3′)-*O*-L-phenylalanylnebularine (Io, ▲).

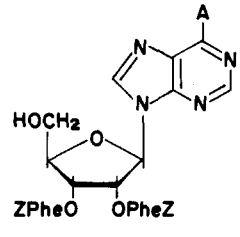
compound It. In the case of the 6-MeS derivative Io, the presence of the sulfur atom precluded catalytic hydrogenation in the last deblocking step. Therefore, Boc-Phe was employed instead of Z-Phe in the reaction with II. Intermediate Im was detritylated in the usual fashion to give In. The removal of the Boc group from In was achieved using 90% CF_3COOH for 10 min at room temperature (Waller et al., 1966). The latter approach was also used in the preparation of the 8-bromoadenosine derivative Ir. It was established in an independent experiment that 8-bromoadenosine gives quantitatively adenosine under the conditions of hydrogenolysis of the *N*-benzyloxycarbonyl group (80% acetic acid, 3 hr, 0°). Analogously to the case of tubercidin, 8-bromo-*N*-dimethylaminomethyleneadenosine was used in the preparation of the corresponding trityl derivative Ip. However, the *N*-dimethylaminomethylene group was removed prior to the condensation with Boc-Phe by treatment with methanolic ammonia (Žemlička and Holý, 1967). The MeOTr and Boc groups of Iq were then simultaneously removed with CF_3COOH to give Ir. The isomeric composition of the 2′(3′)-*O*-(*N*-protected)-L-phenylalanyl derivatives (Table II) was determined by NMR spectroscopy from the corresponding $H_{1'}$ signals (Sonnenbichler et al., 1964).

It should be noted that 2′ ⇄ 3′ isomerism can also be reflected in signals other than that of $H_{1'}$. Thus in compound In, whose NMR spectrum was particularly well resolved, the signals for H_2 for the 2′ and 3′ isomers are distinctly different. All these protons are less deshielded in the 2′ isomer than in the 3′ derivative, in contrast to $H_{1'}$, where the situation is just the opposite (Sonnenbichler et al., 1964; Fromageot et al., 1966). However, in the case of 8-bromoadenosine derivative Iq which has the base residue in a



I

Ia: R′ = MeOTr, R^2 = A = X = H, B = N
Ib: R′ = MeOTr, R^2 = ZPhe, A = X = H, B = N
Ic: R′ = A = X = H, R^2 = ZPhe, B = N
Id: R′ = A = X = H, R^2 = Phe, B = N
Ie: R′ = MeOTr, R^2 = X = H, A = MeO, B = N
If: R′ = MeOTr, R^2 = ZPhe, A = MeO, B = N, X = H
Ig: R′ = X = H, R^2 = ZPhe, A = MeO, B = N
Ih: R′ = X = H, R^2 = Phe, A = MeO, B = N
Ii: R′ = MeOTr, R^2 = X = H, A = Me_2N, B = N
Ij: R′ = X = H, R^2 = ZPhe, A = Me_2N, B = N
Ik: R′ = X = H, R^2 = Phe, A = Me_2N, B = N
Il: R′ = MeOTr, R^2 = X = H, A = MeS, B = N
Im: R′ = MeOTr, R^2 = BOCPhe, A = MeS, B = N, X = H
In: R′ = X = H, R^2 = BOCPhe, A = MeS, B = N
Io: R′ = X = H, R^2 = Phe, A = MeS, B = N
Ip: R′ = MeOTr, R^2 = H, A = NH_2, B = N, X = Br
Iq: R′ = MeOTr, R^2 = BOCPhe, A = NH_2, B = N, X = Br
Ir: R′ = H, R^2 = Phe, A = NH_2, B = N, X = Br
Is: R′ = MeOTr, R^2 = X = H, A = N = CH-NMe_2, B = CH
It: R′ = X = H, R^2 = ZPhe, A = NH_2, B = CH
Iu: R′ = X = H, R^2 = Phe, A = NH_2, B = CH
Iv: R′ = X = H, R^2 = $C_6H_5CH_2CH_2CO$, A = NH_2, B = N



II

IIa: A = H
IIb: A = MeO
IIc: A = Me_2N

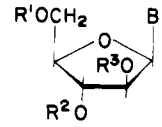
Table V: Acceptor Activity of Some Moderately Active Substrates in the Ribosome-Catalyzed Release of Ac-Phe from *N*-Ac-Phe-tRNA.[a]

Substrate	Concn (m*M*)	Release (%)
Ir	1	24.1
	0.1	4.6
IIIl	1	6.2
	0.1	4.9
Iv	1	6.4
IIIg	1	20.1
	0.7	18.2
	0.3	5.9

[a] Peptidyltransferase activity was determined as described in Materials and Methods.

syn conformation (Tavale and Sobell, 1970), the H_2 in the 2′ isomer is more deshielded than in the 3′ derivative. It should be stressed that although the chemical shifts of the heterocyclic protons of pyrimidine ribonucleoside 2′- and 3′-*O*-acyl derivatives are frequently different (Fromageot et al., 1966; Chládek and Žemlička, 1974), a similar situation in the purine series has not been observed to our knowledge. The NMR spectrum of In thus represents an example where the heterocyclic protons (H_2) of the 2′- and 3′-*O*-acyl derivatives of a purine ribonucleoside are clearly resolved.

The 3′-*O*-L-phenylalanyl derivatives of araC and araA (IIIg and IIIl) were similarly prepared. *N*-Dimethylamino-

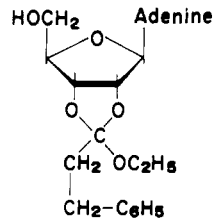


III

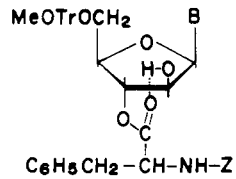
IIIa : B = N^4-TCECcytosine, R^1 = Tr, R^2 = R^3 = H
IIIb : B = N^4-TCECcytosine, R^1 = Tr, R^2 = ZPhe, R^3 = H
IIIc : B = N^4-TCECcytosine, R^1 = Tr, R^2 = R^3 = ZPhe
IIId : B = N^4-TCECcytosine, R^1 = Tr, R^2 = ZPhe, R^3 = Ms
IIIe : B = cytosine, R^1 = R^3 = H, R^2 = ZPhe
IIIf : B = cytosine, R^1 = H, R^2 = R^3 = ZPhe
IIIg : B = cytosine, R^1 = R^3 = H, R^2 = Phe
IIIh : B = adenine, R^1 = MeOTr, R^2 = R^3 = H
IIIi : B = adenine, R^1 = MeOTr, R^2 = ZPhe, R^3 = H
IIIj : B = adenine, R^1 = R^3 = H, R^2 = ZPhe
IIIk : B = adenine, R^1 = H, R^2 = R^3 = ZPhe
IIIl : B = adenine, R^1 = R^3 = H, R^2 = Phe

nomethylene-araA was converted to the corresponding MeOTr derivative of araA (IIIh), and condensed with Z-Phe using DCC in pyridine to give monoaminoacyl derivative IIIi which was separated from the accompanying diaminoacyl derivative by preparative TLC. Both products were detritylated in 80% CH_3COOH to give compounds IIIj and IIIk. In the case of araC, it was necessary to block the cytosine amino group to prevent aminoacylation during condensation with Z-Phe (cf. Shabarova et al., 1957). As a convenient starting material, N^4-(2,2,2-trichloroethoxycarbonyl)-5′-*O*-trityl-araC (IIIa, Gish et al., 1971) was used. The latter was condensed in the usual fashion with Z-Phe using DCC in pyridine to give a mixture of monoaminoacyl and diaminoacyl derivatives IIIb and IIIc which were, in turn, deblocked with zinc in 95% acetic acid (2–6 hr at room temperature for the removal of the TCEC group) and, subsequently, the MeOTr group was removed with 80% CH_3COOH (24 hr at room temperature). The resultant *N*-benzyloxycarbonyl derivatives IIIe and IIIf were purified by preparative TLC. Compound IIIe was hydrogenated to give 3′-*O*-L-phenylalanyl-araC (IIIg).

It is of interest to note that with either IIIa or IIIh we have been unable to isolate the corresponding 2′-aminoacyl derivative although the corresponding 2′,3′-diaminoacyl compounds were readily obtained. This represents a difference relative to the situation in ribonucleosides where the 2′-hydroxy group is supposed to be the site of the kinetically controlled reaction while the more stable 3′ isomer is formed under conditions of thermodynamic control (Chládek et al., 1973). Moreover, it is of interest to note that acylation (acetylation) of uracil ribo-, xylo-, and lyxonucleosides leads always to the predominant substitution of the 2′-hydroxy group (Johnston, 1968). Although our acylating reagent is different, the reaction conditions are quite similar and it is therefore surprising that arabinofuranosyl derivatives IIIb and IIIi constitute an exception. Formylation of araC using HCOOH also did not afford any 2′-*O*-formyl derivative but only the 5′- and 3′-*O*-formylated products (Repta et al., 1975). Framework molecular models show that the intramolecular 2′ ⇄ 3′-*O*-acyl migration (Wolfenden et al., 1964) is not possible in the corresponding arabinofuranosyl derivatives. Therefore the 3′-*O*-aminoacyl derivatives IIIb and IIIi represent most likely primary, kinetically controlled products. It is evident that the 2′-hydroxy group in arabino nucleosides is more sterically hindered by the adjacent base than in ribonucleosides. This, however, does not explain the substantial amount of the diaminoacyl derivatives formed with both araA and araC whereas the 2′-monoaminoacyl isomer is absent. It may be argued that the introduction of the 3′-*O*-aminoacyl group changes the conformation of the arabinofuranosyl moiety making the 2′-hydroxyl more accessible than in the parent compounds IIIa or IIIh. However, the $J_{1',2'}$ values of the diaminoacyl derivatives IIIc, IIIf, and IIIk do not differ significantly from those of the corresponding 3′-*O*-aminoacyl compounds IIIb, IIIg, and IIIj. Therefore, a more likely explanation may be seen in the fact that in both 3′-*O*-aminoacyl derivatives IIIb and IIIi the formation of a hydrogen bond between the 2′-hydroxy group and carbonyl (ester) group of the aminoacyl residue is possible (structure V). Such hydro-



IV



V

gen bonding would render the 2′-hydroxy group more nucleophilic. Consequently, acylation of the 2′-hydroxy group would be easier in 3′-*O*-aminoacyl derivatives IIIb and IIIi than in the corresponding parent nucleosides IIIa and IIIh.

Structural assignment of compounds IIIe and IIIj as the 3′-*O*-aminoacyl derivatives were made from the NMR spectra. In agreement with the ribose series (Chládek et al., 1970), the above derivatives have the $H_{1'}$ signal at a higher field than the corresponding diaminoacyl compounds IIIf and IIIk. In the ribose series the $H_{1'}$ chemical shifts of 2′,3′-di-*O*-aminoacyl derivatives are very close to those of the 2′-*O*-aminoacyl derivatives (Chládek et al., 1970). In the case of IIIb, the reaction with methylsulfonyl chloride in pyridine readily afforded the corresponding 2′-*O*-methylsulfonyl derivative IIId whose $H_{1'}$ signal, as expected, is

substantially shifted downfield from that of the parent compound IIIb. It is interesting that trityl derivatives IIIa and IIIh have more deshielded $H_{1'}$ relative to the 3′-*O*-aminoacyl compounds IIIb and IIIi.

Finally, 2′(3′)-*O*-(3-phenyl)propionyladenosine (Iv) was prepared by the orthoester exchange procedure (Žemlička and Chládek, 1966). Adenosine was reacted with ethyl 3-phenylorthopropionate in dimethylformamide using CF_3COOH as catalyst to give the 2′,3′ cyclic orthoester IV which, in turn, was hydrolyzed with 88% HCOOH for 20 min at room temperature to give compound Iv. The NMR spectrum of IV indicates a mixture of two diastereoisomers. Thus, two well-separated doublets for $H_{1'}$ were found which made it possible to calculate the amount of both diastereoisomers from the height of corresponding signals (64.6% exo and 35.4% endo,[4] respectively). Framework molecular models show that the distance between the oxygen atom of the ethoxy group and $H_{1'}$ in IV is smaller for the endo than for the exo diastereoisomer. It is therefore reasonable to assume that the $H_{1'}$ will be more deshielded in the endo (less abundant) diastereoisomer. This is in agreement with the tentative assignment for the diastereoisomers of 2′,3′-*O*-dimethylaminomethylene ribonucleosides (Žemlička, 1975). It is also of interest to note that NMR spectra have shown that compounds Ij and Iv are ca. 90% pure 3′ isomers.

Biochemical Results. The substrates studied in this work can be divided into two groups relative to their activity in releasing the *N*-Ac-Phe residue from *N*-Ac-Phe-tRNA. The first group included 2′(3′)-*O*-L-phenylalanyl ribonucleosides whose activity is comparable to that of A-Phe, the terminal sequence of natural Phe-tRNA (6-substituted purine nucleosides Id, Ih, Ik, Io, and the derivative of tubercidin Iu). The results are summarized in Figure 1. It can be seen that the replacement of the N-7 atom in A-Phe by the CH group (Iu) has little effect on acceptor activity and the concentration curve of Iu almost coincides with that of A-Phe (Figure 1A). Thus, the differences between Iu and A-Phe are less pronounced than those between the Val-tRNA containing tubercidin as its 3′ terminal nucleoside and the corresponding native Val-tRNA; the latter was found to be more efficient in a ribosome-catalyzed polypeptide synthesis (Uretsky et al., 1968). It is also of interest that the carbocyclic analogue of 8-azapuromycin is a substrate for ribosomal peptidyltransferase and its activity is comparable to that of puromycin (Duquette et al., 1974). Thus, the replacement of N by CH or vice versa in the imidazole portion of a given substrate does not appreciably influence the acceptor activity.

Greater changes have been observed in the series of 2′(3′)-*O*-L-phenylalanyl derivatives of the 6-substituted purine ribonucleosides Id, Ih, Ik, and Io (Figures 1B and 1C). However, in all five compounds investigated, the observed variations in the acceptor activity stay well within the limit of one order of magnitude. The acceptor activity of these derivatives is approximately one to two orders of magnitude higher than that of I-Phe (Rychlík et al., 1969). It is perhaps of interest to note that the electronic configuration of the purine moiety in all five derivatives, assuming that A-Phe is in tautomeric amino form, is similar and different from that in inosine where the keto tautomer is predominant (Evans and Sarma, 1974). Thus, the possibility of stabilization by resonance appears to be more restricted in inosine than in the parent nucleosides derived from Id and, particularly, Ih, Ik, Io, and A-Phe.

An interesting trend has been observed in the latter series of 6-substituted purine derivatives. The acceptor activity roughly follows the electron-donating property (mesomeric effect, +M-effect, Ingold, 1969) of the substituent in position 6 of the purine: NH_2 or Me_2N > MeO or MeS > H (Figures 1A to 1C). Two possible factors can be invoked to explain these results: (a) changes in the electron density of the heterocyclic moiety can influence the extent of hydrogen bond formation between the 2′-hydroxy group and the N-3 atom of the purine moiety or some appropriate region on the ribosome (intramolecular vs. intermolecular hydrogen bond). This possibility has already been discussed in more detail previously (Rychlík et al., 1969); (b) higher acceptor activity of purine derivatives having a strongly electron-donating 6 substituent may reflect a more favorable hydrophobic interaction with some protein or nucleic acid portion of the A site of the ribosomal peptidyltransferase or eventually the peptidyl (donor) tRNA itself. The latter possibility seems particularly intriguing because CPK molecular models indicate that a stacking of both terminal adenosine residues of peptidyl- and aminoacyl-tRNA ("acceptor-donor stacking") in the situation just prior to the peptide bond formation is a possible alternative (Raacke, 1971; Žemlička and Owens, manuscript in preparation).

The second group of substrates includes compounds whose acceptor activity is moderate (derivatives of araC and 8-bromoadenosine, IIIg or Ir) or very low (derivatives of araA IIIl and compound Iv). The results are summarized in Table V. It is of interest that the acceptor activity of the araC derivative IIIg at 10^{-3} *M* corresponds very closely to that of C-Phe (Rychlík et al., 1969). Thus, surprisingly, very little influence of the configuration of the 2′-hydroxy group on the acceptor activity can be seen. On the other hand, the acceptor activity of araA derivative IIIl is negligible even at the high (10^{-3} *M*) concentration and resembles that of dA-Phe (Rychlík et al., 1969). Therefore, in this case, the change of the configuration of the hydroxy group at the C-2′ has the same effect on acceptor activity as its removal.[5]

A profound drop in acceptor activity relative to A-Phe was also found with the 8-bromoadenosine derivative Ir. In view of the syn conformation of the base residue in 8-bromoadenosine (Tavale and Sobell, 1970), the most likely interpretation of this effect would be the requirement of ribosomal peptidyltransferase for a substrate having the anti conformation. However, our results indicate that such a requirement is not absolute because at 10^{-3} *M* compound Ir exhibited the acceptor activity comparable to that of araC derivative IIIg or C-Phe. It should be pointed out that further studies of substrates with a fixed conformation (anti or syn) are necessary to eliminate possible inductive influence of the bromine atom on the acceptor activity of Ir.

Compound Iv, which contains all the necessary structural factors but lacks the amino group in the aminoacyl portion, exhibited neither a significant acceptor activity nor inhibited the puromycin reaction at 10^{-3} *M*. This is, along with other findings (Černá et al., 1970; Pozdnyakov et al., 1972),

[5] Recently, it has been stated (Holý et al., 1974) that compound IIIl is inactive as an acceptor of the peptide chain during ribosome-catalyzed peptide bond formation. A reference (Fisher et al., 1970) cited in support of this statement does not, however, contain any mention of compound IIIl or acceptor activity in a ribosomal system. The above reference pertains solely to the synthesis of arabinofuranosyl analogues of puromycin and their testing in the murine leukemia L1210 system.

at variance with the hypothesis which invokes 2′-peptidyl-3′-aminoacyladenosine as an intermediate in the peptide bond formation process (Neumann et al., 1968).

Compounds Ic, Ig, Ih, Ir, IIIf, and IIIg inhibited DNA synthesis in murine leukemia L1210 in vitro system at 10–100 γ/ml level. The results which were already reported in a preliminary form (Horwitz et al., 1974) will be published elsewhere.

Conclusion

In summary, the following conclusions can be drawn from our results regarding the substrate specificity of ribosomal peptidyltransferase. (a) The activity of A-Phe depends on the configuration of the 2′-hydroxy group, as shown for the arabinofuranosyl derivative IIIl which is practically inactive. On the other hand, the same configurational change in moderately active C-Phe produced no effect (cf. arabinofuranosyl derivative IIIg). (b) The activity of 2′(3′)-*O*-L-phenylalanylpurine ribonucleosides is not substantially influenced by the nature of the substituent in the 6 position provided the basic electronic configuration of the purine moiety is not changed. Thus, activity in the series Id, Ih, Ik, Io, and A-Phe stays within the limit of one order of magnitude and roughly follows the M-effect of the substituent. Replacing of N-7 in A-Phe by isosteric and isoelectronic CH (tubercidin derivative Iu) has not produced any effect on the activity. (c) Electronic factors, cf. (b), appear to have less importance than conformational changes. Thus, 8-bromoadenosine derivative Ir with the purine residue in a syn conformation is, unlike A-Phe, a substrate of only moderate activity. Although experiments with more refined conformational models are necessary, it appears that the requirement for an anti conformation of the adenine residue in A-Phe is not absolute. (d) The presence of the amino group in the aminoacyl moiety of A-Phe is essential. Thus, compound Iv is almost inactive.

Acknowledgments

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The Mechanism of Action of Dipeptidyl Aminopeptidase. Inhibition by Amino Acid Derivatives and Amines; Activation by Aromatic Compounds†

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ABSTRACT: A variety of amino acid and peptide amides have been shown to be inhibitors of dipeptidyl aminopeptidase. Among these compounds derivatives of strongly hydrophobic amino acids are the strongest inhibitors (Phe-NH_2, K_i = 1.0 ± 0.2 m*M*), while amides of basic amino acids were somewhat less effective (Lys-NH_2, K_i = 36 ± 3 m*M*). Short chain amino acid amides are notably weaker inhibitors (Gly-NH_2, K_i = 293 ± 50 m*M*). The interaction of the side chains of compounds with the enzyme appears to be at a site other than that at which the side chain of the amino-penultimate residue of the substrate interacts since the specificity of binding is different. Primary amines have been shown to inhibit, e.g., butylamine, K_i = 340 ± 40 m*M*, and aromatic compounds have been shown to stimulate activity toward Gly-Gly-NH_2 and Gly-Gly-OEt (phenol, 35% stimulation of activity at a 1:1 molar ratio with the substrate). The data suggest that inhibition involves binding at the site occupied by the free α-amino group and the N-terminal amino acid.

Dipeptidyl aminopeptidase (dipeptidyl aminopeptidase I, dipeptidyl peptidase, dipeptidyl-transferase, cathepsin C; EC 3.4.14.1) is a lysosomal exopeptidase which is capable of removing dipeptides sequentially from the amino terminus of a peptide chain (McDonald et al., 1969). The specificity of the enzyme has been studied in some detail (Jones et al., 1952; Izumiya and Fruton, 1956; Fruton and Mycek, 1956; Wiggans et al., 1954; Planta et al., 1964; Voynick and Fruton, 1968). From these studies it appeared that dipeptidyl aminopeptidase had a rather narrow specificity, substrate largely being confined to compounds of the type NH_2-C(R_1)H-CO-NH-C(R_2)H-CO-X, in which R_2 was the side chain of a strongly hydrophobic amino acid. Planta et al. (1964) and Metrione et al. (1966) had reported that the enzyme had no activity toward proteins. However, McDonald et al. (1969) demonstrated that the enzyme possesses a much broader specificity and is capable of hydrolyzing the peptide bonds of proteins in the presence of chloride ions. It is now clear that in the presence of suitable activators the CO–X bond can be hydrolyzed provided that: (1) the α-amino group is free; (2) the amino terminal amino acid (with the side chain R_1) is not basic (Arg or Lys[1]); (3) that the penultimate amino acid (with side chain R_2) is not proline; and (4) that the dipeptide is not linked through a peptide bond to a proline. The usefulness of the enzyme for protein sequence studies has been described (Callahan et al., 1972).

Highly purified dipeptidyl aminopeptidase (Metrione et al., 1966) has been shown to be composed of eight subunits of 25000 molecular weight each (Metrione et al., 1970) and to require sulfhydryl (Fruton and Mycek, 1956) and chloride activation (McDonald et al., 1966). There is some evidence that dipeptidyl aminopeptidase is an allosteric enzyme (Gorter and Gruber, 1970).

Fruton and Mycek (1956) reported on the ability of amino acid derivatives to inhibit dipeptidyl aminopeptidase. Their results indicated that Phe-NH_2 is a much better inhibitor than D-Phe-NH_2; Tyr-NH_2 is a weaker inhibitor than Phe-NH_2; Phe-Phe is an inhibitor; and that Ac-Phe, Phe, β-phenylethylamine, and Leu-NH_2 were not inhibitors. The authors concluded that the requirements for competitive inhibitors included an L-amino acid (stereochemical specificity), a free amino group, and a linked (amidated or esterified) carboxyl group, and that inhibition is favored by an amino terminal aromatic amino acid.

Experiments in our laboratory intended to develop an affinity chromatographic method for the separation and isolation of intracellular proteolytic enzymes have led us to a reinvestigation of the inhibition of beef spleen dipeptidyl aminopeptidase.

Experimental Procedure

Isolation of Enzyme and Determination of Enzyme Activity. The transamidation assay was employed in the manner described by Metrione et al. (1966). For routine assays, Gly-Phe-NH_2 or Gly-Gly-OEt was the substrate unless otherwise indicated. The hydrolysis assay was the method of

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[1] Abbreviations are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature Symbols for Amino-Acid Derivatives and Peptides (1972).