2′/3′-*O*-peptidyl Adenosine as a General Base Catalyst of its Own External Peptidyl Transfer: Implications for the Ribosome Catalytic Mechanism

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Although the presence of a 2′-OH in the 3′-terminal adenosine of peptidyl tRNA has long been known to be crucial for protein biosynthesis,[1] the nature of its rate-enhancement effect is still poorly understood. A hydrogen-bond donation to the adjacent acyl group,[2] a hydrogen-bond acceptance from the attacking amine nucleophile,[3] a role in positioning the ribosome catalytic group (N3 of A2486),[4] and even no participation at all in the elongation step[5] have all been suggested. These hypotheses, however, have never been tested experimentally because of the supramolecular character of the ribosome and its complex with aminoacyl tRNA and peptidyl tRNA. The approaches of modern chemical biology, however, permit the supramolecular complexity to be reduced to a level that allows understanding of the chemical mechanism of this 2′-OH assistance on a fully chemical basis. Hence, we used a linear free-energy relationship (LFER) of the Brønsted type and kinetic isotope effect to study the external peptidyl transfer within a series of 2′/3′-*O*-peptidyl adenosine derivatives as peptidyl tRNA mimics. Herein we report an intramolecular general base catalysis by the vicinal 2′/3′-oxyanion of 2′/3′-peptidyl adenosine. This finding implies that a similar catalytic role of the partially deprotonated 2′-OH of the peptidyl tRNA A76 is possible, provided this P-site substrate group partially protonates the adjacent 3′-oxygen in a cyclic hexagonal transition state involved in a substrate-assisted catalytic mechanism of the ribosome.

The study became possible after Velikian et al. demonstrated that the acidity of 2′/3′-OH in ribonucleosides is steered by the nucleobase structure.[6] Hence, we carried out modifications including atomic and group substitutions in the adenin-9-yl group of adenosine in order to obtain adenosine derivatives with varying 2′/3′-OH pK_a values. Then we probed the pK_a dependence of the rate of external transesterification (ethanolysis) of the corresponding 2′/3′-*O*-benzyloxycarbonyl-L-*p*-nitrophenylalanyl 5′-*O*-trityl adenosine derivatives **1**–**11** (Scheme 1). The transesterification reaction is known to be catalyzed by the ribosome.[7] The substrates were designed so as to be soluble in organic media (tritylated at 5′-OH as well as functionalized with a hydrophobic 2′/3′-*O*-peptidyl group), to possess a suitable signal for HPLC monitoring (*p*-nitrated phenylalanine) and to undergo a peptidyl transfer reaction (transesterification instead of aminolysis) with a measurable rate. The solubility in aprotic organic media is required in order to mimic the environment of the ribosome active site, which must be nonaqueous in order to prevent premature hydrolysis.[5b,8] In hydrogen-bond-donor solvents such as water, the adjacent 2′/3′-OH group is preferentially solvated separately by water molecules, thus preventing its potential catalytic effect.

External transesterification of the adenosine derivatives **1**–**11** in acetonitrile does not occur in the absence of an organic base. It is promoted, however, by non-nucleophilic tertiary amines like 1,8-diazabicyclo[5.4.0]-undec-7-en (DBU).The measured pseudo-first-order rate constants k_{obs} are summarized in Table 1. It can be seen that this apparent rate constant strongly depends on the presence of both a free 2′/3′-OH and a nucleobase, as well as on the nucleobase's structural integrity. Actually, the rate of transesterification of the adenosine derivative **1** is more than 300-fold faster than that of the 2′-deoxyadenosine (**2**), 3′-deoxyadenosine (**3**), or 2′-methyladenosine (**4**) derivatives; this is in agreement with the earlier observation that the presence of an intact 2′/3′-OH is crucial to the reactivity.[1–3] The absence of a nucleobase (1-deoxy ribose derivative **5**) results in a more than one order of magnitude decrease of the rate as compared to the parent nucleoside **1** but is still approximately ten times faster than that of the 2′- or 3′-deoxy derivatives. Substitution in and of the nucleobase also affects the rate. N6-benzoylation of adenosine **1** (N6-benzoyl adenosine **6**), substitution of adenin-9-yl by guanin-9-yl group (**7**) and uracil-1-yl (**10**) significantly reduce the k_{obs} values. *N*3-methylation of uridine derivative **10** (*N*3-methyluridine derivative **11**), however, increases $k_{obs.}$ The substitution of *N*3 for C3-H (3-deazaadenosine derivative **8**) and deletion of the pyrimidine ring (1-imidazolyl riboside (Im) derivative **9**), surprisingly have no effect on k_{obs}.

Since **8** and **9** were designed to probe the plausible participation of N3 in the peptidyl transfer, we tried to find out whether such an effect really does not exist or whether it is masked. The lack of reactivity of 2′/3′-*O*-peptidyl adenosine derivatives **1**–**11** in the absence of a strong base, such as DBU, suggests that DBU promotes the transesterification reaction

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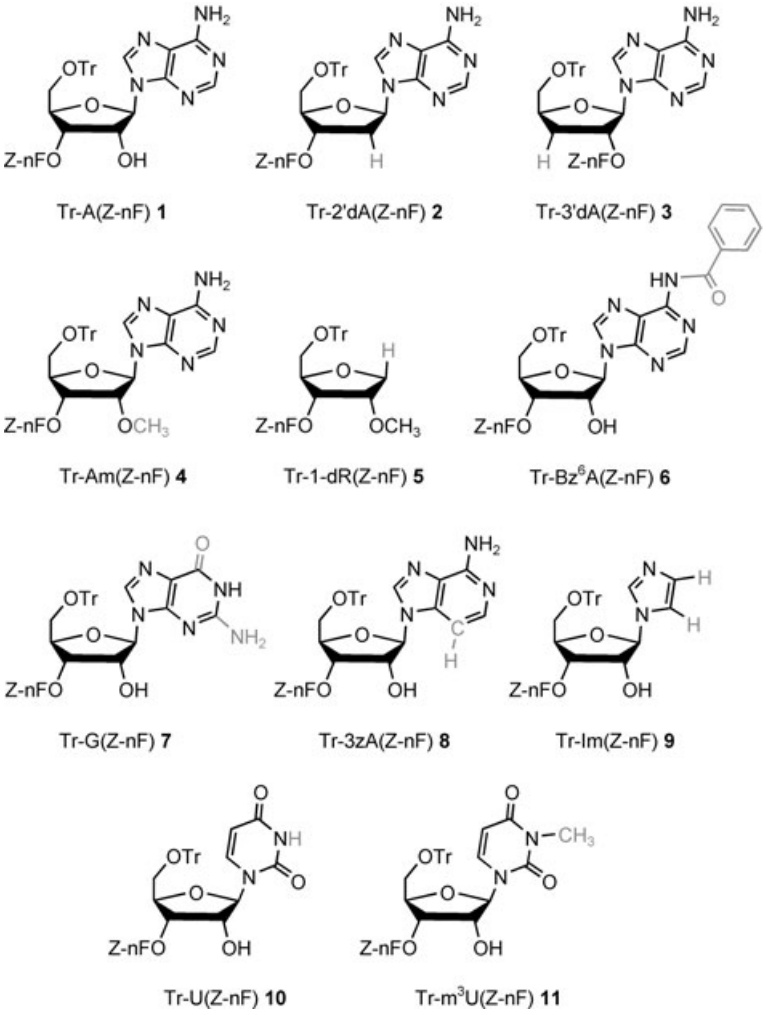


(Scheme 1) by deprotonation of the reagents. Due to the delocalization of the positive charge in its protonated form $DBUH^+$, DBU is a very strong, non-nucleophilic (because of steric hindrance), tertiary amine base. The important sites for proton abstraction in **1** and **5**–**10** are the amide N6H in the N6-benzoyl derivative **6**, the N1-H and N3-H amides of the guanosine and uridine analogues **7** and **10** and the 2′/3′-hydroxyl group in adenosine itself (**1**) and adenosine derivatives **5**–**11**. The deprotonation of the second reagent, ethanol, results in formation of the ethoxide anion, EtO^-. When these ionizations were considered, the second-order rate constants k_R were calculated[9] and the values obtained are also included in Table 1.

Perusal of the $k_R^{\tau_1}$ values (Table 1) indicates that when the ionization of the important deprotonation sites is considered, the "degeneracy" (small difference) for **1**, **8**, **9** and **11** in k_{obs} is removed since the rate constants are now related to the oxyanion concentration. The kinetic data were used to obtain a Brønsted correlation (Figure 1) between the reactivity of 2′/3′-*O*-peptidyl adenosine derivatives with only one (2′/3′-OH) deprotonation site (**1**, **5**, **8**, **9** and

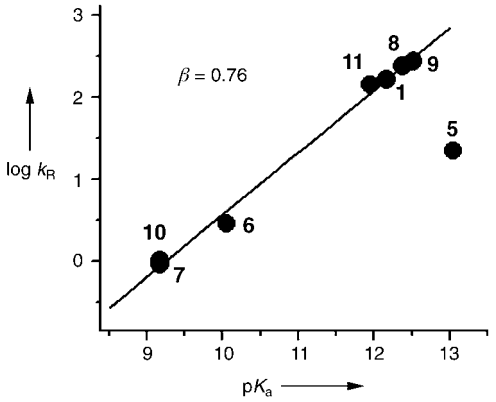
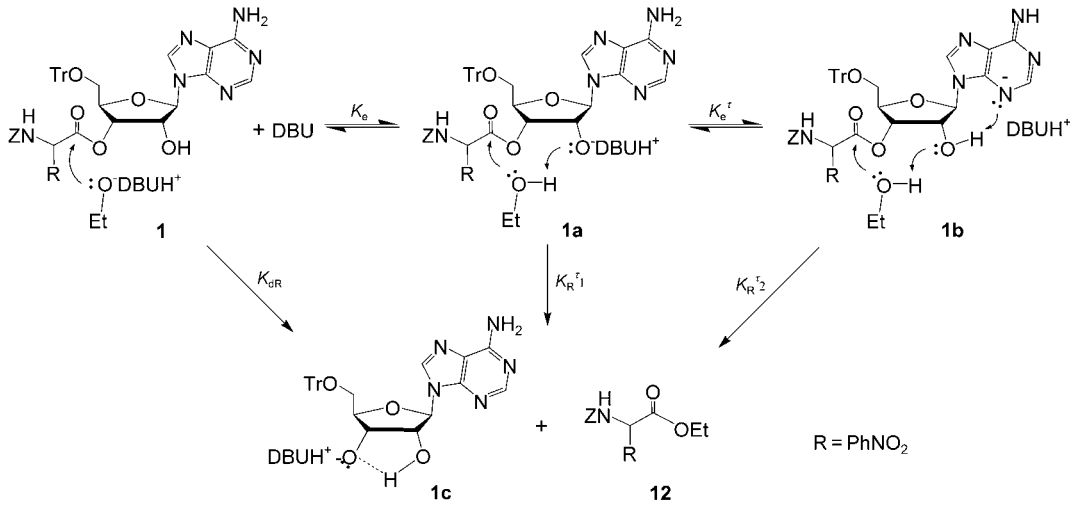


Figure 1. Brønsted plot of the transesterification (ethanolysis) of 2′/3′-O-*peptidyl* adenosine derivatives **1, 5**–**11**; the logarithms of the second-order rate constants k_R are plotted against the pK_a's of 2′/3′-oxyanions **1a, 5a, 8a, 9a** and **11a**, and 6-, 1- and 3-nitranions **6a, 7a** and **10a**.



Scheme 1. Proposed mechanisms of transesterification (ethanolysis) in the presence of 1,8-diazabicyclo[5.4.0]-undec-7-en (DBU) and chemical structure of 2′/3′-*O*-benzyloxycarbonyl-*p*-nitrophenylalanyl 5′-trityl adenosine derivatives **1**–**11**.

Table 1. Kinetic data for the ethanolysis of 2′/3′-*O*-peptidyl adenosine derivatives **1**–**11** and pK_a of the first acid ionization of the corresponding adenosine derivatives **1c**–**11c** (Scheme 1) at 25 °C, $[DBU]_0 = 10^{-2}$ M.

Adenosine derivative	Nucleoside	k_{obs} [10^2 min^{-1}]	$k_R^{T_1}$ [min^{-1} M^{-1}]	$k_R^{T_2}$ [min^{-1} M^{-1}]	pK_a[a]
1	A (**1c**)	64.3 ± 4.1	162.7 ± 8.1		12.17[c]
		46.6 ± 3.1[b]	117.8 ± 7.0[b]		
2	2′dA (**2c**)	0.1 ± 0.004			
3	3′dA (**3c**)	0.2 ± 0.004			
4	Am (**4c**)	0.2 ± 0.003			
5	1-dR (**5c**)	3.4 ± 0.1	22.6 ± 1.4		13.05[c]
6	Bz^{6}A (**6c**)	9.5 ± 0.7		2.9 ± 0.01	10.05[d]
		5.7 ± 0.4[b]		1.7 ± 0.01[b]	
7	G (**7c**)	5.1 ± 0.3		0.9 ± 0.005	9.17[e]
8	3zA (**8c**)	76.4 ± 4.2	243.6 ± 7.1		12.36[c]
9	Im (**9c**)	73.1 ± 4.1	272.7 ± 6.2		12.52[c]
10	U (**10c**)	5.8 ± 0.4		1.0 ± 0.003	9.17[f]
11	m^{3}U (**11c**)	71.8 ± 5.0	142.2 ± 5.1		11.95[c]

[a] pK_a values in aqueous solutions. [b] Measured by using EtOD instead of EtOH. [c] 2′/3′-OH. [d] N^{6}H. [e] N^{1}H. [f] N^{3}H.

11) and their pK_a measured by a method previously described.[6] The derived k_R values increase with increasing pK_a or the basic strength of the 2′/3′-oxyanion; this suggests a positive slope of the Brønsted plot. Although it would have been preferable to have a wider range of pK_a, it was impossible to further perturb the pK_a values of 2′/3′-OH without introducing secondary steric effects. This is supported by the negative deviation of the value for the 1-deoxy derivative **5**, which is known to have a different conformational preference[10] and therefore it is not considered to be in the same series as the other adenosine derivatives. Furthermore, if the $k_R^{T_2}$ values for derivatives **6**, **7** and **10** are calculated by using the known pK_a values 10.05, 9.16 and 9.17 for their amide N6H N1-H and N3-H amide ionization,[11] respectively, the values obtained fit (R = 0.998) a Brønsted plot with a slope of $\beta = 0.76$ well (Figure 1).

The pK_a dependence of the rate data (Figure 1) suggests participation of the 2′/3′-oxyanion in the transesterification of 2′/3′-peptidyl adenosine derivatives **1** and **5**–**11**. The rate acceleration increases with increasing oxyanion base strength. Such a Brønsted relationship is characteristic of general base-catalyzed ester hydrolysis[12] and, in this particular case, of intramolecular general base catalysis by the neighbouring 2′/3′-oxyanion ($k_R^{T_1}$ reaction mechanism in Scheme 1). This conclusion is supported by the kinetic isotope effect of $k_H/k_D = 1.38$ observed when EtOH is substituted by EtOD. The positive Brønsted β value and the primary kinetic isotope effect suggest a proton-in-flight in the rate-limiting transition state, consistent with concerted deprotonation of the attacking neutral alcohol molecule by the 2′/3′-oxyanion (Scheme 1). Moreover, the large positive β value and the small kinetic isotope effect suggest a significant movement of a proton towards the 2′-oxyanion concurrent with attack of the neutral ethanol molecule on the carbonyl carbon. We have recently probed a similar mechanism for the aminolysis of catechol monoesters and found that the proximity of a vicinal *o*-oxyanion greatly enhances the rate of peptide-bond formation.[13]

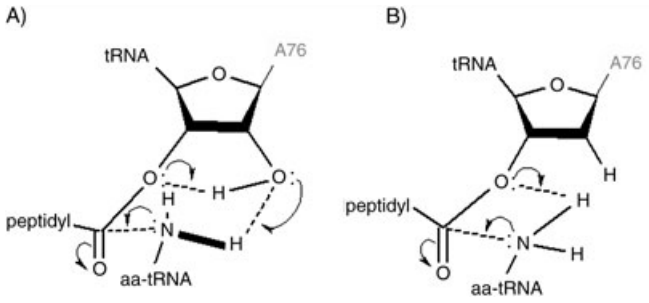
The transesterification of N6-benzoyl adenosine, guanosine and uridine derivatives **6, 7** and **10** deserves special attention. Due to the much lower pK_a values of ionization of their N6H, N1-H and N3-H amides than the pK_a of 2′/3′-OH (Table 1) and under the conditions used (much lower concentration of the substrate than the base), the concentrations of the 6-, 1- and 3-nitranions is much higher than those of the 2′/3′-oxyanions **6a, 7a** and **10a**. Furthermore, we assume a tautomerization of these nitranions to the 3-nitranions **6b** and **7b** (Scheme 1) and to the corresponding tautomeric 2-oxyanion **10b** (not shown in Scheme 1) that can be stabilized by a strong anionic hydrogen bond with 2′-OH. The presence of such a hydrogen-bonding interaction between 2′-OH and the adenin-9-yl N3 has been documented.[14] This intramolecular hydrogen bonding, favoured by the low-dielectric acetonitrile medium, induces a negative charge on the 2′-oxygen similar to that of 6- and 1-nitranions. An indication that this is what happens is the observation that the derived $k_R^{T_2}$ values for **6, 7** and **10** fit the Brønsted correlation line well (R = 0.998) when using the known pK_a values for amide NH. Therefore, the slower transesterification of N6-benzoyl adenosine and guanosine derivatives **6** and **7** can be attributed to the lower efficacy of the intramolecular general base catalysis by 2′-OH, hydrogen bonded to the 3-nitranion ($k_R^{T_2}$ reaction mechanism in Scheme 1). This interpretation is also supported by the kinetic isotope effect k_H/k_D of 1.66 observed for the proteoethanolysis and deuteroethanolysis of **6**.

Although the through-H-bond interaction of the nucleobase with 2′-OH (Scheme 1) is not possible in the case of **8** and **9** because of the N3 substitution and pyrimidine ring deletion, the pK_a dependence of their rate still holds (Table 1). This suggests a through-C1′–C2′-bond (inductive) effect of the nucleobase on the acidity of 2′-OH. Actually, the higher basicity of the 2′/3′-oxyanion in **9a** than in **8a** and **1a** can be attributed to its lower through-C1′–C2′-bond stabilization by the 1-imidazolyl aglycone. In the absence of the fused electron-deficient aromatic pyrimidine (as in **1a**) and pyridine (as in **8a**) moieties, the imidazolyl moiety in **9a** cannot drain its π charge and is therefore a poorer electron acceptor; this accounts for the lower acidity of 2′/3′-OH and therefore the enhanced basicity of the corresponding 2′/3′-oxyanion. Thus, the nucleobase is able to steer the general base efficiency of the 2′/3′-oxyanion by inductive stabilization of its negative charge. It is noteworthy that this through-C1′–C2′-bond interaction provides a negative charge on the 2′/3′-oxygen already in the neutral molecule that is not changed after proton loss of 2′/3′-OH.[15]

For the lack of a 2′/3′-OH group or a free 2′/3′-OH, the transesterification of 2′/3′-deoxy and 2′-*O*-methyl derivatives **2**, **3** and **4**, cannot proceed by the intramolecular general base-catalytic mechanism proposed for the rest of the adenosine derivatives studied. In the case of these substrates, general base catalysis by an external general base is required for an effective transesterification.

After this paper has been submitted, Weinger et al.[16] reported important molecular biological evidence for peptidyl tRNA A76 2′-OH playing an essential catalytic role in the ribosome catalytic activity. This finding is complementary to the results of this paper since the latter underlines the mechanism of the substrate-assisted catalytic effect of the peptidyl tRNA A76 2′-OH.

While mutations of many universally conserved nucleotides (A2451, U2506, U2585 and A2602) in the active site of the large subunit of the ribosome have a limited effect on the rate of the peptidyl transferase reaction when the substrates are intact aminoacyl-tRNAs,[5d] substitution of the 3′-terminal adenosine 2′-OH of the genuine peptidyl tRNA by 2′-H or 2′-F gives rise to a more than 1 000 000-fold rate decrease.[16] Therefore, the main contribution to the overall catalysis of the ribosomal peptide-bond formation comes from the catalytic action of the 3′-terminal 2′-OH of peptidyl tRNA. This large difference in reactivity, however, is not observed in the model reaction studied here: the rate diminishes by only about 600 times when 2′/3′-OH in peptidyl adenosine **1** is substituted for 2′/3′-H (deoxy derivatives **2** and **3**) and 300 times when it is substituted for 2′-OCH_3 (derivative **4**; Table 1). If the role of the 2′-OH were to act as a general base catalyst only, the 2′-F derivative in the ribosome reaction and 2′-OCH_3 derivative **4** in the model reaction (Scheme 1) ought to react faster than the corresponding 2′-deoxy derivatives since both can serve as a general base.[16] That this is not the case suggests that the proton of the 2′-OH group participates in the acceleration of the reaction. Its absence in the peptidyl adenosine 2′-oxyanion **1a** or its substitution by methyl group in the 2′-OCH_3 derivative **4** prevents the expected acceleration in the model reaction (Scheme 1). Moreover, without this proton, the fluorine atom is not able to assist the ribosomal reaction.[16] These findings are consistent with the possibility of the 3′-terminal adenosine 2′-OH of the peptidyl tRNA acting both as general acid and general base. This happens in a cyclic hexagonal transition state (Scheme 2A) in which proton abstraction from the α-NH_2 of



Scheme 2. *A)* Proposed hexagonal transition state for the ribosome-catalyzed aminolysis of genuine peptidyl tRNA and B) tetragonal transition state for the ribosome aminolysis of 2′-deoxy peptidyl tRNAp.

aminoacyl tRNA by the A76 2′-OH is concerted with a proton donation by the same group to the incipient leaving tRNA 3′-oxyanion. This hydrogen-bond interaction has long been suggested.[2] Thus, the peptidyl tRNA A76 2′-OH appears to act as a proton shuttle in the substrate-assisted mechanism of the ribosome, providing a facile pathway for a proton transfer from the incoming α-amino group of aminoacyl tRNA to the leaving tRNA A76 3′-oxyanion. A similar substrate-assisted catalytic mechanism was originally proposed by Das et al.[17] based on an in silico study and found some experimental support very recently.[3a,c] This catalytic role of A76 2′-OH as a proton shuttle is not unique in biocatalysis: the imidazole of histidine is a similar bifunctional general acid–base catalyst in the active site of serine and cysteine proteases.[18] On the other hand, the aminolysis of 2′-deoxy derivatives in the absence of an external general base proceeds through a higher-energy tetragonal transition state (Scheme 2B, M.A.R, G. Vayssilov, D.D.P., unpublished data), here the abstraction of the α-NH_2 proton is carried out directly by the incipient leaving tRNA 3′-oxyanion.

Quite recently Sievers et al.[5c] compared the measured activation parameters for ester aminolysis in the ribosome and in solution, and, on the basis of a small difference in the activation enthalpy, came to the conclusion that "general acid–base catalysis does not play a significant role in peptidyl transfer in the ribosome". Their reference reaction (ester aminolysis by tris(hydroxymethyl)aminomethane; TRIS), however, is not congruent to the ribosome ester aminolysis reaction. Unlike aminolysis by amino acid amides, aminolysis by TRIS proceeds with NH_2-assisted alcoholysis by the vicinal OH group, followed by O–N acyl group migration in the resulting TRIS ester to form the final TRIS amide.[19] Since the pK_a of the aminoacyl tRNA amino group (ca. 8) is much lower than that of the leaving tRNA 3′-OH (ca. 12), a general base catalyst switching on the attack of the amine anion (p$K_a > 30$) is a fundamental requirement for the acceleration of peptide-bond formation.

In conclusion, the results of the present physical organic study including LFER and kinetic isotope effects reveal general base catalysis by the 2′-oxyanion of the peptidyl adenosine transesterification. The finding raises the mechanistic possibility for a general base catalysis by the partially deprotonated 2′-OH of the peptidyl tRNA A76 during the ribosome-catalyzed peptide-bond formation. This is possible provided it donates a proton to the adjacent 3′-oxygen in a cyclic hexagonal transition state (Scheme 2A). Thus, the peptidyl tRNA A76 2′-OH appears to act as a proton shuttle in the substrate-assisted mechanism of the ribosome, providing a facile pathway for transfer of the aminoacyl tRNA α-NH_2 proton to the leaving tRNA A76 3′-oxyanion. Hence, one of the roles of the ribosome active site is to recognize this cyclic hexagonal transition state. The aminolysis of 2′-deoxy and 2′-fluoro derivatives of the peptidyl tRNA is not catalyzed by the ribosome,[16] since the latter is not complementary to the cyclic tetragonal transition state (Scheme 2B) required for this reaction in the absence of an external general base.

Experimental Section

Preparation of the 2′/3′-*O*-peptidyl adenosine derivatives 1–11: 5′-*O*-trityl-2′/3′-*O*-(*N*-benzyloxycarbonyl-L-*p*-nitrophenylalanyl) nucleosides **1**–**11** (Tr-N[Z-nF]) were prepared by transesterification of *N*-(benzyloxycarbonyl)-L-*p*-nitrophenylalanine cyanomethyl ester (Z-nF-OCH_2CN) by the general method described previously.[20] 5′-

O-Trityl adenosine derivatives **1c**–**11c** were obtained by using the best methods in the literature for 5′-*O*-tritylation of nucleosides. Adenosine (A), 2′-deoxyadenosine (2′-dA), dA, 3′-deoxyadenosine (cordycepin, 3′-dA), 2′-*O*-methyladenosine (Am), guanosine (G), uridine (U) and *N*3-methyl uridine (m^3U) were from Sigma. 1-deoxy-D-ribofuranose (1-dR),[21] 3-deazaadenosine (3zA),[22] 1-β-D-ribofuranosyl imidazole (Im)[23] and N6-benzoyl adenosine (Bz^6A)[24] were prepared as previously described. The general procedure used for 2′/3′-*O*-monoaminoacylation of 5′-*O*-tritylated adenosine derivatives **1c**–**11c** and characterization data for the resulting 2′/3′-peptidyl 5′-O-trityl adenosine derivatives **1**–**11** are found in the Supporting Information.

Kinetic studies: The transesterification (ethanolysis) of the 2′/3′-*O*-peptidyl adenosine derivatives **1**–**11** was followed by monitoring the changes both in the concentration of the substrate **1**–**11** itself (HPLC retention time RT ca. 15 min depending on the substrate) and the two products, *N*-benzyloxycarbonyl-L-*p*-nitrophenylalanine ethyl ester **12** (RT ca. 7 min) and the corresponding 5′-*O*-trityl adenosine derivatives **1c**–**11c** (RT ca. 5 min) by using a Waters Liquid Chromatograph equipped with absorbance detector model 441 set at 254 nm and a Nucleosil 100-5C_{18} analytical column (12.5 cm × 4.6 mm). Reactions were carried out in stoppered tubes, which were immersed in a water bath thermostated at 25 °C. A description of a typical kinetic experiment is found in the Supporting Information.

Isotope-effect studies: Kinetic experiments for determination of the kinetic isotope effect were carried out by simple substitution of EtOH by deuteroethanol (EtOD).

pK_a determination: The pK_a values of the 2′/3′-OH of 3-deazaadenosine (3zA) **8c**, 1-β-D-ribofuranosyl imidazole (Im) **9c** and *N*3-methyl uridine **11c** (m^3U) in aqueous solutions were determined by using pH-dependent proton chemical shifts measured at 25 °C and 500 MHz (Brucker AMX 500) in D_2O as described previously for adenosine **1c** and 1-deoxyribose **5c**:[6] the pK_a of DBU in acetonitrile is known to be 23.9.[25] The pK_a^N values of the vicinal OH in 2′/3′-*O*-peptidyl adenosines **1**–**11** in this solvent, however, have never been measured since as esters they are hydrolytically unstable at high pH. To this end, these pK_a^N values in acetonitrile were estimated by using the pK_a values of the corresponding ribonucleosides in aqueous solutions[6] and $\Delta pK_a = 0.5$ and $\Delta pK_a = 14$ for the decrease of diol acidity after 2′/3′-O-monoacylation in water[6] and in acetonitrile,[26] respectively.

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