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Linear free energy relationships and kinetic isotope effects reveal the chemistry of the Ado 2′-OH group

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Abstract—Using kinetic isotope effects (KIE) and Hammett correlations, we show that the main role of the adenosine 2′-OH group on deprotonation by the non nucleophilic base DBU during external acyl group transfer is to generate enhanced electron density on the attacking nucleophile through ionization. The small primary KIEs (1.2 and 1.6) and the large Hammett reaction constants (+2.25 and +3.19) obtained for the ethanolysis of 2′/3′-*O*-*p*-substituted benzoyl 5′-*O*-trityl adenosines and 2′-deoxyadenosines are consistent with an $A_N + D_N$ reaction mechanism. The implications of our results are discussed in terms of chemical contributions of the 2′-OH group in the ribosome catalysis of peptide bond formation.

Ribosome catalyzes peptide bond formation between aminoacyl-tRNA (aa-tRNA)[1] bound to the A site of the ribosome and peptidyl-tRNA at the P-site. The peptide bond is formed as a result of nucleophilic attack by the α-amino group of aminoacyl-tRNA on the ester carbonyl group of peptidyl-tRNA. The first step is deprotonation of the α-NH_3^+ group to create the nucleophilic NH_2 group. Subsequent nucleophilic attack of the α-NH_2 group on the electrophilic carbonyl group leads to the formation of the zwitterionic tetrahedral intermediate, which by deprotonation forms a negatively charged tetrahedral intermediate. The breakdown of the tetrahedral intermediate is initiated by donating a proton back to the leaving oxygen to form the products. The 2′-OH group might be involved in proton transfer, acting either as a general-base to extract a proton from the amine or as a general acid to provide a proton to the 3′-OH group. A recent study presented crystal structures of A-site and P-site substrates bound simultaneously, as well as an analogue of the tetrahedral intermediate that includes the A76 2′-OH providing the most complete picture of the active site to date.[2,3] According to these structures, the 2′-OH hydrogen bonds with the α-NH_2 group in the substrate structure. The 2′-OH is most certainly involved in aligning the nucleophile prior to reaction; however, it is also noted that the 2′-OH is the only functional group positioned to act as a general-base in the intermediate structure. In principle, ribosome can catalyze peptide bond formation via several mechanisms, such as proper positioning of the peptidyl and aminoacyl substrates in the active site, general acid–base catalysis during deprotonation and protonation, or electrostatic stabilization of the transition states.[1,4–9] Substrate assisted catalysis,[10] ribosome being an entropy trap[11] in which the increase of ΔS^* is a primary thermodynamic motivation for the catalysis and 2′-OH acting as a 'proton shuttle' catalyst[12] have also been proposed. Here, we reveal in detail the reaction mechanism and the role of the 2′-OH group as a vicinal mediator in the proton transfer for a model transesterification reaction. For the ethanolysis of 2′/3′-*O*-*p*-substituted benzoyl 5′-*O*-trityl adenosines and 2′-deoxyadenosines (Scheme 1), we obtained Hammett plots with two different reaction constants, $\rho_A = 3.19$ and $\rho_{dA} = 2.25$, respectively. These values imply that the reaction for the two types of substrates proceeds through a similar $A_N + D_N$ mechanism but with different transition state structures. The latter is strongly supported by the primary kinetic isotope effects (KIE) of 1.2 and 1.6 observed for the ethanolysis of the two types of substrates.

External transesterification is known to be catalyzed by the ribosome,[13,14] in which the P-site excludes water until the moment when release factors interact with a stop codon.[2] To mimic the environment of this site we studied the ethanolysis of the substrates in the aprotic polar organic solvent acetonitrile using the strong, bulky organic base 1,8-diazabicyclo[5.4.0]-undec-7-en (DBU)

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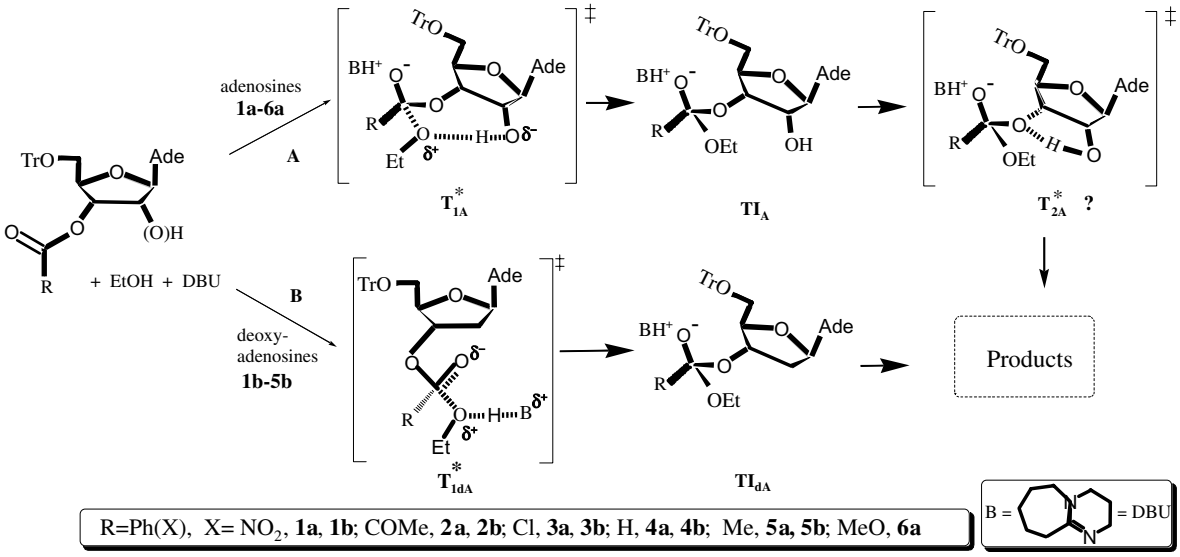

Scheme 1. Proposed $A_N + D_N$ mechanism of the ethanolysis of 2′/3′-*O*-*p*-X-substituted benzoyl 5′-*O*-trityl adenosines (**1a**–**6a**), **A** and 2′-deoxyadenosines (**1b**–**5b**), **B**, pK_a of DBU in acetonitrile is 23.9 pK_a units.[25]

as basic catalyst. k_{obs} constants (Table 1) were obtained from the pseudo-first order decrease of the substrate concentration in HPLC chromatograms. Normal kinetic isotope effects, k_H/k_D, were obtained for the ethanolysis of both types of substrates when EtOH was substituted with EtOD. The pseudo-first order rate constant ratio k_{obsX}/k_{obsH} for the DBU-catalyzed ethanolysis of the 2′/3′-*O*-*p*-X-substituted benzoyl 5′-*O*-trityl adenosines and the 2′-deoxyadenosine derivatives were determined and used to construct the Hammett plots shown in Figure 1. They fit the plots ($R = 0.993$ and 0.999, respectively) with a slope $\rho_A = 3.19$ for the adenosine series and $\rho_{dA} = 2.25$ for the deoxyadenosine series.

These values show that there is a substantial change in the charge of the reaction centre from the ground state to the transition state at the rate-determining step. The large positive reaction constants ($\rho_A = 3.19$ and $\rho_{dA} = 2.25$) and the high pK_as for both the nucleophile (p$K_a = 16$) and the leaving group (p$K_a \sim 12.5$) unambiguously demonstrate that the transesterification of the 2′/3′-*O*-*p*-X-substituted benzoyl 5′-*O*-trityl adenosines and 2′-deoxyadenosines proceeds through tetrahedral addition intermediates $\mathbf{TI_A}$ and $\mathbf{TI_{dA}}$ shown in Scheme 1. Their formation is rate-determining since the nucleofuge for both substrates is less basic and is a better leaving group than the attacking nucleophile. There is no doubt from the Hammett plots obtained and the pK_a values that the transesterification of both the adenosine and deoxyadenosine substrates proceeds through a similar stepwise $A_N + D_N$ mechanism. It is unlikely that the transesterification of these substrates would proceed through a concerted A_ND_N mechanism. There are many reports of tetrahedral adducts between acyl functions and nucleophiles,[15,16] indicating that a stepwise addition–elimination AE ($A_N + D_N$) mechanism is involved in the transfer of an acyl group between strong nucleophiles. There is substantial evidence from both substituent effect[17] and isotope effect[18] studies that the carbonyl group transfer reactions proceed through a concerted A_ND_N mechanism when the nucleophile and the leaving group are only weakly basic. Acyl functions with very good leaving groups and possessing features stabilizing an acylium ion favour stepwise displacement reactions with a $D_N + A_N$ mechanism[19] whereas poor leaving groups confer an $A_N + D_N$ mechanism.[20] Since 2′/3′-*O*-*p*-substituted benzoyl 5′-*O*-trityl adenosines **1a**–**6a** are mixtures of 3′ and 2′-isoforms (see Supplementary data) one could suppose that they compete during the ethanolysis. However, it has long been known that acyl migration in the 2′,3′-*cis*-diol system of nucleosides takes place very rapidly.[21] The respective half times of hydrolysis and equilibration of 2′(3′)-*O*-acetyluridine in 0.1 M phosphate buffer at 20 °C are ca. 30 days and

Table 1. Kinetic data and kinetic isotope effects at 25 °C for the ethanolysis of 2′/3′-*O*-*p*-substituted benzoyl 5′-*O*-trityl adenosines (**1a**–**6a**) and 3′-*O*-*p*-substituted benzoyl 5′-*O*-trityl 2′-deoxyadenosines (**1b**–**5b**) in acetonitrile

p-X	Adenosine derivatives k_{obs} (min^{-1})		KIE	Deoxyadenosine derivatives k_{obs} (min^{-1})		KIE	σ
	EtOH	EtOD		EtOH	EtOD		
NO_2 (**1**)	27.92 ± 1.12	22.75 ± 0.68	1.2	0.052 ± 0.002	$0.0324 \pm 9.7 \times 10^{-4}$	1.6	0.78
COMe (**2**)	1.54 ± 0.06	1.28 ± 0.05	1.2	0.009 ± 0.003	$0.0060 \pm 1.5 \times 10^{-5}$	1.5	0.50
Cl (**3**)	0.42 ± 0.02	$0.35 \pm 8 \times 10^{-3}$	1.2	$0.0025 \pm 7 \times 10^{-5}$	$0.0016 \pm 4.2 \times 10^{-5}$	1.6	0.23
H (**4**)	0.06 ± 0.002	$0.05 \pm 1 \times 10^{-3}$	1.2	$0.0007 \pm 2 \times 10^{-5}$	$0.0004 \pm 1.6 \times 10^{-5}$	1.6	0.0
Me (**5**)	0.03 ± 0.002	$0.025 \pm 1 \times 10^{-3}$	1.2	$0.0003 \pm 1 \times 10^{-5}$	$0.0002 \pm 6 \times 10^{-6}$	1.5	−0.17
MeO (**6**)	0.007 ± 0.0004	$0.0057 \pm 3 \times 10^{-4}$	1.2	—	—	—	−0.27

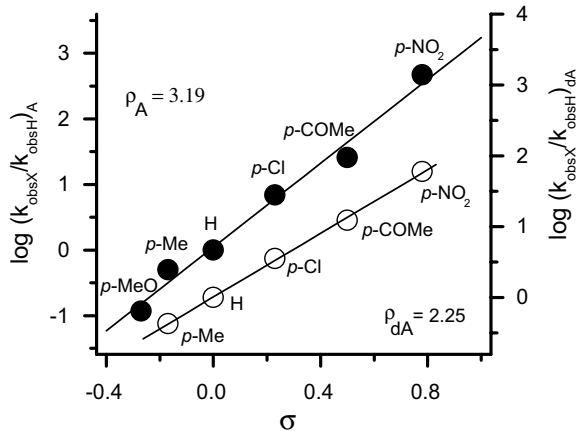

Figure 1. Hammett plots of the ethanolysis of 2′/3′-*O*-*p*-X-substituted benzoyl 5′-*O*-trityl adenosines (●) and 2′-deoxyadenosines (○).

7.5 s, respectively, their ratio being 350,000. The latter implies that there is no competition between 3′ and 2′-isoforms during the external acyl group transfer (ethanolysis).

Qualitatively, the fundamental mechanism of enhancing the nucleophilic power of any given oxygen atom is to enhance the electron density of the nucleophilic lone electron pair. This leads to three possible molecular mechanisms for generating enhanced electron density in the reactant state of the nucleophile[22] by altering the coordination and/or bonding of the nucleophile: (1) desolvation or more precisely the stripping away of H-bond donors to the nucleophilic lone pair, (2) coordination of the alcohol proton, that is, H-bonding to a general-base and (3) ionization. In addition to these three mechanisms, an electrical field generated at the enzyme active site can polarize the bond to the nucleophile enhancing its nucleophilicity.

In our model reaction, we observed normal primary kinetic isotope effects for the ethanolysis of both adenosines **1a**–**6a** and deoxyadenosine **1b**–**5b** substrates of 1.2 and 1.6, respectively. The normal kinetic isotope effects are indicative of a proton 'in flight' at the transition state of the rate-determining step; however, the observed kinetic isotope effects for the ethanolysis of 2′/3′-*O*-*p*-substituted benzoyl 5′-*O*-trityl adenosines (**1a**–**6a**) are well below 2 (Table 1), which is the generally accepted threshold for distinguishing general-base catalysis. More likely, the reason in this case for the generated enhanced electron density on EtOH is ionization by the DBU-deprotonated 2′-OH group; however, general-base catalysis could not be entirely excluded, as the pK_a of the 2′-OH group complies well with Jencks' libido rule[23] and can act as a general-base. The 2′-oxyanion is obviously more efficient in increasing the nucleophilic power of EtOH than the external nonnucleophilic base DBU. The generated enhanced electron density of the 2′-oxyanion results in a greater bond order between the nucleophile and the electrophilic carbonyl group, which leads to the formation of a full negative charge on the carbonyl oxygen, as indicated by the large Hammett reaction constant of 3.19. The small kinetic isotope effect of 1.2 for the adenosine substrates is in agreement with the larger reaction constant of 3.19 since the first shows significant movement of the ethanol OH proton towards the 2′-oxyanion at the transition state $\mathbf{T}^{*}_{\mathbf{1A}}$ and the latter advanced bond formation between the attacking nucleophile (EtO^{-} anion) and the carbonyl carbon at that rate-determining transition state (Scheme 1 **A**). The greater kinetic isotope effect of 1.6 for the deoxyadenosine substrates reveals a more symmetric transition state $\mathbf{T}^{*}_{\mathbf{1dA}}$ (Scheme 1 **B**) in which the total change in the zero-point energies is greater than that in the $\mathbf{T}^{*}_{\mathbf{1A}}$ state and an increased kinetic isotope effect is observed for the transesterification of the deoxyadenosine substrates. It is in good agreement with the reaction constant of 2.25 showing a more planar transition state $\mathbf{T}^{*}_{\mathbf{1dA}}$ in which the bond order between the nucleophile and the carbonyl group has a lower extent than that in the transition state $\mathbf{T}^{*}_{\mathbf{1A}}$. The good agreement between the reaction constants and the kinetic isotope effects proves that the proton transfer and the nucleophilic attack are concerted for the ethanolysis of both kinds of substrates. Tetrahedral intermediate $\mathbf{TI_A}$ breakdown is likely to proceed through transition state $\mathbf{T}^{*}_{\mathbf{2A}}$ in which departure of the leaving group is facilitated by the 2′-OH as the latter donates a proton back to the leaving oxygen, making it a better leaving group. Adenosine is a better leaving group than deoxyadenosine; however, we did not observe a break in the Hammett plot for the ethanolysis of the deoxyadenosine substrates. This shows that there is no change in the rate-determining step for the transesterification of the deoxyadenosines **1b**–**5b**, and it follows a similar $A_N + D_N$ mechanism. These studies clearly show that the role of the deprotonated 2′-OH group in the transesterification is to enhance the nucleophilicity of the attacking nucleophile generating enhanced electron density on it either by ionization or by general-base catalysis. It can be seen from the kinetic data (Table 1) that the amount of anchimeric assistance by the vicinal OH group is enormous, up to 600 fold. Probably A 76 2′-OH group has a similar role in peptide bond formation, enhancing nucleophilic power of the attacking nucleophile. The pK_a of the α-NH_3^+ group in aa-tRNA is estimated to be around 8 and it is likely that the proton to be accepted by a water molecule[24] followed by subsequent nucleophilic attack of the α-NH_2 group is facilitated by the 2′-OH, the latter is supported by the recently presented crystal structures.[2,3] Taking into account the high pK_a of the activated α-NH_2 group and that of the depeptidylated tRNA A76 2′/3′-OH as a leaving group, we can speculate that the peptide bond formation could proceed through a similar $A_N + D_N$ mechanism. It involves the formation of a highly associative transition state in which the proton transfer to the A76 2′-OH and the nucleophilic attack of the α-NH_2 group are concerted. An additional tetrahedral intermediate is formed as its structure resembles strongly that of the transition state. We propose a mechanism of peptide bond formation quite similar to that of the transesterification of 2′/3′-*O*-*p*-substituted benzoyl 5′-*O*-trityl adenosines **1a**–**6a** depicted in Scheme 1 **A**. Although the proposed mechanism for peptidyl transferase reaction is certainly an oversimplification, it nevertheless provides a description of the transition state

and the reaction path of peptide bond formation which will not be altered greatly by the specificities of the EtOH and the α-NH_2 group as O and N nucleophiles.

This study shows that the main role of the deprotonated 2′-OH group in our model transesterification reaction is to enhance the nucleophilic power of the attacking nucleophile. The Hammett correlations and kinetic isotope effects support a stepwise $A_N + D_N$ reaction mechanism in which attack of the nucleophile is rate-determining. Arguments show that a similar $A_N + D_N$ mechanism (Scheme 1 **A)** probably operates for the peptide bond formation.

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Supplementary data

Experimental procedures and characterization of the compounds. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2007.01.139.

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