Reactions of Adenosine with Bromo- and Chloromalonaldehydes in Aqueous Solution: Kinetics and Mechanism

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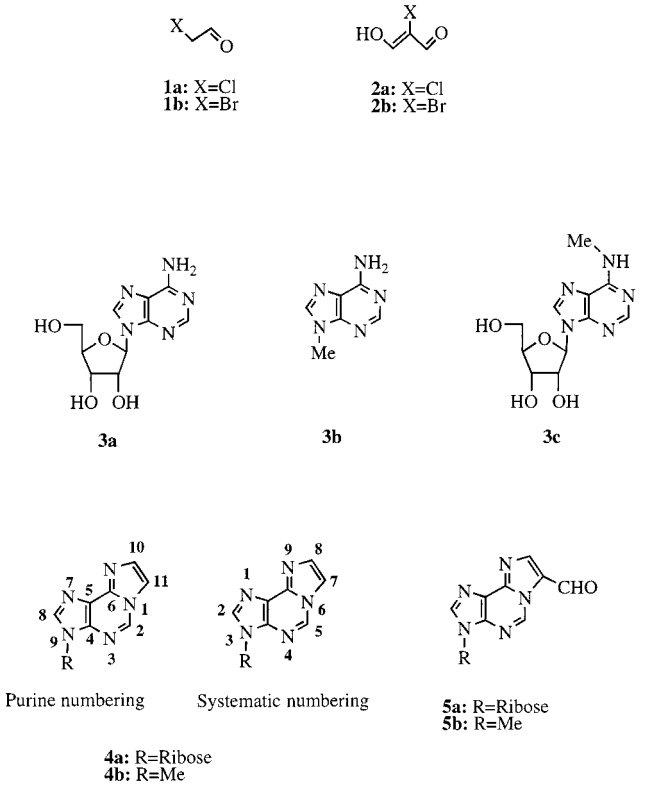
Reactions of adenosine nucleosides with halogen substituted acetaldehydes and malonaldehydes have been studied and pseudo first-order rate constants have been determined. All the reactions yield 1,N^6-etheno adducts, and with malonaldehydes, in addition to this, 11-formyl-1,N^6-etheno adducts are also formed. Particular attention has been paid to the formation of the formyletheno products. The results obtained suggest that the reactions of adenine base with halogenated acetaldehydes and malonaldehydes are basically similar. It also seems that in reactions of halomalonaldehydes with adenosine, the etheno and formyletheno products are formed through the same initial reaction pathway i. e. the attack of the 6-amino group of the adenine base at the carbonyl carbon atom of the aldehyde.

Introduction

Formation of etheno adducts of nucleic acid bases has a dual role in the chemistry of DNA. Firstly, miscoding in DNA synthesis owing to such adducts has been established both in vitro and in vivo,[1] and their formation has been identified as the reason for mutagenicity of several bifunctional halo compounds including vinyl chloride,[1a,1d,2] a widely used industrial chemical, and chlorinated hydroxyfuranones,[3] side products of chlorination of tap water. Many of the mutagenic halo compounds are converted intracellularly to halogenated acetaldehydes or malonaldehydes, which are assumed to react with DNA bases.[4]

In addition to the undesired mutagenic effects, the etheno adducts of nucleobases also have properties that make them useful tools for nucleic acid chemistry. Since the etheno adducts are fluorescent, they are extensively used as surrogates of natural nucleotides in biochemical studies. Ever since the early works of Kochetkov,[5] the rapid and clean reactions of haloacetaldehydes **1a**,**b** with nucleic acid bases, yielding ethenonucleosides, have been utilized extensively in the probing of nucleic acid structures.[6] The corresponding reactions of halomalonaldehydes **2a**,**b** appear even more attractive, since the reactive formyl function of the resulting formyletheno adduct allows for the further derivatization of the polynucleotide chain.

The kinetics and mechanisms of the reactions of haloacetaldehydes **1a**,**b** with nucleosides and DNA have been studied rather extensively.[7] Chloro- and bromoacetaldehydes have been shown to react with adenine, cytosine and guanine nucleosides giving 1, N^6-ethenoadenine,[3c,6,7] 3,N^4-ethenocytosine[3c,6,7a–7c] and 1,N^2-or 2,N^3-ethenoguanine[8] adducts, respectively. It has been suggested that the carbonyl carbon atom of the aldehyde is initially attacked by the exocyclic amino function of a nucleobase and the halogen substituent is then displaced by intramolecular nucleophilic attack of the neighboring ring nitrogen (Scheme 1). The cyclic carbinolamine intermediate is finally dehydrated to the etheno product.



The reactions of halomalonaldehydes **2a**,**b** with nucleobases have been less thoroughly studied. The reaction with adenosine (**3a**) has been reported to give two etheno prod-

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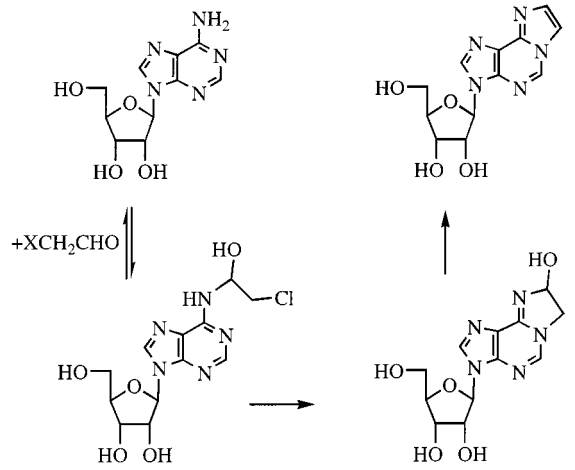
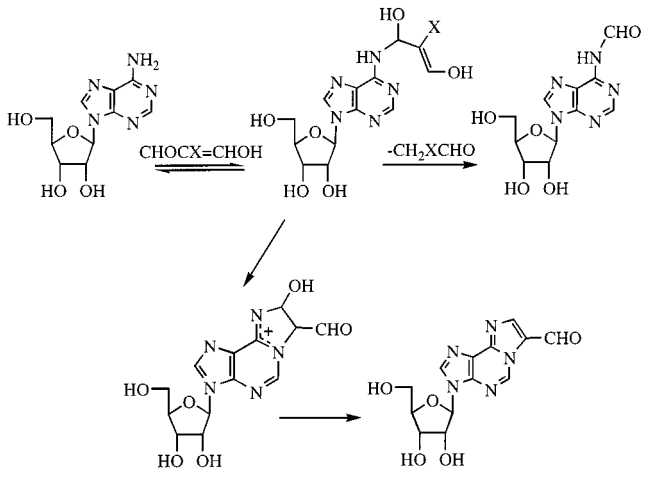


Scheme 1

ucts 1,N^6-ethenoadenosine (**4a**) and 11-formyl-1,N^6-ethenoadenosine (**5a**),[2d,3b] but the mechanism is not known in detail. According to Nair et al.,[2d] the carbonyl carbon atom of the aldehyde is initially attacked by the 6-amino group of adenosine, and the carbinolamine intermediate is either cyclized and dehydrated to **5a** or decomposed to haloacetaldehyde and N^6-formyladenosine (Scheme 2). Compound **4a** is then formed as a secondary product by the reaction of adenosine with the haloacetaldehyde as depicted in Scheme 1. The suggestion is, however, based only on product analysis and the fact that secondary amines are known to accelerate the cleavage of bromomalonaldehyde to bromoacetaldehyde by attacking the carbonyl carbon atom.[9] Kronberg et al.[3a,10] have more recently proposed a similar mechanism, but regarded spontaneous cleavage of halomalonaldehyde as a reasonable alternative for the formation of the haloacetaldehyde. The present paper describes our kinetic studies on the reactions of adenosine (**3a**) with chloromalonaldehyde (CMA; **2a**) and bromomalonaldehyde (BMA; **2b**). For comparative purposes, and to facilitate the mechanistic interpretation of the kinetic results, the reactions with chloroacetaldehyde (CAA; **1a**) and bromoacetaldehyde (BAA; **1b**) were also studied under identical conditions. Special attention was paid to the competition between the formation of **4a** and **5a** under various conditions and detection of unstable intermediates. Comparative measurements were also carried out with 9-methyladenine (**3b**) to simplify the LC-MS analysis of the reaction intermediates observed, and with N^6-methyladenosine (**3c**) to facilitate the detection of possible carbinolamine intermediates. Replacement of one of the N^6-hydrogens of adenosine with a methyl group may be expected to retard the dehydration of carbinolamine intermediates, and hence such intermediates are in all likelihood accumulated to a larger extent.



Scheme 2

Results

The reactions of the adenine substrates **3a**–**c** with halomalonaldehydes **2a**,**b** and haloacetaldehydes **1a**,**b** were studied at 60 °C by determining the composition of aliquots withdrawn at appropriate intervals by RP-HPLC. The measurements were carried out under pseudo first-order conditions, the concentration of **3a**–**c** being 0.1 mM, and that of the aldehyde at least 10 times higher. Under these conditions, the decrease in the concentration of the aldehyde can be considered to become insignificant. The decrease in the concentration of the adenine derivative was followed as a function of time, and a first-order rate constant was calculated by applying the integrated first-order rate law. The products formed in the reactions of adenosine (**3a**) were identified by spiking with authentic samples. Those from the reactions of 9-methyladenine (**3b**) and N^6-methyladenosine (**3c**) were isolated by semipreparative RP-HPLC and identified by ^{1}H- and/or ^{13}C NMR spectroscopy and LC-ES/MS analysis. Consistent with previous reports,[6,7] the reactions of **3a**,**b** with halomalonaldehydes were observed to give both 1,N^6-etheno (**4a**,**b**) and 11-formyl-1,N^6-etheno (**5a**,**b**) adducts, and those with haloacetaldehydes only the etheno adducts. It is worth noting that the formation of **4b** by the reaction of **3b** with BMA (**2b**) is in contradiction with a previous report.[2d] The products of the subsequent decomposition of the etheno adducts were not fully characterized in the present work, but it has been previously shown that **4a** is cleaved under acidic[7d] and basic[11] conditions to form bisimidazole derivative **6**.

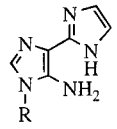


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Figure 1 shows the pseudo first-order rate constants for the disappearance of adenosine (**3a**) as a function of the concentration of the halomalonaldehyde or haloacetaldehyde. As seen, the reactions of adenosine with BMA are about one order of magnitude slower than the reactions with BAA. With chlorosubstituted aldehydes such reactivity difference was observed only at high aldehyde concentration. With both types of aldehydes, the bromosubstituted compounds seem to be more reactive than the chloro-substituted ones. The higher reactivity of BAA relative to CAA

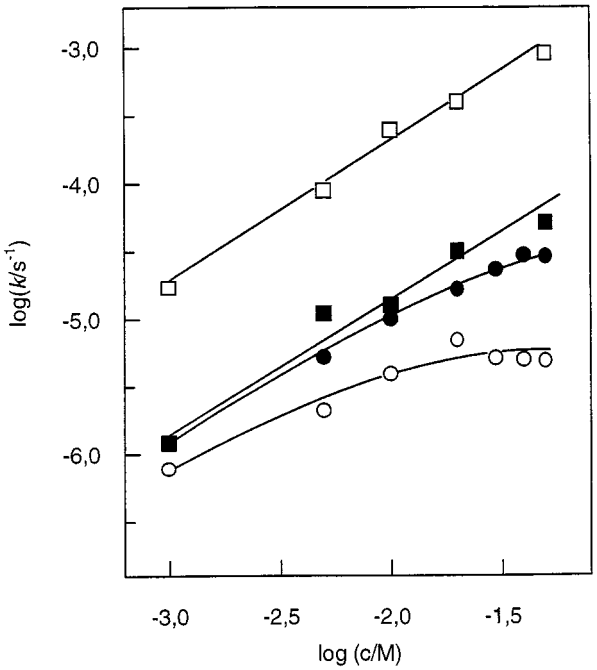


Figure 1. Logarithmic rate constants for the reactions of adenosine (**3a**) with haloacetaldehydes (**1a, b**) and halomalonaldehydes (**2a, b**) as a function of the aldehyde concentration at pH 4.7 and 60 °C. I = 0.1 M ($NaNO_3$); notation: □ bromoacetaldehyde; ■ chloroacetaldehyde; ● bromomalonaldehyde and ○ chloromalonaldehyde

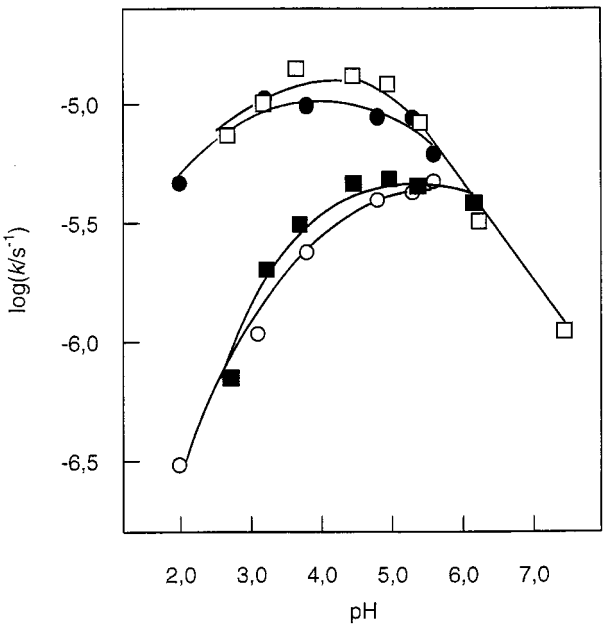


Figure 2. Logarithmic rate constants for the reactions of adenosine (**3a**) and 9-methyladenine (**3b**) with halomalonaldehydes (**2a, b**) as a function of pH at 60 °C; [aldehyde] = 10 mm, I = 0.1 M ($NaNO_3$); notation: □ 9-methyladenine with BMA; ■ 9-methyladenine with CMA; ● adenosine with BMA and ○ adenosine with CMA

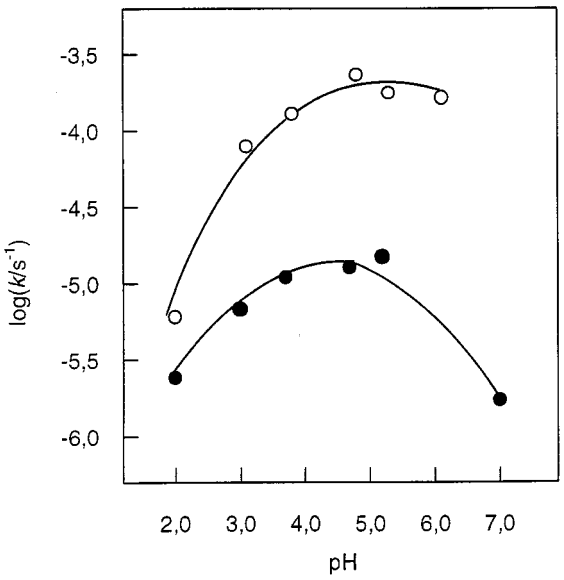


Figure 3. Logarithmic rate constants for the reactions of adenosine (**3a**) with BAA (○) and with CAA (●) as a function of pH at 60 °C; [aldehyde] = 10 mm, I = 0.1 M ($NaNO_3$)

has also previously been observed on reacting these aldehydes with ATP.[12] The reactivity difference between BMA and CMA is, however, smaller than that between BAA and CAA. The dependence of reaction rate on the aldehyde concentration is also different with halomalonaldehydes and haloacetaldehydes. While the reaction with haloacetaldehydes is approximately first-order in the aldehyde concentration over the entire concentration range studied, a clear deviation from the first-order dependence is observed with halomalonaldehydes. With CMA, the rate constants obtained seem to level off to a constant value at high aldehyde concentrations, whereas with BMA the phenomenon is much less pronounced.

Figure 2 shows the pH rate profiles for the reactions of the adenine substrates **3a, 3b** with CMA and BMA. The rate profiles of all the reactions share a common feature: the reaction rate passes through a broad maximum under mildly acidic conditions, and hence only a rather moderate effect of pH on the rate is observed. The greatest dependence on pH was observed in reactions with CMA, where an increase of three units in pH resulted in only a ten-fold rate enhancement. Similar behaviour has previously been observed with haloacetaldehydes.[5,6b,7a,12] This was also seen in the present work (Figure 3). The pH at which the maximum reactivity is observed seems, to some extent, to depend on the identity of the reactants in question, in particular on the halogen substituents of the aldehyde. The maximum value is observed between pH 4 and 5 with BMA and between pH 4.5 and 5.5 with CMA. The reaction of N^6-methyl adenine with BMA is almost entirely pH-independent (data not shown). The buffer concentration had practically no effect on the rate of the disappearance the adenine substrate. The pseudo first-order rate constant for the reaction of adenosine with BMA, for example, remained unchanged (1.2×10^{-5} s^{-1}) on increasing the concentration of a 1:1 formic acid/sodium formate buffer from 0.1 M to 0.5 M (data not shown).

The rate constants for the breakdown of 1,N^6-ethenoadenosine (**4a**) as a function of pH are shown in Figure 4. The dependence of the rate of the reaction on [H^+] seems to become more significant as the pH decreases. The reaction is nearly pH-independent at pH > 3; on going from pH 6

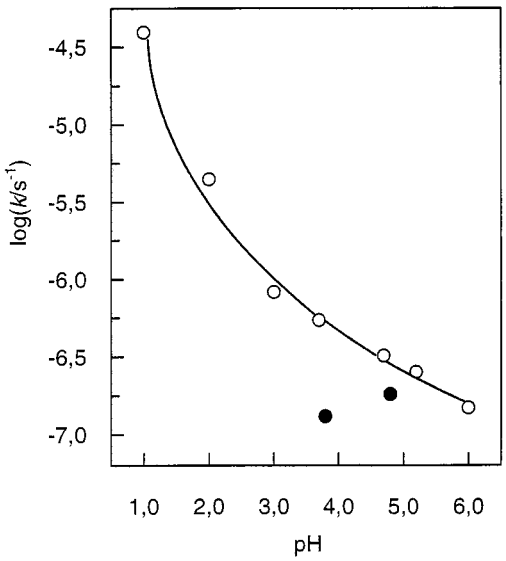


Figure 4. Logarithmic rate constants for the disappearance of ethenoadenosine (**4a**) and formylethenoadenosine (**5a**) in buffer solutions (c = 0.1 M) as a function of pH at 60 °C; I = 0.1 M ($NaNO_3$); ○ ethenoadenosine; ● formylethenoadenosine

to pH 3, the rate increases only by a factor of less than ten. Below pH 3 the dependence approaches a first-order dependence on [H^+], an indication of an acid-catalyzed process. Under slightly acidic conditions, the 1,N^6-formyletheno adduct (**5a**) decomposed 2 to 4 times less readily than **4a**.

As mentioned above, the reactions of adenosine and 9-methyladenine with BMA yield two stable products, viz. the 1,N^6-etheno (**4a**,**b**) and 11-formyl-1,N^6-etheno (**5a**,**b**) adducts. The products were formed in parallel, obeying first-order kinetics. No intermediates could be detected. Compounds **5a**,**b** were not converted into **4a**,**b** under the reaction conditions. Figure 5 shows as an illustrative example

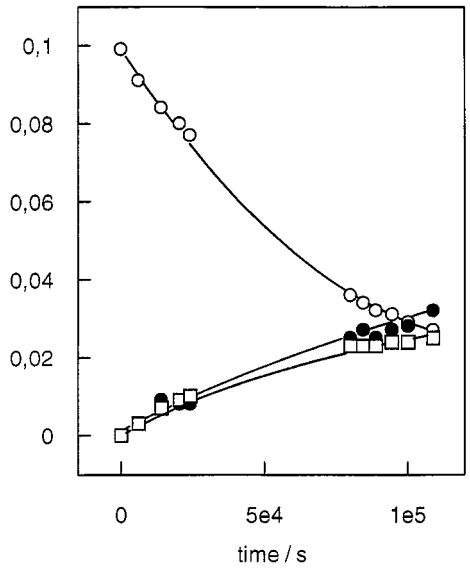


Figure 5. Time-dependent product distribution in the reaction of 9-methyladenine (**3b**) with BMA at pH 2.7 at 60 °C. I = 0.1 M ($NaNO_3$); notation: ■ 9-methyladenine; ○ etheno product (**4b**); ● formyletheno product (**5b**)

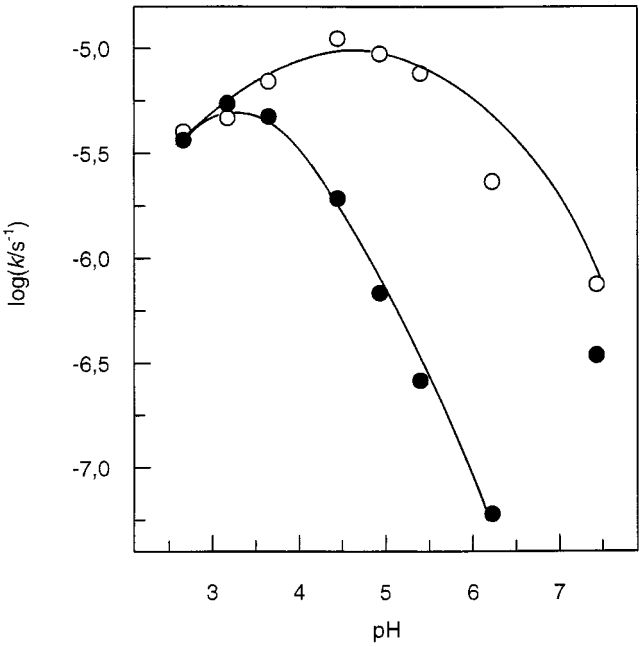


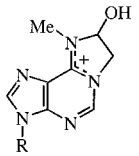
Figure 6. Logarithmic rate constants for the formation of the etheno product (**4b;** ○) and the formyletheno derivative (**5b**; ●) in the reaction of 9-methyladenine with 10 mM BMA as a function of pH at 60 °C; I = 0.1 M ($NaNO_3$)

the time-dependent product distribution for the reaction of **3b** with BMA at pH 2.7. At longer reaction times, breakdown of the etheno adduct **4b** is observed. The pH-dependence of the pseudo first-order rate constants observed for the formation of **4b** and **5b** is depicted in Figure 6. It can be seen that in the reaction of **3b** with BMA, the etheno (**4b**) and formyletheno (**5b**) adducts were formed in an approximately equimolar ratio at pH < 3.5, while at higher pH, **4b** predominated. The optimum pH for the formation of **4b** (pH 4.7) is more than one unit greater than that for the formation of **5b** (pH 3.3). The pH-dependent product distribution of the reaction of adenosine with BMA was observed to be very similar (data not shown). The concentration of BMA does not seem to affect the product ratio, as is shown by the rate contants in Table 1. The formyletheno adduct (**5a**,**b**) was accumulated to a clearly lesser extent when using CMA as starting material. With CMA, the formation of **5a**,**b** only accounts for up to 10% of the total disappearance of **3a**,**b** over the entire pH range (data not shown).

Table 1. Rate constants of formation of the etheno (**4a**) and formyletheno (**5a**) derivatives of adenosine in the reaction of adenosine (**3a**) with BMA at pH 4.7 and 60 °C

[BMA]/mol dm^{-3}	k (**4a**)/10^{-6} s^{-1}	k (**5a**)/10^{-6} s^{-1}
0.005	2.3±0.1	2.9±0.6
0.010	6.3±0.1	3.5±0.4
0.020	10.4±0.1	6.1±0.6
0.030	14.0±0.9	9.4±0.6
0.040	16.0±0.9	13.1±0.6

The reaction of N^6-methyladenosine (**3c**) with BMA and CMA yielded only one major stable product in the pH range from 3 to 6. This compound was characterized by ^1H and ^{13}C NMR spectroscopy and FAB mass spectroscopy and assigned as the hydrated 1,N^6-etheno product of **3c**, i. e. 7,8-dihydro-8-hydroxy-9-methyl-3-(β-D-ribofuranosyl)-imidazo[2,1-*i*]purine (**7**). Additionally, the formation of a small amount of another stable product was observed at pH 6. The HPLC-MS data suggests that this compound could be the 11-formyl-1,N^6-etheno adduct of N^6-methyladenosine (**8**). While N^6-methyladenosine reacted with halomalonaldehydes at pH 4 to 5 as rapidly as adenosine, its reaction rate with CAA was only one third of that of adenosine.



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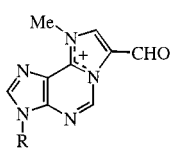

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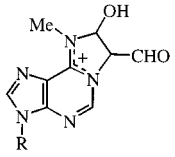

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Discussion

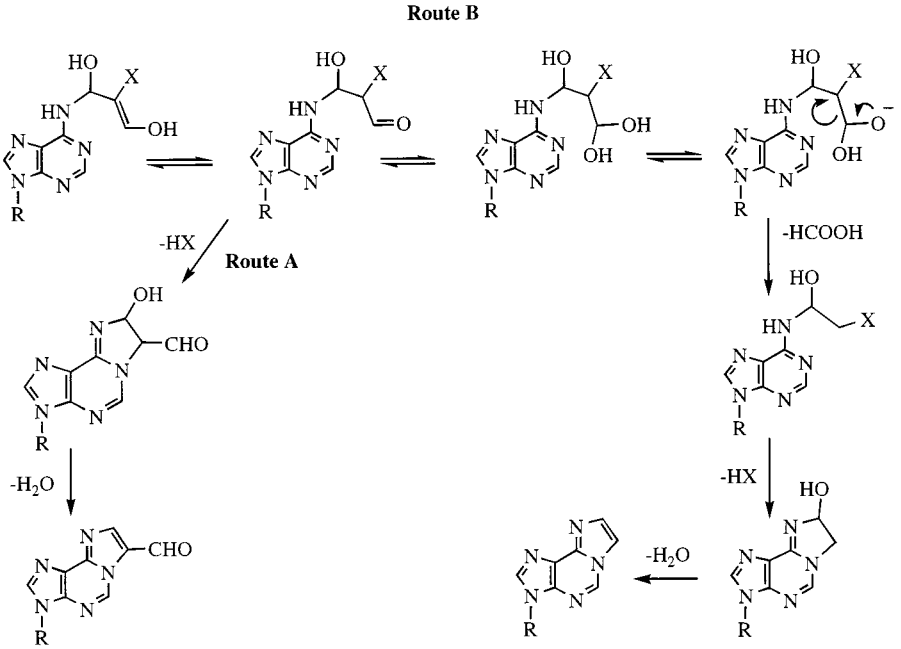
Reaction Order in the Aldehyde Concentration

As indicated in the introduction, the reaction of adenosine with halogenated acetaldehydes has been suggested to be a three-step process.[7a−7c] A cyclic carbinolamine intermediate is obtained by two consecutive reactions: initial nucleophilic addition of the 6-amino group to the carbonyl group and subsequent S_N2 substitution of the halogen substituent by the *N*1 atom of the adenine ring (Scheme 1). While the cyclic carbinolamine intermediate is detectably accumulated, no direct observation of the existence of the acylic carbinolamine has ever been reported. The suggested regioselectivity, i. e. the attack of N^6 on the carbonyl carbon atom and not on *C*2 of the aldehyde, receives support from the observation that the reaction of α-bromopropionaldehyde[7c] or α-chlorobutyraldehyde[13] with adenosine yields only one product, viz. the one having the original carbonyl carbon bonded to N^6. The first nucleophilic attack has been suggested to be the rate-limiting step of the formation of the cyclic carbinolamine intermediate: once the linear carbinolamine has been formed, it is assumed to undergo virtually irreversible cyclization which is faster than the collapse of the linear carbinolamine back to starting materials. The etheno product is then formed by dehydration of the cyclic carbinolamine. This final step is sufficiently fast to prevent extensive accumulation of the cyclic carbinolamine.

The kinetic results of the present study are consistent with the mechanism presented above. The reaction of adenine substrates **3a**,**b** with haloacetaldehydes **1a**,**b** was observed to be first-order in the aldehyde concentration, consistent with the assumed rate-limiting attack on the carbonyl carbon atom. Only a very modest downward curvature could be observed on plotting the pseudo first-order rate constants against the aldehyde concentration (Figure 1). On using BAA (10 mM) as starting material, accumulation of the cyclic carbinolamine at a level up to 10% of the initial concentration of **3a** (10 mM) was observed by HPLC-MS. With CAA, the cyclic carbinolamine is not clearly accumulated. This is expected since the intramolecular displacement of chlorine, i. e. the cyclization step, is undoubtedly slower than that of bromide, while the subsequent breakdown of the intermediate is naturally as rapid in both cases. The proposed mechanism is also consistent with the observed reactivity order of CAA and BAA; replacing of chlorine with bromine may well accelerate the nucleophilic attack on the neighbouring carbonyl carbon atom. Consistent with this, the base-catalysed hydrolysis of α-bromo acetic acid ethyl ester, for example, is faster than that of the corresponding chloro compound.[14] In comparison to the situation with α-halo esters, the reactivity difference observed in this work is, however, unexpectedly large (more than one order of magnitude). Accordingly, the possibility that the cyclization step is partially rate-limiting should not be strictly excluded.

Analogously, the reactions of halomalonaldehydes **2a**,**b** with the adenine substrates **3a**,**b** may also be assumed to be initiated by an attack of the 6-amino group on one of the carbonyl carbon atoms. The formyletheno product (**5a**,**b**) could then be obtained by subsequent cyclization by the displacement of the halogen substituent with the *N*1 atom and dehydration of the resulting cyclic carbinolamine (Scheme 2). To obtain the etheno product **4a**,**b**, the halomalonaldehyde must undergo deformylation. As discussed below in more detail, we believe that this branching of the reaction pathway takes place at the initially formed acyclic carbinolamine (Scheme 3). Accordingly, while formation of the acyclic carbinolamine intermediate may, in principle, be reversible, its subsequent cyclization or deformylation/cyclization must be virtually irreversible processes in dilute aqueous solution. This forms the basis for the following discussion concerning the dependence of the overall reaction rate on the concentration of the halomalonaldehyde.

On the basis of the kinetic results obtained, it can be suggested that in reactions of adenosine (or its analogs) with halogenated malonaldehydes, the first step is no longer rate limiting. The formal kinetics of the reactions of halomalonaldehydes differ from those of haloacetaldehydes in one important aspect: The reaction is not first-order in the aldehyde concentration but the rate increase observed at low aldehyde concentration begins to level off at high aldehyde concentrations (Figure 1). This kind of kinetic behavior is a consequence of saturation of the adenine substrate with the aldehyde. It seems that the acyclic carbinolamine is accumulated in a pre-equilibrium, and its cyclization or deformylation/cyclization constitutes the rate-limiting step for the formation of the cyclic carbinolamine intermediate. In other words, the subsequent reactions of initially formed intermediate are slower than the collapse of the intermediate back to the starting materials. The lower reactivity of halomalonaldehydes **2a**,**b** relative to haloacetaldehydes **1a**,**b** would hence result from the less rapid subsequent reactions of the acyclic carbinolamine intermediate.



Scheme 3

The kinetic saturation is more marked with CMA than with BMA. Since the intramolecular displacement of chlorine is more difficult than that of bromine, and since chlorine may also be expected to facilitate the hydrolytic deformylation less efficiently than bromine, the cyclization step alone may well be rate-limiting with CMA. The reaction of BMA may still represent a transition from rate-limiting formation to rate-limiting breakdown of the acyclic carbinolamine intermediate.

The fact that methylation of the 6-amino group of adenosine (conversion of **3a** to **3c**) retards the reaction of adenosine with CAA but not with CMA may also be accounted for by the proposed change in the rate-limiting step. For steric reasons, the 6-methylamino group of **3c** may be expected to be a less efficient nucleophile than the 6-amino group of **3a**. Since with CAA the initial nucleophilic attack of the amino group constitutes the rate-limiting step, a rate-retardation is observed. With CMA, the amino and carbonyl groups react in pre-equilibrium step, and hence the steric hindrance caused by the methyl group plays a less important role.

pH-Rate Profiles

As an indication of the overall likeness of the reactions of halomalonaldehydes and haloacetaldehydes with adenine substrates, the pH-dependence of all the reactions are rather similar: the pH-rate profiles are bell shaped exhibiting a broad maximum in the pH range 3−5. The maximum with BMA occurs at a lower pH than with CMA. The maximum rate is hence achieved under conditions where the adenine substrates are converted from neutral species to monocations. The pK_a values of **3a** and **3b** are 3.7 and 4.0, respectively, the site of protonation being *N*1.[15] This protonation might be expected to reduce dramatically the nucleophilicity of **3a**,**b**. However, the reactions are only moderately retarded on the acidic side of the pK_a values of **3a**,**b**; the ascending parts of the pH-rate profiles (pH < 3.5) exhibit a reaction-order of less than 0.5 in the hydroxide ion concentration. At pH > 5, the reactions are, in turn, decelerated with increasing pH, although no change in the ionic form of the adenine substrate takes place. Although the interpretation of the pH-rate profiles of multistep reactions, such as the present ones, is a complicated task, it appears clear that the protolytic equilibrium of the adenine substrate alone does not explain the observed pH-rate profiles. The protolytic equilibria of the malonaldehyde also affect the pH-rate profiles. The pK_a values of BMA and CMA are not known, but the pK_a values of their 2-methyl and 2-methoxy counterparts have been reported to be 4.7 and 4.1, respectively.[16] Accordingly, the pK_a values of BMA and CMA bearing a more electronegative 2-substituent than 2-methoxymalonaldehyde may be expected to lie around 3.5 and BMA is quite probably more acidic than CMA. In other words, the adenine substrate and the aldehyde both undergo deprotonation on passing pH 3.5: adenine substrate from a cationic form to a more nucleophilic neutral species, and halomalonaldehyde from a neutral molecule to a less electrophilic enolate anion. The influences of these two deprotonations on the overall reaction rate evidently cancel each other at least partially.

Mechanisms of the Formation of the Etheno and Formyletheno Products

As indicated above, the reactions of adenine substrates **3a**,**b** with BMA or CMA give both etheno (**4a**,**b**) and formyletheno (**5a**,**b**) products, although with CMA the etheno products clearly predominate. It has previously been suggested that the etheno product is actually formed by initial

conversion of halomalonaldehyde to haloacetaldehyde: either the malonaldehyde is cleaved spontaneously to the corresponding acetaldehyde,[9] or the acetaldehyde is formed by initial nucleophilic attack by the 6-amino function of the adenine substrate onto the carbonyl carbon of the malonaldehyde (Scheme 2).[2d] Neither of these mechanisms is, however, consistent with the kinetic data of the present study. The formation of 1,N^6-ethenoadenosine (**4a**) from CMA and adenosine is clearly too fast to be explained by a mechanism involving intermediate formation of CAA by a reaction of CMA with adenosine. If this kind of mechanism is followed, the maximum concentration of CAA present is of the order of 0.1 mM, i. e. equal to the initial concentration of adenosine. Otherwise adenosine should produce CAA catalytically, which means that adenosine should be rapidly regenerated from N^6-formyladenosine. This cannot be the case, since the *N*-formyl derivatives of pyrimidines are known to be hydrolytically rather stable.[17] It is also worth noting that no sign of accumulation of N^6-formyladenosine during a kinetic run could be detected. Accordingly, the pseudo first-order rate constant for the formation of **4a** from CMA and adenosine may be estimated to be of the order of 10^{-7} s^{-1} at pH 4.7, if one assumes that **4a** is actually formed by the reaction of CAA with adenosine (refers to [CAA] = 0.1 mM). The rate constant observed for the reaction between CMA and adenosine under these conditions is, however, 3×10^{-6} s^{-1}. In other words, the mechanism depicted in Scheme 2 for the formation of the etheno product cannot be the major pathway.

The mechanism where the halomalonaldehyde spontaneously releases haloacetaldehyde seems equally unlikely. The stability of BMA was studied by NMR spectroscopy in several different buffer solutions from pH 4.5 to 7.2 at 60 °C. Under these conditions, no decomposition was observed. The results of the kinetic experiments are also inconsistent with this mechanism. The formation of 1,N^6-ethenoadenosine (**4a**) follows first-order kinetics. This could not be the case if the haloacetaldehyde was formed simultaneously with **4a**; the continuous increase of the concentration of one of the starting materials (BAA or CAA) could be expected to lead to a sigmoidal dependence of the concentration of **4a** on time.

Our results suggest that rather than being formed through two separate pathways, the etheno and formylletheno products are formed through a common initial intermediate. The pH-rate profiles obtained for the overall reaction of the adenine substrates with BMA and CMA are rather similar (Figure 2). The profiles differ only slightly with respect to the position of the rate maximum, which can probably be attributed to higher pK_a value of CMA. Yet at a low pH, a large proportion of the reaction of adenosine with BMA yields the formyletheno product, while only a barely detectable amount of this product is formed with CMA. The fact that the shape of the pH-rate profiles of the overall disappearance of the adenine substrate remains similar with BMA and CMA, even though the pH-dependent product distribution is different, suggests that the reaction pathways leading to the two products branch from a common intermediate which still contain the halogen substituent. We tentatively suggest that the branching takes place at the acyclic carbinolamine intermediate, which may either undergo cyclization by displacement of the halogen substituent by the adenine *N*1 atom (Route A in Scheme 3), or hydrolytic deformylation prior to cyclization to a hydroxyethano adduct (Route B in Scheme 3). Since bromine undergoes nucleophilic displacement more readily than chlorine, formation of the formyletheno adduct is favoured with BMA. In all likelihood, the proposed hydrolytic deformylation is base-catalyzed, proceeding by initial deprotonation of one of the hydroxy functions of the covalent hydrate, and hence the formation of the formyletheno adduct is most marked under acidic conditions.

It is also worth noting that if the reaction were initiated by displacement of the halogen substituent of CMA or BMA with the *N*1 atom of the adenine ring, and not by the attack of the 6-amino function on the carbonyl carbon atom, N^6,N^6-dimethyladenosine could be expected to be alkylated by CMA and BMA. No such reaction was observed.

The structural information obtained on the intermediates formed upon reacting N^6-methyladenosine (**3c**) with BMA is consistent with the mechanistic suggestion depicted in Scheme 3. The major intermediate formed was identified as the hydrated etheno product (**7**) by HPLC-MS, and by ^{1}H and ^{13}C NMR spectroscopy. Another minor intermediate that could be isolated and characterized was the formylletheno product **8**. Furthermore, an early intermediate having *m*/*z* = 352 was detected by HPLC-MS. This mass number can naturally be attributed to a number of different structures, but it is clear that this intermediate has already lost the bromine at C2, since the isotope peaks resulting from the presence of bromine were not observed. The differences between the mass number of this intermediate and those of **7** and **8** are 28 and 18, respectively. Such differences may be accounted for by replacement of one hydrogen atom in **7** with a formyl group and addition of a molecule of water to **8**. Accordingly, the early intermediate may have structure **9**.

In conclusion, it seems that the reactions of adenine base with halogenated acetaldehydes and malonaldehydes proceed through similar pathways. The exocyclic amino function of adenine first attacks the carbonyl carbon atom of the aldehyde. While this step is rate-limiting in reactions with acetaldehyde derivatives, in reactions with malonaldehyde derivatives the first step can be regarded as a pre-equilibrium. In the case of haloacetaldehydes, the cyclization and the subsequent dehydration yield the etheno product. With halogenated malonaldehydes, the acyclic intermediate may react in two different ways: A deformylation, followed by cyclization and dehydration yield the etheno products, whereas cyclization and dehydration of the initial intermediate result in the formation of a formyletheno derivative. It hence seems that not only α-haloacetaldehydes, but also other halogenated carbonyl compounds may have mutagenic properties under physiological conditions. Such compounds are, for example, α-halomalonaldehydes,

known to be formed as decomposition products of α-hydroxy furanones.[3] In in vitro experiments, however, the malonaldehyde derivatives appear to be less reactive than the corresponding acetaldehyde compounds. As for the chemical modification of DNA bases, the reaction with bromoacetaldehyde under slightly acidic conditions seems to be the most efficient way to produce ethenobases, whereas bromomalonaldehyde below pH 3 gives the highest yield of formyletheno products.

Experimental Section

Equipment: The kinetic experiments were carried out by using Perkin−Elmer Integral 4000 HPLC equipped with a diode array detector. HPLC-MS analysis was performed on a Perkin−Elmer Sciex API 365 LC/MS/MS triple quadrupole mass spectrometer. A JEOL JNM-A 500 Fourier Transform NMR spectrometer was employed for the NMR characterization of the isolated compounds. A VG Analytical organic Mass Spectrometer and a Fisons Intruments VG ZapSpec were used for HRMS and FAB-MS analysis, respectively.

Materials: The solutions of chloroacetaldehyde were prepared from a commercially available (Merck) 45% aqueous stock solution, the concentration of which was determined as reported in the literature.[18] Bromoacetaldehyde was synthesized from diethyl acetal as described before.[12] The preparation of chloromalonaldehyde[19] and bromomalonaldehyde[20] has been reported previously. The buffer constituents employed were of reagent grade.

Adenosine (3a) and 1,N^6-Ethenoadenosine (4a) were products of Sigma, and were used as received. 11-Formyl-1,N^6-ethenoadenosine (**5a**) was a generous gift of Dr Kronberg, Åbo Akademi University, Turku. 9-Methyladenine was synthesised as described previously, and purified by repeated washes with a mixture of hot acetone and 40% aqueous tetrabutylammonium hydroxide (98:2, v/v).[21] The compound exhibited a molecular ion signal $[M + 1]^+$ at m/z = 149.2 in HPLC-MS. The ^{1}H NMR and UV spectra were identical to those reported previously.[22]

N^6-Methyladenosine (3c): N^6-Methyladenosine was synthesised by a procedure modified from that of Johnson et al.[23] Accordingly, a solution of 6-chloropurine riboside (0.78 mmol; 250 mg) in aqueous methylamine (40%, 12.5 mL) was heated in a Parr reactor at 70 °C and 1.5 bar for 16 hours. The solution was concentrated down to a small volume, and the precipitate was filtered. The filtrate was evaporated to dryness at 40 °C, and the residue was recrystallized from methanol. The yield of pure crystalline product was 141 mg (56%). The methanolic solution was evaporated, and the recrystallization procedure was repeated. After three days at −25 °C, more pure product (80 mg, 30%) was obtained. ^{1}H NMR ([D$_6$]DMSO): δ = 8.32 (s, 1 H, H-2); 8.21 (s, 1 H, H-8); 7.77 (br s, 1 H, NH); 5.87 (d, 1 H, ^1J = 5.6 Hz, H-1′); 5.40 (m, 2 H, 2 × OH); 5.14 (d, 1 H, OH); 4.61 (dd, 1 H, ^1J = 4.7 Hz, H-2′); 4.15 (dd, 1 H, H-3′); 3.96 (ddd, 1 H, H-4′); 3.67 (m, 1 H, H-5′); 3.56 (m, 1 H, H-5′′); 2.95 (d, 3 H, CH$_3$). ^{13}C NMR ([D$_6$]DMSO, ppm from TMS): δ = 155.2 (C-6); 152.5 (C-2); 148.1 (C-4); 139.7 (C-8); 119.9 (C-5); 88.0 (C-1′); 86.0 (C-4′); 76.3 (C-3′); 70.7 (C-2′); 61.5 (C-5′); 34.2 (CH$_3$).

3-Methylimidazo[2,1-*i*]purine (1,N^6-Etheno-9-methyladenine; 4b) and 7-Formyl-3-methylimidazo[2,1-*i*]purine (1,N^6-Etheno-11-formyl-9-methyladenine; 5b): A solution of bromomalonaldehyde (1.51 mmol; 230 mg) and 9-methyladenine (1.50 mmol; 230 mg) in acetic acid buffer (pH 4.7, 100 mL), was heated for three days at 60 °C, and for one day at 90 °C. The progress of the reaction was monitored by RP-HPLC. The reaction solution was then concentrated, and purified on a semipreparative RP-HPLC, using a LiChrospher RP-18 (250 × 10 mm, 5 μm) column. The eluent employed was a mixture of 0.1 M NH$_4$OAc and acetonitrile (88:12). The collected fractions were concentrated, and desalted on the same semipreparative column. The eluent was aqueous acetonitrile containing 14% and 16% acetonitrile for purification of **4b** and **5b**, respectively. For **4b:** ^{1}H NMR (D$_2$O): δ = 8.65 (1 H, s, H-5); 8.07 (1 H, s, H-2); 7.65 (1 H, s, H-8); 7.30 (1 H, s, H-7); 3.62 (3 H, s, CH$_3$). HPLC-MS: m/z 174.2 (M + 1). − For **5b:** ^{1}H NMR (D$_2$O, ppm from TMS): δ = 9.70 (1 H, s, CHO); 9.67 (1 H, s, H-5); 8.26 (1 H, s, H-2); 8.13 (1 H, s, H-8); 3.79 (3 H, s, CH$_3$). − HPLC-MS: m/z = 202.0 (M + 1). − HRMS of **5b:** HMRS(EI) M^+ found 201.064970, $C_9H_7N_5O$ requires 201.065060. The UV spectra of **4b** and **5b** were identical to those of the corresponding ribosides **4a** and **5a**, respectively.

7,8-Dihydro-8-hydroxy-9-methyl-3-(β-D-ribofuranosyl)imidazo[2,1-*i*]purine (7): N^6-Methyladenosine (**3c**; 0.071 mmol; 20 mg) was reacted with BMA (**2b**; 0.115 mmol; 17.4 mg) in aqueous solution (3.4 mL) at pH 4.5. After 48 h incubation under nitrogen at 60 °C, the mixture was cooled and the major product (**7**) was separated by RP-HPLC. ^{1}H NMR ([D$_6$]DMSO): δ = 8.88 (s, 1 H, H-5); 8.83 (s, 1 H, H-2); 6.00 (d, 1 H, ^1J = 5.2 Hz, H-1′); 5.89 (dd, 1 H, ^1J = 4.4 and 7.0 Hz, H-8); 4.77 (dd, 1 H, ^1J = 12.5 and 7.0, H-7); 4.48 (m, 2 H, H-7 and H-2′); 4.16 (dd, 1 H, ^1J = 4.2 and 4.2 Hz, H-3′); 3.99 (m, 1 H, H-4′); 3.68 (dd, 1 H, ^1J = 12.1 and 3.8 Hz; H-5′); 3.57 (dd, 1 H, ^1J = 12.1 and 3.2 Hz, H-5′′); 3.57 (s, 3 H, CH$_3$). − ^{13}C NMR ([D$_6$]DMSO, ppm from TMS): δ = 149.9 (C-2); 148.2 (C-9a); 144.9 (C-3a); 143.5 (C-9b); 116.1 (C-5); 88.1 (C-1′); 85.9 (C-4′); 84.6 (C-8); 74.5 (C-2′); 69.9 (C-3′); 60.8 (C-5′); 54.8 (C-7), 30.3 (CH$_3$). FAB-MS: m/z = 324 (M^+, 100%); 192 (71%).

Kinetic Measurements: The reaction solutions were prepared in sterilized water. The pH was adjusted with formic acid (pH 3−4), acetic acid (pH 4−5.2), MES (pH 5.2−6.5) or HEPES (pH 6.5−7.5) buffers. Below pH 3, aqueous solutions of hydrochloric acid were employed. The pH of the buffer based reaction solutions was measured on a pH meter at 60 °C prior to the kinetic experiments. The ionic strength was adjusted with NaNO$_3$.

The reactions of adenosine (**3a**), 9-methyladenine (**3b**), N^6-methyladenosine (**3c**) and 1,N^6-ethenoadenosine (**4a**) were followed at 60 °C. Reactions were carried out in stoppered tubes, which were immersed in a thermostated water bath. The initial concentration of the starting material was 0.1 mM. Aliquots of 100 μL were withdrawn at appropriate intervals to cover approximately two half-lives of the reaction. The aliquots were cooled down in an ice bath to quench the reaction. When the aliquots could not be analyzed immediately, they were frozen until analyzed.

The HPLC analysis was performed on Hypersil ODS RP-18 column (250 × 5 mm, 5μm particle size). The eluents were mixtures of acetonitrile and 0.05 M acetic acid buffer (pH 4.3, I = 0.1 M with NH$_4$Cl). The acetonitrile content varied depending on the substrate: with adenosine (**3a**) it was 5%, with ethenoadenosine (**4a**) 3.5%, and with N^6-methyladenosine (**3c**) 10%. Aliquots from reactions of 9-methyladenine were analyzed by using a mixture of 0.1 M NH$_4$OAc and acetonitrile (93:7, v/v). UV-detection at wavelengths of 260 and 325 nm was employed.

The concentrations of the adenine substrate (**3a**,**b**), the etheno product (**4a**,**b**) and the formyletheno product (**5a**,**b**) were deter-

mined by using calibration curves. The sum of the concentrations generally remained constant during the reaction. As the breakdown of the etheno products is an acid-catalyzed process, a slight decrease in the total concentration (substrate + etheno products) was observed under acidic conditions.

Calculation of the Rate Constants: Pseudo first order rate constants for the reaction of adenosine (**3a**), 9-methyladenine (**3b**), N^6-methyladenosine (**3c**) and 1,N^6-ethenoadenosine (**4a**) were calculated by using the integrated first-order rate law. The calculation was based on the decrease of the concentration of the starting material as a function of time.

The rate constants obtained for the disappearance of adenosine and 9-methyladenine in the presence of BMA were divided into the rate constants for the formation of the etheno product (**4a**,**b**) and formyletheno product (**5a**,**b**) by Equation (1) and (2).[24] Here k_1 refers to the formation of an etheno product, **4a** or **4b**, k_2 to the formation of a formyletheno product, **5a** or **5b,** k_3 to the overall disappearance of the starting material, **3a** or **3b**, and k_{d2} to the disappearance of the etheno product.

$$x(\mathbf{4a},\mathbf{b}) = [k_1/(k_3-k_{d2})]\,[\exp(-k_{d2}t)-\exp(-k_3t)] \quad (1)$$

$$k_3 = k_1 + k_2 \quad (2)$$

With adenosine, the calculation was based on the mole fraction of ethenoadenosine (**4a**) as a function of time. The rate constants for the overall disappearance of adenosine and the disappearance of **4a** were known, and the rate constant for the formation of **4a** was obtained by nonlinear fitting. In the case of 9-methyladenine (**3b**), only the rate constant for the overall disappearance of the starting material was known, and the rate constants for the formation and disappearance of the products were obtained by fitting.

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