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Mechanism of Inhibition of Enzymatic Deoxyribonucleic Acid Methylation by 2-(Acetylamino)fluorene Bound to Deoxyribonucleic Acid†

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ABSTRACT: Binding of 2-(acetylamino)fluorene (AAF) to C-8 of guanine induces a local destabilization of the DNA helix. A relationship was observed where the degree of DNA modification by AAF was inversely proportional to its methyl acceptor capacity from *S*-adenosyl-L-methionine in the presence of rat brain DNA cytosine 5-methyltransferase. Moreover, substituted DNA (DNA-AAF) behaves as a methylation inhibitor of native DNA. This inhibition is of the mixed type. The substituted DNAs have higher affinities for the enzyme than native DNA. The inhibition is irreversible. Addition of DNA-AAF to the enzyme preincubated with native DNA inhibits methylation, but only after a lag period. This agrees with the model in which the methylase "walks" along the strand to methylate cytosine residues before being detached from the DNA. AAF bound to guanine residues may block the movement of the enzyme along the helix. Single-stranded DNA has an affinity for the methylase 1.6 times lower than that of native double-stranded DNA. On the other hand, single-stranded DNA-AAF is more methylated than double-stranded DNA-AAF. A tentative model taking into account these observations is presented under Discussion. The in vitro hypomethylation of DNA-AAF could explain the in vivo observations made by several authors.

It is presently widely known that chemical carcinogens are either electrophilic reactants or compounds which become activated to electrophilic metabolites (Miller, 1970; Kriek, 1974) able to bind covalently to DNA, RNA, or proteins.

2-(Acetylamino)fluorene (AAF)[1] is a potent liver carcinogen which after metabolic activation binds to liver DNA, RNA, and proteins when administered in vivo [for a review, see Miller (1970)]. 2-(*N*-Acetoxyacetylamino)fluorene (*N*-AcO-AAF), a model ultimate metabolite of AAF, has been shown to react in vitro with DNA to give two adducts, *N*-(deoxyguanosin-8-yl)-AAF and 3-(deoxyguanosin-N^2-yl)-AAF (Kriek et al., 1967; Kriek, 1972; Westra et al., 1976). The first of these adducts represents about 80% of the total. It has been shown

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[1] Abbreviations used: AAF, 2-(acetylamino)fluorene; *N*-AcO-AAF, 2-(*N*-acetoxyacetylamino)fluorene; SAM, *S*-adenosyl-L-methylmethionine; dGuo, deoxyguanosine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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that AAF bound to native DNA induces a conformational change in the vicinity of the adduct in which the fluorene ring is accommodated between the adjacent base pairs leading to a local denaturation of the double helix (Fuchs & Daune, 1972). This conformational change has been called the insertion–denaturation model (Fuchs et al., 1976). A similar model has been proposed by others [for a recent review, see Grunberger & Weinstein (1979)].

How can the carcinogen-modified DNA perform its biological functions? Both in vitro transcription and replication have been studied. It has been found that DNA modified with AAF is impaired in its template activity for bacterial DNA polymerase (Millete & Fink, 1975) and for bacterial RNA polymerase (Berthold et al., 1978). In the latter case, analyses of the RNA products demonstrated that this inhibition is due to premature chain termination. We investigated how the enzymatic methylation of DNA was affected by the presence of the covalently bound AAF residues. For this purpose, DNA–AAF with different amounts of modified bases was used as a substrate for the DNA cytosine 5-methyltransferase from rat brain.

Our first observations indicated that DNA which had been modified by the carcinogen had a decreased methyl acceptor capacity. In fact, we demonstrated that the level of DNA methylation is inversely proportional to the percentage of the bases substituted by AAF (Salas et al., 1979). Moreover, use of the 7-iodo derivative of 2-(*N*-acetoxyacetylamino)fluorene, which does not insert into the DNA like AAF but is fixed outside the helix (outside binding model) (Fuchs et al., 1976), has shown that the exact position of the carcinogen has no effect on the inhibition of the methylation (Salas et al., 1979).

In this study, a mechanism underlying the inhibition of the DNA enzymatic methylation by the bound AAF residues is proposed.

Materials and Methods

All chemicals used were reagent grade. Chicken erythrocyte DNA was a gift from Professor Daune and was prepared as described elsewhere (Kay et al., 1952). Pancreatic RNase was from Worthington Biochemicals, *S*-adenosyl-L-methyl[^{3}H]-methionine (SAM) was from New England Nuclear, and nonradioactive SAM was from Boehringer Mannheim (Mannheim, GFR). Proteinase K was from Merck (Darmstadt, GFR). Unisolv was from KochLight. Diethylaminoethylcellulose (DE-52) was from Whatman. *N*-AcO-AAF was synthesized as described elsewhere (Lotlikar et al., 1966). Reaction of DNA with *N*-AcO-AAF and subsequent purification of modified DNA were described previously (Fuchs & Daune, 1972), and 8-(2-fluorenylacetamido)deoxyguanosine as well as its 5′-phosphate derivative (dGMP-AAF) was synthesized and purified by high-pressure liquid chromatography as described by Fuchs (1978). Denatured DNA was prepared by incubation in a boling water bath for 3 min followed by rapid chilling in ice.

Preparation of Rat Brain DNA Cytosine 5-Methyltransferase. Nuclei were prepared according to Pogo et al. (1966) from 7-day-old Wistar rats brain cortex, an organ which exhibits during early postnatal development high levels of methyltransferases (Salas & Sellinger, 1978). DNA methylase was extracted as described by Simon et al. (1978) for rat liver methylase. However, instead of fractionation by DEAE-Sephadex A-50 batch chromatography, the enzyme was precipitated with ammonium sulfate (80% saturation) at 4 °C, pelleted at 17000*g* for 15 min, resuspended in 10 mM Tris-HCl, pH 7.8, and dialyzed overnight against the same buffer. The solution was applied to a column of DE-52 (1.5 × 15 cm) which had been equilibrated with 20 mM Tris, 20 mM NaCl, and 0.5 mM dithioerythritol (DTE), pH 7.4, and then eluted with 200 mL of a linear gradient from 20 to 750 mM NaCl in the same buffer. Essentially three protein peaks were obtained, the methylase being localized in the second peak. The methylase-containing fractions were pooled and dialyzed against the following buffer: 20 mM Tris, 0.5 mM DTE, and 50% glycerol, pH 7.4. The enzyme was stored as small aliquots at −20 °C. The purified enzyme was about 70-fold more active than the crude brain extract. Protein concentration was determined according to Lowry et al. (1951) by using bovine serum albumin as standard.

Methylation of Chicken Erythrocyte DNA by Rat Brain DNA Cytosine 5-Methyltransferase. The standard assay mixture contained in 0.2 mL of 50 mM Tris-HCl, pH 7.8, and 0.5 mM DTE 40 μg of DNA, 0.35 μg of pancreatic RNase, 50 μg of purified enzyme preparation, 3 μCi of [^{3}H]SAM (50–75 Ci/mmol), and 9 nmol of SAM. The reaction was incubated at 37 °C for various times after which 20 μL of proteinase K (1 mg/mL of 10 mM Tris-HCl, 10 mM EDTA, 10 mM NaCl, and 0.5% $NaDodSO_4$) was added and the tubes were reincubated 30 min at 60 °C. Following proteinase K treatment, 1 mL of carrier salmon sperm DNA at 1 mg/mL and 120 μL of 2 M sodium acetate, pH 5, were added, followed by 2 volumes of ethanol at −20 °C. After 30 min at −20 °C, the pellet was recovered by centrifugation and freed of RNA by dissolution in 0.5 mL of 0.5 M NaOH and incubation for 10 min at 60 °C. The DNA was precipitated by 2 mL of Cl_3AcOH (15% w/v). After 10 min in ice, the precipitate was collected on GFC filters (Watman, England) and washed 6 times with 2 mL of 5% Cl_3AcOH and twice with ethanol. The filters were counted with 4 mL of Unisolv. With this method, the recovery of methylated DNA was about 85%. Blanks without DNA gave about 150 cpm and were subtracted from reaction values greater than or equal to 500 cpm.

Results

Methylation of Native DNA and of DNA–AAF. Chicken erythrocyte double- or single-stranded DNA is a good methyl acceptor substrate in vitro in the presence of the methyl donor *S*-adenosyl-L-methionine (SAM) and rat brain DNA cytosine 5-methyltransferase. In the presence of an optimal concentration of SAM (45 μM), the reaction is linear for about 2 h after which it slows down, probably due to an inhibition by *S*-adenosyl-L-homocysteine, and reaches a plateau at about 20 h. The maximum degree of methylation was about 1 methyl group per 200 cytosines in double-stranded chicken erythrocyte DNA (43% G + C). However, if the nucleic acid is modified with 2-(*N*-acetoxyacetylamino)fluorene, giving 2-(acetylamino)fluorene-substituted DNA (DNA–AAF), both the initial velocity of methylation and the overall methylation plateau are decreased. The results show an inverse proportionality between the degree of DNA modification by AAF and its methyl acceptor capacity (Figure 1). However, a certain degree of methylation is always observed even in DNAs where 5% of the bases are modified.

Inhibition of Native DNA Methylation by DNA–AAF. To study the effect produced by DNA–AAF when present in the reaction mixture containing nonmodified DNA, we added increasing amounts of DNA–AAF containing 4.6% of modified bases to normal DNA followed by *S*-adenosyl-L-methyl[^{3}H]methionine and enzyme. Figure 2 shows the level of methylation obtained after 45-min incubation. As DNA–AAF can itself be methylated, the counts per minute due to its methylation were subtracted as shown in the insert of Figure 2. The latter shows that 1 μg of DNA–AAF inhibits about

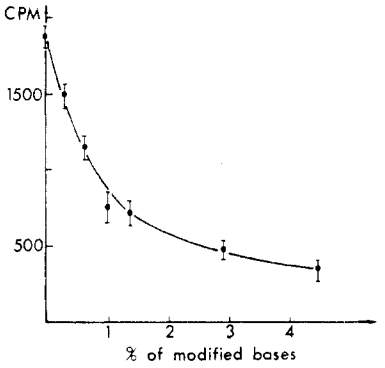

FIGURE 1: Methylation of DNA–AAF. Chicken erythrocyte DNA modified by various amounts of *N*-AcO-AAF was incubated with *S*-adenosyl-L-methyl[^{3}H]methionine and rat brain DNA cytosine 5-methyltransferase as described under Materials and Methods for 120 min at 37 °C.

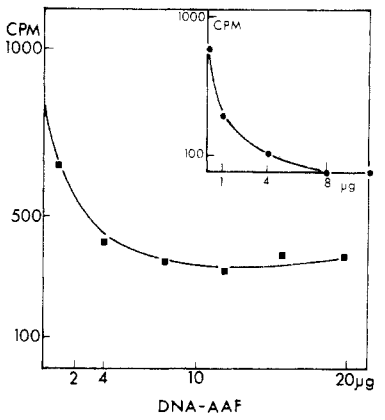

FIGURE 2: Inhibition of native DNA methylation by DNA–AAF. Chicken erythrocyte DNA (20 μg) and variable amounts (0–20 μg) of DNA–AAF (4.6% modified bases) were incubated for 45 min at 37 °C with *S*-adenosyl-L-methionine and brain enzyme under the experimental conditions described under Materials and Methods. Results are the average of two experiments with less than 7% variation. The insert gives the cpm due to the methylation of DNA–AAF.

50% the rate of methylation of 20 μg of nonmodified DNA and 4 μg inhibits about 88%. To see whether the inhibitor is DNA–AAF or AAF bound to dGMP or to dGuo, these two products were synthesized as indicated under Materials and Methods. They were added to the reaction mixture containing native DNA at concentrations corresponding to those present in 4 μg of DNA–AAF (2,5 μM) which causes 88% of inhibition. No inhibition of methylation was observed.

For elucidation of the type of inhibition caused by DNA–AAF, the initial methylation reaction velocities of variable amounts of nonmodified DNA were measured in the presence and absence of DNA–AAF modified to variable extents. Plotting I/v vs. I/S gives a K_m of 250 μM, expressed in phosphate residues, for nonmodified DNA. With DNAs having different percentages of modified bases, a mixed type of inhibition was always obtained (Figure 3). The K_i can be calculated from the curves obtained. Values of 29 μM, 37 μM, 46 μM, and 66 μM for 0.6%, 1.4%, 2.8%, and 4.6%, respectively, of modified bases were obtained. These results were confirmed by the determination of the K_m of the modified DNAs for the enzyme (results not shown). Thus the affinity of the enzyme for the substituted DNA is between 8.7 and 3.8 times higher than that for native DNA.

Stepwise Addition of the Different Substrates (DNA or DNA–AAF). All the above experiments were done by mixing DNA and DNA–AAF before adding the enzyme. In additional experiments, either the DNA or the DNA–AAF was

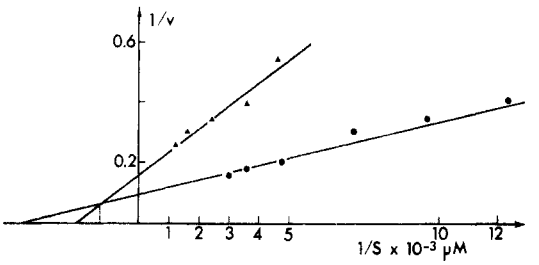

FIGURE 3: Lineweaver–Burk plot of inhibition of native DNA methylation by DNA–AAF. The mixture contained variable amounts of native DNA [5–150 μg/mL in the absence of DNA-AAF (●) or in the presence of 30 μg/mL DNA–AAF 1.4% of modified bases (▲)]. The incubation was for 45 min at 37 °C; v is the number of picomoles of CH_3 incorporated into DNA; S is the concentration of DNA expressed as micromoles of phosphate.

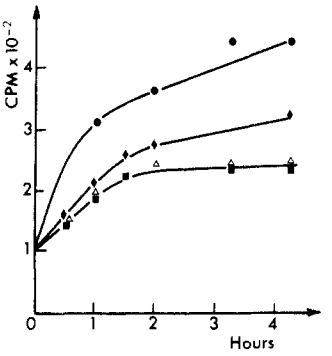

FIGURE 4: Irreversible inhibition of native DNA methylation by DNA–AAF. DNA–AAF (4 μg) (1.8% modified bases) was preincubated at 37 °C in the presence of *S*-adenosyl-L-methionine and enzyme (40 μg). After 5 min, (△) 40 μg of native DNA was added and the methylation was measured. Control without DNA–AAF (●), control with DNA–AAF (■), and control with native DNA mixed with DNA–AAF at zero time (♦).

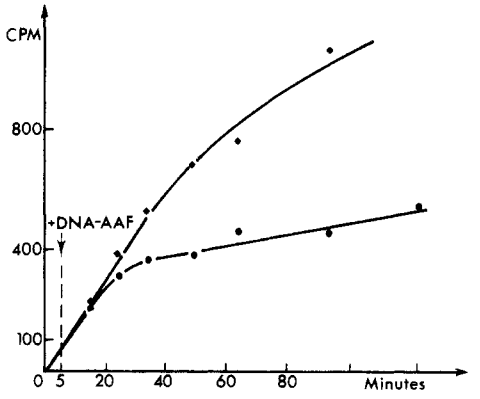

FIGURE 5: Inhibition of methylation of native DNA by late addition of DNA–AAF. Native DNA (40 μg) was preincubated at 37 °C in the presence of *S*-adenosyl-L-methionine and 60 μg of enzyme. After 5-min incubation, 4 μg of DNA–AAF (1.8% modified bases) was added and the incubation continued. (♦) Control without DNA–AAF. (●) DNA–AAF added after 5 min.

mixed with the enzyme prior to the addition of the other nucleic acid. Figure 4 shows the results obtained when 4 μg of DNA–AAF (1.8% of modified bases) was incubated with SAM and limited amounts of DNA methyltransferase for 5 min followed by addition of 40 μg of native DNA. The results (Figure 4) show that when native DNA is added after the methylase is bound to DNA–AAF, the methylation does not increase and remains the same as in the control where only DNA–AAF is present. Thus native DNA does not displace the enzyme from DNA–AAF. We therefore concluded that the binding of the enzyme to DNA–AAF is irreversible. On the other hand, if one preincubates native DNA with the

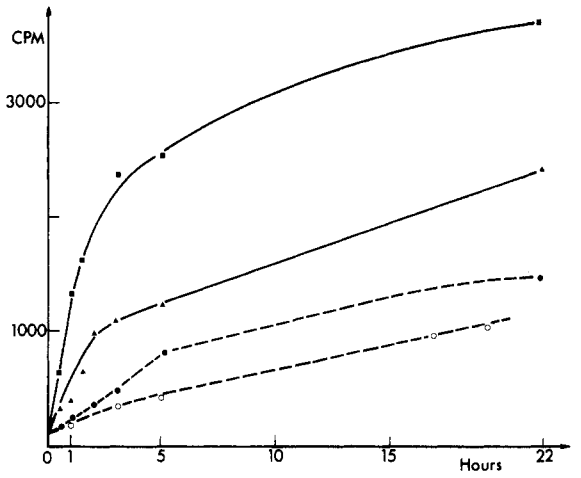

FIGURE 6: Comparison of methylation of single- and double-stranded DNAs substituted or not with AAF. The conditions are as indicated under Materials and Methods. When DNA-AAF was used, both single- and double-stranded DNAs have 4.6% modified bases. (■) Native DNA. (▲) Heat-denatured DNA. (○) DNA-AAF. (●) Heat-denatured DNA-AAF.

enzyme and adds DNA-AAF afterward, one finds that the inhibition of methylation starts only after a lag time of at least 15 min (Figure 5).

Methylation of Single-Stranded DNAs. The higher affinity of the enzyme for DNA substituted by AAF could be due to the presence of single-stranded regions in the DNA. In fact, Fuchs & Daune (1971, 1972) have shown that AAF bound to native DNA induces a local destabilization of the helix. We therefore tested the affinity of the DNA methyltransferase for heat-denatured chicken erythrocyte DNA obtained as indicated under Materials and Methods. The Lineweaver-Burk plot of these results showed that, in fact, the affinity of the single-stranded DNA is 1.6 times lower (K_m = 400 μM) than that of the native double-stranded DNA (not shown). Thus the higher affinity of the DNA-AAF does not come from its local single strandedness. Finally, if one compares the methylation of single-stranded DNA-AAF to double-stranded DNA-AAF, one sees (Figure 6) that the single-stranded DNA-AAF is more methylated.

Discussion

In this study, we have analyzed in detail in vitro enzymatic cytosine methylation of DNA modified with the carcinogen 2-(*N*-acetoxyacetylamino)fluorene which results mainly in the substitution of C-8 of guanines with (acetylamino)fluorene (AAF). The more the DNA is substituted with AAF the less it is methylated. By use of chicken erythrocyte DNA-AAF that contained about 5% of modified bases, the methylation level was about 15% of the control. However, even highly modified DNA maintained a low but nonnegligible capacity for methylation. The analysis of the methylated base formed in DNA revealed only 5-methylcytosine, excluding the possibility that this low methylation level was due to the presence of other methylated bases.

The substituted DNA not only is less methylated but also inhibited the methylation of native DNA. Added simultaneously with native DNA, it caused a mixed-type inhibition and had 3.8-8.6 times higher affinity for the enzyme than the native DNA. DNA-AAF bound irreversibly to the enzyme since native DNA could not displace it. These two results could explain the mixed inhibition of the initial velocity of the reaction. However, the higher affinity of the DNA-AAF for the enzyme seems not to be due to the local destabilization of the DNA by the presence of AAF since unmodified single-stranded DNA has a lower affinity for the enzyme than unmodified double-stranded DNA.

The lower methylation of AAF-substituted DNAs can be explained if we consider the model proposed for the methylation reaction (Kalousek & Morris, 1969; Drahovsky & Morris, 1971a,b) which assumes that the enzyme "walks" along the DNA strands to methylate several cytosine residues along its way before being detached from the nucleic acid. We may therefore expect that AAF bound to the guanine residues prevents the enzyme from moving along the helix. This explanation agrees with the finding that the more the DNA (whether single- or double-stranded) is substituted with AAF the more it will pose obstacles to the enzyme action, resulting in less DNA methylation. Once blocked at the AAF-substituted site of the DNA, the enzyme is irreversibly bound. The different responses of single- and double-stranded DNA-AAF toward the enzyme (Figure 6) can be tentatively explained as follows: double-stranded DNA-AAF contains some cytosine residues which are potential methylation sites. The presence of AAF in the guanine residues opposite those cytosine methylation sites suppresses the methylation of the pyrimidine bases. This mechanism would only occur in double-stranded DNA and could explain the lower degree of methylation of double-stranded DNA-AAF as compared to the single-stranded one.

Methylation of cytosine is the only postreplicational modification so far detected in DNA of higher eucaryotes and thus has been the basis of several proposed mechanisms of gene activity, cellular differentiation, and oncogenesis (Borek & Srinivasan, 1969; Scarano, 1971; Drahovsky & Morris, 1972; Vanyushin et al., 1973; Holliday & Pugh, 1975; Riggs, 1975). In the case of the globin genes (Waalwijk & Flavell, 1978; McGhee & Ginder, 1979) and of DNA from *Herpes virus saimiri* (Desrosiers et al., 1979) a correlation between site-specific DNA methylation and the activity of these genes has recently been shown. A chemical carcinogen, L-ethionine in the form of *S*-adenosylethionine, was shown to inhibit the DNA methylase activity from Novikoff hepatoma (Sneider et al., 1975) and the methylation of inverted DNA repeats in P_{815} mastocytoma (Boehm & Drahovsky, 1979). Finally, Christman et al. (1977) have observed that DNA from erythroleucemic cells grown in the presence of ethionine is hypomethylated. In an in vitro system, this DNA is a better methyl acceptor for the homologous DNA methylase than DNA of the same cells grown without this compound. This inhibition of methylation promotes the expression of globin genes. Our results could explain the hypomethylation recently found in DNA from premalignant nodules and primary hepatocellular carcinomas induced by exposure to 2-(acetylamino)fluorene (Lapeyre & Becker, 1979).

The correlation between the degree of DNA hypomethylation and mitotic activity is not yet established, but chemical carcinogens which, like AAF, bind covalently to DNA and inhibit its methylation could also act through hypomethylation on gene activity, cellular differentiation, and even oncogenesis.

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Affinities of Nucleic Acid Bases for Solvent Water†

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ABSTRACT: Equilibria of transfer of pyridine and benzene derivatives, from the vapor phase to dilute aqueous solution, are enhanced by the introduction of exocyclic amino and hydroxyl substituents. Much larger increases are associated with the introduction of imino and keto substituents. Purine derivatives exhibit comparable behavior. These observations are discussed in relation to group transfer potentials, the observed affinities of nucleic acid bases for the active sites of proteins, environmental influences on the occurrence of rare tautomers that lead to errors in base pairing, and hypotheses concerning the origins of the genetic code.

The polar character of amino acids serves as an important determinant of their tendencies to be found at the surface of globular proteins (Kauzmann, 1959; Perutz, 1965), and these tendencies are closely correlated with the absolute affinities of amino acid side chains for solvent water (Wolfenden et al., 1981). Similarly, it seems evident that base pairing and stacking interactions in nucleic acids must usually occur in competition with solvent interactions with the participating groups (Ts'o, 1970). Furthermore, the strengths and specificities of binding of purine and pyrimidine derivatives by biological receptors, including the active sites of enzymes, presumably reflect the requirement that solvent water be stripped away (at least in part) from both the ligand and the receptor. It would therefore be of interest to have information concerning the affinities of nucleic acid bases for watery surroundings. This information would also be useful for determining the influence of changing solvation on the observed equilibria of metabolic transformation of purine and pyrimidine derivatives in aqueous solution and for testing certain hypotheses concerning factors that may have influenced the early evolution of the genetic code.

Affinities of organic compounds for solvent water can be evaluated, in an absolute sense, by measuring equilibrium constants for their transfer from dilute aqueous solution to the vapor phase. The hydrophilic character of complex molecules, determined in this way, can generally be predicted with reasonable accuracy from their constituent groups (Butler, 1937; Hine & Mookerjee, 1975). In the present study, purine and pyrimidine bases were found to be insufficiently volatile for detection in the vapor phase at room temperature, and pyridine derivatives were therefore examined as models. Derivatives of the nucleic acid bases themselves were examined in solvent–solvent distribution experiments in order to investigate the validity of these models.

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