DOI: 10.1002/ejoc.200600799

The Structural Identification and Conformational Analysis of the Products from the Reaction of Acrolein with 2′-Deoxycytidine, 1-Methylcytosine and Calf Thymus DNA

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Keywords: NMR spectroscopy / DNA alkylation / Nucleobases / Acrolein

LC-MS analysis of the reaction mixture of 2′-deoxycytidine and acrolein in phosphate buffer under physiological conditions showed the formation of three major product peaks. The products were characterized as cyclic adducts comprised of one or two units derived from acrolein. The first (**8**) and third (**9**) eluting peaks were each found to be a pair of diastereomers of a two-ring-fused adduct, 6,9-dihydroxy-2-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-2,5,6,7,8,9-hexahydro-4*H*-2,3a,6a-triazaphenalen-3-one, whilst the second eluting peak (**7**) was identified as a one-ring-fused adduct, 2-hydroxy-7-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-2,3,4,7-tetrahydropyrimido[1,6-*a*]pyrimidin-6-one. The reaction of 1-methylcytosine with acrolein was also examined resulting in the formation of an additional regioisomeric two-ring-fused adduct, 2-hydroxy-7-methyl-3,4,7,9,10,10a-hexahydro-2*H*-1-oxa-4a,7,8a-triazaphenanthren-8-one, not observed in the corresponding reaction with 2′-deoxycytidine. The structures, stereochemistry and conformational analysis of the adducts and their decomposition products were resolved by spectrometric and spectroscopic studies. From the incubation of acrolein with double- and single-stranded DNA and subsequent LC-MS/MS analysis (in multiple reaction monitoring mode) of the hydrolysate, compounds were detected corresponding (i.e. identical R_t and MS) to the peaks that represented the pair of diastereomers **7** and **9**.

Introduction

α,β-Unsaturated compounds are important industrial chemicals, natural products and widely dispersed environmental contaminants. This is of concern as many of them have been found to possess mutagenic and carcinogenic properties. The simplest α,β-unsaturated aldehyde is acrolein (**1**), a by-product formed during the combustion of fossil fuels, wood, tobacco and the heating of cooking oils.[1] Endogenously, **1** is generated via oxidation of polyamines and as a product of lipid peroxidation.[2–4] It is also a metabolite formed in the biotransformation of the chemotherapeutic alkylating drug cyclophosphamide.[5–8] It has been shown that **1** possesses significant mutagenic activity towards both bacterial[9,10] and mammalian cells.[11,12]

As a strong electrophile, acrolein (**1**) reacts with biomolecules to form a variety of adducts. It has been demonstrated to form protein adducts at the lysine residues by Schiff base and Michael addition pathways[3,13] and it may also lead to the formation of DNA-protein cross-links.[14] It has been established that it does react with DNA bases in vitro to form DNA adducts.[15–17] For example, the deoxyguanosine–acrolein adduct, γ-hydroxy-1,N^2-propano-2′-deoxyguanosine (γ-HOPdG), has been detected in DNA in various tissues[2,7,18,19] suggesting its potential role as endogenous DNA lesions in rodents and humans. However, VanderVeen et al.[20] have shown that γ-HOPdG does not induce mutations in *Eschericha coli* in vivo at the sensitivity of the used assay. It has been proven that γ-HOPdG exists predominantly in the ring-opened form in duplex DNA[21] and that this form appears to be less mutagenic than the ring-closed form. In contrast to prokaryotic data, it has been found that γ-HOPdG can contribute to the mutagenicity of acrolein (**1**) in a mammalian system.[22,23] Furthermore, the γ-HOPdG adduct is capable of forming an interchain cross-link to the N^2 position of the opposing guanine in a 5′-CpG sequence.[24] The minor α-HOPdG adduct has also been found to possess mutagenic activity and has been shown to be even more genotoxic than the major γ-HOPdG adduct.[25,26] Therefore, it is suggestive that the mutagenic effects of acrolein (**1**) can be mediated also by other acrolein–DNA adducts even though they occur at lower levels in genomic DNA. Earlier studies have shown that four structurally different adducts are formed in the reaction of acrolein (**1**) with 2′-deoxyadenosine[16,17,27–29] and these adducts may very well all contribute to the observed muta-

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genic effects of **1** in the various assays. Also of interest is the reactivity of **1** with other DNA bases. The reaction of **1** with the cytosine base unit has been studied by several groups[29–32] leading to the identification of a cyclic adduct comprised of one unit of acrolein (**1**). Some investigators[29–31] noticed the formation of an adduct comprising two units of acrolein (**1**), but this finding was not explored further and thus the identity of the adduct has not been determined. In work on structural elucidation of adducts, not only do the stereochemistries of the products need to be determined, but also the conformational analyses have to be performed as biological activities are determined to various degrees by the adopted conformations of the substrates.[33]

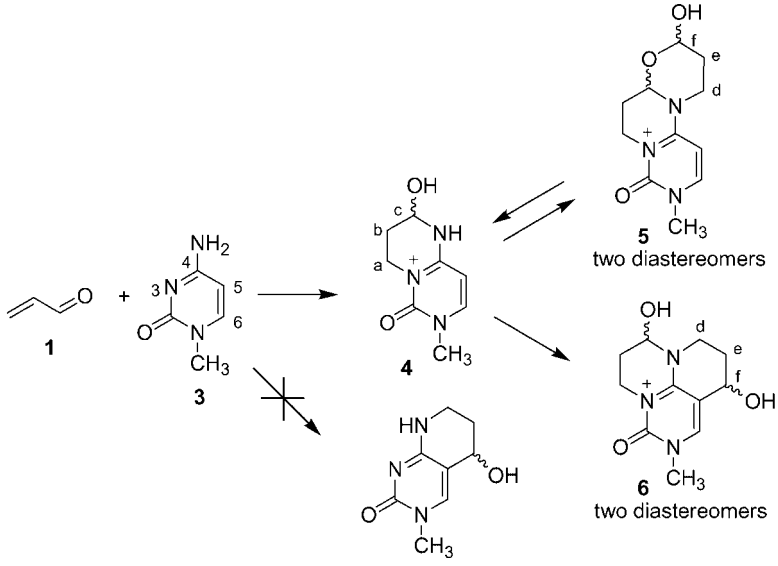
We have now re-examined the reaction of acrolein (**1**) with 2′-deoxycytidine (**2**) and the products that are formed, with particular emphasis placed on scrutinizing the stereochemistries and adopted conformations. LC-MS analysis of the reaction mixture and NMR studies of the isolated products showed the formation of four diastereomers of an adduct consisting of two acrolein units in addition to the one-ring-fused adduct studied previously. To assist in the identification and assignment of the adducts containing two fused rings, the substitution of 1-methylcytosine (**3**) for 2′-deoxycytidine[34] (**2**) in the reaction with acrolein (**1**) was also surreptitiously examined. This reaction resulted in the formation of adducts analogous to those formed from 2′-deoxycytidine (**2**) but, in addition, another regioisomeric two-ring-fused adduct was also obtained. In the current work, we report on the complete structural analysis of the various adducts and their degradation products. We also show that some of these adducts are formed in calf thymus DNA and thus the reaction under study may be of biological significance.

Results and Discussion

Reaction of Acrolein with 1-Methylcytosine

To facilitate the amenable interpretation of the NMR spectroscopic data and the structural assignments of the 2′-deoxycytidine adducts—especially those containing two acrolein units—the adducts from 1-methylcytosine (**3**) were examined first, a strategy that has been successfully adopted previously.[28,34] In both cases, the resulting adducts were isolated by semipreparative chromatography from the reaction mixtures. The formation of either the one-ring-fused adducts or the two-ring-fused adducts was clearly evident by MS and further confirmed by gross NMR structural features. The refined structures and conformational features were elucidated by comprehensive NMR studies using a standard set of experiments and methodology[28,35–37] including the routine acquisition of ^{15}N as this nucleus has been demonstrated to be of great utility in determining the structures of nucleobase adducts[35,36,38–40] and, in this scenario in particular, the state and site of protonation of the molecules.[28] Importantly, prior to spin simulation of the ^{1}H spin systems, the spin systems of each diastereomer in cases where a pair of diastereomers co-eluted were assigned based on persistent consistencies in the various spectra, e.g. 1-D selective TOCSY, 1-D NOESY, HMBC, etc. With limited sample amounts, the aromatic proton signals usually provided the best means of evaluating the number of diastereomers present in a sample and verifying structures by admixture and subsequent ^{1}H NMR analysis.

The composition of the 1-methylcytosine (**3**) reaction mixture revealed three main products, from which the second-eluting compound **4** was quickly identified as the anticipated one-ring-fused adduct akin to the one-ring-fused ad-



Scheme 1. Structures of adducts formed in the reaction between acrolein (**1**) and 1-methylcytosine (**3**). The products are racemic but for this representation, substituents below the idealized plane of the molecule are designated as α and so on. The reaction **5**→**4** occurs during workup.

duct described previously for 9-ethyladenine[28] (Scheme 1). The adduct was isolated as a protonated species and the formal location of the charge is N-3 but with the labile proton (not observed) located on N^4 (i.e. analogous to the previous study) based on the chemical shifts of the nuclei (^{1}H, ^{13}C and ^{15}N) pertaining to the adduct moiety, particularly in comparison with previous results.[28] In **4**, the expected differences in the adduct segment in comparison to the acrolein (**1**) adduct of 9-ethyladenine[28] due to the proximity of the carbonyl group were not apparent, e.g. the deshielding of H-aα, and -β by the anisotropy of the carbonyl group, thus indicating that the flexible mobility of the bicyclic ring system positions the nuclei in the intermediate zone between shielding and deshielding. Consistent with this, only a minimal increase in the geminal coupling constant magnitude between H-aα and H-aβ was evident indicating that the π-bond of the carbonyl group does not adequately bisect the appropriate bond angle. Based on the similarities of the coupling constants to the structures examined previously,[28] the conformation adopted by the adduct ring is essentially the same, viz. $^{c}H_b$, placing the hydroxy group once again in an axial position for this racemate.

Similarly, the third-eluting compound **5** was readily identified as a two-ring-fused adduct counterpart to the two-ring-fused adduct observed in the previous study,[28] viz. where a second ring formation has occurred with fusion onto the first adduct ring (Scheme 1). As expected, two racemic diastereomers were formed and these co-eluted as the one peak. As before, the conformational mobility and geometrical flexibility of nitrogen can permit the permissive participation of a number of ring conformations in which the nitrogens are situated, or it can lead simply to the confluence of a single structure,[41–44] but with the result that the conformational behavior was also analogous to the previous study and thus the C-f epimers differ not only by configuration, but also in the orientation of the HO-f group (axial or equatorial, **5*t*** and **5*c***, respectively) by maintaining an axial orientation for the O-c group. The conformation of the adduct rings for each diastereomer is thus $^{c}H_b$,$^{e}C_c$. The products are salts and the charge is formally placed on N-3 in each.

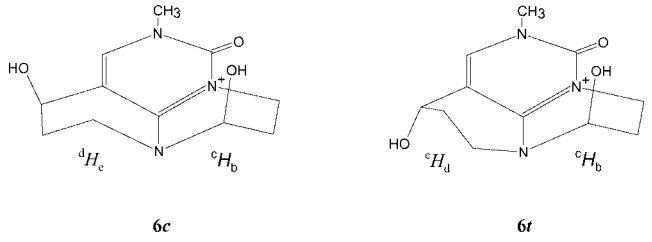
The striking feature of the first-eluting compound **6**, also with the clear incorporation of two acrolein units and the presence of two co-eluting racemic diastereomeric salts, was the lack of the H-5 pyrimidine proton thereby indicating C-5 as an attachment site of the second adduct ring. This was further confirmed by correlations of H-f to C-6, C-5 and C-4. The extraction of the coupling constants indicated similar conformational behavior ($^{c}H_b$) of the first adduct ring (a-b-c) as to the corresponding adduct ring in **4**. Interestingly, conformational interconversion ($^{d}H_e \leftrightarrow {}^{e}H_d$) of the second adduct ring (d-e-f) occurred with a change of configuration at C-f. Thus all hydroxys (i.e. both hydroxy groups in both compounds) are oriented (pseudo)axially. A direct comparison of NOEs to assign the stereochemistry was not possible due to extensive overlap arising from, in the main, the similarity of corresponding nuclei from the two structures. The structural assignment was based on the combined NOE from H-c to H-dax and H-d′eq (signals overlapped) and to H-d′ax and H-deq (signals close but separable). In the *cis* diaxial compound, the d equatorial proton is further away from H-c compared to the d equatorial proton in the *trans* diaxial structure, the converse is true for the d axial protons in relation to H-c. Therefore one spectrally close pair (an axial d proton from one structure and an equatorial d proton from the other structure) is ex-

Table 1. Stereochemical and conformational data for compounds **4**–**11** in D_2O at 25 °C.

	Stereochemistry	Ring conformations a-b-c	d-e-f	Diastereomer ratio	Comments
4	c*R**	$^{c}H_b$	–	–	racemic, HO axial
5*t*	c*R**,f*S**	$^{c}H_b$	$^{d}C_c$	54:46, **5*t*/5*c***	racemic diastereomer isolated with **5*c*** as a pair of C-f epimers, H-c and H-f *trans*, O axial to a-b-c ring, HO axial
5*c*	c*R**,f*R**	$^{c}H_b$	$^{d}C_c$		racemic diastereomer isolated with **5*t*** as a pair of C-f epimers, H-c and H-f *cis*, O axial to a-b-c ring, HO equatorial
6*c*	c*R**,f*R**	$^{c}H_b$	$^{d}H_e$	53:47, **6*c*/6*t***	racemic diastereomer isolated with **6*t*** as a pair of C-f epimers, OHs *cis* and both axial
6*t*	c*R**,f*S**	$^{c}H_b$	$^{e}H_d$		racemic diastereomer isolated with **6*c*** as a pair of C-f epimers, OHs *trans* and both axial
7	1′*R*,3′*S*,4′*R*,c*RS*	$^{c}H_b$/$^{b}H_c$	–	52:48	isolated together as a pair of C-c epimers, spectroscopically inseparable, HO axial in each isomer
8*c*	1′*R*,3′*S*,4′*R*,c*R*,f*R*	$^{c}H_b$	$^{d}H_e$	53:47, **8*c*/8*t***	isolated with **8*t*** as a pair of C-c epimers, OHs *cis* and axial
8*t*	1′*R*,3′*S*,4′*R*,c*S*,f*R*	$^{c}H_b$	$^{e}H_d$		isolated with **8*c*** as a pair of C-c epimers, OHs *trans* and axial
9*t*	1′*R*,3′*S*,4′*R*,c*R*,f*S*	$^{c}H_b$	$^{e}H_d$	49:51, **9*t*/9*c***	isolated with **9*c*** as a pair of C-c epimers, OHs *cis* and axial
9*c*	1′*R*,3′*S*,4′*R*,c*S*,f*S*	$^{c}H_b$	$^{d}H_e$		isolated with **9*t*** as a pair of C-c epimers, OHs *trans* and axial
10[a]	1′*R*,3′*S*,4′*R*,f*R*	–	$^{d}H_e$	–	stereochemically pure, HO axial
11[a]	1′*R*,3′*S*,4′*R*,f*S*	–	$^{e}H_d$	–	stereochemically pure, HO axial

[a] In [D_6]DMSO.

pected to, and does, give rise to a stronger NOE [i.e. H-dβ(eq) in the *trans* diaxial (**6*t***) and H-dβ(ax) in the *cis* diaxial (**6*c***)] than the other spectrally close pair. This result provides the assignment of the signal sets. Table 1 summarizes the stereochemical and conformational data of the products and Figure 1 portrays the conformational preferences of the adduct rings.

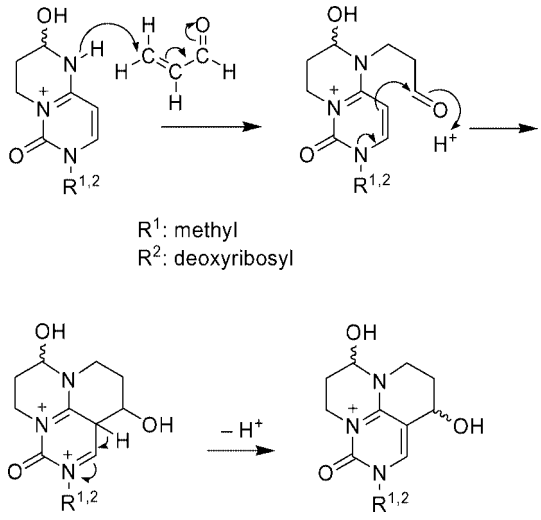


6*c* **6*t***

Figure 1. The conformation and configuration of the adduct rings of **6**. The HO-c groups are axial in both **6*c*** and **6*t*** and the HO-f groups, with the conformational change upon change of configuration, are pseudoaxial in both compounds.

Concerning the mechanistic course of the reaction, **4** is formed by initial Michael reaction at N-3 followed by cyclization at the exocyclic amine N^4, as elaborated previously.[28] Similarly, for **5**, formation follows in a manner akin to the two-ring-fused adduct formation of 9-ethyladenine[28] whereby the second cyclization results from attack of the hydroxy group of the primarily formed ring onto the carbonyl carbon of the second acrolein moiety after a second Michael addition by N^4. Of note, the subsequent attack of an alcohol onto an aldehyde group to form the final product has also been reported in other studies.[34,45]

Although formation of the ring at C-5 of the pyrimidine has not been observed very often,[46–48] it nevertheless has been reported that C-5 can be reactive towards electrophiles. For example, it has been found that glyoxal can react at C-5 of cytidine indicating nucleophilic attack of C-5 on the electrophilic carbonyl carbon,[47] and this occurs even with the open availability of the N-3 site for reaction. Therefore, compound **6** is likely to be obtained by a second Michael addition of another unit of **1** on **4** at N^4 followed by the nucleophilic attack of the pyrimidine C-5 on the residual carbonyl group and hence formation of the second propano bridge (Scheme 2). The reaction is assisted no doubt by the participation of N-1 through the induction of its lone pair to facilitate the nucleophilicity of C-5 in conjunction with the fact that the reaction is intramolecular. It is these two factors coupled together that render the unusual pathway feasible. Since the one-ring-fused adduct resulting from C-5 attack was not observed, this reaction pathway to **6** is unlikely to be a significant contributor to the formation of **6** baring an unforeseen set of esoteric reaction rates. Furthermore, the explicit reaction of the deoxyribose derivative **7** with acrolein (**1**) provided the analogous two-ring-fused product to **6**, thus confirming this reaction pathway. Finally, the conversion of **5** to **6** was also not observed.



Scheme 2. The reaction mechanism leading to the formation of **6**.

Both of the two products **5** and **6** are stable in aqueous solution, though **5** decomposes during workup to **4** (identified by addition of authentic **4** to the degraded sample and subsequent ^{1}H NMR analysis) together with the release of free acrolein (**1**). For some NMR samples, even though dilute, aggregation was evident by spin diffusion and/or dynamic effects with obvious consequences for the adopted adduct conformations. For this reason and in consideration of their co-elution on analytical HPLC columns, to avoid potential self-disproportionation of the stereoisomers,[49] care was taken to collect the entire elution from the semi-preparative columns after it was ascertained that pure stereoisomers could not be obtained.

Reaction of Acrolein with 2′-Deoxycytidine

LC-MS analysis of the reaction between equimolar amounts of acrolein (**1**) and 2′-deoxycytidine (**2**) at pH 7.4 showed that three major adducts were formed within 3 d of reaction. Whilst only a moderate increase of the one-ring-fused adduct **7** could be observed when the reaction was performed with a fivefold excess of **1**, the yields of the two-ring-fused adducts **8** and **9** increased markedly (Figure 2). Under these conditions, the molar yields of **7**, **8** and **9** were 7.7%, 41.6% and 29.3%, respectively, and this reaction system was thus used for the isolation of **8** and **9**. At pH 4.6, **7** was the main product with only small or negligible amounts of **8** and **9**, hence this system was utilized for the procurement of **7**. The structure elucidation and conformational analysis effectively follows on from the previous examination of the 1-methylcytosine adducts as appropriate.

Compound **7** was assessed as being identical with the 3,N^4-hydroxypropano-2′-deoxycytidine adduct previously identified by Smith et al.[30] (Scheme 3) and it was evident by NMR that a mixture of diastereomers is present with a $^{c}H_{b}$ conformation for the adduct ring. The diastereomers could not be distinguished and, furthermore, due to their great similarity, could not even be separated into sets of

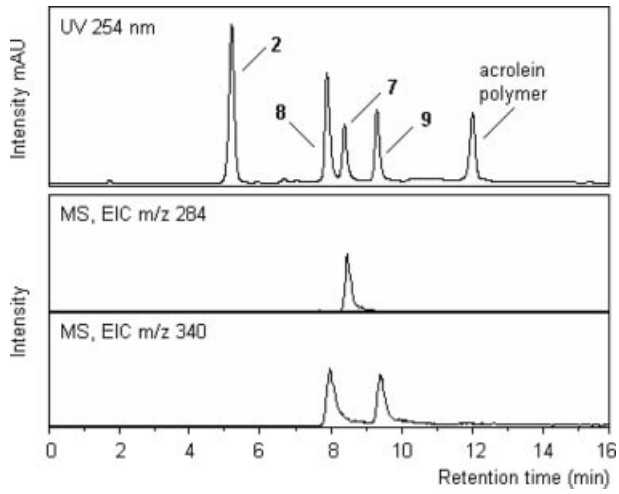


Figure 2. LC-MS chromatogram of the reaction mixture of acrolein (**1**) with 2′-deoxycytidine (**2**) in 0.5 M phosphate buffer pH 7.4 held at 37 °C for 3 d.

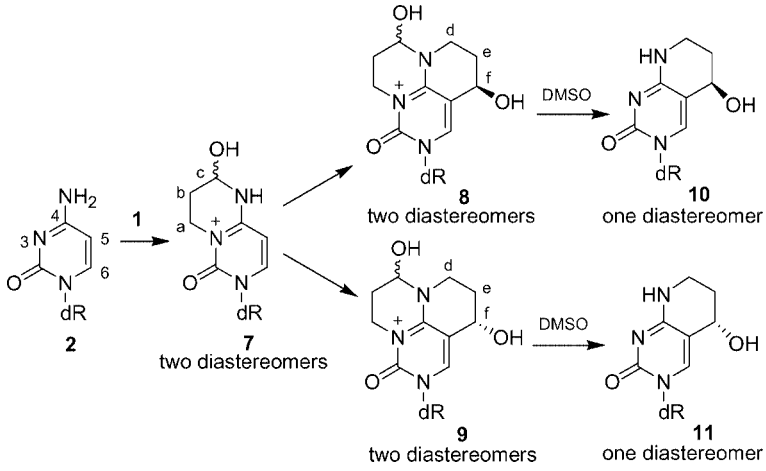
signals. For the adducts **8** and **9**, identical UV spectra and mass fragmental patterns were observed. The NMR spectra of both isolates were also very similar and both were a mixture of a pair of diastereomers. Since the H-5 pyrimidine proton was lacking for both, the identification of the basic structure in each case as the two-ring-fused adduct analogous to **6** was apparent and readily confirmed. Thus, the anticipated four diastereomers were chromatographically separated into two pairs. This was confirmed by admixture of the two samples and ^{1}H NMR analysis which confirmed the presence of a total of four diastereomers. The structural determination, conformational analysis and signal assignment of **8** and **9** follow analogously to the 1-methylcytosine studies as *per* above and the results are summarized in Table 1.

The strong divergence in the retention times of the diastereomeric pairs is remarkable and warrants some comment. One of the configurations at C-f permits a close spatial hydrogen bonding relationship to occur between the hydroxy group at C-f and the hydroxy at C-5′ of the deoxyribose unit, in part this is also dependent on the strong preference for axial orientation of the OH group in the d-e-f adduct ring. The reduced polar character that this interaction imparts to the molecule and/or the reduction in solvation leads to a longer retention on the column under reversed-phase conditions. Furthermore, it implies that **8** and **9** are each a pair of C-c epimers, subsequently confirmed (vide infra). The existence of this interaction and for it being responsible for the chromatographic behavior is underscored by the co-elution of the C-f epimers **6**.

Of note, under physiological conditions, we observed a higher reactivity of **7** towards acrolein (**1**) in comparison to 2′-deoxycytidine (**2**). This finding validated our previous conclusion[17] that the former exocyclic amino nitrogen of the nucleoside base is more reactive towards a Michael addition with a second unit of **1** than an unmodified nucleoside is towards **1**.

An adduct analogous to the third adduct **5** from the 1-methylcytosine reaction could not be detected in the reactions performed with 2′-deoxycytidine (**2**) under conditions which were considered to be near-identical to those applied to 1-methylcytosine (**3**) with respect to pH, time, and temperature.

Both two-ring-fused adducts **8** and **9** were effectively indefinitely stable in aqueous solution but were found to decompose when stored in DMSO in room temperature over the course of several days to new one-ring-fused adducts **10** and **11**, respectively, whereupon they remained as stable entities. The decomposition products were readily identified by NMR whereby for the ^{1}H spectra of **10** and **11**, the H-5 signal from the pyrimidine ring remained *in absentia* thus implying that the adduct ring on the other side was lost in each case. This was confirmed in **10** where the observable hydroxy proton displayed long-range correlations to both C-5 and C-e. In **11**, a C–H long-range correlation between H-6 and C-f was observed. These observations indicate the position of the hydroxy group at C-f. More importantly, each compound was diastereomerically pure (the co-addition of **10** and **11** and subsequent ^{1}H NMR analysis highlighted their diastereomic relationship), thereby confirming



Scheme 3. Structures of adducts formed in the reaction between acrolein (**1**) and 2′-deoxycytidine (**2**).

Table 2. Analytical data of nucleoside adducts found in the DNA hydrolysate.

Nucleoside adduct	Retention time (min)	Transition	Mode of formation	Cone voltage (V)	Collision energy (eV)
7	7.8	284→168	$MH^+ - dR + H$	9	12
9	8.5	340→224	$M^+ - dR + H$	9	15

the aforementioned supposition that **8** and **9** are each a pair of C-c epimers. The divergence in retention times between the epimers **10** and **11** mirrored that of their respective parents with essentially the same chromatographic properties for each parent-product set. The absolute configuration for **10** was assigned as (1′*R*,3′*S*,4′*R*,f*R*) and the conformation adopted by the adduct ring was $^{d}H_{e}$ (Figure 1); for **11**, the absolute configuration was assigned as (1′*R*,3′*S*,4′*R*,f*S*) and the conformation adopted by the adduct ring was $^{e}H_{d}$. Determination of the stereochemistry was based on observation of an NOE between H-1′ and HO-f in **11** after evaluation of the preferred rotamer. Monte Carlo and Molecular Dynamics simulation runs on the geometry-optimized structure in conjunction with direct evaluation of the r_{NOE} (subroutines available in Perch[50]), an important consideration for the correctness of the structural interpretation based on NOE data,[51] revealed that misinterpretation due to the system dynamics could be safely neglected.

Both compounds **10** and **11** were isolated as unprotonated species. Evidently the strong solvation of the salts, in particular the charge, in aqueous solution stabilizes the structures of **8** and **9** but this is insufficient in the organic solvent resulting in decomposition.

The Reaction of Acrolein with DNA

The reaction of acrolein (**1**) with single- and double-stranded calf thymus DNA was performed at pH 7.4 for 3 d. The positive ion electrospray LC-MS/MS analysis of the hydrolysate by multiple reaction monitoring mode in each case revealed the presence of two adduct peaks at precisely the same retention times as **7** and **9**. The monitored ion peaks were the protonated molecular ion and the cation of the salt, respectively, and fragment ion peaks were obtained by loss of the deoxyribosyl unit (m/z = 116 with concomitant H transfer) from the parent ions (Table 2). The level of the adducts formed in double-stranded DNA corresponded to 33 pmol/mg DNA (46 adducts/10^5 nucleotides) for **7** and 22 pmol/mg DNA (31 adducts/10^5 nucleotides) for **9**. In single-stranded DNA, **7** was detected at a level of 68 pmol/mg DNA (94 adducts/10^5 nucleotides) and **9** at a level of 45 pmol/mg DNA (63 adducts/10^5 nucleotides). Due to the co-elution of the two diastereomers comprising **9**, it is not possible to discern whether only one diastereomer is formed or both are formed in the reaction with DNA. Since the amounts were too small to examine even by ^{1}H NMR, it thus remains an open question at present. Similarly for **7**, the presence of one or two diastereomers could not be evaluated.

The creation in DNA of an adduct comprised of two acrolein (**1**) moieties is surprising. However, as we observed previously[17] in the case of adenine in DNA, the N^4 nitrogen of the one-ring-fused adduct reacts faster with another unit of **1** than does the original nucleoside.

Conclusions

The current study has shown that acrolein reacts with 2′-deoxycytidine to form one- and two-ring-fused adducts. However, it became evident that the appearance and distribution of products were dependent on experimental parameters such as pH, reaction time and quantity of the substrates, hence there are similarities but also differences to previous workers′ results. Quite different regioisomeric products were obtained in the case of the very minor base modification reactions [1-methylcytosine (**3**) vs. 2′-deoxycytidine (**2**)] though the same experimental conditions were used (pH reaction time, and temperature). The reaction with 1-methylcytosine results in the formation of an additional two-ring-fused adduct not observed in the reaction with 2′-deoxycytidine (**2**). Furthermore, it was shown that one- and two-ring-fused adducts, **7** and **9**, were formed in double- and single-stranded calf thymus DNA when incubated with acrolein (**1**). These one- and two-ring-fused adducts can potentially obstruct Watson–Crick base pairing and thus they are anticipated to be obstacles to replication and represent potential pre-mutagenic lesions. Therefore, cyclic adducts of 2′-deoxycytidine (**2**), if formed in DNA in vivo, may contribute to the genetic effects of acrolein (**1**).

Experimental Section

General: LC-DAD analyses were carried out using an Agilent Technologies 1100 series liquid chromatographic system consisting of a quaternary pump, a vacuum degasser, an autosampler, a thermostatted column compartment, a diode-array detector (UV) and Agilent ChemStation data handling program (Agilent Technologies, Espoo/Esbo, Finland) using an analytical reversed-phase CN column (5 μm, 4.6 × 150 mm, Zorbax SB-CN, Agilent Technologies), which was eluted with a gradient from 1% acetonitrile in 0.01 M ammonium acetate (pH 7.0) to 30% acetonitrile at a flow rate of 1 mL/min over the course of 20 min. Preparative isolation of the products was conducted on a semipreparative reversed-phase C-18 column (5 μm, 10 × 250 mm, Hypersil BDS-C18, Krotek, Tampere/Tamerfors, Finland). The column was coupled to an Agilent HPLC system equipped with an Agilent 1100 Series G1364C (analytical scale) fraction collector (Agilent Technologies). NMR spectra of adducts were recorded on NMR spectrometers equipped with a *z*-axis field gradient 5 mm inverse broadband probe operating at 600 (and 500) MHz for ^{1}H, 150 (and 125) MHz for ^{13}C and 60 MHz for ^{15}N. Spectra of adducts **10** and **11** were recorded at 25 °C in [D_6]DMSO and the solvent was used as the internal standard (δ = 2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C). All other adducts were

recorded at 25 °C in D_2O referenced internally to DSS (0.015 ppm for ^1H and 0.0 ppm for ^{13}C). ^{15}N spectra were referenced externally to 90% nitromethane in CD_3NO_2 (δ = 0.0 ppm). ^{15}N signals were only observed indirectly whereby the f1 dimension taken from a ^1H-^{15}N HMBC experiment was forward linear predicted to 11 Hz pt^{-1} (acquisition resolution 90 Hz pt^{-1}). In some cases, weak correlations were observed between H-6 and N-1 in HMBC spectra but were consequently confirmed by a 1D selective inverse correlation experiment (selincor[52]) which was adapted from magnitude mode HMQC by substitution of the second 90° ^{15}N hard pulse with a Gaussian-shaped selective pulse of 20 ms and utilizing a coupling evolution optimized for 8 Hz and an f1 evolution time set to 3 μs. Spin analysis was performed using Perch[50] iteration software for the extraction of ^1H chemical shifts and $J_{H,H}$ coupling constants on the complete spectra where possible but otherwise partially. Full experimental details for all NMR experiments are given in refs.[35–37] The UV spectra of the compounds were recorded with a Shimadzu UV-160A spectrophotometer and as the peaks eluted from the LC column by the diode-array detector. LC-ESI-MS/MS analyses were performed on an Agilent 1100 Series LC/MSD Trap SL instrument (Agilent Technologies) equipped with an electrospray source and operated in the positive ion mode. Ionization was carried out using nitrogen as both nebulizer gas (40 psi) and drying gas (12 L/min) heated to 350 °C. The capillary exit offset had a value of 115.3 V and the skim 1 voltage was set at 40 V. The maximum ion accumulation time was 2.00 ms and the target value was 20,000. Collision-induced dissociation experiments coupled with multiple tandem mass spectrometry (MS^n) employed helium as collision gas with the fragmentation amplitude varied between 0.7 and 1.0 V. The compounds were introduced through the LC system using an analytical reversed-phase C-18 column (5 μm, 4 × 125 mm, Hypersil BDS-C18, Agilent Technologies) and the same chromatographic conditions as for LC-DAD analyses were used with the exception of the flow rate which was reduced down to 0.5 mL/min and the use of an Agilent binary LC pump. For the analyses of the DNA adducts, a Quattro Micro triple-quadrupole mass spectrometer with an electrospray interface was used. Additional details concerning the general procedure are available in ref.[17] High resolution mass spectra were recorded on a Fisons ZABSpec-oa TOF instrument (Manchester, U. K.) operating at a resolution of 7,000. Ionizations were carried out using nitrogen as both nebulizer and bath gas; a potential of 8.0 kV was applied to the ESI needle; the temperature of the pepperpot counter-electrode was 90 °C; and PEG 200 was used for the supply of reference mass standards.

Determination of Deoxynucleoside Adduct Yields

Quantitative ^1H analysis, using methanol as an internal standard, was performed on aliquots of adducts. Standard solutions were prepared for LC-DAD analyses by taking a precise volume of the NMR sample and diluting it with an appropriate volume of water. Yield quantification was then made by comparing the adduct peak areas in the standard solutions with the adduct peak areas in the reaction mixtures using UV detection at 254 nm. Molar yields were calculated from the original amount of 2′-deoxycytidine (**2**).

Synthesis of 1-Methylcytosine (3): 1-Methylcytosine (**3**) was obtained according to the method of Hosmane and Leonard.[53]

Preparation of 2-Hydroxy-7-methyl-2,3,4,7-tetrahydropyrimido[1,6-*a*]pyrimidin-6-one (4), 2-Hydroxy-7-methyl-3,4,7,9,10,10a-hexahydro-2*H*-1-oxa-4a,7,8a-triazaphenanthren-8-one (5) and 6,9-Dihydroxy-2-methyl-2,5,6,7,8,9-hexahydro-4*H*-2,3a,6a-triazaphenalen-3-one (6): 1-Methylcytosine (**3**, 175 mg, 1.4 mmol) was allowed to react with acrolein (**1**, 784 mg, 14 mmol) in 0.5 M phosphate buffer (70 mL, pH 7.4) at 37 °C for 3 d. The volume of the aqueous phase was then concentrated to about 5 mL prior to purification of the mixture using the semipreparative C-18 column. The column was eluted isocratically for 2 min with 1% acetonitrile in 0.01 M phosphate buffer (pH 4.6) followed by a gradient to 10% acetonitrile over 20 min at a flow rate of 3 mL/min. The adducts **4** and **6** were then repurified on the semipreparative column eluted with a gradient from 1% to 5% acetonitrile in phosphate buffer (pH 4.6) over the course of 11 min. Adduct **5** was repurified by washing the column with a gradient from 1% acetonitrile/0.01 M formic acid to 5% acetonitrile over the course of 14 min and isocratically with 10% acetonitrile for 2 min at a flow rate of 3 mL/min.

Adduct 4: λ_{max}(HPLC eluent, ammonium acetate/acetonitrile)/nm 218, 284; λ_{min}(HPLC eluent)/nm 246; *m*/*z* (ESI) MS: 182 (100%, M + H^+), MS^2 of 182: 164 (100%, $MH^+ - H_2O$), 126 (34%, $MH^+ - C_3H_4O$); [HRMS: calcd. for ($C_8H_{11}N_3O_2 + H^+$): *m*/*z* 182.0929; found: *m*/*z* 182.0922]; for ^1H,^{13}C and ^{15}N NMR spectroscopic data: see supporting information.

Adduct 5: λ_{max}(HPLC eluent, ammonium acetate/acetonitrile)/nm 222, 294; λ_{min}(HPLC eluent)/nm 254; *m*/*z* (ESI) MS: 238 (100) [M^+], MS^2 of 238: 182 (100%, $M^+ - C_3H_4O$), MS^3 of 182: 126 (100%, $M^+ - C_6H_8O_2$); [HRMS: calcd. for ($C_{11}H_{16}N_3O_3^+$): *m*/*z* 238.1192; found: *m*/*z* 238.1194]; for ^1H,^{13}C and ^{15}N NMR spectroscopic data: see supporting information.

Adduct 6: λ_{max}(HPLC eluent, ammonium acetate/acetonitrile)/nm 222, 294; λ_{min}(HPLC eluent)/nm 254; *m*/*z* (ESI) MS: 238 (100) [M^+], MS^2 of 238: 220 (45%, $M^+ - H_2O$), 182 (100%, $M^+ - C_3H_4O$), MS^3 of 182: 164 (100%, $M^+ - C_3H_4O - H_2O$); [HRMS: calcd. for ($C_{11}H_{16}N_3O_3^+$): *m*/*z* 238.1192; found: *m*/*z* 238.1189]; for ^1H, ^{13}C and ^{15}N NMR spectroscopic data, see supporting information.

Preparation of 2-Hydroxy-7-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-2,3,4,7-tetrahydropyrimido[1,6-*a*]pyrimidin-6-one (7): Acrolein (**1**, 308 mg, 5.5 mmol) was added to 0.5 M phosphate buffer (100 mL, pH 4.6) containing 2′-deoxycytidine (**2**, 250 mg, 1.1 mmol) and the resulting solution left to incubate at 37 °C for 1 d after which the reaction was stopped with **7** as the main reaction product. The volume of the aqueous phase was then concentrated to about 5 mL prior to purification of the mixture using the semipreparative C-18 column. The column was eluted with a gradient from 1% acetonitrile in 0.01 M ammonium acetate (pH 7.0) to 30% acetonitrile over the course of 20 min at a flow rate of 3 mL/min. The collected fractions were combined, evaporated to dryness, and dried under vacuum to yield **7**.

Adduct 7: λ_{max} (H_2O)/nm 218 and 284 (ε/$dm^3\ mol^{-1}\ cm^{-1}$ 9120 and 17500); λ_{min} (H_2O)/nm 246 (3540); *m*/*z* (ESI) 284 (100%, M + H^+), MS^2 of 284: 168 (100%, MH^+ – deoxyribosyl + H), MS^3 of 168: 150 (100%, MH^+ – deoxyribosyl – H_2O + H); [HRMS: calcd. for ($C_{12}H_{17}N_3O_5 + H^+$): *m*/*z* 284.1246; found: *m*/*z* 284.1248]; for ^1H, ^{13}C and ^{15}N NMR spectroscopic data, see supporting info and also, $J_{H1',H2'\alpha}$ = 6.54/6.55, $J_{H1',H2'\beta}$ = 6.07/6.12, $J_{H'2\alpha,H2'\beta}$ = –14.25/–14.26, $J_{H'2\alpha,H3'} \approx 4.5$, $J_{H'2\beta,H3'}$ = 6.56/6.53, $J_{H'3,H4'}$ = 4.12/4.14, $J_{H'4,H5'proS}$ = 3.31/3.48, $J_{H'4,H5'proR}$ = 5.11/5.11, $J_{H'5proS,H5'proR}$ = –12.55/–12.51, $J_{H'5proS,HO5'}$ = 5.14, $J_{H'5proR,HO5'}$ = 5.19.

Preparation of 6,9-Dihydroxy-2-(2′-deoxy-β-D-*erythro*-pentofuranosyl)-2,5,6,7,8,9-hexahydro-4*H*-2,3a,6a-triazaphenalen-3-one (8 and 9): Acrolein (**1**, 308 mg, 5.5 mmol) was added to 0.5 M phosphate buffer (100 mL, pH 7.4) containing 2′-deoxycytidine (**2**, 250 mg, 1.1 mmol) and the resulting solution left to incubate at 37 °C for 3 d after which the reaction was worked up. The volume of the

aqueous phase was concentrated to about 5 mL prior to purification of the mixture using the semipreparative C-18 column. The column was eluted with a gradient from 1% acetonitrile in 0.01 M formic acid to 5% acetonitrile over the course of 14 min and then isocratically for 2 min with 10% acetonitrile at a flow rate of 3 mL/min. The collected fractions were evaporated to dryness and dried under vacuum to yield **8** and **9**. LC-DAD analysis of the reaction mixture provided yields for **7**, **8** and **9** of 7.7%, 41.6% and 29.3%, respectively.

Adducts 8 and 9: λ_{max} (H_2O)/nm 222 and 294 (ε/dm^3 mol^{-1} cm^{-1} 9020 and 15370); λ_{min} (H_2O)/nm 254 (3000); *m*/*z* (ESI) 340 (100) [M$^+$], MS^2 of 340: 224 (100%, M$^+$ – deoxyribosyl + H), MS^3 of 224: 168 (100%, M$^+$ – deoxyribosyl – C_3H_4O + H), MS^4 of 168: 150 (100%, M$^+$ – deoxyribosyl – C_3H_4O – H_2O + H); [HRMS: calcd. for ($C_{15}H_{22}N_3O_6^+$): *m*/*z* 340.1509; found: *m*/*z* 340.1520]; ^{1}H, ^{13}C and ^{15}N NMR spectroscopic data: see supporting information.

Decomposition Products of 8 and 9 (10 and 11, respectively): Approximately 4 mg of each of the products **8** and **9** were separately dissolved in 1 mL of DMSO and stored at room temperature for 3 d. The products were then separated on the semipreparative column eluted with the gradient consisting of water and acetonitrile starting from 1% acetonitrile and ending after 14 min at 21% acetonitrile. The flow rate was 3 mL/min.

Compounds 10 and 11 (diastereomers): λ_{max}(HPLC eluent, ammonium acetate/acetonitrile)/nm 282; λ_{min}(HPLC eluent)/nm 242; *m*/*z* (ESI) 284 (100%, M + H$^+$), MS^2 of 284: 168 (100%, MH$^+$ – deoxyribosyl + H), MS^3 of 168: 150 (100%, MH$^+$ – deoxyribosyl – H_2O + H); for **10** [HRMS: calcd. for ($C_{12}H_{17}N_3O_5$ + H$^+$): *m*/*z* 284.1246; found: *m*/*z* 284.1245]; for **11** [HRMS: calcd. for ($C_{12}H_{17}N_3O_5$ + H$^+$): *m*/*z* 284.1246; found: *m*/*z* 284.1239]; for ^{1}H, ^{13}C and ^{15}N NMR spectroscopic data, see supporting information and also, for **10**: $J_{H1',H2'\alpha}$ = 5.88, $J_{H1',H2'\beta}$ = 7.61, $J_{H'2\alpha,H2'\beta}$ = –13.19, $J_{H'2\alpha,H3'}$ = 3.06, $J_{H'2\beta,H3'}$ = 5.97, $J_{H'3,HO3'}$ = 4.14, $J_{H'3,H4'}$ = 2.96, $J_{H'4,H5'proS}$ = 4.22, $J_{H'4,H5'proR}$ = 4.14, $J_{H'5proS,H5'proR}$ = –11.69, $J_{H'5proS,HO5'}$ = 5.14, $J_{H'5proR,HO5'}$ = 5.19, for **11**: $J_{H1',H2'\alpha}$ = 5.97, $J_{H1',H2'\beta}$ = 7.67, $J_{H'2\alpha,H2'\beta}$ = –13.12, $J_{H'2\alpha,H3'}$, 3.13, $J_{H'2\beta,H3'}$ = 6.09, $J_{H'3,HO3'}$ = 4.15, $J_{H'3,H4'}$ = 3.00, $J_{H'4,H5'proS}$ = 4.09, $J_{H'4,H5'proR}$ = 3.89, $J_{H'5proS,H5'proR}$ = –11.97, $J_{H'5proS,HO5'}$ = 5.11, $J_{H'5proR,HO5'}$ = 5.14.

Reaction of Acrolein with Single- and Double-Stranded Calf Thymus DNA: The procedure was followed as described in ref.[17]

Supporting Information (see also the footnote on the first page of this article): The detailed ^{1}H, ^{13}C and ^{15}N NMR spectroscopic data for compounds **4**–**11**.

[1] International Agency for Research on Cancer. *IARC Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*, **1995**, *63*, p. 337.
[2] F.-L. Chung, R. G. Nath, M. Nagao, A. Nishikawa, G.-D. Zhou, K. Randerath, *Mutat. Res.* **1999**, *424*, 71–81.
[3] K. Uchida, M. Kanematsu, Y. Morimitsu, T. Osawa, N. Noguchi, E. Niki, *J. Biol. Chem.* **1998**, *273*, 16058–16066.
[4] J. Pan, F.-L. Chung, *Chem. Res. Toxicol.* **2002**, *15*, 367–372.
[5] International Agency for Research on Cancer, *IARC Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans* **1981**, *26*, 165–202.
[6] T. R. Crook, R. L. Souhami, A. E. McLean, *Cancer Res.* **1986**, *46*, 5029–5034.
[7] M. A. McDiarmid, P. T. Iype, K. Kolodner, D. Jacobson-Kram, P. T. Strickland, *Mutat. Res.* **1991**, *248*, 93–99.
[8] K. Ramu, L. H. Fraiser, B. Mamiya, T. Ahmed, J. P. Kehrer, *Chem. Res. Toxicol.* **1995**, *8*, 515–524.
[9] R. A. Parent, H. E. Caravello, R. H. C. San, *J. Appl. Toxicol.* **1996**, *16*, 103–108.
[10] L. J. Marnett, H. K. Hurd, M. C. Hollstein, D. E. Levin, H. Esterbauer, B. N. Ames, *Mutat. Res.* **1985**, *148*, 25–34.
[11] R. D. Curren, L. L. Yang, P. M. Conklin, R. C. Grafstrom, C. C. Harris, *Mutat. Res.* **1988**, *209*, 17–22.
[12] M. Kawanishi, T. Matsuda, A. Nakayama, H. Takebe, S. Matsui, T. Yagi, *Mutat. Res.* **1998**, *417*, 65–73.
[13] L. M. Kaminskas, S. M. Pyke, P. C. Burcham, *Chem. Res. Toxicol.* **2005**, *18*, 1627–1633.
[14] A. J. Kurtz, R. S. Lloyd, *J. Biol. Chem.* **2003**, *278*, 5970–5976.
[15] F.-L. Chung, R. Young, S. S. Hecht, *Cancer Res.* **1984**, *44*, 990–995.
[16] R. A. Smith, D. S. Williamson, R. L. Cerny, S. M. Cohen, *Cancer Res.* **1990**, *50*, 3005–3012.
[17] A. J. Pawłowicz, M. Munter, Y. Zhao, L. Kronberg, *Chem. Res. Toxicol.* **2006**, *19*, 571–576.
[18] R. G. Nath, J. E. Ocando, F.-L. Chung, *Cancer Res.* **1996**, *56*, 452–456.
[19] R. G. Nath, J. E. Ocando, J. B. Guttenplan, F.-L. Chung, *Cancer Res.* **1998**, *58*, 581–584.
[20] L. A. VanderVeen, M. F. Hashim, L. V. Nechev, T. M. Harris, C. M. Harris, L. J. Marnett, *J. Biol. Chem.* **2001**, *276*, 9066–9070.
[21] C. de los Santos, T. Zaliznyak, F. Johnson, *J. Biol. Chem.* **2001**, *276*, 9077–9082.
[22] M. Kanuri, I. G. Minko, L. V. Nechev, T. M. Harris, C. M. Harris, R. S. Lloyd, *J. Biol. Chem.* **2002**, *277*, 18257–18265.
[23] I. G. Minko, M. T. Washington, M. Kanuri, L. Prakash, S. Prakash, R. S. Lloyd, *J. Biol. Chem.* **2003**, *278*, 784–790.
[24] I. D. Kozekov, L. V. Nechev, A. Sanchez, C. M. Harris, R. S. Lloyd, T. M. Harris, *Chem. Res. Toxicol.* **2001**, *14*, 1482–1485.
[25] I.-Y. Yang, G. Chan, H. Miller, Y. Huang, M. C. Torres, F. Johnson, M. Moriya, *Biochemistry* **2002**, *41*, 13826–13832.
[26] A. M. Sanchez, I. G. Minko, A. J. Kurtz, M. Kanuri, M. Moriya, R. S. Lloyd, *Chem. Res. Toxicol.* **2003**, *16*, 1019–1028.
[27] Y. Kawai, A. Furuhata, S. Toyokuni, Y. Aratani, K. Uchida, *J. Biol. Chem.* **2003**, *278*, 50346–50354.
[28] A. J. Pawłowicz, T. Munter, K. D. Klika, L. Kronberg, *Bioorg. Chem.* **2006**, *34*, 39–48.
[29] R. S. Sodum, R. Shapiro, *Bioorg. Chem.* **1988**, *16*, 272–282.
[30] R. A. Smith, D. S. Williamson, S. M. Cohen, *Chem. Res. Toxicol.* **1989**, *2*, 267–271.
[31] R. A. Smith, I. A. Sysel, T. S. Tibbels, S. M. Cohen, *Cancer Lett.* **1988**, *40*, 103–109.
[32] A. Chenna, C. R. Iden, *Chem. Res. Toxicol.* **1993**, *6*, 261–268.
[33] L. C. James, P. Roversi, D. S. Tawfik, *Science* **2003**, *299*, 1362–1367.
[34] L. Gingipalli, P. C. Dedon, *J. Am. Chem. Soc.* **2001**, *123*, 2664–2665.
[35] J. Mäki, P. Tähtinen, L. Kronberg, K. D. Klika, *J. Phys. Org. Chem.* **2005**, *18*, 240–249.
[36] P. Virta, A. Koch, M. U. Roslund, P. Mattjus, E. Kleinpeter, L. Kronberg, R. Sjöholm, K. D. Klika, *Org. Biomol. Chem.* **2005**, *3*, 2924–2929.
[37] E. Balentová, J. Imrich, J. Bernát, L. Suchá, M. Vilková, N. Prónayová, P. Kristian, K. Pihlaja, K. D. Klika, *J. Heterocycl. Chem.* **2006**, *43*, 645–656.
[38] J. Mäki, K. D. Klika, R. Sjöholm, L. Kronberg, *J. Chem. Soc., Perkin Trans. 1* **2001**, 1216–1219.
[39] M. U. Roslund, P. Virta, K. D. Klika, *Org. Lett.* **2004**, *6*, 2673–2676.
[40] K. D. Klika, J. Mäki, R. Sjöholm, L. Kronberg, *ARKIVOC* **2006**, *xii*, 65–74.
[41] A. Rosling, K. Klika, F. Fülöp, R. Sillanpää, J. Mattinen, *Heterocycles* **1999**, *51*, 2575–2588.
[42] A. Rosling, K. D. Klika, F. Fülöp, R. Sillanpää, J. Mattinen, *Acta Chem. Scand.* **1999**, *53*, 103–113.
[43] A. Rosling, M. Hotokka, K. D. Klika, F. Fülöp, R. Sillanpää, J. Mattinen, *Acta Chem. Scand.* **1999**, *53*, 213–221.
[44] P. Tähtinen, A. Bagno, K. D. Klika, K. Pihlaja, *J. Am. Chem. Soc.* **2003**, *125*, 4609–4618.

[45] M. C. Byrns, D. P. Predecki, L. A. Peterson, *Chem. Res. Toxicol.* **2002**, *15*, 373–379.
[46] D. Florea-Wang, E. Haapala, J. Mattinen, K. Hakala, J. Vilpo, J. Hovinen, *Chem. Res. Toxicol.* **2004**, *17*, 383–391.
[47] R. Olsen, P. Molander, S. Ovrebo, D. G. Ellingsen, S. Thorud, Y. Thomassen, E. Lundanes, T. Greibrokk, J. Backman, R. Sjöholm, L. Kronberg, *Chem. Res. Toxicol.* **2005**, *18*, 730–739.
[48] H. Kasai, N. Iwamoto-Tanaka, S. Fukada, *Carcinogenesis* **1998**, *19*, 1459–1465.
[49] For example, a) R. M. Carman, K. D. Klika, *Aust. J. Chem.* **1991**, *44*, 895–896; b) V. A. Soloshonok, *Angew. Chem. Int. Ed.* **2006**, *45*, 766–769 and references cited therein.
[50] See for example: R. Laatikainen, M. Niemitz, U. Weber, J. Sundelin, T. Hassinen, J. Vepsäläinen, *J. Magn. Reson. Ser. A* **1996**, *120*, 1–10. See also: *Peak Research NMR Software*, Perch Solutions Ltd., Kuopio, Finland, **2003** (http://www.perchsolutions.com).
[51] K. D. Klika, J. Imrich, I. Danihel, S. Böhm, P. Kristian, S. Hamuľaková, K. Pihlaja, A. Koch, E. Kleinpeter, *Magn. Reson. Chem.* **2005**, *43*, 380–388.
[52] S. Berger, *J. Magn. Reson.* **1989**, *81*, 561–564.
[53] R. S. Hosmane, N. J. Leonard, *Synthesis* **1981**, *2*, 118–119.

Received: September 14, 2006
Published Online: December 8, 2006

