Molecular Mechanisms of DNA Damage Initiated by $\alpha,\beta$-Unsaturated Carbonyl Compounds As Criteria for Genotoxicity and Mutagenicity

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$\alpha,\beta$-Unsaturated carbonyl compounds are important not only from a theoretical but also a practical standpoint. These ubiquitous compounds can interact with DNA through various mechanisms. The predominant interaction is the formation of cyclic 1,N$^2$-deoxyguanosine adducts; 7,8-cyclic guanine adducts are also found. We have synthesized and characterized the stereoisomers of adducts formed by about 20 $\alpha,\beta$-unsaturated carbonyl compounds. The different types of adducts and the mutagenic and genotoxic response can be explained by the molecular structures of the agents. Compounds forming saturated cyclic adducts are mutagenic in S. typhimurium strain TA100 and to a lesser extent in TA1535. Substances with a leaving group at the C-3 position form unsaturated conjugated cyclic adducts and are mutagenic only in the His D9052 frameshift strains with an intact excision repair system (no arcA mutation). Metabolic epoxidation of the double bond and other metabolic activation, e.g., activation of the nitrogroups via nitroreductases, were also found to contribute to genotoxic and mutagenic activities. Our results have further elucidated the genotoxic mechanisms of these compounds; however, additional investigations are required for a complete understanding of the genotoxic activity of this class of compounds.

Introduction

$\alpha,\beta$-Unsaturated carbonyl compounds are frequently occurring industrial chemicals and environmental pollutants. The worldwide industrial production of acrolein, for example, is estimated to be more than 500,000 tons yearly (1). Some members of this group, such as ethylvinyl ketone, are found in fruits and vegetables and in other foodstuffs (2). Others such as acrolein are formed during biological processes, for instance, in the formation of humic acid. Furthermore, acrolein and its congeners are combustion products found in considerable amounts in automobile exhausts, tobacco smoke, and flue gases (3). Some of them are produced during the preparation of foods. $\alpha,\beta$-Unsaturated carbonyl compounds are also used as pesticides or formed as degradation products of pesticides (4,5). Since our group first published structure mutagenicity relationships for these compounds (6–8), many other studies have confirmed that these compounds are mutagenic (9–11). Crotonaldehyde has been found to induce liver cancer in rats (12), and it should be considered as a potential human carcinogen.

The formation of cyclic 1,N$^2$-deoxyguanosine adducts has been reported (13–15) and could at least in part be responsible for the mutagenic activity and genotoxic effects as expressed in the induction of the sfiA function in the SOS Chromotest. To date all data published are consistent with the assumption that this widespread class of compounds represents a potential risk to human health. The diverse testing protocols do not, however, allow uniform interpretation of the underlying mechanisms.

In spite of a common structural feature, $\alpha,\beta$-unsaturated carbonyl compounds can undergo different interactions with DNA, which lead to different genotoxic and mutagenic responses. The following genotoxic mechanisms are conceivable: formation of cyclic adducts, frameshift interaction, strand breaks, and cross-linking. In addition to direct interactions, other metabolic activations are conceivable, such as metabolic epoxidation and formation of radicals or activation of nitro groups or amino groups. The purpose of our studies is to provide a better understanding of the genotoxic, mutagenic, and possible carcinogenic activities of this class of compounds.
Materials and Methods

Chemicals
The chemicals, biochemicals, and reagents used were purchased in the highest purity available from Merck (Darmstadt, FRG), Serva (Heidelberg, FRG), Roth (Karlsruhe, FRG), or Boehringer (Mannheim, FRG).

The α,β-unsaturated carbonyl compounds were either bought or synthesized according to the literature or our own methods. All synthesized compounds were characterized by spectroscopic methods, in particular by NMR spectroscopy, and checked for purity by gas chromatography or HPLC. The substances tested in the SOS Chromotest and Ames test were carefully purified immediately before use and in general had purities greater than 99.5%.

Synthesis of Adducts
1, N2-Cyclic deoxyguanosine adducts and 1, N2-cyclic deoxyguanosine 5'-monophosphate adducts were synthesized by reaction of the respective α,β-unsaturated carbonyl compound with deoxyguanosine in phosphate buffer or dimethylsulfoxide. After separation from polymers on a Sephadex LH20 column, the stereoisomers were isolated via MPLC/HPLC on a RP18 column using methanol/water as eluant and characterized using 1H-NMR, 13C-NMR spectroscopy, mass spectrometry, and 3-D-fluorescence spectroscopy. The NMR spectra of the acrolein adducts are shown as representative examples in “Results.” The corresponding 5'-deoxynucleotides were synthesized and characterized as described by Hoffman et al. (15).

To synthesize unsaturated 1, N2-cyclic guanine adducts, guanine (deoxyguanosine) was treated with a 3-fold excess of the respective α,β-unsaturated carbonyl compound in 0.2 N HCl solution at 60°C. After the end of the reaction the mixture was neutralized with 1 N NaOH, and the adducts were precipitated. The tricyclic adducts were purified via MPLC chromatography using an RP18 column and water/methanol (80:20) as eluant.

Mutagenicity and Genotoxicity Testing
The preincubation Salmonella test according to Maron and Ames (16) was used as described previously (17). In a few cases a modification of the liquid assay according to Rannung et al. (18), as described elsewhere (19), was also carried out.

The SOS Chromotest using E. coli strain PQ37 was performed according to Quillardet and Hofnung (20) as previously described (21). The method used for the SOS Chromotest with E. coli strain PM21 was described recently (22).

| Compound          | Type of constitution isomers | Number of adducts identified |
|-------------------|-------------------------------|-------------------------------|
| Acrolein          | 2 regioisomers                | 3                             |
| Crotonaldehyde    | 1 regioisomers                | 2                             |
| Methacrolein      | 2 regioisomers                | 5                             |
| Pentenal          | 1 regioisomers                | 2                             |
| Hexenal           | 1 regioisomers                | 2                             |
| 3,3-Dimethylacrolein | 1 regioisomers            | 1                             |
| α-Chloroacrolein  | 2 regioisomers                | 6                             |
| α-Bromoacrolein   | 2 regioisomers                | 6                             |
| Methylvinyl ketone| 1 regioisomers                | 2                             |
| Ethylvinyl ketone | 1 regioisomers                | 1                             |

**Table 1. Chemicals that form type I adducts.**

**Figure 1.** (A) HPLC of deoxyguanosine cyclic nucleosides Ia, b, and c with acrolein. Separation on a RP18 column, length 25 cm, internal diameter 4 mm, eluted with MeOH/H2O, 11/89 isocratic at a flow rate of 1 mL/min. Wavelength of the UV detection was 254 nm. (B) HPLC of deoxyguanosine cyclic 5'-nucleotides Ia, b, and c. Conditions: Supelco LC18 column, 10 min isocratic with sodium acetate, pH 5.3, 50 mM, then 30 min with a gradient 0–50% MeOH at a flow rate 1 mL/min; photo diode array. (C) Same conditions as in B, however, enlarged scale from 25–27 mins.
The method used for DNA binding studies in bacteria has been described previously (23).

**Results**

From our results, we can distinguish at least five main types of α,β-unsaturated carbonyl compounds. The results are presented here in preliminary form and will be published in detail in forthcoming papers.

**Substances Forming Saturated 1,N²-Deoxyguanosine Adducts**

We have synthesized and characterized the stereoisomers of deoxyguanosine adducts of 11 α,β-unsaturated carbonyl compounds and their corresponding 5’-deoxynucleotides [Eq. (1)]. An overview is given in Table 1. The chromatographic and NMR-spectroscopic data of the acrolein adducts are given as an example in

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**Figure 2.** (A) 1H-NMR spectra of acrolein adducts 1a and b (400 MHz) and 1c (250 MHz) using hexadeutero DMSO (DMSOd₆) as solvent and tetramethylsilane (TMS) as internal standard. (B) 13C-NMR spectra of acrolein adducts 1a and b (100 MHz) and 1c (62.5 MHz), DMSOd₆, and TMS as internal standard.
Table 2. Mutagenicity and genotoxicity of type I α, β-unsaturated carbonyl compounds.

| Compound                  | Mutagenicity in TA100, revertants/μmole | Genotoxicity, SOSIP<sup>a</sup> | SOS Chromotest, Imax<sup>b</sup> |
|---------------------------|------------------------------------------|---------------------------------|----------------------------------|
| Acrolein                  | 2,400                                    | 6.5 × 10⁻⁴                      | 1.6                              |
| Crotonaldehyde            | 257                                      | 7.3 × 10⁻³                      | 1.4                              |
| Methacrolein              | 184                                      | 0                               | 1.0                              |
| Pentenal                  | 89 (NS)<sup>c</sup>                     | 0                               | 1.0                              |
| Hexenal                   | 65 (NS)                                  | 0                               | 1.0                              |
| 3,3-Dimethacrolein        | 78 (NS)                                  | 4.8 × 10⁻⁴                      | 1.7                              |
| Methylvinyl ketone        | 472                                      | 7.3 × 10⁻³                      | 2.1                              |
| 2-Chloroacrolein          | 1,512,571                                | 11.20                           | 6.0                              |
| 2-Chloro-3-methylacrolein | ND<sup>d</sup>                           | 5.92                            | 10.5                             |
| 2-Chloro-3,3-dimethylacrolein | 38,344                             | 0.08                            | 11.0                             |
| 2-Bromoacrolein           | 1,236,534                                | 25.2                            | 8.8                              |

<sup>a</sup>SOSIP, SOS inducing potency according to the definition of Quillardet and Hofnung (20).

<sup>b</sup>Imax, maximal SOS induction factor (20); genotoxicity is significant at Imax ≈ 1.5.

<sup>c</sup>NS, not significant.

<sup>d</sup>ND, not determined.

**Figure 3.** Three-dimensional fluorescence spectra of cyclic adducts produced by reaction of 2'-deoxyguanosine with 3-chloro-2-methylacrolein (a) and 2-chloroacrolein (b) registered on a Hitachi 650-40 fluorescence spectrophotometer combined with an Atari 520 ST computer using a self-designed computer program. Excitation maximum of (a) 321 nm, emission maximum 514 nm. Excitation maximum of (b) 235 and 305 nm, emission maximum 350 nm.

**Figure 4.** (A) ¹H-NMR spectrum, 200 MHz, of the guanine adduct of 3-chloro-2-methylacrolein in DMSOδ and TMS as internal standard. (B) ¹³C-NMR spectrum, 100 MHz, of the guanine adduct of 3-chloro-2-methylacrolein in DMSOδ and TMS as internal standard.
Figures 1 and 2. Besides the 1,N2-cyclic adducts, trace amounts of 7,8-cyclic guanine adducts have also been detected in these studies. These type I compounds are mutagenic in the His G46 strain TA100 and to a lesser extent in TA1535, and they induce the sfiA function in the SOS Chromotest (Table 2). 2-Chloro and 2-bromo substituted congeners are extremely mutagenic in TA100.

We have synthesized and characterized seven adducts of this type. The spectroscopic data of the 1,N2-adduct of 3-chloro-2-methacrolein are presented as an example (Figs. 3 and 4). In contrast to the saturated adducts, these conjugated unsaturated adducts possess a strong fluorescence. Therefore, these adducts should be easily detected in biological material via HPLC using a fluorescence detector. We could isolate and detect the respective adduct of 3-chlorocrotonaldehyde from bacterial DNA after incubating a bacterial suspension with this substance.

These type II compounds are mutagenic in the His D3052 frameshift Salmonella strain containing an intact excision repair system, but they are negative in the frameshift strains TA1538 and TA98 and also negative in the His G46 strains TA1535 and TA100. The 2-alkyl substituted compounds, e.g., 3-chloro-2-methacrolein, show only borderline activity in strain His D3052 (Table 3). Nearly all the compounds tested, even those possessing poor leaving groups (alkoxy groups), induce the sfiA function in the SOS Chromotest (Table 3).

Other Compounds

Evidence has been found that α,β-unsaturated ketones such as methylenyld ketone or ethylvinyl ketone are activated to epoxides (2,24). Although the specific mutagenicity (revertants per µmole) are not higher, the peak revertant rate is clearly higher in the presence of S9 mix than in its absence. Addition of SKF 525, an inhibitor of monoxygenases, led to a loss in mutagenicity, whereas addition of TCPO (trichloropropene oxide), an inhibitor of the epoxide hydrolases, resulted in an increase in mutagenicity.

2-Chloro- and 2-bromocinnamaldehydes do not form cyclic adducts although they are highly mutagenic in the strains TA100, TA98, and TA1538, but not in TA1535, and these compounds are also positive in the SOS Chromotest. The relatively high mutagenicity and genotoxicity of 2-bromocinnamaldehyde, which is about 20 times higher than that of acrolein, is remarkable. Cinnamaldehyde and its derivatives, which are substituted at the phenyl ring, e.g., p-chlorocinnamaldehyde or o-methoxycinnaldehyde, do not show any mutagenic or genotoxic activities.

Cinnamaldehydes containing nitro groups are evidently metabolically activated via the nitro groups (Table 4). p-Nitrocinnamaldehyde and p-nitrocinamnic acid are highly mutagenic in TA100 strains containing nitroreductase but not mutagenic or only poorly mutagenic in such TA100 strains that lack nitroreductase activity, e.g., TA100 TN5 DNP6 or NR TA100. The fact that the acid as well as the aldehyde are mutagenic strongly suggests that the formation of cyclic adducts is not responsible for this mutagenicity.

Discussion

The predominant interaction of α,β unsaturated carbonyl compounds with DNA components is the formation of cyclic adducts. Two different types of adducts are formed. The absence or presence of a suitable leaving group at the C-3 position of the acrolein moiety decisively determines the mutagenic response. It is remarkable that these rather small differences in the structure (Fig. 5) of the molecule produce marked differences in mutagenicity.

Compounds of type I are mutagenic in His G46 S. typhimurium strains. A lack of excision repair (uvr mutation) and the presence of the pkM101 plasmid (error prone repair) increases markedly the sensitivity of the His G48 strains toward the type I compounds. In contrast, the type II compounds, showing frameshift activity in His D3052 strains, require an intact excision repair system for the induction of back mutation. Basu and Marnett (9) have explained this effect by the high toxicity of these type of compounds toward the bacteria,
Table 3. Mutagenicity and genotoxicity of 3-chloro- and 3-ethoxyacroleins.

| Acrolein compound          | Structure | Genotoxicity in the SOS Chromotest | Mutagenicity in His D3052, revertants/μmole |
|---------------------------|----------|------------------------------------|---------------------------------------------|
|                           |          | SOSIP<sup>a</sup> | I<sub>max</sub><sup>b</sup> |                                      |
| 3-Chloroacrolein          |          | 5.8 × 10^-4 | 1.25 | ND<sup>c</sup>       |
| 3-Chloro-2-methyl-acrolein|          | 0          | 1.00 | 0                     |
| 3-Chloro-3-methyl-acrolein|          | 3.5 × 10^-4 | 1.60 | 184 (S9 -) 123 (S9 +) |
| 3-Ethoxy-2-methyl-acrolein|          | 9.94 × 10^-4 | 1.81 | NS<sup>d</sup>      |
| 3-Ethoxy-2-propyl-acrolein|          | 2.4 × 10^-3 | 2.73 | NS                    |
| 3-Ethoxy-2-pentyl-acrolein|          | 8.26 × 10^-3 | 1.71 | 0                     |
| 2,3-Dihydro-4H-pyran-5-carbaldehyde| | 4.4 × 10^-3 | 5.01 | 13/μL                 |

<sup>a</sup>SOSIP, SOS inducing potency according to the definition of Quillardet and Hefnung (20).
<sup>b</sup>I<sub>max</sub>, maximal SOS induction factor (20).
<sup>c</sup>ND, not determined.

Table 4. Mutagenicity in *S. typhimurium* TA100 and genotoxicity in the SOS Chromotest of nitrocinnamaldehyde and related compounds.<sup>a</sup>

| Substances               | TA100 - S9 | TA100 + S9 | TA100 TN5 | DNP6 | NRTA100 - S9 | NRTA100 + S9 | SOS Chromotest |
|--------------------------|-----------|-----------|----------|------|-------------|-------------|---------------|
| p-Nitrocinnamaldehyde    | +         | +         | NS       | +    | NS          | +           | +             |
| o-Nitrocinnamaldehyde    | +         | +         | ND       | ND   | ND          | +           | +             |
| p-Nitrocinnamic acid     | +         | +         | +        | ND   | ND          | +           | +             |
| o-Nitrocinnamic acid     | +         | +         | +        | ND   | ND          | +           | +             |

<sup>a</sup>(+ +) Strong positive; (+) positive; (-) negative; NS, not significant; ND, not determined.

which survive only if they possess an excision repair system.

A further interesting point is the extremely high mutagenicity of 2-bromo- and 2-chloro-substituted congeners in the His G48 strains TA100 and TA1535. These results demonstrate that the mutagenicity of the type I compounds does not depend exclusively on the pkM101 plasmid-mediated error prone repair but, at least in some cases, also depends on base pair substitution. It is, however, not yet clear whether and to what extent other processes in addition to binding at GC hot spots are involved, e.g., second site mutations.

The reason for the enormous mutagenicity-enhancing effect of the chlorine or bromine substituents in position 2 of the acrolein moiety presently remains unclear. It is, however, remarkable that such substituents at either the C-2 or the C-3 position of the acrolein moiety strongly influence the type of mutagenic response as well as the strength of mutagenicity. Whereas in the case of type I compounds it is most likely that formation of cyclic adducts leads to mutagenicity and genotoxicity, in the case of the type II compounds a clear relationship between formation of adducts and frameshift mutagenicity is not clear. Several other interactions such as strand breaks or the formation of crosslinking are also conceivable. These possibilities are now under investigation in our laboratory. Nevertheless, formation of DNA adducts is a clear indicator of genotoxicity, mutagenicity, and presumably carcinogenicity. There is a strong correlation between the type of adducts formed
in vitro and the type of mutagenic/genotoxic response. Therefore, more sensitive methods must be developed that allow the detection of adducts from biological studies such as the \( ^{32}P \)-postlabeling HPLC technique or the monoclonal antibody technique.

In addition to type I and type II compounds, there are other \( \alpha,\beta \)-unsaturated carbonyl compounds that may act by different mechanisms. A possible mechanism is the epoxidation of the double bond via monooxygenases. We have found evidence for an epoxidation only for \( \alpha,\beta \)-unsaturated ketones, e.g., methylvinyl ketone (24) or ethylvinyl ketone (2). It should, however, be noted that these ketones are mutagenic per se as type I compounds and that reactions of the epoxides are involved additionally.

When investigating 2-chloro- and 2-bromocinnamaldehyde, cyclic adducts were not formed although these compounds are highly mutagenic in \( S.~typhimurium \) TA100, TA98, and TA1538, but not in TA1585. Due to the conjugation of the aromatic system of the phenyl group with the \( \alpha,\beta \)-unsaturated \( \pi \) electron system, a frameshift interaction of these compounds is conceivable, although their structures are different from the classical intercalators such as acridine dyes. An intercalating effect has been proposed for the \( p \)-methoxyphenyl moiety of puromycin intercalating between two adenines (25).

Nitrocinnamaldehydes and nitrocinnamic acids can evidently be activated via their nitro groups. We found an interesting structure mutagenicity relationship: \( p \)-nitrocinnamaldehyde and \( p \)-nitrocinnamic acid showed high mutagenic activities in strain TA100 (possessing nitroreductases) without addition of metabolizing enzymes (S9 mix). When using TA100 strains lacking nitroreductase activity, the compounds exerted mutagenic activities only in the presence of S9 mix. \( o \)-Nitrocinnamaldehyde was only slightly mutagenic in strain TA100 in the absence of S9 but clearly mutagenic in the presence of S9 mix, whereas \( o \)-nitrocinnamic acid was nonmutagenic irrespective of the presence or absence of S9 mix.

A question which to date has not been addressed is the role of radical formation (radicals are possibly formed in the reductive metabolism of halosubstituted acrolein congeners via the monooxygenases) and the production of reactive oxygen species, which may also contribute to the genotoxicity of these compounds.

Although our investigations and those of others have elucidated the relationship between structure, interaction mechanism, and genotoxic activities, many aspects of the genotoxicity of the \( \alpha,\beta \)-unsaturated carbonyl compounds are still not entirely understood and deserve further investigation.

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