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# Mutagenicity of oxaspiro compounds with Salmonella

J.E. Sinsheimer, P.K. Chakraborty, E.A. Messerly and V. Gaddamidi

*College of Pharmacy, University of Michigan, Ann Arbor, MI 48109-1065 (U.S.A.)*

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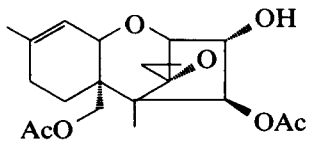
**Summary**

The spiro attachment of an epoxide group to a tetrahydropyran ring in the trichothecene mycotoxins has prompted this study of the mutagenicity and alkylation rates of the trichothecene, anguidine, and 5 related model oxaspiro compounds. While the model compounds were weak alkylating agents of 4-(4-nitrobenzyl)pyridine as a test nucleophile, anguidine lacks such activity. Also, while mutagenicity was not established for anguidine in Salmonella TA100, 3 of the oxaspiro compounds were weakly mutagenic and 2 compounds were toxic to the bacteria. The toxicity and mutagenicity of the model compounds are more related to their polarity than to their alkylation rates.

The presence of an epoxide group in a spiro attachment to a tetrahydropyran ring is a predominate feature of trichothecene mycotoxins. With the growing awareness of the contribution of trichothecenes to the potential health hazards of toxic mold contamination of grains, a large number of these mycotoxins have been isolated and characterized. Furthermore, their biological properties, especially their acute toxicity, have been examined (Bamburg and Strong, 1971; Ueno, 1983; Betina, 1984). One of the trichothecenes, anguidine (**1**), has been evaluated in phase II clinical trials as an antineoplastic agent where its acute toxicity prevented continued testing (Roush et al., 1985).

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There has also been considerable attention directed towards the trichothecenes as possible chemical warfare agents in regard to the yellow rain affair (Ember, 1984).

The carcinogenicity, teratogenicity and mutagenicity of the mycotoxins in general have been reviewed and only limited positive results are indicated for trichothecene compounds (Stark, 1980; Hayes, 1981). In a study of fusarium toxins, 6 trichothecenes have been tested for their mutagenicity in Salmonella strains TA98, TA100, TA1535 as well as TA1537 and found not to be mutagenic with or without the presence of an S9 liver fraction (Wehner et al., 1978). This lack of mutagenicity in the Ames test (Maron and Ames, 1983) is

Correspondence: Dr. J.E. Sinsheimer, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109-1065 (U.S.A.).

not unexpected based upon the reduced mutagenicity of 1,1-disubstituted epoxides (Wade et al., 1978).

However, since the epoxide moiety of trichothecenes has been reported to be essential for the biological activity of these compounds (Ueno, 1977), it was of interest to compare the mutagenicity of model oxaspiro compounds where the epoxide would be in a less complex structure. Indeed, as part of a previous structure–mutagenicity study of cycloaliphatic epoxides, we reported that cyclohexylidene oxide (1-oxaspiro[2,5]octane) was weakly mutagenic with Salmonella strain TA100 (Frantz and Sinsheimer, 1981). It is the purpose of the present investigation to extend this previous study by synthesizing additional model compounds which together with cyclohexylidene oxide and anguidine could be compared as to their mutagenicity in the Ames test for an initial evaluation of the genotoxicity of the oxaspiro moiety.

## Materials and methods

*Test compounds*

The starting materials for the synthesis of the oxaspiro compounds were purchased from Aldrich Chemical Company (Milwaukee, WI). Cyclohexylidene oxide was obtained from the Alfred Bader Chemicals Division of Aldrich and was used without further purification.

Anguidine (4$\beta$,15-diacetoxy-3$\alpha$-hydroxy-12,13-epoxytrichothec-9-ene; diacetoxyscirpenol) was a gift from Dr. M.A. Marletta, University of Michigan. The desired oxaspiro compounds were prepared by the procedure of Corey and Chaykovsky (1965) by reaction of the corresponding ketone with dimethyloxosulfonium methylide. The required ketone for 3-tetrahydropyranylidene oxide was synthesized from 3,4-dihydro-2*H*-pyran by hydroboration (Brown et al., 1985) followed by oxidation of the resulting 3-pyranol with Jones reagent (Eisenbraun, 1965). These intermediates and all final oxaspiro compounds were purified by distillation under reduced pressure and if necessary were redistilled until their IR and NMR spectra were consistent with their structures. The synthesis of 4-*tert.*-butylcyclohexylidene oxide has been previously reported by Corey and Chaykovsky (1965), and that of 2-decalenylidene oxide by Carlson and Behn (1967).

TLC was used to monitor reactions, determine $R_f$ values and examine the purity of test compounds. Analtech (Newark, DE) prescored silica-GF Uniplates (2 cm × 10 cm, 250 $\mu$m) and $CH_2Cl_2$–MeOH (98 : 2) as the solvent were employed. A compound was considered suitable for mutagenicity testing when a sample (2 $\mu$l, 10% $CH_2Cl_2$ solution) after TLC development showed one homogeneous spot upon alkylation of 4-(4-nitrobenzyl)pyridine (Hammock et al., 1974). Partition coefficients were obtained by reversed phase HPLC (Carlson et al., 1975) with the system as previously described (Rosman et al., 1986) but with an Altex (Berkeley, CA) model 156 refractive index detector. The mobile phase was methanol–water (1 : 1) at a flow rate of 1 ml/min.

A comparison of the chemical reactivities of the oxaspiro compounds was obtained by reacting 0.2 $\mu$mole of each compound with 4-(4-nitrobenzyl) pyridine (Aldrich) at 60 °C for 60 min as previously described (Nelis et al., 1982). It was necessary with some lots of 4-(4-nitrobenzyl)pyridine, in order to obtain a satisfactory blank in this colorimetric test, to remove traces of a color-producing impurity by extracting an ether solution of the reagent with water prior to recovery of the reagent from the ether solution.

*Mutagenicity assay*

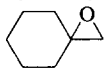
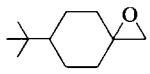
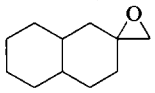
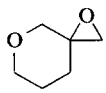
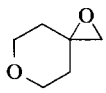
Dose–response relationships were determined using *Salmonella typhimurium* strain TA100 without S9 activation in the plate incorporation assay as described by Maron and Ames (1983). Following evaluation of each compound for dose–response relationships, doses of 2.5 and 5.0 $\mu$moles/plate were selected for concurrent testing of the relative mutagenicities of the oxaspiro compounds using bacteria from the same overnight culture. All tests were run in triplicate.

## Results and discussion

The structure and physical–chemical properties of the model oxaspiro compounds are given in Table 1 and include NMR data in confirmation of the desired structures. The HPLC-partition coefficient data in Table 1 in comparison to cyclohe-

TABLE 1

PROPERTIES OF OXASPIRO COMPOUNDS

| idene oxides | bp/mm Hg | TLC [a] $R_f$ | HPLC $T_R$ [b] min : sec | $^{\pi}$HPLC [c] | Nitrobenzyl pyridine reaction [d] | NMR [e] $\delta$ (assignments) |
|---|---|---|---|---|---|---|
| Cyclohexyl  | – | 0.79 | 7 : 24 | 0.212 | 0.09 ± 0.014 | 1.4–1.8 (10H, m, $CH_2$)<br>2.60 (2H, s, $^{O}\!>\!CH_2$) |
| 4-*tert.*-Butylcyclohexyl  | 52–54 °/0.8 | 0.67 | 45 : 12 | 1.231 | 0.219 ± 0.003 | 0.9 (9H, s, $CH_3$)<br>1.0–1.9 (9H, m, $CH_2$ + CH)<br>2.65 (2H, s, $^{O}\!>\!CH_2$) |
| 2-Decalenyl  | 70–72 °/0.8 | 0.82 | 35 : 0 | 1.109 | 0.132 ± 0.004 | 0.9–2.0 (16H, m, $CH_2$ + CH)<br>2.63 (2H, s, $^{O}\!>\!CH_2$) |
| 3-Tetrahydropyranyl  | 57–59 °/10 | 0.65 | 4 : 26 | −0.376 | 0.264 ± 0.015 | 1.63–2.0 (4H, m, $CH_2$)<br>2.70 (2H, s, $^{O}\!>\!CH_2$)<br>3.45–3.81 (4H, m, $OCH_2$) [f] |
| 4-Tetrahydropyranyl  | 50–52 °/9.3 | 0.53 | 4 : 10 | −0.506 | 0.274 ± 0.027 | 1.5–1.94 (4H, m, $CH_2$)<br>2.69 (2H, s, $^{O}\!>\!CH_2$),<br>3.76–3.93 (4H, m, $OCH_2$) |

[a] Elution was with $CH_2Cl_2$–MeOH (98 : 2).

[b] Retention time ($T_R$) includes a void time of 3 min 16 sec.

[c] $^{\pi}$HPLC=log ($k'$ test compound/$k'$ cyclohexene oxide). See Carlson et al. (1975).

[d] Absorbance (± SD) at 560 nm after 60 min at 60 °C with $n = 6$.

[e] At 270 MHz in $CDCl_3$.

[f] NMR included trace extraneous peaks at $\delta$ 2.4 and 4.5. Repeated distillation of this compound while not removing these bands did produce a 4-(4-nitrobenzyl)pyridine TLC puritv test consistent with the presence of only 1 alkylating agent.

xene oxide, indicated that we were dealing with compounds of 3 sets of polarity. 3-Tetrahydropyranylidene and 4-tetrahydropyranylidene oxide are more polar than cyclohexene oxide while 4-*tert.*-butylcyclohexylidene as well as 2-decalenylidene oxide are considerably less polar. Cyclohexylidene oxide is of intermediate polarity. Reactivity with 4-(4-nitrobenzyl)pyridine as a measure of the chemical alkylating ability of these compounds is also reported in Table 1. The relatively strong reaction conditions of 60 °C for 1 h were used for this set of compounds after determining color development was exceedingly slow at 37 °C with the strongest alkylating compound, 4-tetrahydropyranyl oxide, only reaching an absorbance of 0.075 after 1 h. The conditions used for our alkylation comparison were found to be on the linear portion of the reaction rate curves in regard to both concentration and reaction time for these compounds. Among the model oxaspiro compounds, the weakest alkylating agent in the present series, cyclohexylidene oxide, is stronger than the 1,2-disubstituted epoxides cyclohexene oxide (0.024 ± 0.003) and cyclopentene oxide (0.011 ±

0.002) while the trichothecene, anguidine, was inactive ($0.003 \pm 0.003$) when run under the same conditions. Although the alkylating ability of the oxaspiro compounds is in the same order of magnitude as our previous series of 1,1-disubstituted epoxides, the *para*-substituted $\alpha$-methylstyrene oxides (Rosman et al., 1986), they are less active in this regard than the monosubstituted propylene and butylene oxides (Rosman et al., 1987) or the glycidyl ethers (Rosman et al., 1988).

The results for the mutagenicity testing with Salmonella TA100 of our model oxaspiro compounds in comparison to anguidine are shown in Table 2. Differences in toxicity to the bacteria is a dominant feature in attempting comparative mutagenicity testing in this series of compounds. Two of the compounds, 4-*tert.*-butylcyclohexylidene and 2-decalenylidene oxides could only be tested at the lower concentrations before there was a noticeable reduction in background lawn. Cyclohexylidene oxide also showed signs of toxicity but only at the exceptionally high concentration of 60 $\mu$moles/plate while 3- and 4-tetrahydropyranylidene oxide were not toxic even at this dose.

Direct comparisons of mutagenicities were made at the same time from the same overnight culture of bacteria with compound concentrations of 2.5 and 5.0 $\mu$moles/plate. While statistically significant differences in revertants per plate compared to the negative controls were established for the 3- and 4-tetrahydropyranylidene and cyclohexylidene oxides at the 2.5-$\mu$mole concentration, none of these cyclic 1,1-disubstituted epoxides are strong mutagens. In this regard they are similar to the cyclic 1,2-disubstituted epoxides (Jung et al., 1981; Frantz and Sinsheimer, 1981) and exhibit the previously observed reduction in mutagenicity of the 1,1-disubstituted aliphatic epoxides in comparison to their mono-substituted analogues (Wade et al., 1978). Our finding of a lack of mutagenicity for anguidine at even higher concentrations con-

TABLE 2

MUTAGENICITY DOSE RESPONSE FOR OXASPIRO COMPOUNDS WITH *Salmonella typhimurium* STRAIN TA100

| Dose ($\mu$moles) | Revertants [a] | | | | | |
|---|---|---|---|---|---|---|
| | Anguidine | Cyclohexylidene oxide | 4-*tert.*-Butylcyclohexylidene oxide | 2-Decalenylidene oxide | 3-Tetrahydropyranylidene oxide | 4-Tetrahydropyranylidene oxide |
| 0 | $110 \pm 11$ | $110 \pm 3$ | $125 \pm 10$ | $125 \pm 10$ | $123 \pm 8$ | $123 \pm 8$ |
| | $(99 \pm 11)$ [b] | $(99 \pm 11)$ | $(99 \pm 11)$ | $(99 \pm 11)$ | $(99 \pm 11)$ | $(99 \pm 11)$ |
| 0.01 | $102 \pm 6$ | – | $125 \pm 25$ | $133 \pm 4$ | – | – |
| 0.1 | $94 \pm 9$ | – | $113 \pm 17$ | $119 \pm 11$ | – | – |
| 1.0 | $117 \pm 10$ | – | $138 \pm 10$ [c] | $116 \pm 6$ | – | – |
| 2.5 | – | – | – | – | – | – |
| | $(99 \pm 8)$ | $(113 \pm 10)$ * | $(74 \pm 12)$ [c] | $(86 \pm 10)$ [c] | $(161 \pm 12)$ ** | $(165 \pm 8)$ ** |
| 5.0 | – | – | – | – | – | – |
| | $(90 \pm 6)$ | $(117 \pm 6)$ ** | (0) [c] | (0) [c] | $(220 \pm 21)$ | $(206 \pm 6)$ |
| 10 | $85 \pm 7$ | – | 0 [d,e] | 0 [d,e] | – | – |
| 15 | $97 \pm 6$ | $149 \pm 7$ | – | – | $485 \pm 4$ | $379 \pm 22$ |
| 30 | – | $185 \pm 12$ | – | – | $880 \pm 27$ | $742 \pm 49$ |
| 45 | – | $204 \pm 21$ | – | – | $1525 \pm 157$ | $944 \pm 27$ |
| 60 | – | $189 \pm 21$ [c] | – | – | $2277 \pm 30$ | $1225 \pm 98$ |

[a] The mean of revertants for 3 plates $\pm$ S.D.

[b] The values in parentheses are for the concurrent comparisons at 2.5 and 5.0 $\mu$moles in which the bacteria were from the same overnight culture.

[c] Reduction in background lawn.

[d] No background lawn.

[e] Dose showed slight precipitation in agar.

* Indicates lowest dose for which the means of revertants are significantly greater than the mean of the controls ($p < 0.05$) based on square-root-transformed data using Dunnett's control group comparison test (one-sided).

** Same as above where $p < 0.01$.

firms the report of Wehner et al. (1978) of the absence of mutagenicity for this and similar trichothecene toxins.

As we have previously observed with monosubstituted epoxides (Rosman et al., 1987, 1988), relative reactivity with 4-(4-nitrobenzyl)pyridine is not a reliable predictor of mutagenicity within the present oxaspiro series. Certainly, the relative polarity of these compounds has to be considered. The more polar compounds (the compounds with the lowest HPLC-partition coefficients), 4-tetrahydropyranylidene and 3-tetrahydropyranylidene oxides, were the most mutagenic compounds. Cyclohexylidene oxide with intermediate mutagenicity and toxicity has intermediate polarity. In contrast, 4-*tert.*-butylcyclohexylidene and 2-decalenylidene oxides, the most non-polar compounds, were the most toxic to the Salmonella and no mutagenicity could be established for these two compounds.

The fact that the 2 compounds with the epoxide attached to a tetrahydropyran ring, as is the case for the trichothecenes, have the greatest mutagenicity is of interest. However, a broad conclusion cannot be made from this observation because of the onset of toxicity at the lower doses for two of the non-pyran derivatives which limits the direct comparison of mutagenicity for these 4 compounds. It is concluded from this study of model compounds that the epoxide moiety in the oxaspiro form does not increase the alkylating activity nor mutagenicity with Salmonella over that expected for 1,1-disubstituted epoxides in general. Thus, these aspects of genotoxicity testing do not lead to an explanation for the extreme biological activity reported for the trichothecene mycotoxins.

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## References

Bamburg, J.R., and F.M. Strong (1971) 12,13-Epoxytrichothecenes, in: S. Kadis, A. Ciegler and S.J. Ajl (Eds.), Microbial Toxins. Vol. VII, Academic Press, New York, pp. 207–292.

Betina, V. (1984) Mycotoxins, Production, Isolation, Separation and Purification, Elsevier, New York.

Brown, H.C., J.V.N. Vara Prasad and S.H. Zee (1985) Hydroboration, 71. Hydroboration of representative heterocyclic olefins with boranemethyl sulfide, 9-borabicyclo[3,3.1] nonane, dicyclohexylborane, and disiamylborane, Synthesis of heterocyclic alcohols, J. Org. Chem., 50, 1582–1589.

Carlson, R.G., and N.S. Behn (1967) The stereochemistry of epoxidation of rigid methylenecyclohexane systems, Determination of stereochemistry of exocyclic epoxides in rigid cyclohexanes by nuclear magnetic resonance half band widths, J. Org. Chem., 32, 1363–1367.

Carlson, R.M., R.E. Carlson and H.L. Kopperman (1975) Determination of partition coefficients by liquid chromatography, J. Chromatogr., 107, 219–223.

Corey, E.J., and M. Chaykovsky (1965) Dimethyloxosulfonium methylide ($(CH_3)_2SOCH_2$) and dimethylsulfonium methylide ($(CH_3)_2SCH_2$), Formation and application to organic synthesis, J. Am. Chem. Soc., 87, 1353–1365.

Eisenbraun, E.J. (1965) Cyclooctanone, Org. Synth., 45, 28–32.

Ember, L.R. (1984) Yellow rain, Chem. Eng. News, 62, 8–34.

Frantz, S.W., and J.E. Sinsheimer (1981) Bacterial mutagenicity and toxicity of cycloaliphatic epoxides, Mutation Res., 90, 67–78.

Hammock, L.G., B.D. Hammock and J.E. Casida (1974) Detection and analysis of epoxides with 4-(*p*-nitrobenzyl)-pyridine, Bull. Environ. Contam. Toxicol., 12, 759–764.

Hayes, A.W. (1981) Mycotoxin,Teratogenicity and Mutagenicity, CRC Press, Boca Raton, FL.

Maron, D.M., and B.N. Ames (1983) Revised methods for the Salmonella mutagenicity test, Mutation Res., 113, 173–215.

Nelis, H.J.C.F., S.C. Airy and J.E. Sinsheimer (1982) Comparison of the alkylation of nicotinamide and 4-(*p*-nitrobenzyl)pyridine for the determination of aliphatic epoxides, Anal. Chem., 54, 213–216.

Rosman, L.B., V.G. Beylin, V. Gaddamidi, B.H. Hooberman and J.E. Sinsheimer (1986) Mutagenicity of *para*-substituted α-methylstyrene oxide derivatives with Salmonella, Mutation Res., 171, 63–70.

Rosman, L.B., V. Gaddamidi and J.E. Sinsheimer (1987) Mutagenicity of aryl propylene and butylene oxides with Salmonella, Mutation Res., 189, 189–204.

Rosman, L.B., P.K. Chakraborty, E.A. Messerly and J.E. Sinsheimer (1988) Mutagenicity of aromatic glycidyl ethers with Salmonella, Mutation Res., 206, 115–125.

Roush, W.R., M.A. Marletta, S. Russo-Rodriguez and J. Recchia (1985) Trichothecene metabolism studies: isolation and structure determination of 15-acetyl-3α-(1′-β-D-glucopyranosiduronyl)-scirpen-3,4β,15-triol, J. Am. Chem. Soc., 107, 3354–3355.

Stark, A.-A. (1980) Mutagenicity and carcinogenicity of mycotoxins: DNA binding as a possible mode of action, Annu. Rev. Microbiol., 34, 235–262.

Ueno, Y. (1977) Mode of action of trichothecenes, Ann. Nutr. Alim., 31, 885–900.

Ueno, Y. (1983) Trichothecenes, Chemical, Biological and Toxicological Aspects, Elsevier, New York.

Wade, D.R., S.C. Airy and J.E. Sinsheimer (1978) Mutagenicity of aliphatic epoxides, Mutation Res., 58, 217–223.

Wehner, F.C., W.F.O. Marasas and P.G. Thiel (1978) Lack of mutagenicity to *Salmonella typhimurium* of some Fusarium mycotoxins, Appl. Environ. Microbiol., 35, 659–662.