Alkylating Potential of Oxetanes

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Small, highly strained heterocycles are archetypical alkylating agents (oxiranes, β-lactones, aziridinium, and thiirinium ions). Oxetanes, which are tetragonal ethers, are higher homologues of oxiranes and reduced counterparts of β-lactones, and would therefore be expected to be active alkylating agents. Oxetanes are widely used in the manufacture of polymers, especially in organic light-emitting diodes (OLEDs), and are present, as a substructure, in compounds such as the widely used antimitotic taxol. Whereas the results of animal tests suggest that trimethylene oxide (TMO), the parent compound, and β,β-dimethyloxetane (DMOX) are active carcinogens at the site of injection, no studies have explored the alkylating ability and genotoxicity of oxetanes. This work addresses the issue using a mixed methodology: a kinetic study of the alkylation reaction of 4-(*p*-nitrobenzyl)pyridine (NBP), a trap for alkylating agents with nucleophilicity similar to that of DNA bases, by three oxetanes (TMO, DMOX, and methyloxetanemethanol), and a mutagenicity, genotoxicity, and cell viability study (*Salmonella* microsome test, BTC *E. coli* test, alkaline comet assay, and MTT assay). The results suggest either that oxetanes lack genotoxic capacity or that their mode of action is very different from that of epoxides and β-lactones.

Introduction

Small, highly strained heterocycles are archetypical alkylating agents (Figure 1); oxiranes (*1*), β-lactones (*2*), aziridinium rings from nitrogen mustards, and thiirinium ions from sulfur mustards (*3*) are strong electrophiles that bind directly to DNA, forming adducts that can give rise to mutations and cancer (*4*).

Structurally, oxetanes, which are tetragonal ethers (Figure 1), lie in between two groups of powerful genotoxins; they are higher homologues of oxiranes (epoxides), their rings being one link larger and thus less strained, and they also have a structure similar to that of more oxidized and more electrophilic β-lactones. These two groups of compounds have long been known to be direct alkylating agents and powerful carcinogens without metabolic activation (*5*−*9*). Thus, oxetanes were tested for their carcinogenicity in the late 1960s. Animal tests, in which the compound was delivered by subcutaneous injection, revealed that trimethylene oxide (TMO[1]), the parent compound, (80 mg/kg/week, BD-rats) (*10*), and its dimethyl derivative β,β-dimethyloxetane (DMOX) (*9*) induce tumors at the site of injection. Nevertheless, these results have been quoted as "equivocal".[2]

Like many aliphatic ethers, oxetanes are fairly stable molecules (*11*), in spite of their somewhat strained ring, and, as a rule, are reactive only in the presence of an acid catalyst. TMO, for instance, does not undergo hydrolysis in neutral aqueous medium, its acid-catalyzed hydrolysis being rather slow (*12*), and in aqueous medium, its reaction with amines to form 3-hydroxypropylamines is negligible at room temperature (*13*). Oxetanes are present as a substructure in many chemicals, such as the well-known antimitotic taxol, and are used as monomers in the manufacture of polymers with a variety of applications (*14, 15*), such as organic light-emitting diodes (OLEDs) (*16*−*18*), or as support for enzymatic and solid-phase methods (*19*).

In recent years, the metabolism and genotoxicity of linear and cyclic ethers has attracted widespread attention, and chemicals such as glycolethers (*20*−*24*), gasoline additives such as methyl-*tert*-butyl ether (*25*), ether solvents such as 1,4-dioxane (*26*−*28*), or reagents such as furan (*29*) have been addressed in several studies.

While some authors have concluded that some oxetanes are carcinogenic, a systematic study of their mutagenicity and alkylating ability remains to be performed. Thus, the aim of this work was to investigate the alkylating potential of this type of compound. To do so, we applied a mixed approach to study three model oxetanes, namely, oxetane (OX), trimethylene oxide (TMO), 3,3-dimethyloxetane (DMOX), and methyloxetanemethanol (MOM).

The first approach involved a battery of biological tests, the Ames *Salmonella* assay, the BTC *E. coli* test, the MTT cell viability assay, and the comet assay, to search for muta-

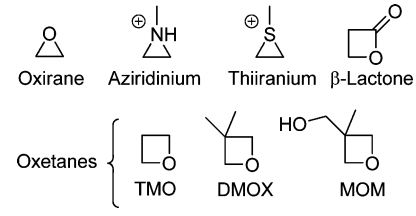

Figure 1. Some small heterocyclic alkylating agents.

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[1] Abbreviations: DMOX, β,β-dimethyloxetane; MOM, methyloxetanemethanol; MMT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; NBP, 4-(*p*-nitrobenzyl)pyridine; OX, oxetane; TMO, trimethylene oxide.

[2] Registry of Toxic Effects of Chemical Substances: RQ6825000.

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genic or toxic effects of these three compounds in both the absence and presence of a microsomal activation system or the human cytchrome P450s CYP2A6 and CYP2E1.

The second approach involved the study of their reactivity with 4-(*p*-nitrobenzyl)pyridine (NBP), a trap for alkylating agents of nucleophilicity similar to that of DNA bases (*30, 31*). NBP is known to react with strong (*32, 33*) and weak (*34*−*36*) alkylating agents, and much insight into the alkylation mechanisms *in vivo* can be gained from the kinetic study of this reaction *in vitro* (*37*−*41*).

Experimental Procedures

TMO (97%), DMOX (98%), and MOM (98%) were from Sigma and were used without further purification.

Ames *Salmonella*/Microsome Assay. The oxetanes were tested for mutagenic activity using the preincubation version of *Salmonella* Mutagenicity Test as described in Mortelmans and Zeiger (*42*). Briefly, 1.5−500 μg of each compound was combined with the *Salmonella* tester strain, a metabolic activation mixture derived from Aroclor 1254-induced rat liver (as required), and incubated for 20 min at 37 °C. The contents were then mixed with molten agar and poured onto glucose minimal medium agar plates. The plates were inverted and incubated at 37 °C for 48 h. Following incubation, the plates were photographed digitally, and the number of revertant colonies per plate was determined using Labworks Software (version 4.6) (UVP. Inc.USA). Two bacterial test strains were used: TA98 and TA100.

Suitable positive controls were used, as suggested by Mortelmans and Zeiger (*42*): 2.5 μg/plate 4-nitro-*o*-phenylendiamine for TA98 without activation, 5.0 μg/plate sodium azide for TA100 without activation, and 2.5 μg/plate 2-aminoanthracene for both TA98 and TA100 with activation.

Samples were tested both with and without a metabolic activation mixture. The S9 metabolic activation mixture (10%) was prepared as suggested by Mortelmans and Zeiger (*42*), using Aroclor 1254-induced rat liver S9 (Moltox Inc.).

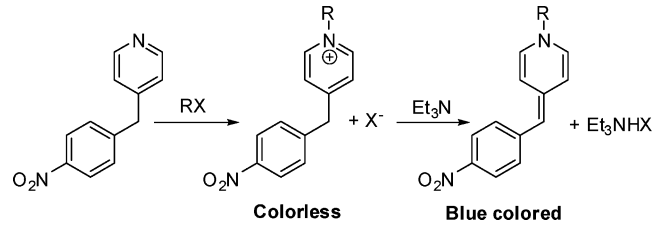
BTC *E. coli* Test. This assay uses *E. coli* strains that are capable of expressing human biotransformation enzymes, in particular cytochrome P450, cytochrome P450 NADPH oxidoreductase, and, optionally, cytochrome b_5 (*43*). These strains have been used to study the role of specific human CYPs in the bioactivation of several premutagens, in particular alkylating agents (*44*), and the importance of human cytochrome b_5 in this bioactivation (*45*).

Briefly, an aliquot of 1.5−500 μg of each compound was combined with the BTC_CYP tester strain and preincubated for 45 min at 37 °C. The contents were then mixed with molten agar and poured onto glucose minimal media agar plates. The plates were then inverted and incubated at 37 °C for 48 h. Following incubation, the plates were photographed digitally, and the number of revertant colonies per plate was determined using Labworks Software (version 4.6) (UVP Inc., USA).

Suitable positive controls were used: 60 μg/plate *N*-nitrosodiethylamine for BTC_2E1_POR and BTC_2E1_b5_POR, and 100 μg/plate 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone for BTC2A6_POR and BTC2A6_b5_POR.

Alkaline Comet Assay. Conventional microscope slides were dipped in a solution of 1% agarose in water and allowed to dry on a flat surface. Chinese hamster cells (MZ) (kindly provided by Professor H. R. Glatt, German Institute of Human Nutrition, Nuthetal, Germany) were cultured in 1 cm^3 of culture medium per well (Ham's F-10 medium, supplemented with 10% newborn calf serum and 1% antibiotic solution [penicillin−streptomycin]) in 12-well plates, and incubated at 37 °C under a 5% CO_2 atmosphere. The cells were grown for 24 h and then exposed to different concentrations of TMO, DMOX, and MOM (dissolved in 10 μL of DMSO), ranging from 1 to 30 mM, for a 1-h period; 250 μM hydrogen peroxide was used as a positive control. After trypsinization and a rinse with PBS, cells were centrifuged at 250*g* for 5 min at 4 °C and were mixed with 500 μL of 1% low-melting-point agarose (LMPA). One hundred microliters of the mixture was applied to each precoated slide and coverslipped. The slides were maintained at 4 °C for 20 min, the coverslip was removed, and the cells were immersed in a freshly made lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, and 1 (v/v) Triton X-100 at pH 10) for 1 h at 4 °C. The slides were then incubated in alkaline buffer (0.3 M NaOH and 1 mM Na_2EDTA) for 20 min at 4 °C in an electrophoresis tank. Electrophoresis was conducted at 0.8 V/cm (20 V) and 300 mA for 20 min at 4 °C, after which the slides were washed three times in neutralizing buffer (400 mM Tris, pH 7.50) for 5 min at 4 °C. The slides were subsequently dried for 5 min in water−ethanol mixtures with increasing ethanol contents, 50%, 70%, and 100%, and stained with 100 μL of ethidium bromide (20 $\mu g/cm^3$).

Scheme 1. NBP Test: Formation of Color after Alkylation



Fifty comets per slide, two slides per treatment, were analyzed at 200× magnification by fluorescence microscopy (Leica DMLB, equipped with a short-arc mercury lamp, HBO 103 W/2) attached to a digital camera (Applied Imaging Corp., now Genetix) and connected to a personal computer, and images of randomly selected cells were captured from each slide using Cytovision (v3.0) capture software (Genetix). The cells were then photographed, and the images were analyzed; tail moment and DNA percentage in tail were scored using CometScore software (Tritek CometScore freeware, v1.5; www.autocomet.com).

MTT Reduction Assay. The MTT assay is based on the reduction of the yellow MTT tetrazolium salt by mitochondrial dehydrogenases to form a blue MTT formazan (*46, 47*) and was performed according to Martins et al. (*48*). Approximately, 7.5 × 10^3 V79 Chinese hamster lung fibroblast cells were cultured in 200 μL of culture medium per well (Ham's F-10 medium, supplemented with 10% newborn calf serum and 1% antibiotic solution [penicillin−streptomycin]) in 96-well plates and incubated at 37 °C under a 5% CO_2 atmosphere. The cells were grown for 16 h and then exposed to different concentrations of the oxetanes (dissolved in DMSO), ranging from 0.10 to 30 mM, for a 24-h period. Hydrogen peroxide (1 mM) was used as a positive control and the appropriate volume of DMSO (10 μL) as a negative control. The cells were washed with culture medium, incubated with MTT (500 $\mu g/cm^3$) for a further 4 h, and then carefully rinsed with PBS. At the end of the incubation period, the medium was discarded and DMSO (200 μL) was added to each well. Absorbance was read at 595 nm in a Zenyth 3100 microplate reader. Four independent experiments were performed with 2 replicates per dose, and four individual cultures were used for each oxetane concentration in each independent experiment.

Kinetic NBP Test. Alkylation reactions were performed in the 2−5 pH range. NBP ($pK_a = 4.40$) was used as a buffer solution to maintain constant pH, and HCl was added to fix the pH value. To render NBP soluble, the reaction mixtures were prepared in 7:3 (v/v) water/dioxane medium. To monitor the alkylation reactions, 2.4 cm^3-aliquots of the alkylation mixture were removed at different times and added to a cuvette containing 0.6 cm^3 of 99% triethylamine (Et_3N) to form the colored compounds (Scheme 1), after which absorbance was measured at the wavelength of maximum absorption (λ = 570 nm). The NBP concentration was in the 0.005−0.05 M range, and oxetane concentrations were in the 0.1−5 × 10^{-3} M range. Detailed reaction conditions are given in the figure and table legends. NBP was from Aldrich (98%), and dioxane (99%) was from Panreac.

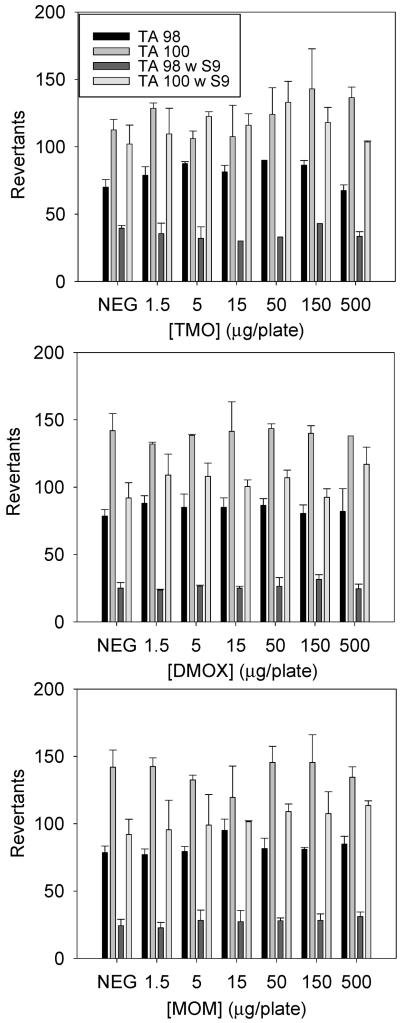


Figure 2. Number of revertants per plate in the Ames test for the three oxetanes, using the TA98 and TA100 strains with and without the S9 fraction. Results for the positive controls are omitted for clarity.

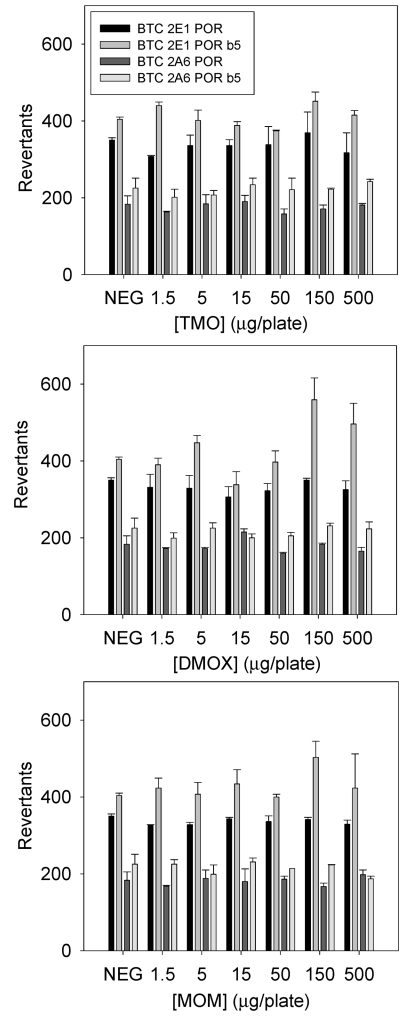


Figure 3. Number of revertants per plate in the BTC *E. coli* test for the three oxetanes. Results for the positive controls are omitted for clarity.

Positive-mode electrospray ionization mass spectra were recorded on a Waters ZQ4000 spectrometer by direct injection, with methanol as the solvent.

Statistical Analyses. Dose-related effects were assessed using linear regression analysis. One-way ANOVA and Student's *t*-test were used to compare treatment levels with the control group. Kinetic profiles were fitted using SigmaPlot 10.0.

Results

Mutagenicity and Genotoxicity. Ames Test. The three oxetanes were tested at concentrations ranging from 1.5 to 500 μg/plate spaced at half-logarithm intervals. The results using strains TA98 and TA100 with and without S9 mix are shown in Figure 2. At all doses tested, the number of revertants in the two strains was not significantly different from the negative control, and no dose−response relationship was observed. This was the case both when using microsomal activation and when not.

BTC *E. coli* Test. The results obtained with the BTC assay are shown in Figure 3. At all doses tested, the number of revertants in the two strains was not significantly different from the negative control. Also, there was a lack of any dose−response relationship. This suggests that neither TMO, DMOX, nor MOM was being activated by CYP2E1 or CYP2A6 (in the presence or absence of cytochrome b_5) to form mutagens.

Comet Assay. The results, measured as % of tail DNA or tail moment, are shown in Figure 4. For the three compounds, the results were not significantly different from the negative control or the S9 activation mix. This suggests that TMO, DMOX, and MOM lack the potential to produce DNA strand breaks.

Cell Viability. The three oxetanes were tested at concentrations ranging from 0.1 to 30 mM, spaced at half-logarithm concentration intervals; the results are shown in Figure 5. At all doses tested, the ratio of survival was not significantly different from that of the negative control, either in the presence or in the absence of microsomal activation. In addition, there was a complete lack of any dose−response relationship, which suggests a very low toxicity of the three oxetanes or their metabolites at below the highest concentrations tested.

Alkylation Mechanism. To search for a chemical interpretation for the lack of mutagenicity of the oxetanes in the absence of microsomal activation, the reaction kinetics of the alkylation of NBP by the oxetanes was studied.

The adducts formed in the reaction of NBP with the oxetanes showed maximum absorption at $\lambda = 570$ nm (Figure 6a).

The alkylation reaction of NBP by the oxetanes only occurred in acidic medium, and no reaction was observed at neutral pH. Since oxetanes are also hydrolyzed in acidic media (k_{hyd}), we propose a reaction mechanism such as that depicted in Scheme 2, in which the acid-catalyzed alkylation reaction competes with

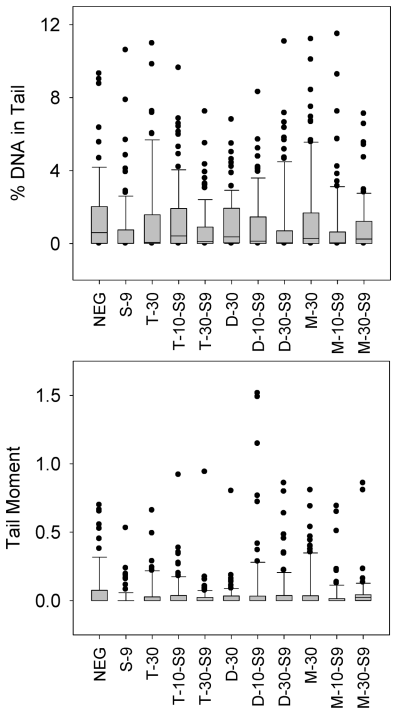


Figure 4. Results of the Comet assay, testing the three oxetanes (T, TMO; D, DMOX; M, MOM) at concentrations of 10 and 30 mM with and without the S9 activation mix. The positive control is omitted for clarity.

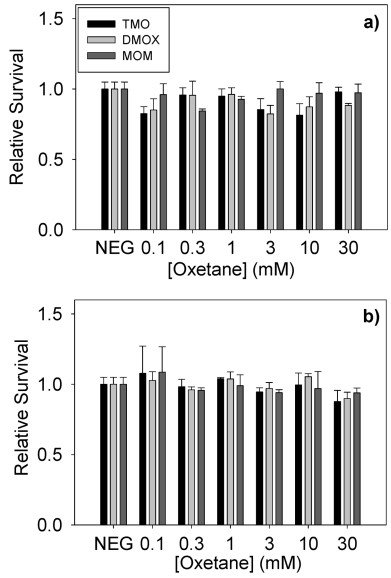


Figure 5. Relative proliferation as measured in the MTT Assay. (a) Absence of the S9 mixture. (b) Presence of the S9 mixture.

acid-catalyzed hydrolysis. From this, we derived the differential rate eqs 1 and 2. Given that NBP is partially protonated in acidic medium, $[NBP]_f$ is the amount of free, nonprotonated NBP.

$$\frac{d[OX]}{dt} = -k_{alk}[OX][NBP]_f[H^+] - k_{hyd}[OX][H^+] \quad (1)$$

$$\frac{d[AD]}{dt} = k_{alk}[OX][NBP]_f[H^+] \quad (2)$$

$[NBP]_f$ depends on pH and the total NBP concentration ($[NBP]_t$), as seen in eq 3; $K_a = 10^{-4.40}$ is the acid dissociation constant of NBP.

$$[NBP]_f = \frac{K_a}{[H^+] + K_a}[NBP]_t \quad (3)$$

Since NBP was in large excess with respect to the particular oxetane under study, its concentration was assumed to remain constant during the reaction. Applying the pseudofirst-order approximation, we define $k_{alk\ exp}$ (eq 4).

$$k_{alk\ exp} = k_{alk}\frac{K_a[H^+]}{[H^+] + K_a}[NBP]_t \quad (4)$$

Integration of eqs 1 and 2 and expression of the resulting rate equation in terms of absorbance yields eq 5:

$$A_t = \varepsilon_{AD} l [OX]_o \frac{k_{alk\ exp}}{k_{alk\ exp} + k_{hyd}[H^+]}(1 - e^{-k_{alk\ exp}t - k_{hyd}[H^+]t}) \quad (5)$$

where ε_{AD} is the molar absorption coefficient of the adduct at $\lambda = 570$ nm and l the cuvette length.

Whereas the alkylation of TMO is moderately slow (with reaction times up to a month in optimal conditions), DMOX and MOM react with NBP very slowly (with reaction times of several months). Accordingly, the integral method could only be applied to TMO. DMOX and MOM were studied using the initial rate method (see below).

Integral Method. Figure 6b shows the fit of the experimental measurements of absorbance to eq 5. From such fits, the global rate constant k_{obs}, which is the sum of the alkylation and hydrolysis experimental rate constants, was obtained (eq 6).

$$k_{obs} = k_{alk\ exp} + k_{hyd}[H^+] \quad (6)$$

Plotting k_{obs} against the total concentration of NBP at constant pH allowed us to calculate k_{alk} from the slope and k_{hyd} from the intercept (Figure 7a). The value obtained for k_{hyd} for TMO at 37.5 °C in 7:3 w/d medium is 2.7×10^{-3} M^{-1} s^{-1}.

Once k_{alk} and k_{hyd} were known, it was possible to calculate the molar absorption coefficient of the adduct using the absorbance value once the plateau had been reached (A_∞), using eq 7 to correct for the fraction of hydrolyzed TMO (Figure 7b).

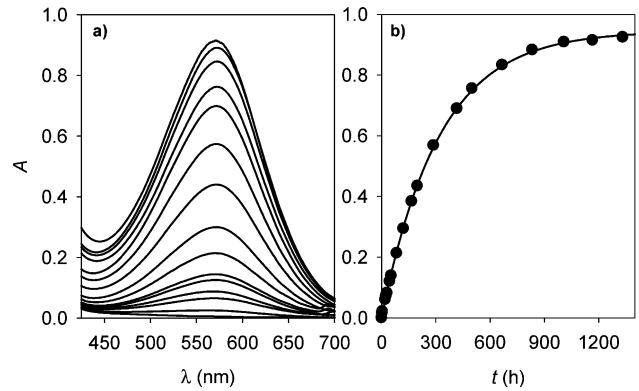
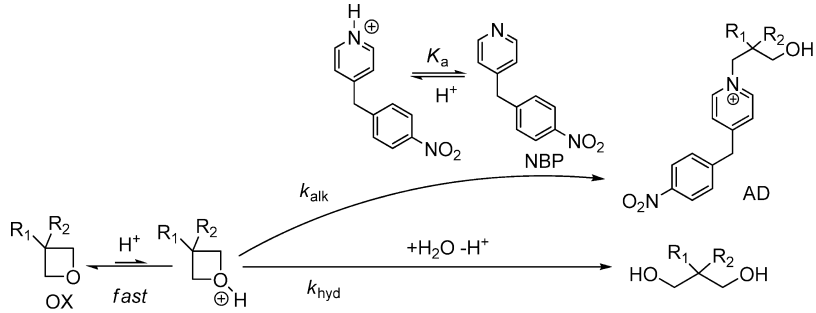


Figure 6. (a) Spectrograms showing the formation of the NBP-TMO adduct over time; (b) formation of the NBP-TMO adduct over time; $\lambda = 570$ nm; 7:3 (v/v) water/dioxane medium. $[NBP]_o = 4.0 \times 10^{-2}$ M; $[TMO]_o = 5.0 \times 10^{-4}$ M; pH 4.00; $T = 37.5$ °C.

Scheme 2. Alkylation of NBP by Oxetanes and Concurrent Hydrolysis



The value obtained is $\varepsilon_{NBP\text{-}TMO} = (4.16 \pm 0.05) \times 10^3\ M^{-1}\ cm^{-1}$.

$$A_{inf} = \varepsilon_{AD} l \frac{k_{alk\ exp}}{k_{alk\ exp} + k_{hyd}[H^+]}[TMO]_o \qquad (7)$$

Initial Rate Method. Study of the NBP alkylation by DMOX and MOM required the use of the Initial Rate Method (*49*) for the calculation of $k_{alk\ exp}$ (eq 8).

$$v_o = \varepsilon_{AD} l k_{alk\ exp}[OX]_o \qquad (8)$$

Since the molar absorption coefficient of the adducts NBP-DMOX and NBP-MOM could not be determined owing to their low reactivity (the plateau could not be reached in feasible time spans), the value obtained for the adduct NBP-TMO was used for all three compounds. This assumption is based on the fact that substituents without significant electronic or conjugative effects have little influence on the molar absorption coefficient. For instance, increasingly substituted 1,3-butadiene, isoprene, and 2,4-hexadiene show molar absorption values differing by less than 10% (*50*). It is known that alkyl 4-(*p*-nitrobenzyl)pyridonium adducts lacking conjugated double bonds show very similar absorption coefficients, such as the adducts formed by acrylamide (*36*) or β-butyrolactone (*32*).

The alkylation rate constants of TMO were calculated using both the integral method and the IRM, and their values were found to be equal. All three compounds were tested to see if they would fulfill eq 8 in IRM conditions (reaction orders and pH dependence). The values obtained for k_{alk} are given in Table 1. From the variation in k_{alk} with temperature, it was possible to calculate the activation energies for the alkylation reaction (Table 2).

Adducts were detected by mass spectrometry as having *m*/*z* = 273.1, 301.1, and 317.2 for TMO, DMOX, and MOM

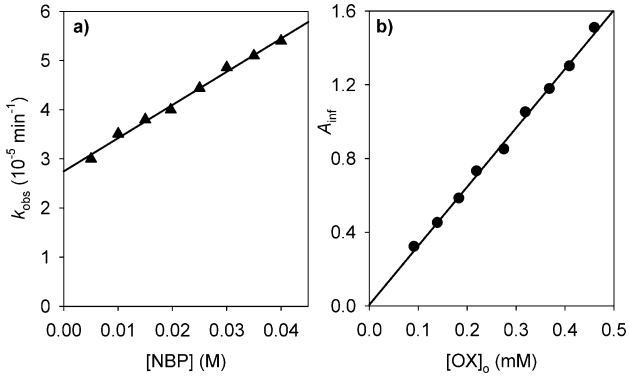


Figure 7. (a) Reaction order with respect to NBP; $[TMO]_o = 3.0 \times 10^{-4}$ M. (b) Calculation of $\varepsilon_{NBP\text{-}TMO}$; $[NBP]_o = 0.040$ M; 7:3 (v/v) w/d, pH 3.80, and $T = 37.5$ °C.

Table 1. Rate Constants of NBP Alkylation by the Three Oxetanes as a Function of Temperature

	k_{alk} (M^{-2} min^{-1})		
T (°C)	TMO	DMOX	MOM
20.0	3.4 ± 0.1	0.52 ± 0.02	
22.5	4.3 ± 0.1	0.68 ± 0.02	
25.0	5.8 ± 0.2	0.92 ± 0.03	0.16 ± 0.02
27.5	7.6 ± 0.3	1.23 ± 0.05	0.21 ± 0.03
30.0	10.8 ± 0.5	1.62 ± 0.07	0.29 ± 0.03
32.5	14.9 ± 0.5	2.12 ± 0.09	0.37 ± 0.5
35.0	17.8 ± 0.8	3.18 ± 0.12	0.46 ± 0.04
37.5	23.1 ± 0.9	4.07 ± 0.15	0.76 ± 0.06

Table 2. Activation Energies for the Alkylation of NBP by the Oxetanes

oxetane	E_a (kJ mol^{-1})
TMO	85 ± 2
DMOX	90 ± 2
MOM	93 ± 4

respectively, which correspond to the positively charged adducts (exact mass = 273.12, 301.16, and 317.15).

The very low rate constants and the high reaction energies suggest that oxetanes lack direct alkylating potential (see below).

Discussion

Mutagenicity and Genotoxicity. The results obtained in the *Salmonella* test suggest that the three oxetanes tested and their metabolites lack mutagenic activities using strains TA98 and TA100, as can be seen in Figure 2. At all doses, the number of revertants was not significantly different from that of the negative control. Similar results were obtained upon using the S9 mix for oxidative metabolism, which together with a lack of a dose-response relationship suggests that neither TMO, DMOX, and MOM nor their metabolites are mutagenic in the test system.

The three compounds also tested negative in the BTC_CYP *E. coli* test, using strains expressing human cytochromes CYP2E1 and CYP2A6 (*44*), both of which are known to be involved in the activation of xenobiotics. Since oxetanes are small and rather polar molecules, CYP2E1 would be a likely candidate involved in their biotransformation (*51*).

While the coexpression of cytochrome b_5 increases the bioactivation of some alkylating compounds by CYP2A6 and 2E1 (*45, 52*), the results were equally negative when the strains BTC_2A6_b5_POR or BTC_2E1_b5_POR were applied.

The three model oxetanes demonstrated no DNA strand breaks in the comet assay, using Chinese hamster cells, either in the presence or absence of the S9 mix. This inability to form strand breaks points further in the direction of oxetanes and their oxidation metabolites having very limited or no genotoxic effects.

These negative results could cast certain doubts on previous positive results obtained in animal tests, which have been called equivocal.[3] For instance, those results may have been influenced by the presence of minor impurities (the boiling point referred to by Druckrey et al. (*10*) differs significatively from the one given in the literature) that became significant at high dose usage or perhaps may have been due to the acute toxicity of oxetanes, which, followed by compensatory cell proliferation, could increase the likelihood of tumor production (mitogenesis).

Toxicity. No toxicity to mammalian cells was observed in the MTT assay, either in the presence or absence of the S9 mix in doses up to 30 mM. This negative result seems to indicate that oxetanes and their oxidation metabolites are very sparingly toxic for mammalian cells, which is consistent with the LD_{50} value reported for DB-rats: 500 mg/kg (*10*). This suggests that previous positive results in animal test systems were possibly not due to the toxicity of the compounds and subsequent cell proliferation but rather to some other factor, such as the presence of impurities.

Alkylation Mechanism. The values of the alkylation rate constants k_{alk} shown in Table 1 are rather low in the case of TMO and extremely low in the cases of DMOX and MOM.

The fact that the alkylation reaction takes place only at acidic pH values is consistent with the general reactivity of oxetanes. For example, trimethylene oxide undergoes hydrolysis only in strongly acidic media. The value obtained by us for k_{hyd} from the fitting of the experimental data to eq 6 is 2.7×10^{-3} M^{-1} s^{-1} in 7:3 w/d at 37.5 °C, which is coherent with the value given in the literature: 1.6×10^{-3} M^{-1} s^{-1} at 25 °C in 6:4 w/d (*12*). The discrepancy is because our value was obtained at a higher temperature in a more polar reaction medium.

The half-reaction times for the alkylation reaction, even under the most favorable conditions, extend up to one month in the case of TMO and are 10- to 40-fold longer in the cases of DMOX and MOM (and were therefore prohibitively time-consuming for study by the integral method). The high values of the activation energies obtained, especially in the case of DMOX and MOM, indicate that a significant energy barrier exists in the alkylation reaction, which is coherent with the generally low reactivity of these ethers (*11*). These lower rate constants and activation energies are consistent with the steric effect of the substituents at position 3; indeed, both compounds are neopenyl-like and thus show very limited reactivity in S_N2 reactions.

At the neutral pH of the cellular medium, these alkylation reactions can be considered to be nonexistent or, at least, insignificant. The kinetic NBP test is extremely sensitive for the detection of alkylating agents, to such an extent that some were positively detected with NBP, albeit slowly, while having very small or almost no genotoxic effects, as has been shown for nonactivated acrylamide or sorbic acid (*34*−*36*). Thus, from the combined kinetic data and the biological results obtained in the absence of microsomal activaction, it can be safely assumed that oxetanes are not direct alkylating agents. This is possibly due to their lower ring-strain when compared to that of oxiranes and their lower electrophilicity when compared to that of β-lactones. Their lack of mutagenicity, genotoxicity, and cytotoxicity in the presence of the S9 mix and human cytochromes CYP2E1 and CYP2A6, with or without coexpression of cytchrome b_5, may also indicate that oxetanes are not activated to alkylating agents or other direct-acting mutagens *in vivo*.

[3] Registry of Toxic Effects of Chemical Substances: RQ6825000.

The apparent contradiction between our results and previous animal tests suggests either that (i) oxetanes are extremely weak genotoxins or nongenotoxic and that previous positive results would have been due to spurious factors such as the use of unrealistically high doses or the presence of impurities or that (ii) their carcinogenic effect is exerted by mechanisms completely different from those of similar compounds, such as oxiranes and β-lactones, which do cause DNA damage.

Conclusions

(i) The oxetanes studied showed no mutagenicity either in the *Salmonella* assay with strains TA 98 and TA 100 both with or without the S9 mix or in the BTC *E. coli* test using strains expressing CYP2E1 and CYP2A6, with or without cytchrome b_5.

(ii) No genotoxicity (comet assay) or cytotoxicity (MTT assay) to V79 Chinese Hamster cells is observed in the presence and absence of the S9 activation mix.

(iii) Oxetanes react with NBP according to the rate equation $r = k_{alk}[OX][NBP][H^+]$. The fact that the reaction only takes place in acidic medium and the very low rate constants suggests that oxetanes are not direct alkylating agents at physiological pH.

(iv) If oxetanes are indeed carcinogenic, their mode of action must be very different from that of similar compounds such as oxiranes and β-lactones, which are direct alkylating agents and are carcinogenic without metabolic activation.

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