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# Structure–activity considerations and in vitro approaches to assess the genotoxicity of 19 methane-, benzene- and toluenesulfonic acid esters

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**Abstract**

Sulfonic acid esters are considered as potentially alkylating agents that may exert genotoxic effects in bacterial and mammalian cell systems. One possible source of human exposure stems from drug synthesis when the salt-forming agents methanesulfonic acid, benzenesulfonic acid or *p*-toluenesulfonic acid are used together with alcoholic solvents such as methanol, ethanol and propanol. In this study computer-assisted structural considerations and in vitro approaches (Ames mutagenicity test using *Salmonella typhimurium* strains TA98 and TA100, and the micronucleus test using L5178Y mouse lymphoma cells) were used to assess the genotoxic properties of 19 sulfonic esters. While all esters may be principally active as genotoxicants based on the presence of the sulfonate moiety, the statistical correlative multiple computer automated structure evaluation (MCASE) system (MC4PC version 1.0) using the Ames mutagenicity A2I module (version 1.54), rank-ordered the activity of the benzenesulfonic acid esters in the Ames test negligible due to an inactivating modulator and a deactivating fragment, whereas the methane- and toluenesulfonic acid esters were predicted to be positive in this test.

In the Ames test, with the exception of the *p*-toluenesulfonic acid ethyl and *iso*-butyl esters, all compounds came out positive in *Salmonella* strain TA100. Methanesulfonic *iso*-propyl, *sec*-butyl and benzenesulfonic acid *iso*-propyl ester also showed mutagenic potential in strain TA98. In general, differences between results seen in Ames tests performed with or without metabolic activation were rather small.

In L5178Y mouse lymphoma cells, benzenesulfonic acid *n*- and *iso*-butyl ester and *p*-toluenesulfonic acid *iso*-butyl ester did not increase the number of cells containing micronuclei. The other esters were positive in this micronucleus test; however, methanesulfonic acid *iso*-butyl ester was found to be only weakly positive at excessively cytotoxic concentrations. These

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compounds were generally found to be more potent with regard to micronucleus induction when tested without metabolic activation (20 h treatment).

In conclusion, the *iso*-propyl esters of the three sulfonic acids under study were found to be the strongest mutagens, either when tested in the Ames test or the micronucleus assay, whereas *p*-toluenesulfonic acid *iso*-butyl ester was the only compound shown to be devoid of a genotoxic potential in both tests.


*Keywords:* Sulfonic acid esters; Genotoxicity; Structure–activity relationship; MCASE; Ames test; Micronucleus test

## 1. Introduction

The synthesis of new pharmaceutical entities involves the use of reactive substances that, as expected from their chemical properties, may show genotoxic activity in short-term in vitro genotoxicity assays. Such compounds, if not efficiently purged during the process, may find their way into the final synthesis step, i.e. towards the (normally non-genotoxic) drug substance. Hence, their presence may need to be controlled at a very low level. An internal evaluation of Novartis' synthesis schemes for active pharmaceutical ingredients in 2002 and 2003 for the presence of genotoxic intermediates demonstrated that genotoxic compounds appear in a significant percentage of all drug substance syntheses at some stage during the synthesis. Genotoxicity testing of these drug substances, however, yielded mostly negative results. Despite such negative tests, there may be a need for control of genotoxic intermediates that cannot be efficiently removed from the final product since they may constitute an undesirable carcinogenic hazard in case of human use.

Since organic chemistry involves the use of reactive chemicals, it is obvious that the presence of genotoxic substances in synthesis pathways is not totally avoidable. Sulfonic acid esters, for example, which are considered to be potentially alkylating agents, are likely formed during drug synthesis when the salt-forming agents, methanesulfonic acid (mesylate), benzenesulfonic acid (besylate) or *p*-toluenesulfonic acid (tosylate) are used together with alcoholic solvents like methanol, ethanol or propanol. Methyl methanesulfonate (CAS 66-27-3) and ethyl methanesulfonate (CAS 62-50-0) are well-known mutagenic impurities, of which the lowest observed effect concentrations (LOEC) in *Salmonella* strain TA100 are 5 and 1500 μg/plate, respectively (data not shown). Moreover, methyl methanesulfonate and ethyl methanesulfonate have both been demonstrated to be carcinogenic in animals and are suspected human carcinogens [1]. Despite the wide use of mesylate, besylate and tosylate salts as pharmaceuticals, literature data on genotoxic or carcinogenic properties of their esters are sparse.

In this study, computer-aided structure–activity reasoning and in vitro approaches are used to assess the genotoxic properties of 19 sulfonic esters with the aim to discriminate between active versus inactive, non-genotoxic sulfonic acid esters, which could consequently influence the use of salt-forming agents and alcohols during the drug synthesis.

## 2. Materials and methods

### 2.1. Test compounds

The name, CAS number, chemical structure, purity and source of each sulfonic acid ester evaluated in this study are given in Table 1. The purity was determined be means of gas chromatography.

### 2.2. Computer-aided test system: MCASE

Multiple computer automated structure evaluation (MCASE) as MC4PC version 1.0 (MULTICASE Inc., Beachwood, OH, USA) is geared to predict a particular toxicity of a compound on the basis of discrete structural fragments found to be statistically relevant to a specific biological activity (biophores). It is assumed that the presence of a biophore previously found in a number of active compounds in the database is indicative of a potential activity, which is considered to be a reasonable basis to assess the potential activity of new molecules.

The system also generates information on deactivating fragments (biophobes), which are fragments detected only in inactive substances in the database. In addition, MCASE provides information on the degree by

Table 1
Sulfonic acid esters used in the study

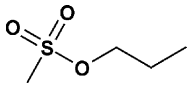
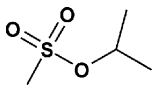
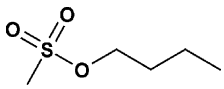
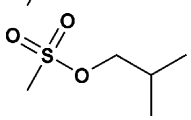
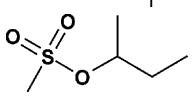
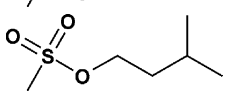
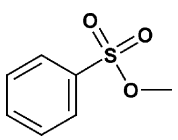
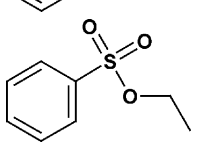
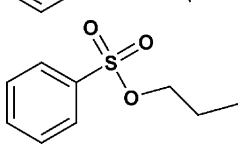
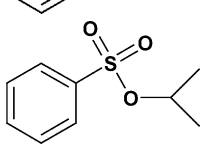
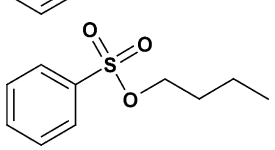
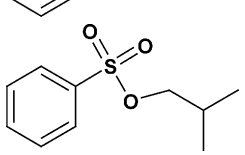
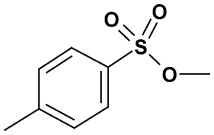
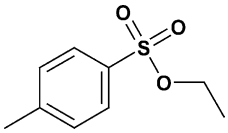
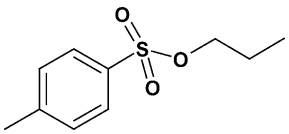
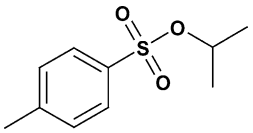
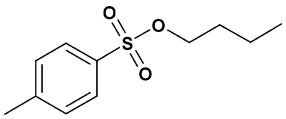
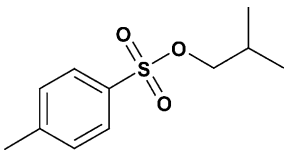
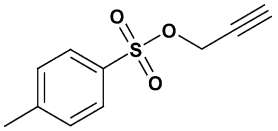
| Name | CAS number | Structure | Purity (%) | Source |
|---|---|---|---|---|
| Methanesulfonic acid esters (ME) | | | | |
| *n*-Propyl ester (MnPr) | 1912-31-8 |  | 99.3 | Novartis[a] |
| *iso*-Propyl ester (MiPr) | 926-06-7 |  | 98.4 | Novartis[a] |
| *n*-Butyl ester (MnBu) | 1912-32-9 |  | 99.0 | Novartis[a] |
| *iso*-Butyl ester (MiBu) | 16156-53-9 |  | 100.0 | Novartis[a] |
| *sec*-Butyl ester (MsBu) | 16156-54-0 |  | >95[b] | Novartis[a] |
| *iso*-Pentyl ester (MiPe) | 16156-55-1 |  | 78.2[c] | Novartis[a] |
| Benzenesulfonic acid esters (BE) | | | | |
| Methyl ester (BMe) | 80-18-2 |  | Not given | TCI America, no. B0033 |
| Ethyl ester (BEt) | 515-46-8 |  | 99.5 | TCI America, no. B0032 |
| *n*-Propyl ester (BnPr) | 80-42-2 |  | 98.2[d] | Novartis[a] |
| *iso*-Propyl ester (BiPr) | 6214-18-2 |  | 99.8[e] | Novartis[a] |
| *n*-Butyl ester (BnBu) | 80-44-4 |  | 98.3[d] | Novartis[a] |
| *iso*-Butyl ester (BiBu) | 24698-43-9 |  | 99.1[d] | Novartis[a] |

Table 1 (*Continued*)

| Name | CAS number | Structure | Purity (%) | Source |
|---|---|---|---|---|
| *p*-Toluenesulfonic acid esters (TE) | | | | |
| Methyl ester (TMe) | 80-48-8 |  | >97 | Fluka, Switzerland, no. 89800 |
| Ethyl ester (TEt) | 80-40-0 |  | >99 | Fluka, Switzerland, no. 89770 |
| *n*-Propyl ester (TnPr) | 599-91-7 |  | 98.7 | TCI America, no. T0726 |
| *iso*-Propyl ester (TiPr) | 2307-69-9 |  | 99.6[d] | Novartis[a] |
| *n*-Butyl ester (TnBu) | 778-28-9 |  | 96.9[d] | Novartis[a] |
| *iso*-Butyl ester (TiBu) | 4873-56-7 |  | 99.0[d] | Novartis[a] |
| Propargyl ester (TPrg) | 6165-76-0 |  | 97 | Fluka, Switzerland, no. 09954 |

[a] Compounds synthesized internally: R—$SO_2$—Cl + R′—OH → R—$SO_2$—O—R′ [2]
[b] Unstable over time.
[c] 18% *iso*-pentanol.
[d] 3–5% toluene.
[e] 7% toluene.

which a molecule is covered via the identification of unknown fragments (i.e. fragments which, in their particular structural environment, are not represented in the database). A prediction is regarded as uncertain if more than two uncovered functionalities are detected within the molecule. By default, structural fragments are defined as substructures of three–nine atoms in length. For the analysis of mutagenicity in the Ames test, the A2I database version 1.54 was used. This database has been constructed on the basis of published Ames test results using different *Salmonella typhimurium* and *E. coli* strains, in the presence as well as the absence of S9-based metabolic activation systems. For this study, no MCASE database was available to predict the results of the micronucleus tests.

### 2.3. Rat liver S9

The rat liver S9 used for metabolic activation was purchased from Molecular Toxicology Inc., 157

Industrial Park Dr. Boone, NC 28607, USA as MOLTOX™ post-mitochondrial supernatant (S9).

The S9-mix used for the *Salmonella* mutagenicity experiments was prepared according to Maron and Ames [3]. For the *Salmonella* mutagenicity experiments, 12.5 μl of an S9-mix containing 10% rat liver S9-fraction per tester plate were used. For the mouse lymphoma micronucleus test the final concentration of the S9-mix in the medium was 2%. The composition of the S9-mix was as follows (for 3 ml S9-mix): glucose-6-phosphate, 108 mg; NADP, 15 mg; KCl 150 mM, 0.6 ml; Aqua dest., 1.2 ml; S9-fraction, 1.2 ml.

### 2.4. Salmonella reverse mutation (Ames) test

The method used followed the recommendations by Maron and Ames [3] and the OECD Guideline [4]. In this study a reduced Ames test design using only *S. typhimurium* strains TA98 and TA100 was applied. Both strains were obtained from B.N. Ames, Biochemistry Department, University of California, Berkeley, CA, USA. A test compound was considered to be mutagenic in the plate test if it produced, in at least one concentration and one strain, a revertant number equal to twice (or more) the control incidence. The test compounds were evaluated using concentrations of 15, 50, 150, 1500 and 5000 μg/plate. Due to bacteriotoxicity of benzenesulfonic acid and *p*-toluenesulfonic acid methyl ester, an additional experiment was performed with strain TA100 only, with concentrations of these two compounds of 312.5, 625, 1250, 2500 and 78.125, 156.25, 312.5, 625, 1250, 2500 μg/plate, respectively.

### 2.5. Micronucleus test in mouse lymphoma (L5178Y) cells

The in vitro micronucleus test is a well-established test for early genotoxicity screening of new compounds in industrial toxicology. For assessing the clastogenic or aneugenic potential of a test compound, the micronucleus induction in mammalian cells has been shown to be a sensitive and specific parameter with an excellent correlation between chromosomal aberration and micronucleus data in vitro, in primary cells [5,6].

Mouse lymphoma cells were obtained from Hofmann-La Roche (Basel, Switzerland). The cells are growing in suspension which facilitates their handling as well as continuous cultivation. Based on the data of a preliminary solubility test and within the limit to test up to 10 mM or 5 mg/ml (whichever was lower), 10 concentrations were selected for the main experiment. All compounds were dissolved in DMSO. The cells were diluted in RPMI-10 medium (Gibco) to 200,000 cells/ml for the 3 h treatment and to 100,000 cells/ml for the 20 h treatment (cell count determined by use of a SYSMEX cell counter), respectively. The desired final concentration was obtained by adding 10 μl of DMSO-dissolved test compound to 1 ml cell suspension (sufficient for four wells of 200 μl each), resulting in 1% DMSO in the treatment medium. Incubation was conducted with 96-well plates with four replicates per test compound concentration. For the micronucleus experiment, the cells were treated with the test compound solution for 3 h (with or without S9) or for 20 h (without S9 only). After the treatment, cells were washed with phosphate-buffered saline and further incubated with fresh medium for 21 h in case of 3 h treatment, or 28 h in case of 20 h treatment. Before sampling, a cell count for one of the replicates of each concentration was determined as cytotoxicity parameter, and the concurrent negative control was set to 100%. Based on the relative cell count, the concentration that inhibited growth by approximately 50–70%, and two lower concentrations showing less cytotoxicity were chosen for analysis. The selected cell suspensions were then spread on glass slides by cytocentrifugation (Shandon Cytospin). Cells were fixed and stained with Schiff's reagent (nucleus staining) and Congo-Red counterstaining for the cytoplasm. Two thousand cells (1000 cells per culture) were analyzed from at least three concentrations of each test compound, as well as from negative and positive controls. For the analysis, the in-house developed fully automated image analyzer ROBIAS was employed [7]. A result was considered positive when the micronucleus frequency was at least twice the historical negative control value as well as twice the actual concurrent negative solvent control value. These criteria are based on an in-house validation study of the assay. The historical average solvent control data for micronucleus induction were as follows: 3 h treatment without S9, $0.73 \pm 0.13$; 20 h treatment without S9, $0.82 \pm 0.18$; 3 h treatment with S9, $0.78 \pm 0.16$.

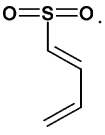
## 3. Results

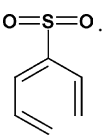
### 3.1. Computer-aided test systems

The results of the computer-aided analyses of the sulfonic acid esters are given in Table 2. Basic chemical reasoning such as the one reflected in rule-based systems predicted all sulfonic acid esters under study to be active as mutagens based on the presence of a sulfonate moiety as a potentially alkylating functionality. MCASE, as a statistically correlative system, predicted the methanesulfonic acid esters as well as the *p*-toluenesulfonic acid esters to be active as mutagens in the Ames tests based on the sulfonate moiety as a biophore. This biophore was found in 14 active and only 4 inactive molecules in the database. Although the sulfonate moiety was detected as a biophore for the benzenesulfonic acid esters as well, their activity

Table 2
Results of the computer-aided analysis

| Compound | Basic SAR rule | MCASE (A2I database) | | |
|---|---|---|---|---|
| | | Result | Biophore | Comment |
| Methanesulfonic acid esters (ME) | | | | |
| MnPr | Alkylating agent | Active | $SO_2$—O— | None |
| MiPr | Alkylating agent | Active | $SO_2$—O— | None |
| MnBu | Alkylating agent | Active | $SO_2$—O— | None |
| MiBu | Alkylating agent | Active | $SO_2$—O— | None |
| MsBu | Alkylating agent | Active | $SO_2$—O— | None |
| MiPe | alkylating agent | Active | $SO_2$—O— | None |
| Benzenesulfonic acid esters (BE) | | | | |
| BMe | Alkylating agent | Inactive | $SO_2$—O— | Inactivating modulator: $SO_2$—C=CH—CH=CH[a]; deactivating fragment: CH=CH—CH=C—CH with $SO_2$ at C4[b] |
| BEt | Alkylating agent | Inactive | $SO_2$—O— | Inactivating modulator: $SO_2$—C=CH—CH=CH[a]; deactivating fragment: CH=CH—CH=C—CH with $SO_2$ at C4[b] |
| BnPr | Alkylating agent | Inactive | $SO_2$—O— | Inactivating modulator: $SO_2$—C=CH—CH=CH[a]; deactivating fragment: CH=CH—CH=C—CH with $SO_2$ at C4[b] |
| BiPr | Alkylating agent | Inactive | $SO_2$—O— | Inactivating modulator: $SO_2$—C=CH—CH=CH[a]; deactivating fragment: CH=CH—CH=C—CH with $SO_2$ at C4[b] |
| BnBu | Alkylating agent | Inactive | $SO_2$—O— | Inactivating modulator: $SO_2$—C=CH—CH=CH[a]; deactivating fragment: CH=CH—CH=C—CH with $SO_2$ at C4[b] |
| BiBu | Alkylating agent | Inactive | $SO_2$—O— | Inactivating modulator: $SO_2$—C=CH—CH=CH[a]; deactivating fragment: CH=CH—CH=C—CH with $SO_2$ at C4[b] |
| *p*-Toluenesulfonic acid esters (TE) | | | | |
| TMe | Alkylating agent | Active | $SO_2$—O— | None |
| TEt | Alkylating agent | Active | $SO_2$—O— | Molecule exists in the database as active |
| TnPr | Alkylating agent | Active | $SO_2$—O— | None |
| TiPr | Alkylating agent | Active | $SO_2$—O— | None |
| TnBu | Alkylating agent | Active | $SO_2$—O— | None |
| TiBu | Alkylating agent | Active | $SO_2$—O— | None |
| TPrg | Alkylating agent | Active | $SO_2$—O— | None |

[a] 

[b] 

was expected to be negligible due to the influence of an inactivating modulator (i.e. a fragment found in the molecules containing the biophore, but which yielded negative Ames test results) and a biophobe (i.e. a fragment present only in inactive molecules). The biophobe was found in seven inactive compounds in the A2I database.

### 3.2. In vitro test systems

An overview over the results of the reduced Ames test (strains TA98 and TA100) and the mouse lymphoma micronucleus test (mouse lymphoma MNT) is given in Table 3.

#### 3.2.1. Methanesulfonic acid esters

All methanesulfonic acid esters under evaluation showed a genotoxic response in both the Ames test and the mouse lymphoma micronucleus test in vitro (Table 3). The methanesulfonic acid esters were all positive in *Salmonella* strain TA100 with or without metabolic activation. The Ames test results obtained with TA100 without or with metabolic activation are shown in Figs. 1 and 2, respectively, and are given as fold increases over the concurrent negative control values.

*iso*-Propyl (MiPr) and *iso*-butyl (MiBu) ester showed a higher increase in the number of revertants when tested without metabolic activation. Dif-

Table 3
Results of the Ames test and the mouse lymphoma MNT

| Compound | Ames test | | Mouse lymphoma MNT | |
|---|---|---|---|---|
| | Result | Mutagenic response in strain(s) ±S9 | 20 h −S9 | 3 h +S9 |
| Methanesulfonic acid esters (ME) | | | | |
| MnPr | + | TA100 ±S9 (1) | + | ± |
| MiPr | ++ | TA98, TA100 ±S9 (1) | + | + |
| MnBu | + | TA100 ±S9 (1) | + | ± |
| MiBu | ++ | TA100 ±S9 (1) | (+) | − |
| MsBu | + | TA98, TA100 ±S9 (1) | + | + |
| MiPe | ++ | TA100 ±S9 (1) | + | − |
| Benzenesulfonic acid esters (BE) | | | | |
| BMe | + | ± in TA100 −S9 (2), + in TA100 +S9 (2) | + (1) | + |
| BEt | + | TA100 ±S9 (1) | + (1) | + |
| BnPr | + | TA100 ±S9 (1) | (+) | ± |
| BiPr | ++ | TA98, TA100 ±S9 (1) | + | + |
| BnBu | + | TA100 −S9 (3), TA100 +S9 (1) | − | − |
| BiBu | + | TA100 +S9 (3) | − | − |
| *p*-Toluenesulfonic acid esters (TE) | | | | |
| TMe | + | TA100 ±S9 (4) | + (1) | + |
| TEt | ± | TA100 ±S9 (1) | + | + |
| TnPr | + | TA100 ±S9 (1) | (+) | − |
| TiPr | + | TA100 ±S9 (1) | + | + |
| TnBu | + | TA100 ±S9 (1) | + | − |
| TiBu | − | None | − | − |
| TPrg | + | TA100 +S9 (5) | n.d. | n.d. |

*Criteria for positivity and potency in the Ames tests*: +, active (two-fold increase in the number of revertants over the concurrent negative control values in at least one strain + or −S9); ++, very active (at least five times the number of revertants of the concurrent negative control values in one strain + or −S9, doubling at ≤5000 μg/plate); −, negative; ±, weakly positive (reproducible dose-dependent increase in the revertant numbers but no doubling in the number of concurrent control revertants); (1), no precipitation or bacteriotoxicity found up to the highest concentration tested (i.e. 5000 μg/plate) 3 h −S9 treatment; (2), no precipitation, bacteriotoxicity seen at ≥2500 μg/plate; (3), no precipitation, bacteriotoxicity seen at 5000 μg/plate (in case of TiBu only in TA100 +S9); (4), no precipitation, bacteriotoxicity seen at 5000 μg/plate (TA98 ±S9), ≥1250 μg/plate (TA100 −S9) or ≥2500 μg/plate (TA100 +S9); (5), no precipitation, bacteriotoxicity seen at ≥1500 μg/plate. *Criteria for positivity and potency in the MN tests*: +, significant increase in the number of cells containing micronuclei at concentrations showing no or at most moderate cytotoxicity; (+), significant increase in the number of cells containing micronuclei at highly/excessively cytotoxic concentrations, genotoxic effect cannot be excluded; ±, only weak effects obtained at 10 mM; −, negative; (1), 3 h −S9 treatment; n.d., experiment not done.

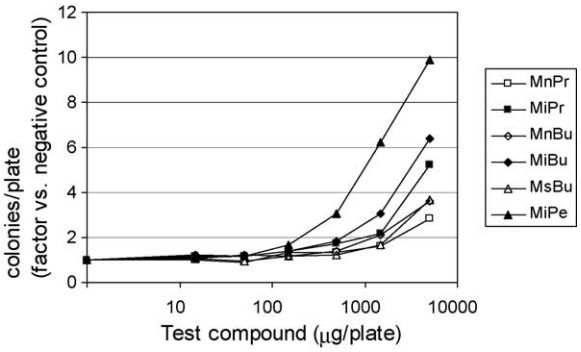



Fig. 1. Ames test results for ME in strain TA100 −S9.

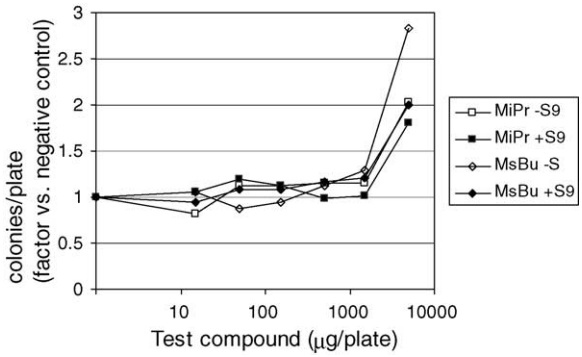



Fig. 3. Ames test results for MiPr and MsBu in strain TA98 ±S9.

ferences between the test results obtained ±S9 for the other methanesulfonic acid esters were either absent or the compounds were slightly more mutagenic with metabolic activation.

Generally, the '*iso*-methanesulfonic acid esters' were more potent mutagens in the Ames test than the *sec*-butyl ester and the '*n*-esters' (with the rank order MiPe ≫ MiBu > MiPr).

MiPr and *sec*-butyl (MsBu) ester additionally yielded positive results in *Salmonella* strain TA98 ±S9 (Fig. 3). However, the mutagenic responses in strain TA98 with both esters were weak, and no doubling in the number of background revertants was reached with MiPr in strain TA98 +S9.

The methanesulfonic acid esters showed positive results in the mouse lymphoma MNT after 20 h treatment without metabolic activation (Fig. 4). However, MiBu induced only a very weak increase in the number of cells containing micronuclei after a 20 h treatment without S9, and the effect was seen at excessively cytotoxic concentrations. The other methanesulfonic acid esters were at most moderately cytotoxic.

MiPr was clearly the most potent of the methanesulfonic esters in inducing micronuclei. It was followed by the '*n*-esters' (MnBu > MnPr) while the *sec*-butyl ester (MsBu) and *iso*-pentyl ester (MiPe) showed the lowest potency in this test. MiPr and MsBu were shown to be positive also after a 3 h treatment +S9, while the other methanesulfonic acid esters yielded at most weakly positive results (MnPr and MnBu) or were negative (MiBu and MiPe) under these conditions.

### 3.2.2. *Benzenesulfonic acid esters*

The benzenesulfonic acid esters yielded positive results in the Ames test in strain TA100, but not all were positive in the mouse lymphoma MNT (Table 3).

The Ames test results of the benzenesulfonic acid esters in strain TA100 without or with metabolic activation are shown in Figs. 5 and 6, respectively. Not all

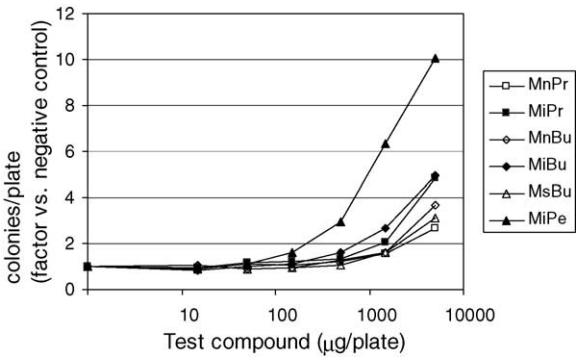



Fig. 2. Ames test results for ME in strain TA100 +S9.

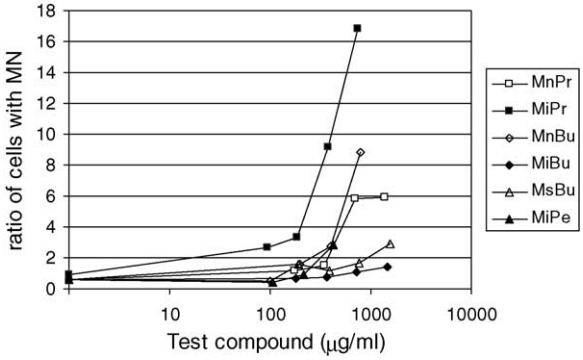



Fig. 4. MN test results 20 h treatment −S9 for ME.

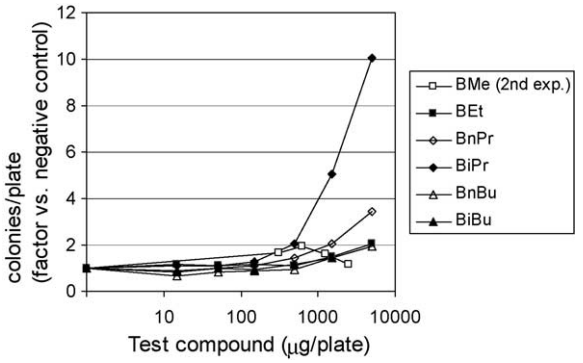



Fig. 5. Ames test results for BE in strain TA100 −S9.

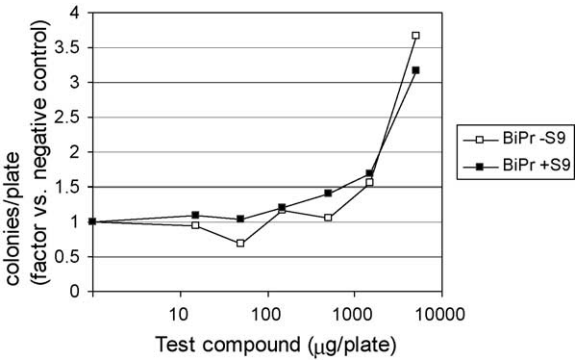



Fig. 7. Ames test results for BiPr in strain TA98 ±S9.

benzenesulfonic acid esters showed a comparable mutagenicity in strain TA100 when tested with or without metabolic activation. Methyl (BMe), *n*-propyl (BnPr), *n*-butyl (BnBu) and *iso*-butyl (BiBu) ester showed higher increases in the revertant number when tested with S9 than without. Due to the excessive bacteriotoxicity of BMe, a second experiment was performed with this compound. In this second experiment a doubling in the revertant number compared with the concurrent controls was reached at 625 μg/plate −S9 and at 312.5 μg/plate +S9. Without metabolic activation BiBu did not reach a doubling in the control colony number in strain TA100. In the case of the ethyl ester (BEt) a doubling of the control revertant number was observed only at the highest concentration tested (i.e. 5000 μg/plate). Of the benzenesulfonic acid esters, *iso*-propyl ester (BiPr) was demonstrated to be the strongest mutagen in the Ames test (followed by BnPr). In addition, it was the only compound that yielded positive results also in strain TA98 ±S9 (Fig. 7).

BMe and BEt were shown to induce micronucleated cells after a 3 h treatment with or without metabolic activation (Table 3). The *iso*- and *n*-butyl esters were negative in the mouse lymphoma MNT. BMe, BnPr and BiPr were the most potent compounds of the benzenesulfonic acid esters regarding the induction of micronuclei (Fig. 8). Due to the cytotoxicity, which prevented BMe and BnPr from being tested at higher concentrations, and the fact that only the methyl ester was tested at 3 h, a more detailed comparison is impossible. However, BiPr was also positive after a 3 h treatment +S9, whereas BnPr was only weakly positive.

### 3.2.3. *p*-Toluenesulfonic acid esters

The methyl (TMe), *n*-propyl (TnPr), *iso*-propyl (TiPr), *n*-butyl (TnBu) and propargyl (TPrg) esters of *p*-toluenesulfonic acid all exerted mutagenic activity

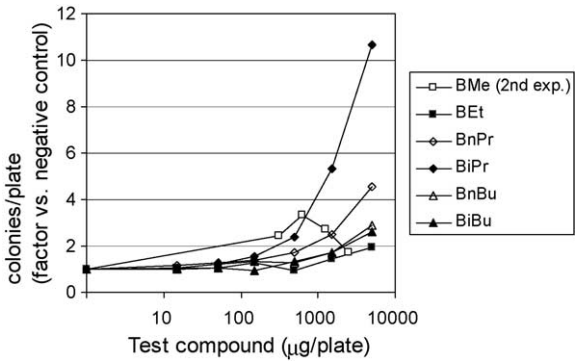



Fig. 6. Ames test results for BE in strain TA100 +S9.

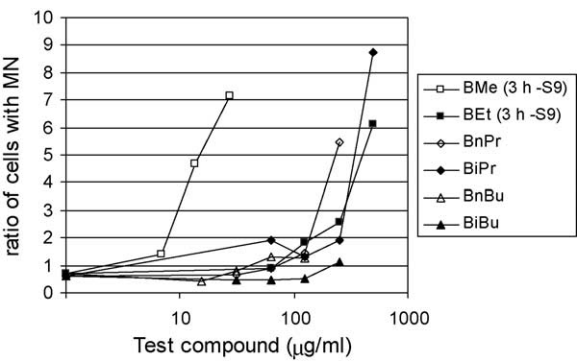



Fig. 8. MN test results 20 h treatment −S9 for BE.

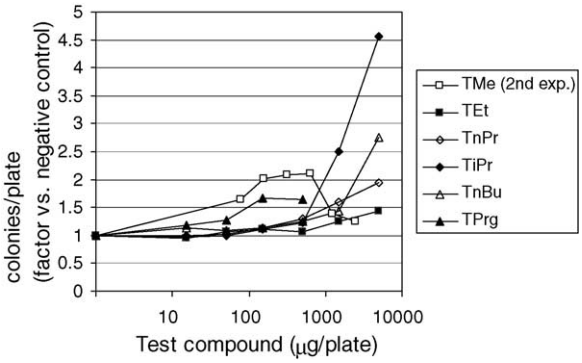



Fig. 9. Ames test results for TE in strain TA100 −S9.

in the Ames test (Table 3). These esters (with the exception of TPrg which was not tested in the MNT) were also positive in the MNT, but TnPr only at highly cytotoxic concentrations. Additionally, the ethyl ester (TEt), which was only weakly positive in the Ames test, was demonstrated to induce micronuclei in mouse lymphoma cells. The *iso*-butyl ester (TiBu) neither showed evidence of mutagenic potential in the Ames test nor was it positive in the mouse lymphoma micronucleus test. The propargyl ester was demonstrated to be positive in strain TA100. None of the *p*-toluenesulfonic esters was found to be active as a mutagen in *Salmonella* strain TA98.

The TMe and TnPr esters showed higher increases in revertant numbers when tested with S9 than without metabolic activation (Figs. 9 and 10). The ethyl ester TEt was only weakly positive in the Ames test: a maximum factor of 1.58 was obtained in strain TA100 with metabolic activation.

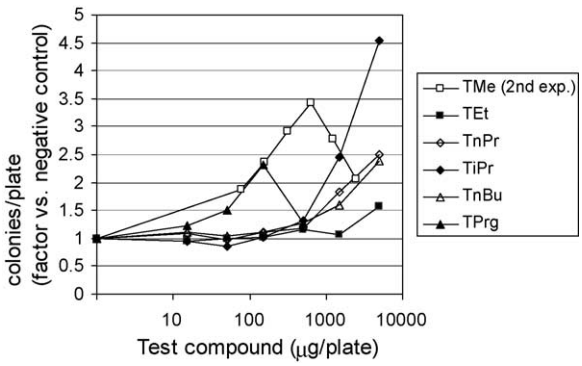



Fig. 10. Ames test results for TE in strain TA100 +S9.

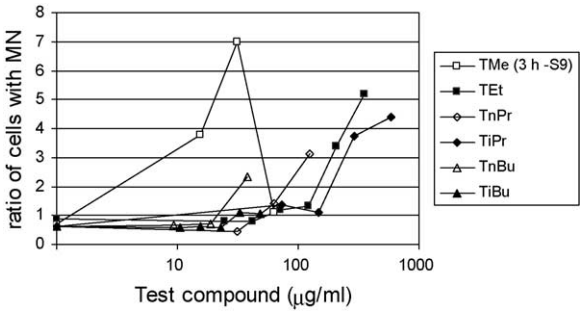



Fig. 11. MN test results 20 h treatment −S9 for TE.

TiPr was the most potent of the *p*-toluenesulfonic acid esters (followed by methyl and *n*-butyl ester), and it showed similar results when tested with or without metabolic activation in the Ames test.

Thr TMe, TEt and TiPr esters were positive in the micronucleus test with or without metabolic activation (Table 3). In addition, these esters were the strongest in their potential to induce micronuclei (Fig. 11). TnPr was positive only at highly cytotoxic concentrations. Both '*n*-esters' (TnPr and TnBu) did not induce micronuclei after 20 h treatment −S9. TiBu did not increase the number of cells containing micronuclei either after a 20 h treatment −S9 or a 3 h treatment +S9.

## 4. Discussion

In structural analogy to methanesulfonic acid methyl and ethyl ester, all sulfonic acid esters are suspected to be alkylating agents. This is reflected in structure–activity reasoning incorporated into the knowledge base of certain computerized toxicity prediction programs. On the basis of such a prediction the sulfonate moiety was correctly identified as a structural alert for mutagenicity in the Ames test in 89.5% (17 out of 19), and for clastogenicity and/or aneugenicity in the micronucleus test with mouse lymphoma (L5178Y) cells in 77.8% (14 out of 18) of all esters evaluated in this study (MiBu was not considered a true positive in the micronucleus test due to the very weak increase in MN frequency at excessively cyctotoxic concentrations).

MCASE (A2I module) using a statistically correlative approach predicted all methanesulfonic and *p*-toluenesulfonic acid esters to be active as mutagens

in the Ames test. According to the program's calculations the mutagenic activity of the benzenesulfonic acid esters was expected to be negligible due to the presence of an inactivating modulator and a deactivating fragment, both structural fragments located in the benzenesulfonate moiety. Inactivating modulators are generally detected in molecules that contain the biophore, but were found to be negative in the Ames test, whereas deactivating fragments are represented in inactive compounds only. The inactivating modulator was derived from 4,4′-(3*H*-2,1-benzoxathiol-3-ylidene)-bis-2,5-dimethyl-phenol and benzenesulfonic acid dodecyl ester, the latter being more structurally related to the benzenesulfonic acid esters evaluated in this study. The deactivating fragment, which was almost identical to the inactivating modulator (see Table 2), was found in seven molecules, four of which were dyes. The remaining molecules were benzenesulfonic acid dodecyl ester, *m*-amino-benzenesulfonic acid and benzenesulfonic acid itself. The presence of the dodecyl ester among the molecules containing the inactivating modulator as well as the deactivating fragment explains the negative Ames prediction of the benzenesulfonic acid esters by MCASE. In this study the benzenesulfonic acid esters were all found to be positive in the Ames test. Hence, MCASE correctly predicted 61.1% (11 out of 18 compounds) to be active in the Ames test. For this calculation *p*-toluenesulfonic acid ethyl ester was not taken into consideration since this ester already exists in the database of MCASE as mutagenic. Published data indicate the compound to be positive in *Salmonella* strain TA1535 without metabolic activation [8], while in this study only *Salmonella* strains TA98 and TA100 were used. The availability of new data in particular on Ames-positive benzenesulfonic acid esters in the MCASE learning set improved the predicitivity of the system. The observed decrease of the mutagenic potential of the benzene and toluenesulfonic acid esters with increase of the (branched) alkyl side chains (as detailed further) contributed to a refined MCASE prediction module which is used in-house.

Monofunctional methanesulfonates have been demonstrated to be directly acting mutagens, which exert their mutagenic activity in *Salmonella* strain TA100 by nucleophilic substitution ($S_N1$ or $S_N2$) reactions [9]. Additionally, in another study the authors suspected the $S_N1$-type methanesulfonates to cause mutations probably by $O^6$-guanine alkylation, subsequently leading to a GC–AT transition mutation [10]. In our study all of the methane-, benzenesulfonic and *p*-toluenesulfonic acid esters, with the exception of ethyl and *iso*-butyl ester of *p*-toluenesulfonic acid, were shown to be mutagenic in *Salmonella* strain TA100. In addition, methanesulfonic acid *iso*-propyl and *sec*-butyl ester as well as benzenesulfonic acid *iso*-propyl ester were positive in strain TA98, but none of the toluenesulfonic acid esters was mutagenic. Among the methane- and benzenesulfonic acid esters the '*iso*-esters' exerted the strongest mutagenic activity in strain TA100. For *p*-toluenesulfonic acid *iso*-propyl a 4.5-fold increase in mutant numbers versus the negative control (±S9) was reached, whereas *iso*-butyl ester was found to be negative in the Ames test. Thus, taken together, there seems to be a tendency for the *p*-toluenesulfonic esters to be less mutagenic than the methane- and benzenesulfonic acid esters. The observed strong mutagenic potential of all *iso*-propyl esters might be related to the methyl group at the α-C-atom of the alkyl chain, which exerts a +I (inductive effect, i.e. an electronic effect due to the polarization of σ-bonds within a molecule) and a +M effect (mesomeric effect attributed to a substituent due to overlap of its π-orbitals with the π-orbitals of the rest of the molecule). Both the +I and +M effects stabilize the partially positive charge on the α-C atom, enhance destabilization of the C–(O–$SO_2$) bond and, thus, result in increased $S_N$-reactivities as shown for allylic and α,β-unsaturated compounds [11].

In general, the differences between the mutagenic activities observed in Ames tests conducted with or without metabolic activation were small. Hence, according to our experiments, metabolic activation seems not to influence the mutagenicity of the sulfonic acid esters significantly. Exceptions are the benezenesulfonic acid *iso*-butyl and *p*-toluenesulfonic acid propargyl esters, which were found to be positive (i.e. showing a doubling in the revertant number) only with S9-mix. This is in contrast to the observed decrease in the mutagenic properties of allylic compounds in *Salmonella* strain TA100 after incubation with S9-mix, an effect that has been associated with the reaction of the directly alkylating allylic substances with nucleophiles such as glutathione in the S9-mix [12]. Generally, the sulfonic acid esters tended to be less mutagenic when tested with metabolic activation (3 h treatment) in the mouse lymphoma micronucleus test than in the absence of S9 (3 h treatment). This is in concordance

with results obtained with methanesulfonic acid ethyl ester, which is a known direct alkylating agent in both bacterial and mammalian cell test systems. Similar to the Ames test results, the *iso*-propyl esters were observed to be highly potent micronucleus inducers. The benzene- and *p*-toluenesulfonic acid methyl esters were also shown to be strongly positive, but extreme toxicity prevented the exact quantitative assessment of the genotoxic potential of both compounds. Methanesulfonic acid *iso*-butyl ester was only weakly positive at excessively cytotoxic concentrations. Benzene- and *p*-toluenesulfonic acid *iso*-butyl esters were shown to be neither clastogenic nor aneugenic in the micronucleus test. Hence, longer (branched) alkyl side chains may lead to a decrease in the mutagenic potential of the sulfonic acid esters. This is in concordance with the observed influence of the chain length of the alkyl substituent on the induction of mutations in diploid human lymphoblasts or *S. typhimurium* (forward and reverse bacterial mutation assay) [13]. However, in our experiments methanesulfonic acid *iso*-pentyl ester was again found to be positive in the MNT. The similarity in the mutation frequency between mammalian and bacterial cells observed in other studies [13] was not entirely confirmed by our data. Thus, for example, the ethyl ester of *p*-toluenesulfonic acid was only weakly positive in the Ames test, but clearly positive the micronucleus test in vitro.

However, in general, despite of the differences between bacteria and mammalian cells, a comparably good correlation of the genotoxic potential of the sulfonic acid esters in the Ames test, using strains TA98 and TA100 and in the L5178Y mouse lymphoma micronucleus test was observed. The *iso*-propyl esters were found to be strong mutagens in both tests whereas *p*-toluenesulfonic *iso*-butyl ester was found to be negative in the Ames as well as in the micronucleus test.

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