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Chemical and toxicological studies of products resulting from sorbic acid and methylamine interaction in food conditions

C. Ferrand[1], **F. Marc**[1], **P. Fritsch**[2], **P. Cassand**[3], and **G. de Saint Blanquat**

[1] Laboratoire d'Analyse et de Valeur Biologique des Aliments, Département Universitaire des Sciences d'Agen, Université Bordeaux I, Agen, France
[2] INSERM, Laboratoire UPS, EA 2405, CHU Rangueil, Université Toulouse III, Toulouse, France
[3] Laboratoire de Toxicologie Alimentaire, ISTAB, Université Bordeaux I, Talence, France

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Summary. Sorbic acid has a system of conjugated double bonds which makes it able to undergo nucleophilic addition reactions with certain functions. The interactions between sorbic acid and an amine present in the endogenous constituents of food were quantified. We demonstrated the formation of new products and studied the underlying mechanisms using ethyl sorbate and various amines. HPLC, GC, GC-SM and NMR analyses of the reaction mixes enabled the isolation and identification of the products. The addition reactions led, at 20°C, to linear monoadducts and, at 50°C, to cyclic derivatives resulting from double addition. Mutagenesis studies, involving Ames test and genotoxicity studies with HeLa cells and on plasmid DNA, in cyclic interaction products, showed that none of the products studied presented neither mutagenic nor genotoxic activities.

Keywords: Amino acids – Sorbic acid – Methylamine – Interaction products – 3-D-test – Ames test

Introduction

Sorbic acid and its potassium and calcium salts are preservative additives used in many processed foods: margarine, fruit preparations, drinks, fresh cheese, etc. They benefit from a large spectrum of microbiocidal action (Kinderlerer and Hatton, 1990). Sorbic acid and sorbates are regularly the subject of requests as regards the development of their use and recent evaluations (Leikanger, 1992) show an increase in their consumption.

From the point of view of structural chemistry, sorbic acid is the *trans-trans* form of hexa 2, 4 dienoic acid. It possesses a system of conjugated double bonds which makes it able to react with many nucleophilic agents: such as sulfites (Khandelwal and Wedzicha, 1990a), nitrites (Kito and Namiki, 1978;

Osawa and Namiki, 1982), thiols (Wedzicha and Brook, 1989; Khandelwal and Wedzicha, 1990a, b), and amines (Shamma and Rosenstock, 1961; Verbiscar and Campbell, 1964; Kheddis et al., 1981).

The reactivity of sorbic acid with amines has rarely been studied in food. On the other hand, using drastic conditions (in an autoclave at 200°C for 6 hours), Kheddis et al., (1981), Verbiscar and Campbell (1963), and Khandelwal and Wedzicha (1990b) described the formation of dihydropyridones ((e) and (f)) (figure 1) derived from sorbic acid and from various primary amines.

We investigated whether such a reaction can become prominent in milder conditions, that is to say close to those of food processing. For this, we used a model system composed of sorbic acid, and a primary amine: methylamine disolved in water at 50°C. The kinetics of formation of each adduct was studied at 20°C and 50°C.

Some reports indicate (Hayastu et al., 1975; Namiki and Kada, 1975; Namiki et al., 1981) that, at high concentrations and at temperatures between 60 and 90°C, sorbic acid reacts with nitrites to produce mutagenic substances. Using Ames test with *Salmonella typhimurium* strain, Namiki and Kada (1975) showed that ethylnitrolic acid had a weak mutagenic activity but using the rec assay test with *Bacillus subtilis* strain, the activity was much higher. Another product that has been identified, 1,4-dinitro-2-methylpyrol (DNMP), is well known as the most mutagenic compound: its mutagenic power is at the top of the scale in Ames test towards *Salmonella typhimurium* strains TA98 and TA100 with no metabolic activation and in the rec assay test (Namiki and Kada, 1975; Namiki et al., 1981). The third compound produced by sorbate-nitrite interactions is a derivative of furoxan. It is devoid of mutagenic activity and a compound E – which structure has not yet been determined – has a weak mutagenic activity in the rec test assay (Namiki et al., 1981). Mutagenic activity has also been shown by Lafont and Lafont (1979) in wine containing sulphur dioxide and sorbic acid after 45 days of storage.

Considering this general background, we decided to pursue our studies on the interactions between sorbic acid and amines and to evaluate the genotoxicity and mutagenesis of the products. The latter are expected to be present in food so their harmlessness must be proved – their biological activity not having been evaluated to date. In the present study we investigated the activity of the reaction products with Ames test using *Salmonella typhimurium* strains TA98 and TA100 (Maron and Ames, 1983) and the genotoxic activity using the damaged DNA detection assay (3D test), with plasmid DNA and genomic DNA (Salles and Provot, 1999).

Material and methods

Chemical studies

Reagents: All products were obtained from Aldrich.

Gas chromatography: The gas chromatograph (Perkin Elmer 8420) was equipped with a DB1 capillary column (25 m $\times$ 0.25 μm internal diameter (I.D.)). The operating

conditions were: oven temperature 60°C to 250°C at 10°C/min; injection port 300°C, detector 300°C, carrier gas pressure 20 psi, split flow 40 to 50 ml/min.

Gas chromatography – Mass spectrometry: A mass spectrometer (Perkin Elmer mass 910), with quadripole separation was coupled to a gas chromatograph (Perkin Elmer 9000 autosystem). It was equipped with a DB1 capillary column (apolaire, formed by a dimethylpolysiloxane film, 25 m × 0.25 μm I.D.)

Nuclear magnetic resonance: NMR analyses were performed, after extractions and purifications of products, at 250 MHz for ^{1}H and at 62.9 MHz for ^{13}C (Brucker Ac 250). The solvent was deuterated chloroform and the reference was tetramethylsilane.

Procedure: In order to study the reaction mechanisms involved, we chose to use the ester form (ethyl sorbate) which permitted us to isolate and characterize some reaction intermediates more easily as recommended by Khandelwal and Wedzicha (1990b) who had shown that the adducts formed from sorbic acid decay spontaneously after acid and basic extractions.

For all our tests, sorbic acid or ethyl sorbate was present in the reaction mixtures (30 ml) at the same concentration of 0.33 Mol/l in absolute ethanol. The methylamine was used at 0.66 Mol/l. Milder reaction conditions were used than those described in the literature in an attempt to remain closer to the conditions of food processing (room temperature: 20°C or 50°C). The mixtures obained were left under mechanical stirring during 15 days. The adducts formed were assayed by GC every day, this gave the reaction kinetics. After extraction and purification of the different components they were characterised by two techniques: mass spectrometry and NMR.

The adducts were extracted by adding diethyl ether and aqueous NH_4Cl to pH 5–6, then the aqueous phase was collected and diethyl ether and aqueous Na_2CO_3 were added to yield pH 9–10. The organic phase was collected, dried with magnesium sulfate, and concentrated by evaporation. When required, the adducts were purified on an alumina column (Fluka, neutral, activity 1.200 × 10 mm ID); the eluents were increasingly polar solutions of diethyl ether – petroleum spirit.

Toxicological studies

We tested the genotoxic and mutagenic activity of cyclic interaction compouds because they are more stable than linear products.

Study of the genotoxic effects of the reaction products: 3D tests

Materials: The equipment (luminometer and microtitration plates) and all the reagents for the 3D test came from the SFRI Co (France), except for methylmethanesulphonate (MMS) which came from Sigma (France). The plasmids were produced by a bacterium *Escherichia coli* pBluescript KS+, 2959 bp, (Stratagene, La Jolla, USA). The cells used were adherent HeLa cells of human origin (European Molecule at Biology Laboratory, Heidelberg, Germany).

The 3D test for DNA damage using plasmid DNA: The method was conducted using the test procedure described by Salles and Provot (1999). The sample of cyclic products was diluted in DMSO (dimethyl sulphoxide) at 400 mg/ml; then, the following dilutions were made in ultrapure water: 10, 1, 0.1, 10^{-2}, and 10^{-3} mg/ml.

Ultrapure undamaged plasmid DNA was placed in contact with the sensitised wells for 30 minutes at 30°C under gentle shaking. The dilutions of the samples were incubated on the DNA for 30 min at 30°C. A positive repair control consisting of UV-damaged plasmid DNA was included along with two further controls, one with the solvent alone and one with a known genotoxic compound: MMS, 2 and 10 mMol/l.

The wells were then rinsed twice with phosphate-buffered saline (PBS) containing 0.1% Tween 20 after which 50 μl of DNA repair mix were added. The repair solution was

composed of protein repair complexes (P2) and nucleotides, one of which was labelled (dUTP-biotin (solution E of the 3D kit from SFRI).

After 3 hours of incubation at 30°C, the plate was washed three times and 50 μl of conjugate solution was placed in each of the wells. The conjugate solution was composed of modified bovine serum albumin and a solution of concentrated avidine-peroxidase conjugate (solution c1 of the SFRI 3D kit). Incubation at 30°C was then continued for 15 minutes under gentle stirring.

The plate was washed five times and 50 μl of developing solution (chemoluminescent substrate: flask D form the SFRI 3D kit) were added to each well under total darkness. After 5 minutes of incubation at 30°C under gentle stirring in the dark, the light emitted was measured with a luminometer. The signal – in relative light units (RLU) – is directly proportional to the number of breaks repaired. The ability of a drug to cause strand breaks in DNA is measured from the *intensity* of the signal at a given level (mean RLU adduct of two wells) to the signal of the solvent alone (RLU DMSO) = repair ratio. Any ratio greater than one indicates an increase of the repair signal and thus an increase in the number of lesions on the DNA. If its value is greater than two, the *drug* tested *should have* a significant genotoxic effect. Each *experiment* was performed in duplicate and the mean of the relative light units obtained was calculated.

The 3D test for DNA damage using genomic DNA: The adherent HeLa cells were cultured for 20 h at 37°C in SFRI 4 medium containing 10% foetal calf serum and 2 mM glutamine under a 10% CO_2-enriched atmosphere. The 96-well culture plates were seeded with 10^4 cells per well. The sample of interaction products was added to the culture supernatant. The HeLa cells were incubated for 1 hour with the various doses. The culture supernatant was eliminated when 100 μl of lysis solution (Solycel, SFRI) was added to each well. Incubation was then continued for 90 min at 30°C under gentle shaking. Genomic DNA was recovered and analysed with the 3D test. Aliquots of 50 μl of each cell lysate were placed in contact with the sensitised wells (washed twice beforehand) for 30 minutes at 30°C under gentle shaking. The other steps of the test were the same as those for the 3D test on plasmid DNA (Salles and Provot, 1999).

Mutagenicity test

Bacterial strains: *Salmonella typhimurium* strains TA98 and TA100, his^- (strains unable to develop on an environment lacking in histidine) were provided by Dr Bruce N. Ames (Departement of Biochemistry, University of California, CA 94720, USA).

Mutagenesis assay: The Ames test was conduced using the test procedure described by Ames et al. (1975). Tester strains were maintained and checked for conservation of properties as recommended by Maron and Ames (1983). A 100 μl of the diluted interaction products solution to be tested / 30 μl DMSO was added to 100 μl of TA100 or TA98 strains and 500 μl of S9 mix (when the reaction products were activated). The mixtures were then homogenized in 2.5 ml of top agar and poured onto the Vogel-Bonner minimal glucose agar plates which were scored for histidine revertants after 48 h incubation at 37°C.

Metabolic activation of the added chemicals was provided by supplementing Aroclor 1254-induced male rat liver microsome preparation enriched with co-factors. The S-9 fraction was used at a concentration of 0.1 ml per ml of S-9 mix. (S-9 mix: KCL 1.65 Mol/l, $MgCl_2$ 0.4 Mol/l, Glc-6P 1 Mol/l, NADP 0.1 Mol/l, phosphate buffer 0.2 M pH 7.4, S9 and distilled water).

With each chemical tested, three assays per concentration were performed with or without metabolic activation The mutagenic activity was defined as the number of bacterial induced revertants his+ per plate.

Results

Chemical study

Results at 50°C: At 50°C, two isomeric adducts; a minor product (e): 30% and a major product (f): 70% (Table 1) in the form of a pair of enantiomeric optical isomers due to the appearence of an asymetric carbon (*) occurred with sorbic acid. The physical and chemical characteristics in ^{1}H and ^{13}C NMR as well as in GC-MS shown in Table 1 agree with those of Kheddis et al., (1981).

The kinetics of the reactions obtained at 50°C are shown in Fig. 2. In the kinetic study, as the adducts appeared, sorbic acid was seen to disappear. The relationship between the two was not strictly proportional because condensation reactions of sorbic acid itself can take place at high temperatures.

Results at 20°C: At 20°C for ethyl sorbate, we detected, isolated, assayed and characterized two isomeric; a major product (B) 80% and a minor product (b): 20% which occurred undoubtedly in the form of pairs of enantiomers (Table 2). From their physical and chemical characteristics the structures of the products were inferred. They are reported in Table 2.

The kinetics of the various reactions at 20°C are shown in Fig. 3. A rather small quantity of cyclic adduct was formed during the reaction, and does not appear in the kinetic curves.

Toxicological study

Genotoxicity of the chemical reaction products: For each concentration the repair ratio was determined (repair ratio = ratio of the signal at a given level (mean RLU adduct of two wells) to the signal of the solvent alone) and results of genotoxicity on plasmid DNA and genomic DNA are shown in Table 3.

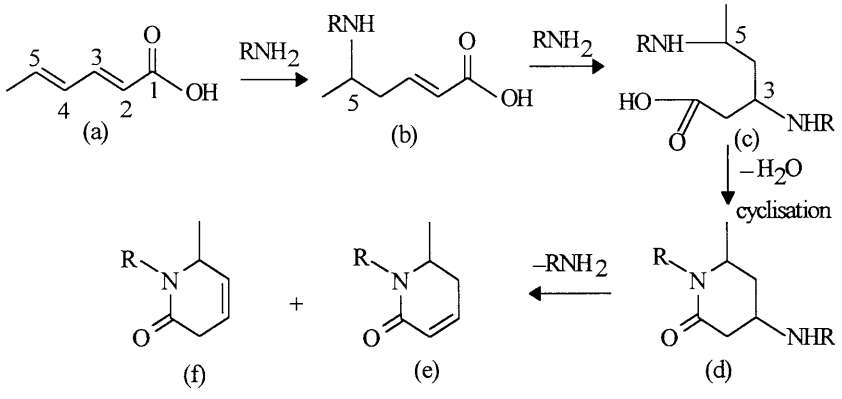
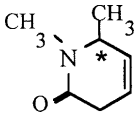
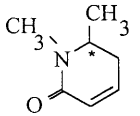


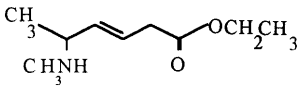
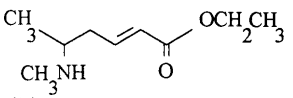
Fig. 1. Nucleophilic addition of amines to sorbic acid resulting in cyclic adducts. **a** Sorbic acid; **b** linear monoadduct; **c** linear diadduct; **d** cyclic diadduct; **e** and **f** dihydropyridones

Table 1. Structures of cyclic adduct formed at 50°C and results of ^{1}H and ^{13}C NMR

Interaction product		^{1}H NMR in $CDCl_3$/TMS (ppm)	^{13}C NMR in $CDCl_3$/TMS (ppm)	GC-MS m/z
	MWt = 125 g/mole	1.28 ($C\underline{H}_3$-CH, d); 2.80 ($C\underline{H}_2$, m); 3.06 ($C\underline{H}_3$-N, s); 3.94 (C<u>H</u>, m); 5.71 (C<u>H</u>=C<u>H</u>, m)	20.79 ($\underline{C}H_3$); 31.75 ($\underline{C}H_2$); 32.30 ($\underline{C}H_3$-N); 56.28 ($\underline{C}H$); 121.27 ($\underline{C}H$=); 127.29 ($\underline{C}H$=); 173.5 ($\underline{C}O$)	125 = $M^{\cdot+}$ (12.7%) 110 = $M^{\cdot+}$ with loss of CH_3. (100%) 82 = ($M^{\cdot+}$ − CH_3) with loss of CO (9.6%) 42 = ($M^{\cdot+}$ − CH_3) with loss of CH_2=C=O (67.2%)
(f) major cyclic	N-methyl-6-methyl-3,6-dihydro-2-pyridone			
	Mwt = 125 g/mole			
(e) minor cyclic	N-methyl-6-methyl-5,6-dihydro-2-pyridone			

s Singlet; *d* doublet; *m* multiplet.

Table 2. Structures of the linear adducts formed at 20°C and results of ^{1}H and ^{13}C NMR and GC-MS

Compound		^{1}H NMR in $CDCl_3$/TMS (ppm)	^{13}C NMR in $CDCl_3$/TMS (ppm)	GC-MS m/z
	MWt = 171 g/mole	1.15 ($C\underline{H}_3$-CH, d); 1.26 ($\underline{C}H_3$-CH_2O, t) 1.9 (N$\underline{H}$, wide s);	14.2 ($C\underline{H}_3$-CH_2O); 21.33 ($\underline{C}H_3$); 33.83 ($\underline{C}H_3$-NH);	171 = $M^{\cdot+}$ (0.1%) 156 = $M^{\cdot+}$ loss of CH_3 (0.3%)
(B) major linear	ethyl 5-(N-methylamino) hex-3-enoate	2.36 ($C\underline{H}_3$-NH, wide s); 3.02 ($C\underline{H}_2$-COO, m); 3.3 (C$\underline{H}$-N, m); 4.14 (-$C\underline{H}_2$-O, q); 5.41 (C$\underline{H}$=, dd); 5.61–5.71 (C$\underline{H}$=, m)	37.8 ($\underline{C}H_2$-COO) 57.43 ($\underline{C}$H-NH); 60.62 ($\underline{C}H_2O$); 122.83 ($\underline{C}$H=); 137.67 (CH=); 173 ($\underline{C}$O)	110 = ($M^{\cdot+}$ − CH_3) with loss of C_2H_5OH (1.7%)
	MWt = 171 g/mole			
(b) minor linear	ethyl 5-(N-methylamino) hex-2-enoate			

s Singlet; *d* doublet; *t* triplet; *q* quadruplet; *m* multiplet.

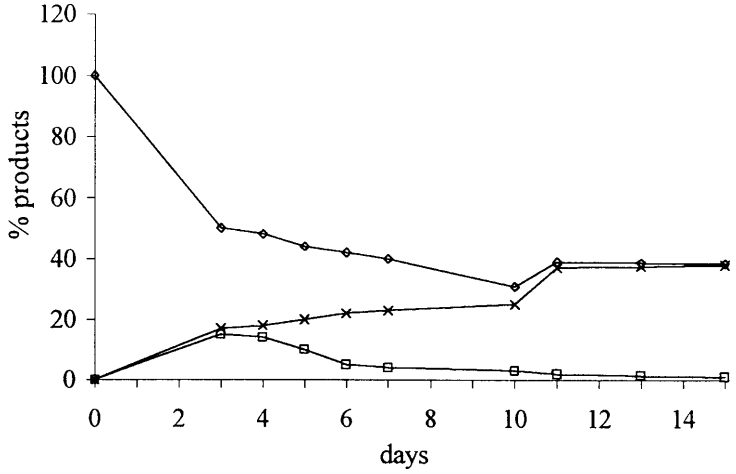


Fig. 2. Kinetics of evolution at 50°C of products present in the sorbic acid/methylamine mixtures. ◇ Sorbic acid, × cyclic adduct (f), □ cyclic adduct (e). Reagents and conditions: methylamine: 0.04 mole; solvent: absolute ethanol: 30 ml, sorbic acid: 0.01 mole, the reaction mixture was mechanically stirred at 50°C for 14 days

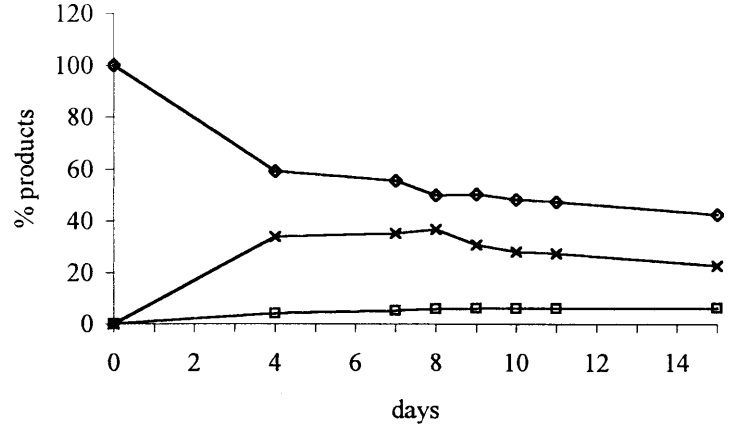


Fig. 3. Kinetics of evolution at 20°C of products present in the ethyl sorbate\methylamine mixtures. ◇ Ethyl sorbate, × linear adduct (B), □ linear adduct (b). Reagents and conditions: methylamine: 0.02 mole, solvent: absolute ethanol: 30 ml, ethyl sorbate: 0.01 mole. The reaction mixture was kept under mechanical stirring at 20°C for 14 days

Mutagenicity of the chemical reaction products: The results of the mutagenesis tests towards *Salmonella typhimurium* strains TA98 and TA100 are reported in Fig. 4 and 5.

Discussion

Chemical study

Study of the mechanism

The formation of this kind of compound B and b is described by Khandelwal and Wedzicha (1990b). The major product results from the attack of a

Table 3. Results of the 3D DNA damage test with plasmid DNA and with genomic DNA from HeLa cells *in vitro*

		Test on plasmid DNA Repair ratio	Test on genomic DNA Repair ratio
Iinteraction products concentration mg/ml	10	0.40 ± 0.02	0.61 ± 0.05
	1	0.79 ± 0.01	0.65 ± 0.09
	0.1	0.81 ± 0.04	0.87 ± 0.10
	10^{-2}	0.91 ± 0.02	1.16 ± 0.15
	10^{-3}	0.96 ± 0.04	0.92 ± 0.09
MMS	10 mM/l	3.64 ± 0.21	8.30 ± 2.9
	2 mM/l	1.82 ± 0.11	0.72 ± 0.23

Repair ratio ratio of the signal at a given level (mean RLU adduct of two wells) to the signal of the solvent alone. If its value is greater than two, the molecule tested *should have* a significant genotoxic effect.

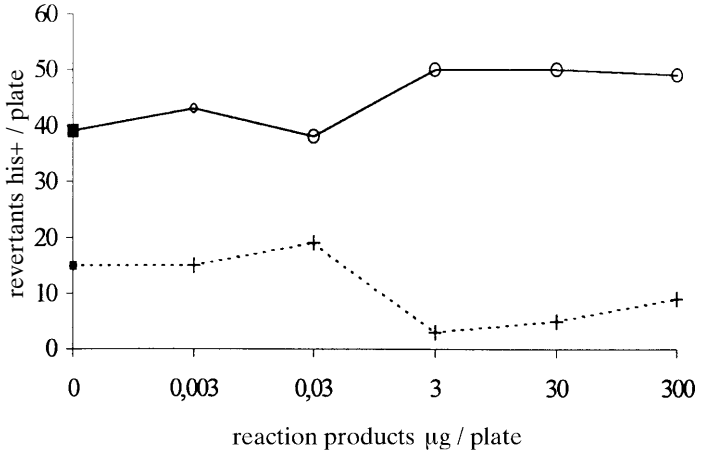


Fig. 4. Mutagenesis in *Salmonella thyphimurium* TA98 of reaction products methylamine – sorbic acid. (○) Mutagenic activity of reaction products in strain TA98 with S9 mix, (+): without S9 mix, (■): blanks (buffer): spontaneous revertants, positive control with S9: 2-aminoantracene (0.1 μg/plate): 236 revertants His+/plate, positive control without S9: 2(2-furyl)-3-(5-nitro-2-furyl) acrylamide (0.5 μg/plate): 500 revertants His+/plate

nucleophile (Nu: methylamine) on the sorbate at position 5 followed by the addition of a proton at position 2. The minor product results from the attack of a nucleophile (Nu) on the linear chain at position 5 followed by the addition of a proton at position 4 (Fig. 6).

According to the mechanism given, the formation of the cyclic structures (e) and (f) is the result of the double addition of amine which led to structure (c). This undergoes cyclisation with the elimination of a water molecule to lead to (d), then the loss of an amine molecule to lead to (e) plus (f). In the present study, the formation of intermediate diadducts (c) and (d) was not proven. They are included to explain the formation of the cyclic products (e) and (f) (Fig. 1), the *trans* geometry of the monoadduct double bond (b) forbids its direct transformation to (e) and (f). So, in our reaction conditions,

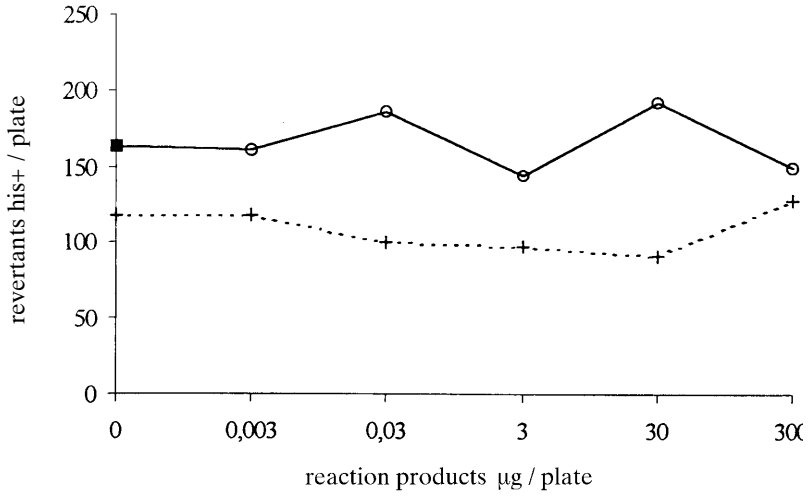


Fig. 5. Mutagenesis in *Salmonella thyphimurium* TA100 of reaction products methylamine – sorbic acid. (○) Mutagenic activity of reaction products in strain TA100 with S9 mix, (+): without S9 mix, (■): blanks (buffer): spontaneous revertants, positive control with S9: 2-aminoantracene (0.001 μg/plate): 612 revertants His+/plate, positive control without S9: 2(2-furyl)-3-(5-nitro-2-furyl) acrylamide (1.0 μg/plate): 780 revertants His+/plate

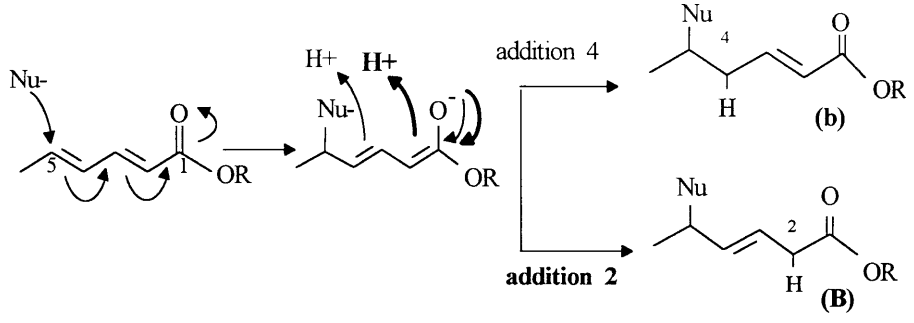


Fig. 6. Nucleophilic addition to a sorbate ester leading to two linear adducts

we think that the diadducts spontaneously give the cyclic unsaturated compounds (e) plus (f) as main products.

Moreover, with such a mechanism, the cyclic products result only from the second addition of an amine to the linear conjugated compound (b). This in fact activates the double bond. This observation enabled us to check the attribution that we made for the double bond position in structures (B) (major) and (b) (minor). On heating (B) and (b) to 50°C with amine only (b) led to pyridones (f) and (e); so (b) is definitely the adduct having the conjugated double bond. The fact that no linear structure was detected when sorbic acid sorbate were used at 20°C instead of ester does not allow us to conclude that no reaction takes place. Indeed, Khandelwal and Wedzicha (1990b) established the instability of sorbic acid-thiol adducts whereas a non-

negligible reactivity exists for ester forms. Furthermore, obtaining cyclic structures at high temperature is strictly due to the formation of adduct (b) and diadducts (c) and (d). Because cyclic structures appear at 50°C, with ethyl sorbate as well as with sorbic acid, the linear adducts, which are reaction intermediates, must also appear. Their instability would provoke either the return to the initial compound at 20°C, or their evolution toward cyclic structures (e) and (f) at higher temperatures.

Toxicological study

Genotoxicity of the chemical reaction products

Whatever the concentrations of the different samples tested, the repair ratios obtained were close to 1 and statistical comparisons were not justified. The samples were not found to be genotoxic with the 3D tests (Table 3); none reached the threshold of significance for genotoxicity i.e. 2.

This absence of direct toxicity does not exclude the possibility of progenotoxicity. It therefore appeared advisable to pursue the study on previously metabolised products with the S9 fraction. A variation of this test consists in using hepatocytes which, due to their capacity to metabolise progenotoxics, will induce damage on their own DNA.

Mutagenicity of the chemical reaction products

The test results obtained by the chemical reaction products were similar than in controls with spontaneous revertants. The chemical reaction products did not appear to be mutagenic with or without the presence of liver S9 fraction towards TA98 (Fig. 4) and TA100 (Fig. 5) *Salmonella thyphimurium* strains. In the presence of S9, the reaction products formed between sorbic acid and amines, gave rise to his+ values close to the threshold values for mutagenicity. However, as the value for spontaneous reversion was never doubled (Chu et al., 1981), none of the compounds tested were mutagenic in this test.

No mutagenic effect was demonstrated for the cyclic products formed by interaction between sorbic acid and methylamine at 50°C, unlike the situation reported for compounds formed between sorbic acid and nitrates (Namiki and Kada, 1975; Namiki et al., 1981) or sorbic acid and sulphates (Lafont and Lafont, 1979). However, we cannot conclude that reaction products studied are totally harmlessness. Indeed, Namiki and Kada (1975) observed that ethylnitrolic acid (addition product of nitrite and sorbic acid) only caused a low mutagenic response with Ames test but this compoud was classified as a strong mutagen in the rec assay test. Likewise, Lafont and Lafont (1979) showed that wine containing SO_2 and sorbic acid coexerts a mutagenic activity in the rec assay test, while Lück (1983) reported no mutagenic effect in the Ames test. It therefore appears preferable to confirm the absence of toxicological risk of the reaction products studied by additional tests such as, the rec assay test, using *Bacillus subtilis* strain (Ozaki et al., 1998) and a test based on *Escherichia coli* strains B/r WP2 trp^- (Kilbey et al., 1984).

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References

Ames BN, McCann J, Yamasaki E (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat Res 31: 347–364

Chu KC, Patel KM, Lin AH, Tarone RE, Linhart MS, Dunkel VC (1981) Evaluating statistical analysis and reproducibility of microbial mutagenicity assays. Mutat Res 85: 119–132

Hayastu H, Chung KC, Kada T, Nakajima T (1975) Generation of mutagenic compound(s) by a reaction between sorbic acid and nitrite. Mutat Res 30: 417–419

Khandelwal GD, Wedzicha B (1990a) Nucleophilic reactions of sorbic acid. Food Addit Contam 75: 685–694

Khandelwal GD, Wedzicha B (1990b) Derivatives of sorbic acid-thiol adducts. Food Chem 37: 159–169

Kheddis B, Bamibah D, Handi M, Perié JJ (1981) Dihydropyridones-2 dérivés de l'acide sorbique: synthèse et analyse conformationnelle. Bull Sci Chim Fr 3–4: 135–140

Kilbey BJ, Legator M, Nichols W, Ramel C (eds) (1984) Handbook of mutagenicity test procedures, 2nd edn. Elsevier, Amsterdam

Kinderlerer JL, Hatton PV (1990) Fungal metabolites of sorbic acid. Food Addit Contam 7: 657–669

Kito Y, Namiki M (1978) A new N-nitropyrrole. 1,4-dinitro-2-methylpyrrole, formed by the reaction of sorbic acid with sodium nitrite. Tetrahedron 343: 505–508

Lafont P, Lafont JJ (1979) Détection d'un produit de réaction de l'anhydride sulfureux sur l'acide sorbique dans le vin, par une méthode microbiologique. Med Nut 15: 195–196

Leikanger S, Bjertness E, Scheie AA (1992) Effect of food preservatives on growth and metabolism of plaque bacteria in vitro and in vivo. Scand J Dent Res 100: 371–376

Lück E (1983) Point de vue sur le dossier analytique et toxicologique de l'acide sorbique. Med Nut 19: 21–26

Maron DM, Ames BN (1983) Revised methods for the Salmonella mutagenicity test. Mutat Res 113: 173–215

Namiki M, Kada T (1975) Formation of ethylnitrolic acid by the reaction of sorbic acid with sodium nitrite. Agric Biol Chem 39: 1335–1336

Namiki M, Udaka S, Osawa T, Tsuji K, Kada T (1981) Formation of mutagens by sorbic acid-nitrite reaction: effect of reaction conditions on biological activities. Mutat Res 73: 21–28

Osawa T, Namiki M (1982) Mutagen formation in the reaction of nitrite with food components analogous to sorbic acid. Agric Biol Chem 46: 2299–2304

Ozaki A, Kitano M, Itoh N, Kuroda K, Furusawa N, Masuda T, Yamaguchi H (1998) Mutagenicity and DNA-damaging activity of decomposed products of food colours under UV irradication. Food Chem Toxicol 36: 811–817

Salles B, Provot C (1998) Methods in molecular biology. In vitro chemiluminescence assay to measue excision repair in cell extracts vol 113, chap 31. D.S. Henderson, Totowa, NJ, p 400

Shamma M, Rosenstock P (1961) The synthesis and properties of some α, β – unsatured valerolactams. J Org Chem 26: 718–725

Verbiscar A, Campbell K (1964) Unsatured six-membered ring lactams. J Org Chem 29: 2472–2474
Wedzicha B, Brook M (1989) Reaction of sorbic acid with nucleophiles: preliminary studies. Food Chem 31: 29–40

Authors' address: (Carine) Ferrand, Laboratoire d'Analyse et de Valeur Biologique des Aliments, Département Universitaire des Sciences d'Agen, Université Bordeaux I, avenue Michel Serres, F-47000 Agen, France,
Fax 33.5.53.48.06.49, E-mail: *dusa@dusagen.u-bordeaux.fr.*

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