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Evidence for the formation of Michael adducts from reactions of (*E*,*E*)-muconaldehyde with glutathione and other thiols

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Abstract

Glutathione induces the rapid isomerization of (*Z*,*Z*)-muconaldehyde to (*E*,*E*)-muconaldehyde via (*E*,*Z*)-muconaldehyde, probably via reversible Michael addition of the thiol to one of the enal moieties of the muconaldehyde. Reactions of (*E*,*E*)-muconaldehyde with glutathione (in the presence and absence of equine glutathione *S*-transferase), phenylmethanethiol, *N*-acetyl-L-cysteine, and *N*-acetyl-L-cysteine methyl ester were investigated using mass spectrometric techniques. In each case, evidence was obtained for the formation of Michael adducts, e.g., reaction between (*E*,*E*)-muconaldehyde and glutathione gave 4-glutathionyl-hex-2-enedial and 3,4-bis-glutathionyl-hexanedial. These experiments suggest that (*Z*,*Z*)-muconaldehyde, a

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putative metabolite of benzene, could lead to the long established urinary metabolite of benzene, (*E*,*E*)-muconic acid, via glutathione-mediated isomerization to (*E*,*E*)-muconaldehyde.

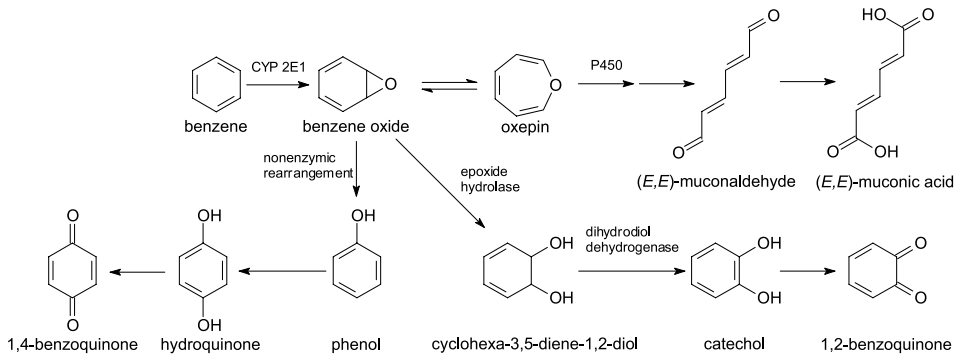
Keywords: (*E*,*E*)-Muconaldehyde; Glutathione; Isomerization; Thiols adducts; Benzene

1. Introduction

The complex metabolism and toxicities of benzene [1,2] are initiated by cytochrome P450 (CYP450)[1] oxidation to benzene oxide–oxepin [3–5], which undergoes further metabolism to an array of intermediates and products (Scheme 1). Attempts to elucidate the mechanisms of benzene toxicities have been focused on the roles of several reactive metabolites including benzene oxide–oxepin [3–5], muconaldehydes (**1**–**3**) [6–9], 1,2- and 1,4-benzoquinones [10], as well as reactive oxygen species [2]. (*E*,*E*)-Muconic acid is a long established ring-opened metabolite of benzene [11–13], the levels of which in the urine of humans and animals exposed to benzene can be used as a biomarker [14,15]. The precursor of (*E*,*E*)-muconic acid is presumed to be (*E*,*E*)-muconaldehyde (**3**), which is derived from its (*Z*,*Z*)-isomer (**1**) via the (*E*,*Z*)-isomer (**2**). The (*Z*,*Z*)-isomer is believed to be formed from benzene oxide–oxepin via a CYP450-dependent oxidation of the oxepin component [16]. In model experiments, it was shown that (*Z*,*Z*)-muconaldehyde slowly isomerizes by an electrocyclic process to (*E*,*Z*)-muconaldehyde, which can undergo either acid- or base-catalyzed conversion to the more stable (*E*,*E*)-muconaldehyde (**3**) [9,17].

(*E*,*E*)-Muconaldehyde (**3**) is comparable to benzene with respect to its toxic effects on bone marrow, and was shown in experiments with bacterial and mammalian cells to be both genotoxic and cytotoxic [6,18,19]. A comparative study on the metabolism of benzene to (*E*,*E*)-muconic acid in DBA/2N and CB57BL/6 mice was consistent with the hypothesis that reactive ring-opened metabolites, such as (*E*,*E*)-muconaldehyde (**3**), play a role in benzene hemotoxicity [20]. The benzene sensitive mouse strain, DBA/2N, excreted significantly more muconic acid than the less sensitive strain, CB57BL/6, after treatment with hemotoxic doses of benzene [20]. It has also been suggested that the mono-reduced and mono-oxidized forms of muconaldehyde, because of their lesser electrophilicity/reactivity, are more likely to clear the liver unconjugated and find their way to target tissues [21], and that reversible thiol conjugation, e.g., with GSH (**4**), is a possible transport mechanism for muconaldehyde [22]. Little evidence for the formation of adducts with thiol nucleophiles has previously been reported and, due to the complex chemistry and reactivity of the muconaldehydes, isolation and identification of products are difficult. The kinetics of the reaction

[1] *Abbreviations used:* GSH, glutathione; GST, glutathione *S*-transferase; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; CYP450, cytochrome P450; PCI, positive chemical ionization; NCI, negative chemical ionization.



Scheme 1. Simplified metabolic pathway for benzene showing major reactive species.

of GSH with (**3**) were studied but adducts were only characterized by UV spectroscopy [22].

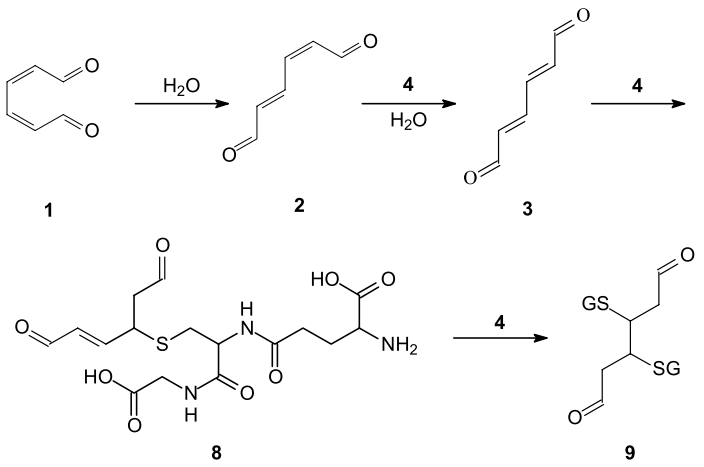
In this paper, we report an investigation of the GSH (**4**)-catalyzed isomerization of (*Z*,*Z*)-muconaldehyde (**1**) to (*E*,*E*)-muconaldehyde (**3**) via (*E*,*Z*)-muconaldehyde (**2**). The reaction of (*E*,*E*)-muconaldehyde with (**4**), phenylmethanethiol (**5**), *N*-acetyl-L-cysteine (**6**), and *N*-acetyl-L-cysteine methyl ester (**7**) were studied using mass spectrometric techniques to detect adducts. Reaction between (**3**) and (**4**) resulted in the formation of 4-glutathionyl-hex-2-enedial (**8**) and 3,4-bis-glutathionyl-hexanedial (**9**) as observed by mass spectrometry.

2. Results and discussion

*2.1. Isomerization of (Z,Z)-muconaldehyde (**1**) in water*

The diastereoselective isomerization of (*Z*,*Z*)-muconaldehyde (**1**) to (*E*,*Z*)-muconaldehyde (**2**) has previously been investigated in the non-aqueous solvents acetonitrile, benzene, and dimethyl sulfoxide [9,17]. A thermally allowed electrocyclic process, via a small equilibrium concentration of 2-formyl-2*H*-pyran, was suggested for the mechanism [9].

To assist in the interpretation of the reactions of muconaldehyde with GSH (**4**), the behavior of (*Z*,*Z*)-muconaldehyde (**1**) in water was also considered. Thus, reaction of (*Z*,*Z*)-muconaldehyde (**1**) in D_2O (pH 6.5) at 25 °C was monitored by ^1H NMR spectroscopy (Scheme 2), by integration of the resonances from aldehyde groups and using 8% (*E*,*E*)-muconaldehyde as internal standard. Within 15 min of preparation of the reaction mixture, 20% of the (*Z*,*Z*)-muconaldehyde (**1**) had undergone isomerization to (*E*,*Z*)-muconaldehyde (**2**) (Fig. 1). The percentage of (*Z*,*Z*)-muconaldehyde (**1**) diminished to 25% within 4 h at 25 °C and to 8% after 10 h. Concurrently, the level of (*E*,*Z*)-muconaldehyde (**2**) increased from 29% at 1 h, to 58% at 4 h, and to 69% after 10 h. Under these conditions, the (*E*,*Z*)-isomer remained stable and did not undergo further isomerization to the (*E*,*E*)-isomer. The half-life



Scheme 2. Isomerization of (*Z*,*Z*)-muconaldehyde (**1**) to (*E*,*E*)-muconaldehyde (**3**) and its subsequent reaction with GSH (**4**) in water solvent.

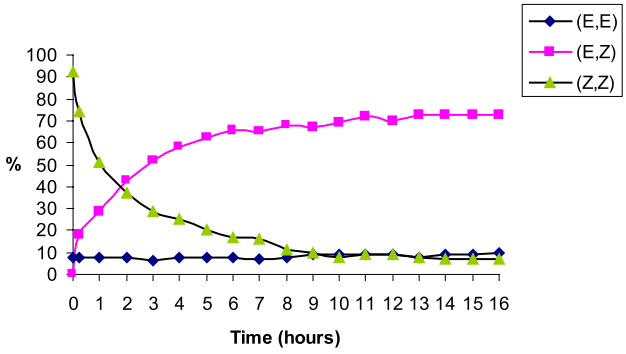


Fig. 1. Isomerization of (*Z*,*Z*)-muconaldehyde (**1**) to (*E*,*Z*)-muconaldehyde (**2**) under aqueous conditions.

for the isomerization was ca. 2 h. Throughout the reaction, there was no change in the concentration of (*E*,*E*)-muconaldehyde (**3**) (8%).

2.2. Isomerization of (Z,Z)-muconaldehyde (1) catalyzed by glutathione (4) in water

The effect of GSH (**4**) on the isomerization of (*Z*,*Z*)-muconaldehyde (**1**) to (*E*,*E*)-muconaldehyde (**3**) via (*E*,*Z*)-muconaldehyde (**2**) at 25 °C under aqueous conditions was investigated. The isomerization is believed to proceed via a Michael addition of the thiol nucleophile to the (*Z*)-enal to afford an intermediate, which eliminates the thiol to give an (*E*)-enal [17]. This process is analogous to the slow triethylamine-catalyzed isomerization of (*Z*,*Z*)-muconaldehyde (**1**) to (*E*,*E*)-muconaldehyde (**3**) via (*E*,*Z*)-muconaldehyde (**2**) [17], but is mechanistically different from the thermally induced route to (*E*,*Z*)-muconaldehyde (**2**) from (*Z*,*Z*)-muconaldehyde (**1**), which occurs via an electrocyclic process [9,23]. ^{1}H NMR was used to monitor the isomerization of (*Z*,*Z*)-

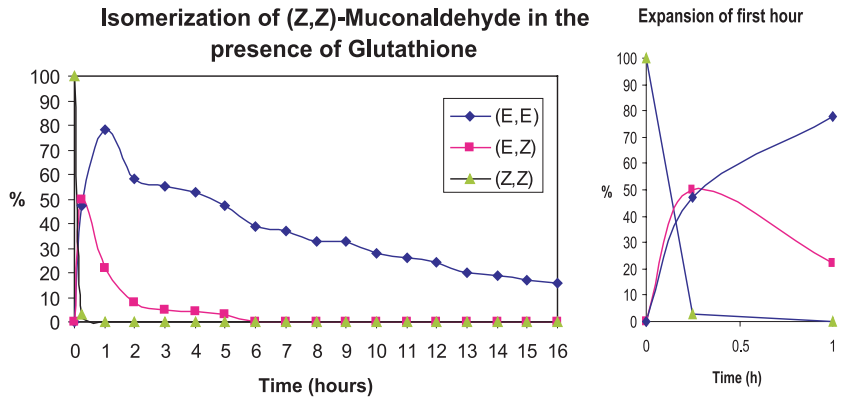


Fig. 2. Isomerization of (*Z*,*Z*)-muconaldehyde (**1**) to (*E*,*E*)-muconaldehyde (**3**) via (*E*,*Z*)-muconaldehyde (**2**) in the presence of GSH (**4**) (expansion showing the data for the first hour of reaction).

muconaldehyde (**1**) over 16 h (Scheme 2). Within 15 min of the addition of (**4**), only 3% of the total muconaldehyde content was accounted for by the (*Z*,*Z*)-isomer, whereas (*E*,*Z*)-muconaldehyde (**2**) accounted for 50% and the (*E*,*E*)-isomer for 47% (Fig. 2). This is in sharp contrast to the isomerization observed in the absence of GSH (**4**) where only 20% of (*Z*,*Z*)-muconaldehyde (**1**) had isomerized after 15 min. It is noteworthy that in the absence of (**4**), conversion of **1** exclusively yields (*E*,*Z*)-muconaldehyde (**2**). However, in the presence of (**4**), within 1 h (*Z*,*Z*)-muconaldehyde (**1**) had completely isomerized to the (*E*,*Z*)- and (*E*,*E*)-isomers (**2** and **3**), or had reacted with (**4**).

The concentration of (*E*,*E*)-muconaldehyde (**3**) was at its maximum (78%) after 1 h, with the isomer mixture containing 22% of the (*E*,*Z*)-isomer (**2**). The (*E*,*E*)-isomer then decreased over time; after 6 h the concentration was half of its maximum. After 6 h only the (*E*,*E*)-isomer was present (39%), while after 16 h it had reduced to 16%. The mechanism of isomerizations presumably involves reversible Michael additions of GSH to an enal moiety of a muconaldehyde. The formation of such adducts was shown by mass spectrometric analysis of solutions of (**3**) with thiols, including GSH (see below).

*2.3. Reaction of (E,E)-muconaldehyde (**3**) with glutathione (**4**)*

Incubation of (*E*,*E*)-muconaldehyde (**3**) with varying molar ratios of GSH (**4**) was carried out at pH 8, in the presence and absence of commercial equine GST. Mass spectrometric analysis (NCI) of these reaction mixtures showed the formation of two adducts (Fig. 3). These were identified as 4-glutathionyl-hex-2-enedial (**8**) [*m*/*z* 416.1 (M − 1)], formed by addition of one equivalent of GSH (**4**) to (*E*,*E*)-muconaldehyde (**3**), and 3,4-bis-glutathionyl-hexanedial (**9**) [*m*/*z* 723.4 (M − 1)] from addition of two equivalents of (**4**) (Scheme 2). Although adducts **8** and **9** are relatively stable they eventually disappear with the formation of unidentified products. The presence of GST had no effect on the formation of adducts **8** and **9** compared with its absence,

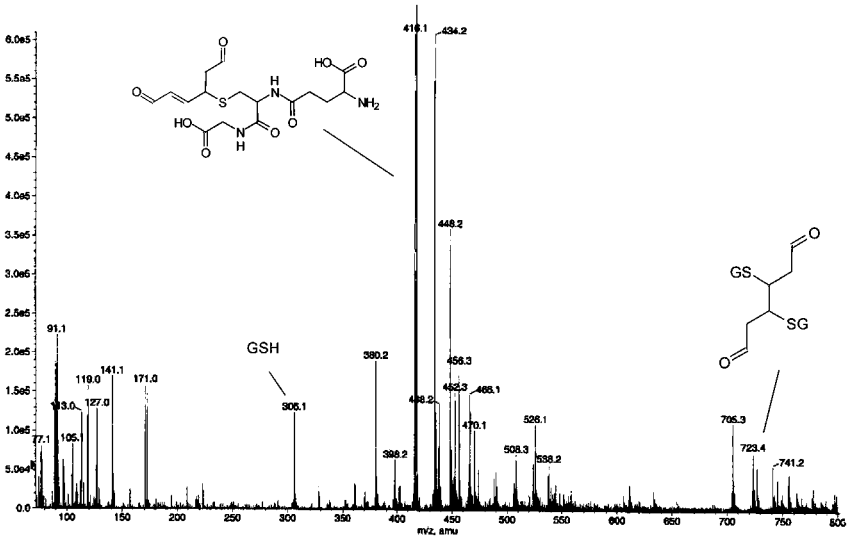


Fig. 3. Mass spectrum of the reaction mixture of (*E*,*E*)-muconaldehyde (**3**) with GSH (**4**) in the presence of GST.

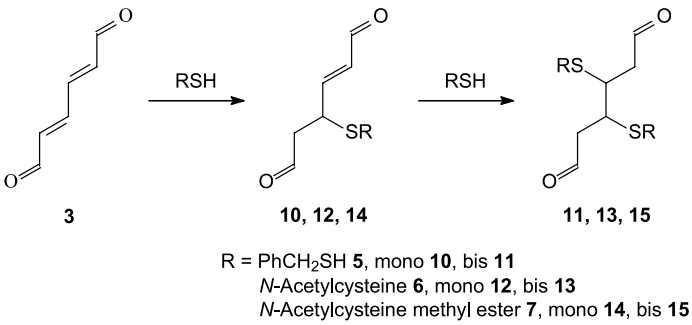
but this could be due to the lack of the appropriate isozyme [24] in the commercial preparations used.

When (**3**) and (**4**) were allowed to reacted in a 1:1 ratio, mono adduct (**8**) was observed with only a trace of the bis adduct (**9**). However, increasing the GSH (**4**) to a 5-fold molar excess caused increases in the levels of (**8**) and (**9**) (8:1, **8**:**9**), but still favoring a greater proportion of the mono adduct (**8**). This is in agreement with a report [22] that a second nucleophilic addition to (*E*,*E*)-muconaldehyde (**3**) is not favored. The reactions of conjugated aldehydes with thiols generally proceed by a 1,4-addition [25]. Although a bis adduct can be formed due to the multi-functional nature of (*E*,*E*)-muconaldehyde, it is expected that the first addition would decrease the reactivity of the remaining enal [18]. Steric hindrance from the bound glutathionyl group would further decrease the likelihood of a second nucleophilic addition.

When a 5-fold excess of (*E*,*E*)-muconaldehyde (**3**) was allowed to react with GSH (**4**) in the presence of GST, only the mono adduct (**8**) (*m*/*z* 416.1, M − 1) was observed. Mass fragmentation of the peak at *m*/*z* 416.1 (M − 1) produced daughter ions at *m*/*z* 306.1 due to released GSH (**4**) and at *m*/*z* 271.9 from loss of the glutamic acid residue from GSH (**4**). Fragmentation analysis of (**9**) showed *m*/*z* 723.4 (M − 1), which appears to be more stable than the *m*/*z* 416.1 peak from mono adduct (**8**). There were weak signals for the daughter ion at *m*/*z* 416.0 (loss of one molecule of GSH) and at *m*/*z* 306.1 (GSH).

*2.4. Reaction of (E,E)-muconaldehyde (**3**) with phenylmethanethiol (**5**)*

Reaction of (*E*,*E*)-muconaldehyde (**3**) with the model thiol phenylmethanethiol (**5**) appeared to result in formation of a mono adduct from a Michael-type addition to



Scheme 3. Reaction between (*E*,*E*)-muconaldehyde (**3**) and thiols (**5**), (**6**), and (**7**).

one of the enal groups (Scheme 3). Analysis of the reaction mixture by GC-MS showed a signal corresponding to 4-benzylsulfanyl-hex-2-enedial (**10**) (t_R 24.6 min, *m*/*z* 234). PCI GC/MS showed an ion at *m*/*z* 235 corresponding to MH^+. Treatment of adduct (**10**) with sodium borohydride resulted in the formation of 4-benzylsulfanyl-hex-2-ene-1,6-diol (*m*/*z* 238) by reduction of the two aldehyde functions. PCI analysis showed MH^+ at *m*/*z* 239. However, no bis adduction was observed when monitoring by GC-MS and may be due to instability of the bis adduct or the reversible conjugation of (**4**) at the elevated temperatures of the GC column.

Mass spectrometric analysis in the negative ion mode showed a signal relating to (**10**) (*m*/*z* 233.1, M − 1) with daughter ions due to benzyl (*m*/*z* 91.0) and $C_7H_7S^-$ (*m*/*z* 122.9) by MS/MS analysis. The bis adduct, 3,4-bis-benzylsulfanyl-hexanedial (**11**) (*m*/*z* 356.2, M − 1) also fragmented to give a daughter ion at *m*/*z* 91.0 corresponding to benzyl. The adducts were observed in the ratio 7:1, **10**:**11**.

*2.5. Reaction of (E,E)-muconaldehyde (**3**) with N-acetyl-L-cysteine (**6**) and N-acetyl-L-cysteine methyl ester (**7**)*

Reactions of (*E*,*E*)-muconaldehyde (**3**) with *N*-acetyl-L-cysteine or *N*-acetyl-L-cysteine methyl ester were studied because the expected adducts might be useful reference standards for the quantitative measurement of cysteine adduct levels in blood using methods similar to those developed for benzene oxide [5,10]. When (*E*,*E*)-muconaldehyde (**3**) was incubated with either *N*-acetyl-L-cysteine (**6**) or *N*-acetyl-L-cysteine methyl ester (**7**) at a range of pH (7, 8.5, and 10) both mono (**12**) and bis (**13**) adducts were observed in the reactions (Scheme 3) in a ratio of 4:1, **12**:**13**.

For *N*-acetyl-L-cysteine (**6**), MS/MS analysis in the negative ion mode showed formation of an adduct, 2-acetylamino-3-[4-oxo-1-(2-oxo-ethyl)-but-2-enylsulfanyl]-propionic acid (**12**) with *m*/*z* 272.1 (M − 1) corresponding to addition of one molecule of *N*-acetyl-L-cysteine (**6**) to (*E*,*E*)-muconaldehyde (**3**) (Scheme 3). The fragmentation of the ion at *m*/*z* 272.1 gave daughter ion signals at *m*/*z* 124.6, corresponding to muconaldehyde with a sulfur attached, whilst the signal at *m*/*z* 108.9 corresponded to muconaldehyde. An apparent hydrate of the adduct showing an ion at *m*/*z* 290.0 (M − 1) was also observed. The bis adduct, 2-acetylamino-3-[(2-acetylamino-2-car-

boxy-ethylsulfanyl)-4-oxo-1-(2-oxoethyl)-butylsulfanyl]-propionic acid (**13**) was observed with an ion at *m*/*z* 435.1 (M − 1) and its hydrate at *m*/*z* 453.2 (M − 1). Product fragmentation of the ion at *m*/*z* 435.1 (**13**) showed a signal due to two sulfurs attached to muconaldehyde (*m*/*z* 143.2, M − 1).

For *N*-acetylcysteine methyl ester (**7**), using MS/MS analysis in the positive ion mode, two adducts were again seen, with the mono adduct, 2-acetylamino-3-[4-oxo-1-(2-oxoethyl)-but-2-enylsulfanyl]-propionic acid methyl ester, (**14**) observed at *m*/*z* 288.3 (M + 1), while the bis adduct, 2-acetylamino-3-[2-(2-acetylamino-2-methoxycarbonyl-ethylsulfanyl)-4-oxo-1-(2-oxoethyl)-butylsulfanyl]-propionic acid methyl ester, (**15**) was observed at *m*/*z* 465.2 (M + 1). The fragmentation of ion *m*/*z* 288.3 (M + 1) (**14**) showed loss of the methyl ester (*m*/*z* 229.0), while the signal at *m*/*z* 144.0 was from sulfur attached to muconaldehyde. There was also a signal due to muconaldehyde at *m*/*z* 111.1. The fragmentation of ion *m*/*z* 465.2 (**15**) (M + 1) showed signals due to muconaldehyde with two sulfurs attached (*m*/*z* 176.0) and muconaldehyde with two SCH_2C^+ attached (*m*/*z* 205.1). For adduct formation of (*E*,*E*)-muconaldehyde (**3**) with *N*-acetylcysteine methyl ester (**7**), the products were in the ratio 2:1, **14**:**15**.

3. Conclusions

These studies have shown that if (*Z*,*Z*)-muconaldehyde is formed as a metabolite of benzene it can undergo an intracellular GSH-catalyzed isomerization to the (*E*,*E*)-isomer. The formation of mono- and bis-adducts from (**3**) and thiols including GSH (**4**) has been demonstrated by the studies described above. The reversibility of the addition of GSH to muconaldehydes indicates that GSH could act as a physiological transporter of (*E*,*E*)-muconaldehyde to target sites. Future work will attempt to isolate purified adducts and to explore the effect of individual GST isozymes [24] on adduct formation.

4. Experimental

4.1. Caution

Benzene oxide–oxepin and muconaldehydes must be handled in a well-ventilated hood by an operator wearing appropriate protective clothing.

4.2. General

All chemicals were obtained from Sigma–Aldrich (Gillingham, Dorset, UK or St. Louis, MO, USA) or Lancaster (Newgate, Lancashire, UK). Chemicals and solvents were either AnalaR grade, which were used directly, or laboratory reagent grade purified further where appropriate. The ^{1}H NMR spectra were run on a Bruker or Jeo1 spectrometer at either 200 or 500 MHz using residual proton signals from the

deuterated solvents as references: acetonitrile (1.95 ppm), chloroform (7.25 ppm). All coupling constants were measured in hertz.

4.3. Mass spectrometry

MS/MS analyses were conducted using a Sciex API3000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA). Analytes were injected via infusion mode at a rate of 10 μL/min. The mass spectrometer was operated in Q1 scan mode to determine parent ions and then in multiple reaction monitoring mode to assess resulting daughter ions. The system was operated with negative and positive turbo ion spray atmospheric pressure ionization.

GC-MS analyses in electron ionization mode were conducted using a HP 5980 series gas chromatograph coupled to a HP 5989A MS engine. The mass spectra were recorded by scanning the mass spectrometer from *m*/*z* 50 to 550. The injector, transfer-line, and ion source temperatures were 250, 280, and 150 °C, respectively, and the electron energy was 70 eV. A DB-5 fused silica capillary column (60 m, 0.25 mm i.d., and 0.25 μm film thickness) was used with helium as the carrier gas at a flow rate of 1.5 mL/min. Injections of 2 μL were made in splitless mode, with the GC oven held at 75 °C for 2 min and then ramped at 10 °C/min to 250 °C where it was held for 30 min.

4.4. Synthesis of benzene oxide and (*E,E*)-muconaldehyde

Benzene oxide–oxepin was prepared essentially as described in the literature [26,27], with modifications as developed by our group [28]. Thus, one double bond of 1,4-cyclohexadiene was brominated and the other was epoxidized. Treatment of the dibromoepoxide with DBU gave benzene oxide–oxepin. (*Z*,*Z*)-Muconaldehyde (**1**) and (*E*,*Z*)-muconaldehyde (**2**) were synthesized according to Golding et al. [17]. Periodate cleavage of *cis*-cyclohexa-3,5-diene-1,2-diol in ice-cold water afforded (*Z*,*Z*)-muconaldehyde (**1**), which was isomerized to (*E*,*Z*)-muconaldehyde (**2**) by heating in acetonitrile at 55 °C in the dark. (*Z*,*Z*)-Muconaldehyde (**1**) was also synthesized from benzene oxide via reaction with dimethyldioxirane [16] (*E*,*E*)-Muconaldehyde (**3**) was prepared as described by Davis and Whitman [29]. Essentially, benzene oxide–oxepin (1 mol equiv.) in DMSO and water (1.1 mol equiv.) was oxidized with *N*-bromosuccinimide (1.1 mol equiv.) and extracted into ether.

4.5. Reactions of (*E,E*)-muconaldehyde

4.5.1. Isomerization of (*Z,Z*)-muconaldehyde (*1*) with water

(*Z*,*Z*)-Muconaldehyde containing 8% (*E*,*E*)-muconaldehyde (5 mg, 0.05 mmol) in D_2O (0.7 mL, pH 6.5) was placed in a NMR tube. The reaction mixture was kept at 25 °C in the absence of light and was monitored hourly by ^1H NMR for 16 h.

4.5.2. Isomerization of (*Z,Z*)-muconaldehyde (*1*) with glutathione (*4*) in water

To (*Z*,*Z*)-muconaldehyde (5 mg, 0.05 mmol) in D_2O (0.7 mL) in a NMR tube was added GSH (13.8 mg, 0.05 mmol). The reaction mixture was kept at 25 °C in the

absence of light and was monitored immediately, and then hourly by ^{1}H NMR for 16 h.

*4.5.3. Reaction of (E,E)-muconaldehyde (**3**) with glutathione (**4**) and glutathione S-transferase*

(*E*,*E*)-Muconaldehyde (3.6 mg, 0.03 mmol) was incubated with varying molar ratios of GSH (molar ratio EE:GSH 1:1, 1:2, or 5:1) in the presence and absence of equine glutathione *S*-transferase (1 mg) at 37 °C for 30 min in ammonium acetate buffer (5 mL, 0.1 M, pH 8). The resulting solution was analyzed by mass spectrometry.

*4.5.4. Reaction of (E,E)-muconaldehyde (**3**) with phenylmethanethiol (**5**)*

To phenylmethanethiol (2 mol equiv.) in acetonitrile (3 mL) was added triethylamine (2 mol equiv.) and the solution was stirred for 10 min. (*E*,*E*)-Muconaldehyde (100 mg, 1 mol equiv.) was added and the solution was stirred for 2 h at room temperature. The reaction mixture was diluted into two aliquots. One aliquot was reduced by adding sodium borohydride (0.5 equivalents). The other was not reduced. Partial purification of each aliquot was achieved by preparative TLC. The fractions were analyzed by GC-MS.

*4.5.5. Reaction of (E,E)-muconaldehyde (**3**) with N-acetyl-L-cysteine (**6**) and N-acetyl-L-cysteine methyl ester (**7**)*

(*E*,*E*)-Muconaldehyde (5 mg, 0.05 mmol) was incubated with *N*-acetyl-L-cysteine (7.4 mg, 0.05 mmol) or *N*-acetyl-L-cysteine methyl ester (8.8 mg, 0.05 mmol) at 37 °C for 30 min in ammonium acetate buffer (5 mL, 0.1 M, various pH) and the solutions were analyzed by mass spectrometry.

Acknowledgments

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