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Review

Structure elucidation of phase II metabolites by tandem mass spectrometry: an overview

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Abstract

The present paper provides a summary of the collision-induced dissociation of protonated and deprotonated phase II metabolites of drugs and pesticides. This overview is based on published literature and unpublished data from the authors. In particular, glutathione conjugates and their biotransformation products are discussed in detail. In addition, the fragmentation of the major classes of conjugates, i.e. glucuronides, glucosides, malonylglucosides, sulfates, acetates, methyl and glycine conjugates, is reported. Collision-induced dissociation, as studied by tandem mass spectrometry, allows the rapid identification of the type of conjugate, whereas the exact conjugation site can in general be determined only by additional NMR experiments.

Keywords: Phase II metabolites; Tandem mass spectrometry; Collision-induced dissociation

Contents

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1. Introduction

The uptake of almost all organic compounds (both natural and xenobiotic) by organisms (animals, plants, microorganisms) is followed by biotransformation reactions (metabolism) catalyzed by a large variety of more or less substrate-specific enzymes. Among the xenobiotic compounds that undergo biotransformation, drugs and pesticides are of particular importance as the biotransformation does not always lead to inactivation (detoxification) of the agent but in some instances, may lead to more active (bioactivation) or even more toxic compounds (biotoxification). Hence, the registration authorities have declared it mandatory that the major metabolites found during drug and pesticide development be identified.

Biotransformation of drugs and pesticides proceeds in (at least) two distinct steps. During the first step (phase I), the xenobiotic compound is functionalized by oxidation, hydrolysis or (less frequently) reduction, leading to the introduction of, e.g. hydroxyl, amino, carboxyl or thiol groups into the molecule (primary metabolites). In a second step (phase II), these primary metabolites undergo conjugation reactions with endogenous agents to form secondary metabolites [1]. In many instances, the phase I biotransformation is a necessary prerequisite for the subsequent conjugation. However, if the above-mentioned functional groups are already present in the parent drug or pesticide they may undergo direct conjugation. Phase II biotransformation does not only (mostly) lead to an inactivation of the original agent and its primary metabolites, but also to increased hydrophilicity and thus enhanced excretion (except acetylation and methylation). Also, conjugation increases the molecular weight and thus makes the xenobiotic compounds more amenable to biliary excretion, particularly as glucuronides and glutathione (GSH) conjugates. The enzymes involved in these conjugation reactions are termed transferases. In contrast to phase I biotransformations, which are exothermic, conjugation reactions as observed in phase II are endothermic processes. Thus, one of the two conjugating partners, either the conjugating agent or (less frequently) the xenobiotic compound, must be present in an activated state, for instance, the conjugating partner bearing an energy-rich moiety such as a nucleotide.

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the most important methods for structure elucidation of drug and pesticide metabolites. While in many instances a full structure elucidation of unknown compounds is only possible by one- or two-dimensional NMR techniques, the limited sensitivity of NMR or HPLC–NMR in general allows for structure elucidation of the major metabolites, whereas metabolites present at low concentrations can only be identified by mass spectrometry. In spite of the limited structural information obtained by mass spectrometry, this technique is used successfully in the structure elucidation of metabolites, since additional information such as the structure of the parent compound and its general biotransformation pathways is usually available.

Originally, mainly gas chromatography–mass spectrometry (GC–MS) was used in metabolism studies. However, the very polar phase II metabolites are not amenable to GC–MS and have to be derivatized prior to analysis. With the advent of new, soft ionization methods such as fast atom bombardment (FAB) [3], thermospray ionization (TSP) [4], atmospheric pressure chemical ionization (APCI) [5] and electrospray ionization (ESI) [6], a direct HPLC–MS analysis of phase II metabolites became possible; the latter two ionization methods, in particular, are used routinely today. However, the mass spectra obtained with these newer ionization techniques are devoid of fragments (and their structure-specific information). Collisional activation (CA) in conjunction with tandem mass spectrometry (MS/MS) [7] can be used to induce and analyze the dissociation of the even-electron quasi-molecular ions preferentially generated by these newer ionization methods. Today, tandem mass spectrometry is used successfully and on a routine basis for structure elucidation of metabolites in drug and pesticide development where mainly triple–quadrupole instruments [8] and ion traps [9], but also hybrid instruments such as combinations of quadrupole and (linear) ion trap [10], quadrupole and time-of-flight (Q-TOF)

[11] or (linear) ion trap and Fourier transform ion-cyclotron resonance (FT-ICR) [12,13] mass analyzers are used. The latter two techniques provide high-resolution data of the (quasi-) molecular and fragment ions (i.e. data on the elemental composition) and are, hence, particularly powerful for structure elucidation of unknown metabolites. Ion traps which allow multiple tandem mass spectrometric experiments (MS^n) are well suited for structure elucidation of conjugates as they permit the differentiation between the fragments of the intact conjugate (recording the MS^2 spectra) and of the non-conjugated compound (the phase I metabolite) by investigation of the collision-induced dissociation in MS^3, MS^4, ... mode, which is important for the structure elucidation of the latter [2]. Unfortunately, ion traps suffer from poor transmission of small fragment ions.

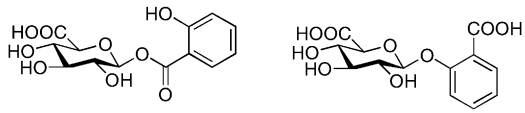
Although tandem mass spectrometry is routinely used in drug and pesticide metabolism studies, only a limited number of detailed tandem mass spectrometric studies on phase II metabolites (but no overview) has been published. Up to now, the reports have concentrated mainly on the well-known fragmentation of glucuronides and sulfates. It is very likely that numerous data and a detailed knowledge on the fragmentation of phase II metabolites exist with drug- and pesticide-producing companies. This knowledge is, however, in general not published. Hence, we here present an overview of the collision-induced dissociation of protonated and deprotonated molecules of the major types of conjugates formed during phase II of the biotransformation of drugs and pesticides based on the one hand, on published literature; and on the other hand, on unpublished data from the Agricultural Center of BASF company and the Fraunhofer ITEM. Some of these data are presented here. In addition further, non-published data were used to corroborate the conclusions derived from the literature.

2. Major conjugation reactions

2.1. Conjugation with glucuronic acid (GlcA)

Conjugation reactions with glucuronic acid occur in animals with glucuronosyltransferase after activation of the acid to uridine-5′-diphosphate glucuronic acid (UDP-5′-glucuronic acid). The glucuronic acid is bound to the corresponding molecule by a glycosidic linkage. Glucuronidation reactions are observed with the following functional groups of the phase I metabolite or the parent compound:

- hydroxyl groups (→ *O*-glucuronides);
- carboxyl groups (→ *O*-glucuronides);
- NHOH-groups (→ *O*-glucuronides);
- amino groups (→ *N*-glucuronides);
- tertiary amines (→ *N*-glucuronides);
- thiol groups (→ *S*-glucuronides);
- 1,3-dicarbonyl compounds (→ C-glucuronides).



(1) (2)

Scheme 1. Glucuronidation of salicylic acid.

Furthermore, a differentiation is made between ester glucuronides and ether glucuronides as shown in Scheme 1 for salicylic acid. C-Glucuronides (formed by substitution of an acidic hydrogen bound to carbon), and in particular, quaternary *N*-glucuronides are observed only rarely [2].

If more than one of the above-mentioned functional groups are present in a phase I metabolite, mass spectrometry in general does not allow to identify the glucuronidation site. This holds true in particular if two of these functional groups are bound to the same aromatic ring [2]. In this case, additional one- and two-dimensional NMR experiments have to be carried out to locate the conjugation site [2].

2.1.1. Quasi-molecular ions

Conjugation leads to a mass shift of the quasi-molecular ion as summarized in Table 1 for the various conjugates discussed in this publication. The table lists the expected mass shift of the $[M+H]^+$ and $[M-H]^-$ ions for various types of conjugates as compared to the non-conjugated metabolite for conjugation by (formally) hydrogen and chlorine substitution, respectively. Moreover, the table indicates characteristic neutral losses from the $[M+H]^+$ and $[M-H]^-$ ions of the conjugate.

Depending on the nature of the metabolite, glucuronides are detected in positive and/or negative-ion mode. Intact glucuronides are best observed under ESI conditions, although in some instances APCI or atmospheric pressure photo ionization (APPI) can also be employed [14]. The masses of $[M+H]^+$ and $[M-H]^-$ ions are 176 Da higher than those of the non-conjugated metabolites.

2.1.2. Collision-induced fragments

In positive-ion mode, an abundant fragment $[M+H-176]^+$ is observed in most instances [2,14–20,38,39,44–47].

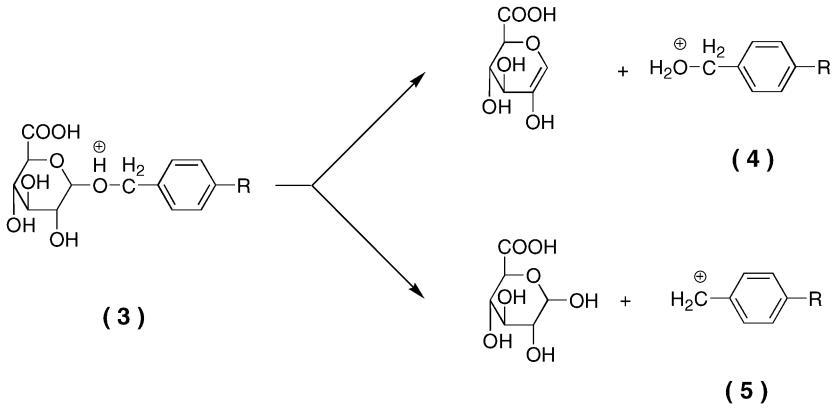
Acyl- or benzylglucuronides, however, undergo a loss of 194 Da (glucuronic acid, GlcA) from the quasi-molecular ions, either in addition to the loss of 176 Da (anhydroglucuronic acid) or even exclusively (see also Scheme 2) [20]. As an example, the MS^2 spectrum of the glucuronide of the fungicide dimoxystrobin is shown in Fig. 1 (triple quadrupole, ESI). Loss of glucuronic acid (194 Da) from the protonated molecule at *m/z* 519 leads to the abundant fragment at *m/z* 325. Loss of 176 Da is not observed.

Other fragments from the intact glucuronide are often missing or of low abundance (except for loss of one or several molecules of water). This allows for the additional study of the phase I metabolite by recording the collision-induced fragments of the $[M+H-176]^+$ ion in MS^3 mode [2].

Table 1
$[M+H]^+$ and $[M-H]^-$ ions and characteristic neutral losses of important types of conjugates

Conjugation reaction/ conjugation with	Substituent	Mass shift (Da) after		Characteristic neutral loss	Mass of neutral lost from $[M+H]^+$	Mass of neutral lost from $[M-H]^-$
		H-sub.	Cl-sub.			
Methylation	CH_3	14		Methyl radical	15	
Acetylation	$COCH_3$	42		Ketene	42	
Glycine	Glycyl	57		Glycine	75	
				$CO+H_2O$	46	
Sulfatation	SO_3H	80	46	SO_2	64	64
				SO_3	80	80
	$(O)SO_3H$	80(96)		SO_2	64	
				SO_3	80	80
Cysteine	Cysteinyl	119	85	Cysteine	121	121
				Alanine[a]	89	
				Formic acid	46	
				NH_3	17	
N-Acetylcysteine	*N*-Acetylcysteinyl	161	127	NAcCys	163	163
				See footnote[b]	129	129
				Acetamide	59	
				Ketene	42	
Glucose (Glc)	Glucosidyl	162		AnhydroGlc	162	
				Glucose	180[c]	
Cys–Gly	Gly-cysteinyl	176	142	CysGly	178	176
				AlaGly[a]	146	144
				Glycine	75	
				NH_3	17	
Glucuronic acid (GlcA)	Glucuronidyl	176		AnhydroGlcA	176	176
				GlcA	194[c]	
GlcNAc	*N*-Acetylglucosaminyl	203		AnhydroGlcNAc	203	203
				GlcNAc	221[c]	
γ-Glu-Cys	γ-Glu-cysteinyl	248	214	See footnote[d]	216	216
				Glutamine	146	
				Anhydroglutamic ac	129	129
Malonyl-Glc	Malonylglucosidyl	248		AnhydromalonylGlc	248	
				CO_2	44	
				MalonylGlc	266	
Glutathione (GSH)	Glutathionyl	305	271	GSH	307	306
				γ-GluAlaGly[a]	275	
				γ-GluAlaGly-2H[a]	273	273
				Glutamine	146	
				Anhydroglutamic ac	129	
				Glycine	75	
GlcGlc		324				
Acetyl-GlcGlc		366				
MalonylGlcGlc		410			410	

[a] Cleavage of the S—CH_2 bond in cysteine leads to alanine.
[b] *N*-Acetyl-2-iminopropionic acid.
[c] Formed with conjugates with benzylic or acylic bond.
[d] *N*-(γ-Glutamyl-iminopropionic acid).



Scheme 2. Collosion-induced fragmentation of benzyglucuronides.

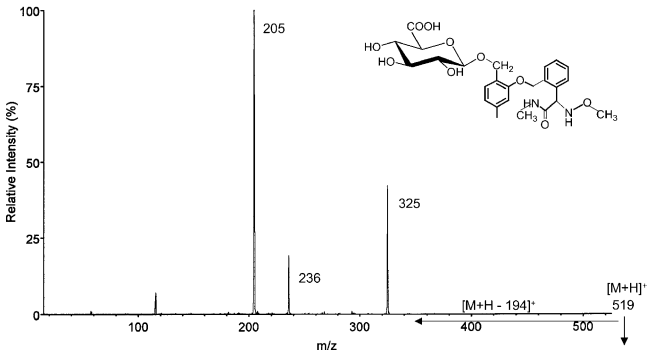


Fig. 1. MS/MS of protonated dimoxystrobin glucuronide, $[M+H]^+ = 519$ (triple quadrupole, electrospray ionization).

In some instances, additional abundant fragments are found which originate directly from the intact glucuronide, e.g. with the drug retigabine [18]. This may complicate the structure elucidation of the phase I metabolite if only MS^2 spectra are available, as with triple–quadrupole instruments under normal operation conditions. However, pseudo MS^3 spectra may also be generated by triple–quadrupole instruments applying in-source collision-induced dissociation, CID (additional fragmentation in the interface region by increasing the cone voltage).

In negative-ion mode an abundant $[M-H-176]^-$ and a usually less abundant ion at *m/z* 175 is generally found. In addition, secondary fragment ions at *m/z* 113 (loss of CO_2 and water from *m/z* 175), and a less intense ion at *m/z* 85 (extrusion of CO from *m/z* 113) appear (Scheme 3). If a dihydroxylated phase I metabolite has been conjugated with two molecules of glucuronic acid (e.g. as observed with benzo[*a*]pyrene [17]), both GlcA moieties may successively be eliminated under CID conditions, i.e. in negative-ion mode both the $[M-H-176]^-$ and $[M-H-2\times176]^-$ ions, plus the ion at *m/z* 175 are observed.

2.2. Glucosidation

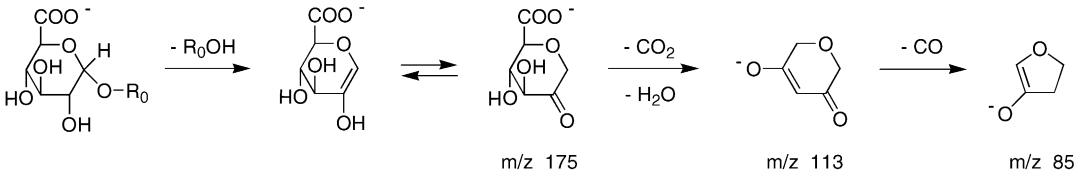
Conjugation of metabolites with glucose (Glc) is frequently observed during the biotransformation of xenobiotica in plants [20]. This conjugation resembles that with glucuronic acid. Conjugation with two (GlcGlc) or more glucose units glycosidically bound to one another may also occur.

2.2.1. Quasi-molecular ions

If conjugation with one glucose unit occurs, a quasi-molecular ion 162 Da higher in *m/z* value than that of the phase I metabolite is observed, while conjugation with two glucose units leads to an increase in the quasi-molecular ion mass by 324 Da (Table 1).

2.2.2. Collision-induced fragments

Similar to glucuronides, an abundant fragment ion $[M+H-162]^+$ formed by loss of anhydroglucose is found for monoglucosidated metabolites, a loss of 324 Da for doubly glucosidated compounds (Table 1) [20]. In contrast to the



Scheme 3. Secondary fragmentation of the glycuronyl moiety in negative-ion MS/MS spectra of glucuronide conjugates.

Fig. 2. MS/MS of protonated fenpropimorph malonyl glucoside, $[M+H]^+ = 568$ (Q-TOF, electrospray ionization).

glucuronides discussed above, no typical fragment ions of the glucose moiety are observed.

2.3. Malonylglucosidation

In biotransformation reactions, e.g. of pesticides in plants conjugation with malonylglucosides is frequently observed [20,21]. The structure of this conjugate for the fungicide fenpropimorph is shown in Fig. 2.

The conjugation leads to a protonated molecule whose *m/z* value is 248 Da higher than that of the phase I metabolite (Table 1). The positive-ion mode MS/MS spectrum of the protonated conjugate (MalonylGlc-fenpropimorph) is shown in Fig. 2 (Q-TOF, ESI). Upon collisional activation, loss of anhydromalonylglucose (248 Da) and (less abundant) loss of malonylglucose (266 Da), leading to *m/z* 320 and 302, respectively, is observed, revealing the endogenous agents involved in this conjugation. In addition, loss of 44 Da (CO_2) is found [20].

Further, conjugation of fenpropimorph with one or two glucose units and malonic acid may occur leading, e.g. to MalonylGlcGlc. In the latter instance, collison-induced dissociation of the protonated conjugate proceeds via loss of anhydromalonylGlcGlc (410 Da) or CO_2 (44 Da).

2.4. Conjugation with N-acetylglucosamine (GlcNAc)

Conjugation with *N*-acetylglucosamine occurs in the cell membranes [20,41,48]. Upon collisional activation, the quasi-molecular ion loses 203 Da (221 Da if the conjugation has occurred in benzylic position) [20].

2.5. Sulfatation

Hydroxyl groups (in particular phenolic groups) are readily conjugated with sulfuric acid (activated as 3′-phosphoadenosine-5′-phosphosulfate (PAPS)) in the presence of sulfotransferases, as shown in Scheme 4 for phenol [14,16,17,19,22,23,38–40]. Less frequently, amino groups (in particular aromatic amines) are conjugated with sulfuric acid.

2.5.1. Quasi-molecular ions

Sulfate conjugates can only be ionized intact using the ESI method [14]. ESI in negative-ion mode is preferred, although for some conjugated metabolites positive-ion ESI spectra were also reported (e.g. in [14,19,23]). Introduction of a sulfate group increases the *m/z* value of the quasi-molecular ion by 80 (Table 1). The ^{34}S isotope can be used to confirm the presence of a sulfate group [24].

2.5.2. Collision-induced fragments

The negative-ion MS/MS spectra of the $[M-H]^-$ quasi-molecular ion of sulfate esters, i.e. $R–O–SO_3^-$, are dominated by the loss of SO_3 (80 Da), leading to the ion $[M-H–SO_3]^-$ and a usually less abundant $[M-H–H_2SO_4]^-$ ion (loss of 98 Da) [17,20,22]. Alternatively, the charge may reside on the sulfate group, leading to the abundant radical ion at *m/z* 80, $SO_3^{-\bullet}$, and a less abundant ion at *m/z* 97, HSO_4^- [17,20,22]. With sulfates other than esters, e.g. $R–NH–SO_3^-$, only the ion pair $[M-H–SO_3]^-$ and $SO_3^{-\bullet}$ is found, which allows to distinguish between both types of conjugates [23].

Similarly, under positive-ion conditions, the ion $[M+H–SO_3]^+$ (loss of 80 Da) is formed. In some reports, other fragment ions were observed at very low abundance, i.e. under positive-ion formation the ion $[M+H–HSO_3^{\bullet}]^+$ [16] and under negative-ion formation the ion $SO_4^{-\bullet}$ [17] have been observed. The discussed signals allow for a ready identification of sulfate conjugates, while additional MS^3 experiments provide information on the structure of the non-conjugated metabolite.

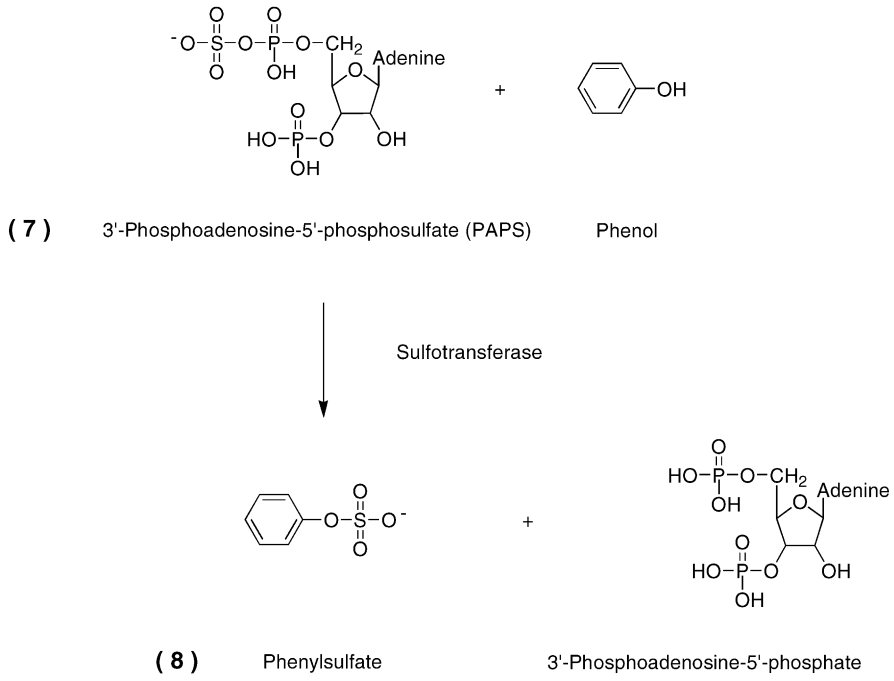
Metabolites with several phenolic OH-groups may form mixed glucuronides and sulfates. Thus in the MS/MS spectrum of the benzo[*a*]pyrene-*O*-sulfate-*O*-glucuronide, recorded in negative-ion mode, the ions $[M-H-80]^-$, $[M-H-177]^{-\bullet}$ and $[M-H-80-177]^{-\bullet}$ were observed, where it is not clear though why a radical of 177 Da instead of an even-electron molecule of 176 Da is lost [17].

2.6. Acetylation

Amino substituents which cannot be biodegraded by an oxidative mechanism are frequently acetylated. In particular, aromatic and aliphatic amino groups are *N*-acetylated, while hydroxylamines may also be *O*-acetylated. Such acetylations may be reversible. They do not improve excretion of phase I metabolites. These conjugates may be more toxic than their precursors. *N*-Acetylation reactions are catalyzed by *N*-acetyltransferases.

2.6.1. Quasi-molecular ions

Depending on the nature of the phase I metabolites, *N*-acetyl conjugates can be ionized by ESI in positive- and/or negative-ion modes. The $[M+H]^+$ or $[M-H]^-$ ions increase in mass by 42 Da as compared to the non-conjugated metabolite (Table 1).



Scheme 4. Sulfatation of phenol.

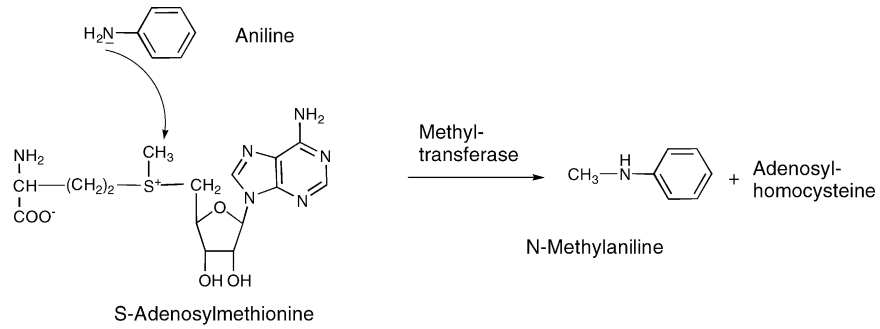
2.6.2. Collision-induced fragments

If an aromatic amino group is acetylated and its MS/MS spectrum is recorded in positive-ion mode (as with the metabolite of the drug retigabin [18]), both the ion $[M + H–CH_2=C=O]^+$ (loss of 42 Da) and the CH_3CO^+ ion at *m*/*z* 43 will be observed, revealing that acetylation has taken place. The structure-specific fragment $[M + H–CH_2=C=O]^+$ is also frequently, but not always, observed if an aliphatic amine has been acetylated. Loss of ketene will not be observed if competing low-energy decomposition pathways (other weak bonds) are accessible [19].

Conjugation of a drug metabolite by glutathione and further biotransformation of this conjugate to the cysteine conjugate are often followed by *N*-acetylation of the amino group of cysteine leading to the *N*-acetyl derivative, as shown in Scheme 9c for the fungicide boscalid. The occurrence of such an *N*-acetylation is again revealed by loss of ketene (42 Da) [20,25,27,56,57] (see below).

2.7. Methylation

Conjugation of phase I metabolites by methylation represents the reverse process to the often-observed oxidative demethylation processes found for phase I metabolites. This biotransformation reduces the polarity of the precursor metabolite and, hence, its excretion. Such methylation reactions usually do not lead to toxic products (however, quaternization of heterocyclic amines can increase their toxicity, e.g. formation of paraquat from bispyridyl [20]). Amino groups, phenolic groups in compounds containing a dihydroxyphenyl group [28], but also thiol groups [29] may be methylated in a phase II reaction leading to an –NH–CH_3,



Scheme 5. Methylation of aniline.

an –O–CH_3, and an –S–CH_3 group. The methylating agent is *S*-adenosylmethionine, the enzyme involved is a methyltransferase. Scheme 5 shows the methylation of aniline.

2.7.1. Quasi-molecular ions

The conjugates are usually ionized under positive-ion conditions, with ESI, but also the APCI and APPI methods are well suited [14]. Upon methylation, the $[M+H]^+$ ion mass increases by 14 Da.

2.7.2. Collision-induced fragments

In contrast to all conjugation products discussed so far, the bond between the endogenous agent and the metabolite involved in this conjugation, i.e. the CH_3–N, CH_3–O or CH_3–S bond, is in general not cleaved. Hence, the conjugation is reflected by the increased mass of the quasi-molecular ion, but not directly evident from the MS/MS spectra (exceptions see below). Rather, all fragments which include the methylation site are also shifted to higher *m/z* values by 14 Da. This way, the methylation site can often be localized, as discussed for flavonoid metabolites in [28]. However, if a phenyl ring is substituted by two hydroxyl groups, MS/MS data can unambiguously prove the methylation of a hydroxyl group, but usually do not allow to differentiate between the two possible methylation sites, as the MS/MS spectra of the isomers differing only in their substitution pattern are almost undistinguishable. In this instance, only additional NMR data allow for an unambiguous assignment of the exact methylation site.

In contrast to the methylation of hydroxyl, thiol, or aliphatic amino groups methylation of an aromatic amino group during phase II biotransformation is reflected in the MS/MS spectrum by an abundant loss of a methyl radical from the protonated conjugate. Thus, the methylation of aniline as shown in Scheme 5 leads to *N*-methyl aniline. The MS^2 spectrum of the $[M+H]^+$ ion of *N*-methyl aniline after electrospray ionization (as recorded in an ion trap mass spectrometer) is shown in Fig. 3. This MS^2 spectrum is dominated by an abundant loss of a $CH_3^\bullet$ radical, while other fragments are of very minor abundance.

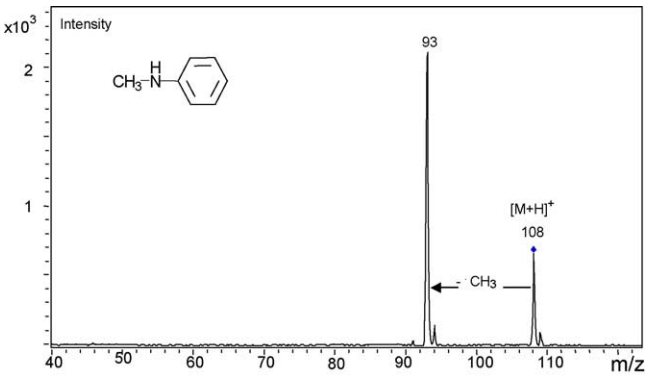


Fig. 3. MS/MS of protonated *N*-methylaniline, $[M+H]^+ = 108$ (ion trap, electrospray ionization).

2.8. Conjugation with glutathione and subsequent degradation of the conjugate

The tripeptide glutathione (γ-L-glutamyl-L-cysteinyl-glycine, Glu–Cys–Gly), GSH, exists at millimolar concentrations in the intracellular fluid of mammalian systems. The structure of GSH is shown in Scheme 6.

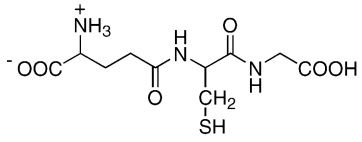
Because of its nucleophilic cysteinyl thiol group, GSH can react with electrophiles during biotransformation of xenobiotic compounds (either with the xenobiotic compound itself or its electrophilic metabolites) and thus affords detoxification.

During conjugation, GSH can be added to an activated double bond, e.g. the ortho position of a phenolic group of an aromatic ring [30], to an epoxide group already present in the molecule or arising from oxidation of an aromatic ring or olefine, to the carbon of an isocyanate group [31], or by substitution of halogen atoms, e.g. in halogenated aliphatic and aromatic hydrocarbons (such as 1,2-dichloroethane [32] or pesticides [20]). Glutathione-*S*-transferase (GST) catalyzes this conjugation.

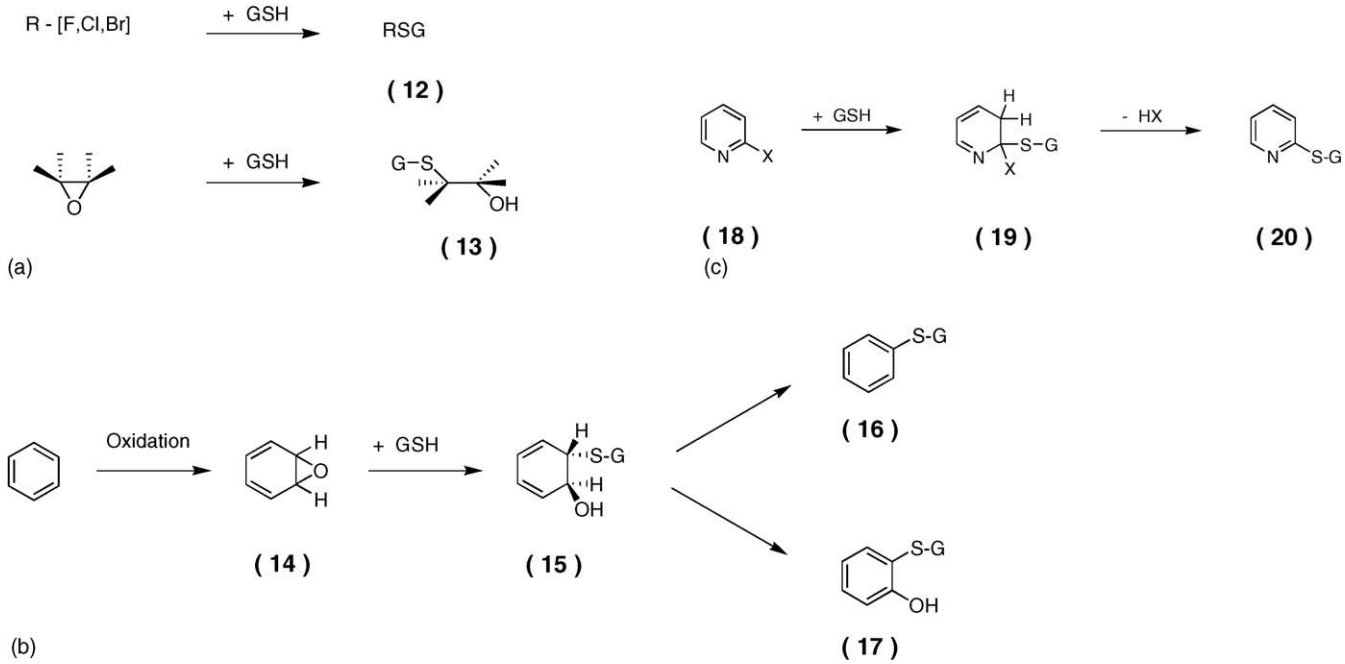
A general overview of the different mechanisms of glutathione conjugation is given in Scheme 7. Conjugation of glutathione (GSH) to haloaliphatic compounds proceeds by nucleophilic substitution as mentioned above and as shown in Scheme 7a (**12**). Scheme 7a also represents the conjugation of aliphatic epoxides with GSH leading to (**13**).

Conjugation of aromatic rings usually occurs via an areneoxide (**14**) as unstable intermediate. If this epoxide is attacked by GSH, a non-aromatic conjugate (**15**) (a cyclohexadiene derivative) with S-bound glutathione and a hydroxyl group at the adjacent position of the ring is generated, as shown in Scheme 7b. The mass of the quasi-molecular ion $[M+H]^+$ in positive-ion mode is 324 Da higher than that of the neutral precursor (in the following abbreviated as R_o), i.e. $[M+H]^+ = [R_o + O + GSH + H]^+ = [R_o + 324]^+$. This product may be stable enough to survive isolation, but quite commonly rearomatizes, either by elimination of water, which only leaves the glutathione residue on the ring (compound (**16**), quasi-molecular ion: $[M+H]^+ = [R_o + GSH - 2H + H]^+ = [R_o + 306]^+$), or by autooxidation/dehydrogenation to compound (**17**) in Scheme 7b with both glutathione and a hydroxyl group (in ortho position to GS) bound to the ring. The quasi-molecular ion is given by $[M+H]^+ = [R_o + O + GSH - 2H + H]^+ = [R_o + 322]^+$.

In halogenated aromatics, the halogen atom is usually exchanged for glutathione. It is not clear whether this exchange



Scheme 6. Glutathione (GSH).



Scheme 7. Conjugation with glutathione (a) aliphatic compounds, epoxides; (b) aromatic compounds; (c) halogenated heterocyclic compounds.

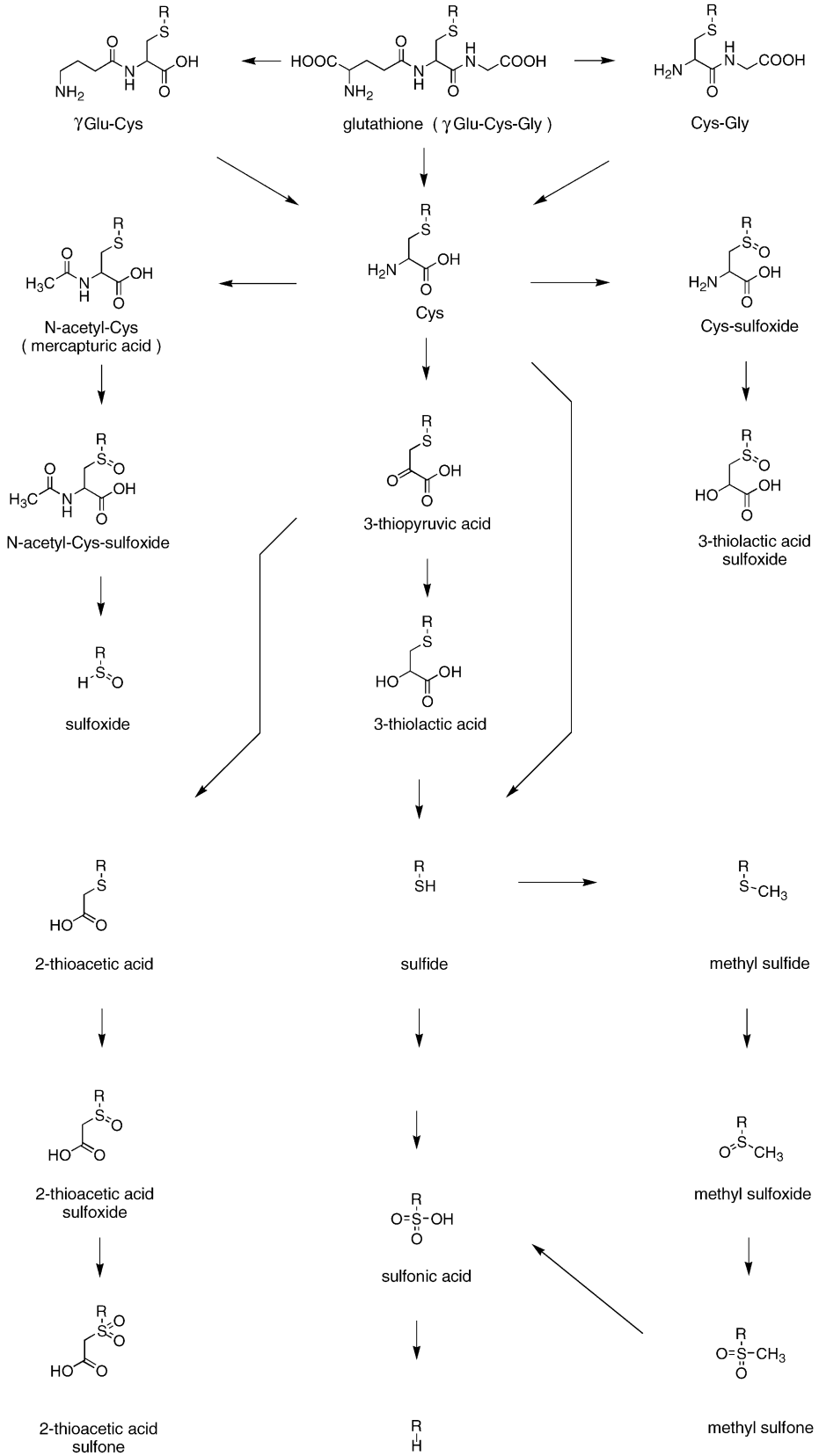
results from direct nucleophilic attack of the sulfur of GSH, which is not very common in aromatic systems, or proceeds via an arene oxide. A direct exchange definitely occurs in activated heterocyclic systems such as 2-halopyridines (see (**18**) in Scheme 7c). A substitution of a halogen X by GSH (under elimination of HX) leads to (**20**) and to the quasi-molecular ion $[M+H]^+ = [R_o - HX + GSH + H]^+ = [R_o + 272]^+$ if X is chlorine. If conjugation with GSH under substitution of halogen occurs via an arene oxide, a hydroxyl group will be introduced in ortho position to the GSH residue (leading to the quasi-molecular ion $[M+H]^+ = [R_o + O - HX + GSH + H]^+ = [R_o + 288]^+$ if X is chlorine).

The initial conjugation of xenobiotica or their metabolites with glutathione is usually followed by further biotransformation reactions. An overview of the various reactions observed is presented in Scheme 8 [1], while Scheme 9 illustrates some of these biotransformation reactions for the fungicide boscalid, where the initial conjugation with GSH proceeds either via substitution of the chlorine at the heteroaromatic ring (see also Schemes 7c and 9a), or via conjugation to one of the two aromatic rings via route (**14**) → (**15**) → (**16**) or route (**14**) → (**15**) → (**17**) in Scheme 7b, as shown for boscalid in Scheme 9b. Subsequent biotransformation reactions result in a loss of glutamic acid to form a glycinylcysteinyl conjugate (**24**), or in a loss of glycine to form a γ-glutamylcysteinyl conjugate (**25**), while successive elimination of both glutamic acid and glycine results in a cysteinyl conjugate (**26**) (see Scheme 9c). In a further step, this cysteinyl conjugate can be acetylated at the amino group of the cysteine, giving rise to the NAcCys conjugate (**27**) in Scheme 9c. Moreover, the cysteine conjugate may be further biodegraded according to the general Scheme 8. Most of these glutathione biodegradation products have been identified in metabolism studies on the fungicide boscalid. Sulfoxides and sulfones may originate from biotransformations or may be artifacts resulting from air oxidation.

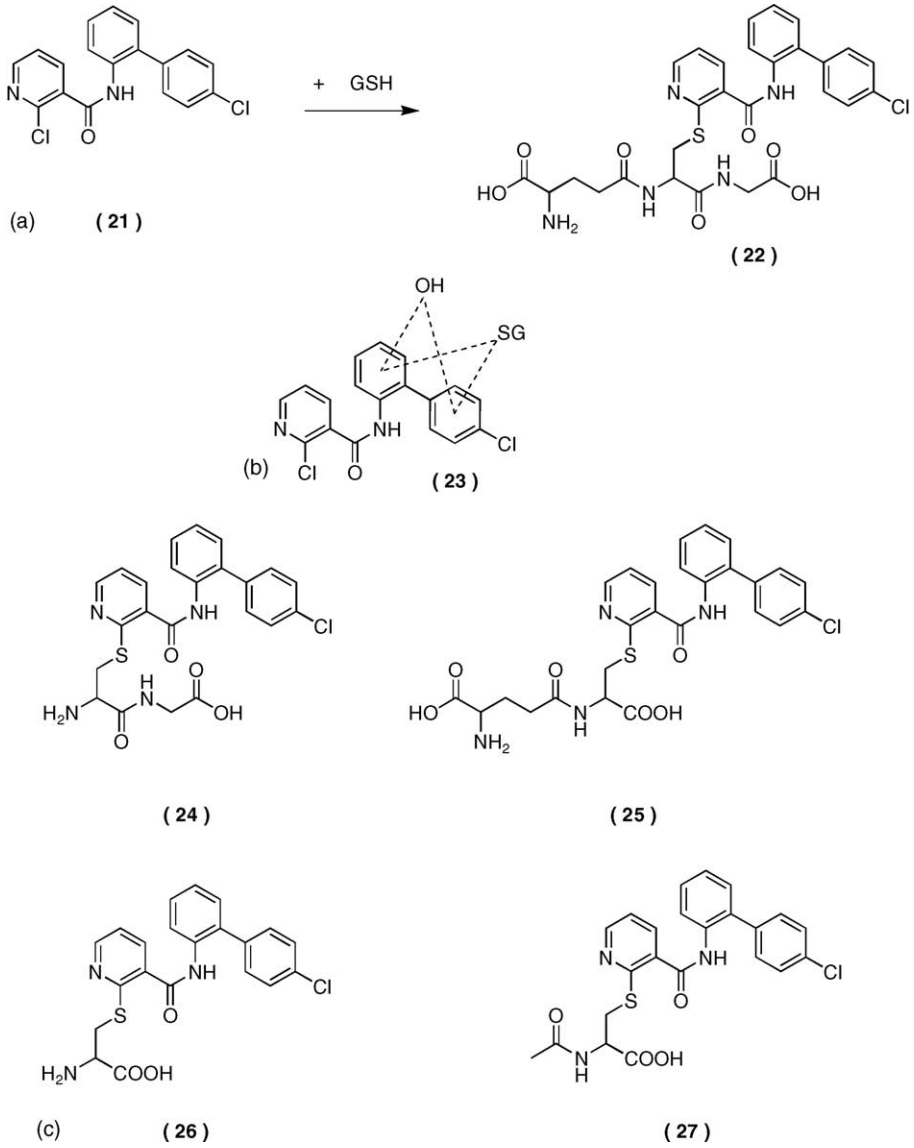
In the case of aromatic compounds, the precursors for these subsequent biotransformation products are the conjugates (**15**)–(**17**), and (**20**) in Schemes 7b and c. The *m*/*z* values of the quasi-molecular ions can be calculated as described above, e.g. for the halogenated aromatic compound (**18**) in Scheme 7c, the quasi-molecular ion of the *GlyCys conjugate* is given as $[M+H]^+ = [R_o - HX + GlyCys + H]^+ = [R_o - 36 + 178 + 1]^+ = [R_o + 143]^+$, if X is chlorine, where GlyCys has the nominal mass of 178 Da. The quasi-molecular ion of the corresponding *GluCys conjugate* is given as $[M+H]^+ = [R_o - HX + GluCys + H]^+ = [R_o - 36 + 250 + 1]^+ = [R_o + 215]^+$, if X = chlorine, where GluCys has the nominal mass of 250 Da.

During the following biotransformation step, a *cysteine conjugate* may be formed (where Cys has a nominal mass of 121 Da) leading to a quasi-molecular ion of $[M+H]^+ = [R_o - HX + Cys + H]^+ = [R_o - HX + 121 + 1]^+ = [R_o + 86]^+$, if X = chlorine. Finally, acetylation of the Cys-conjugate leads to the *NAcCys-conjugate (mercapturic acid)*, the quasi-molecular ion of which is 42 Da higher in mass than that of the preceding Cys-conjugate.

Glutathione conjugates and their above-mentioned further biotransformation products are ionized under positive- or negative-ion conditions. For negative-ion formation, the correponding $[M-H]^-$ ions will have *m*/*z* values which are two mass units lower.



Scheme 8. Metabolic pathway of glutathione conjugates.



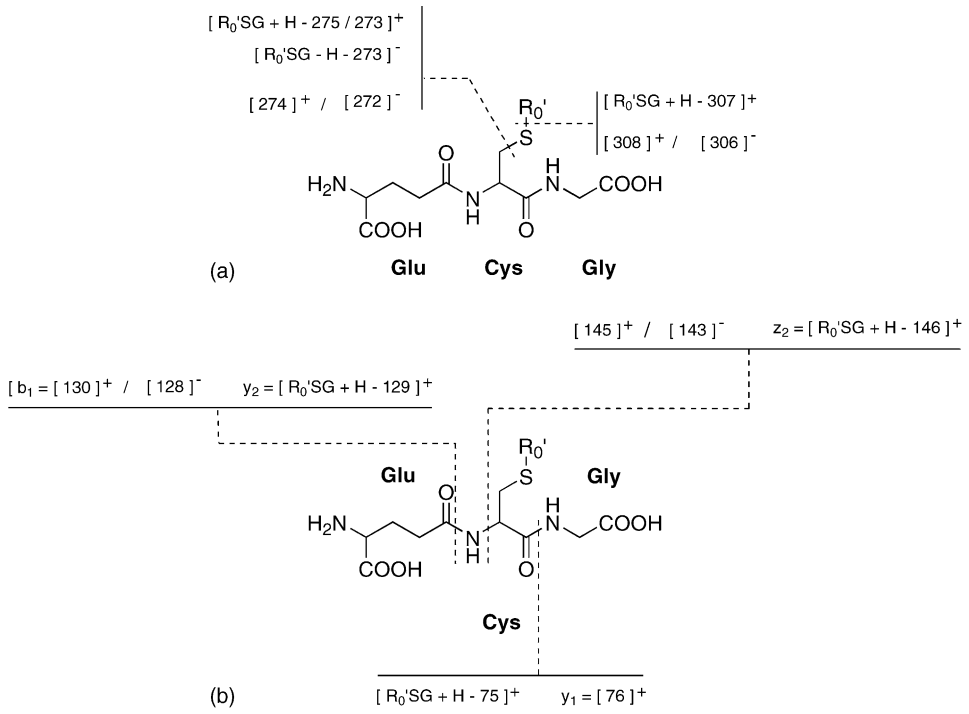
Scheme 9. Conjugation of boscalid with glutathione (a) heterocyclic site and (b) aromatic site. (c) Biotransformation of the GSH conjugate of boscalid.

2.8.1. Collision-induced fragmentation

Under CID conditions, the *glutathione conjugates* show a characteristic fragmentation pattern. A general scheme for the collision-induced dissociation of protonated glutathione conjugates based on FAB-MS/MS measurements was reported by Pearson et al. as early as 1988 [34,35].

As one of two typical fragmentation processes, either the R'_o–S bond between the conjugation site of the precursor metabolite and the sulfur of cysteine and/or the S–CH_2 bond within the cysteine residue is cleaved. As a function of the gas phase basicity or acidity of the precursor metabolite versus glutathione, the charge may reside on the primary metabolite moiety R'_o and/or on the peptide moiety [15,20,25,27,30,31,33,35–38,49–55], and depending on the chemical properties and structure of the precursor R_o, cleavage of the CH_2–S bond is more abundant than cleavage of the R'_o–S bond (or even observed exclusively) [15,20,35–38] or vice versa [25,27,30,31,33]. As a second process, the well-known fragmentation of the peptide either from the C terminus, i.e. y_1, y_2, but also z_2, or from the N terminus, i.e. a_2, a_1, b_1, may occur. These dissociations lead to *m*/*z* values for the fragments as shown in Scheme 10a and b. (In Schemes 10–15, the symbol R'_o instead of R_o is used for the metabolite moiety, since depending on the mechanism of conjugation outlined in Scheme 7, the metabolite moiety may have changed.) The peptide-specific fragmentation may be followed by further cleavage of the S–CH_2 or R'_o–S bonds. Particularly characteristic for protonated glutathione conjugates are loss of glycine (75 Da) and anhydroglutamic acid (129 Da), while other collision-induced fragments (in particular the loss of glutamine, 146 Da) can be used in addition to corroborate the identification of the GSH adduct.

In Fig. 4, the product ion spectrum of the protonated molecule of boscalid, where GSH is conjugated to the het-

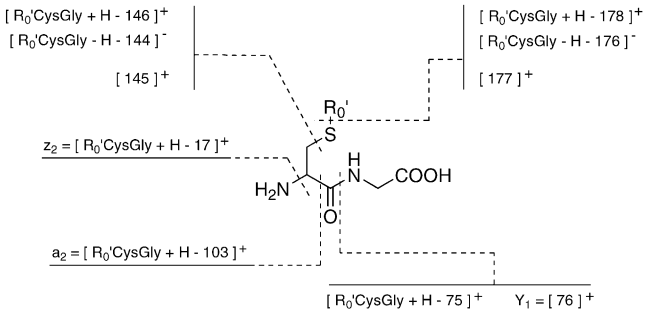


Scheme 10. Fragmentation of (a) glutathione conjugates and (b) peptide moiety in glutathione conjugates.

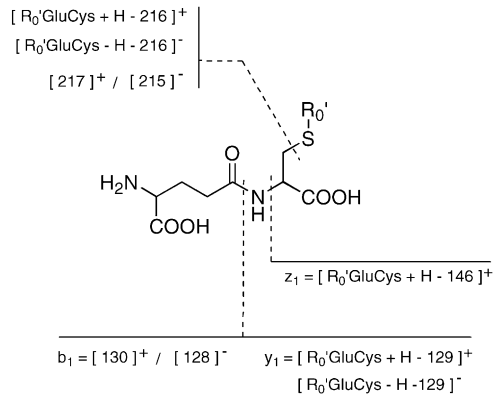
erocyclic ring of boscalid (electrospray ionization, triple quadrupole), is shown as an example (see Scheme 9a). As illustrated in Scheme 10a, cleavage of the S–CH_2 bond leads to a neutral loss of 273 Da. In addition, the abundant losses of 75, 129 and 146 Da further characterize the GSH adducts (see Scheme 10b). Moreover, after the initial loss of anhydroglutamic acid (129 Da), glycine (75 Da) is lost in a second step, leading to the base peak at *m*/*z* 410. In all instances the charge resides on the metabolite moiety while charge retention on the peptide moiety leads to the corresponding fragment ion at *m*/*z* 274 which decomposes further by loss of glycine (75 Da) or anhydroglutamic acid (129 Da) to the fragment ions at *m*/*z* 199 and 145, respectively. Similarly, the MS/MS spectrum of the $[M-H]^-$ ion (not shown here) is characterized by a dominant cleavage of the S–CH_2 bond leading to a loss of 273 Da and formation of the corresponding negative fragment ion at *m*/*z* 272, while dissociation of the peptide moiety is accompanied by neutral loss of 129 Da and formation of the negative fragment ion at *m*/*z* 128.

Note that often in a first step, dissociation of protonated GSH conjugates occurs via a competing low-energy fragmentation (such as loss of water), while the above-discussed fragmentations (characteristic for GSH conjugates) are only observed in a second consecutive step. This also holds true for collision-induced dissociation of the biotransformation products of the GSH adducts discussed below.

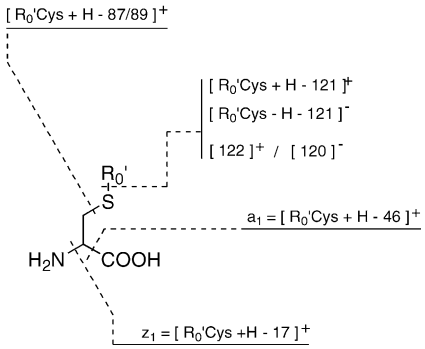
The collision-induced fragments of *CysGly conjugates* can be rationalized in a similar fashion as demonstrated in Scheme 11 for positive ions. Again, depending on the nature of the primary metabolite, cleavage of the R_0'–S bond [25,33] or of the S–CH_2 bond [20] may occur. Cleavage of



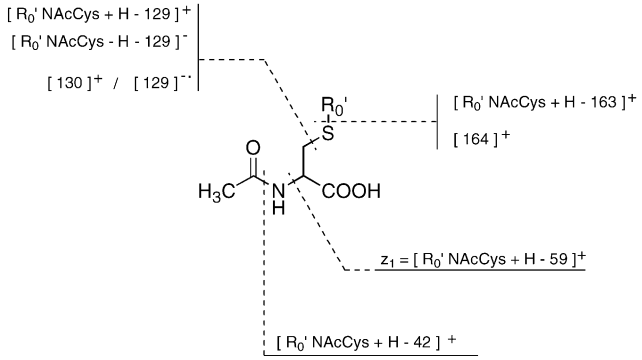
Scheme 11. Fragmentation of CysGly conjugates.



Scheme 12. Fragmentation of GlyCys conjugates.



Scheme 13. Fragmentation of Cys conjugates.



Scheme 14. Fragmentation of *N*-acetylcysteine (NacCys) conjugates (mercapturic acids).

the S–CH_2 bond occurs either with retention of the charge on the metabolite moiety and neutral loss of 146 Da or with retention of the charge on the peptide moiety and formation of an ion at *m/z* = 145, as outlined in Scheme 11. As glutamic acid was split off during biotransformation of GSH, only loss of glycine (75 Da) is observed with this conjugate upon further fragmentation of the peptide moiety, while the collision-induced fragment characteristic for the glutamic acid moiety in GSH adducts (loss of 129 Da) is missing (as expected).

For example, the product ion spectrum of the protonated CysGly conjugate of boscalid bound to the heterocyclic ring, not shown here [20] (i.e. (**24**) in Scheme 9c), shows an abundant loss of 17 Da (NH_3), 75 Da (glycine) and 146 Da in accordance with Scheme 11.

The collision-induced fragments of *GluCys conjugates* [20] follow the same rules and are shown in Scheme 12. Thus, for the GluCys conjugate of boscalid (i.e. (**25**) in Scheme 9c), cleavage of the S–CH_2 bond leads to a neutral loss of 216 Da and formation of the corresponding fragment ion at *m/z* 217. Now only the neutral losses characteristic for the glutamoyl moiety (loss of 129 and 146 Da) are observed while (as expected) loss of 75 Da is not found. The loss of 129 Da (anhydroglutamic acid) is followed by loss of 46 Da (HCOOH).

2.8.2. *Cysteine conjugates*

The fragmentation routes of cysteine conjugates under positive-ion formation are summarized in Scheme 13 [20,27,33]. Again, either cleavage of the R'_0–S bond [27,33] (loss of 121 Da and formation of the fragment at *m/z* 122 in positive ion mode) or cleavage of the S–CH_2 [20] bond is observed, leading to a loss of 87 and/or 89 Da, as exemplified for Cys conjugated to boscalid ((**26**) in Scheme 9c). As often found with aliphatic and aromatic acids and amines under positive ion conditions, collision-induced loss of formic acid (46 Da) and ammonia (17 Da) further characterize the conjugate.

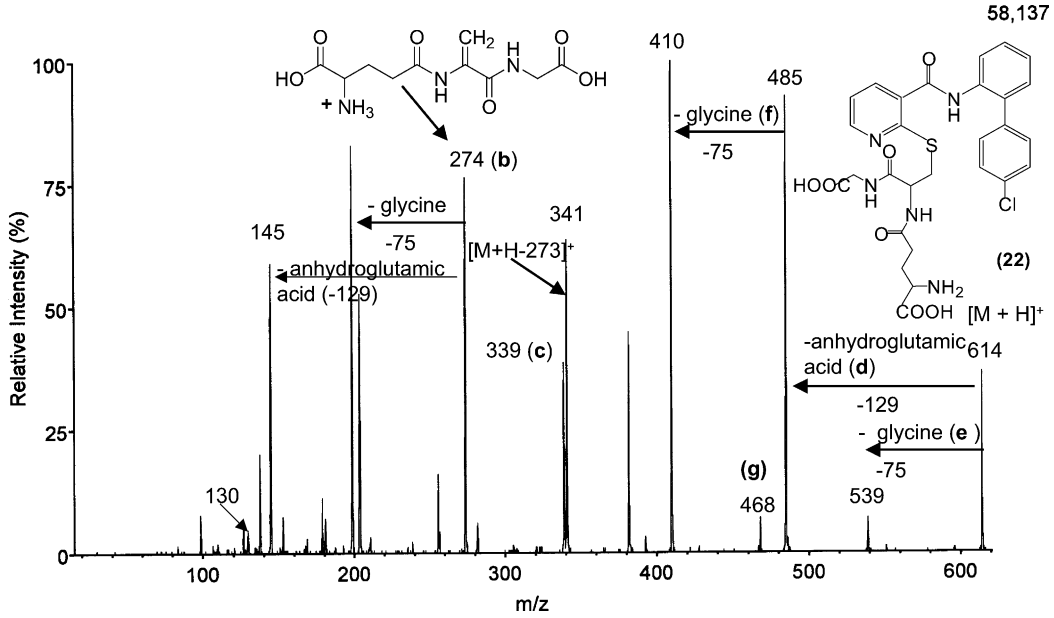
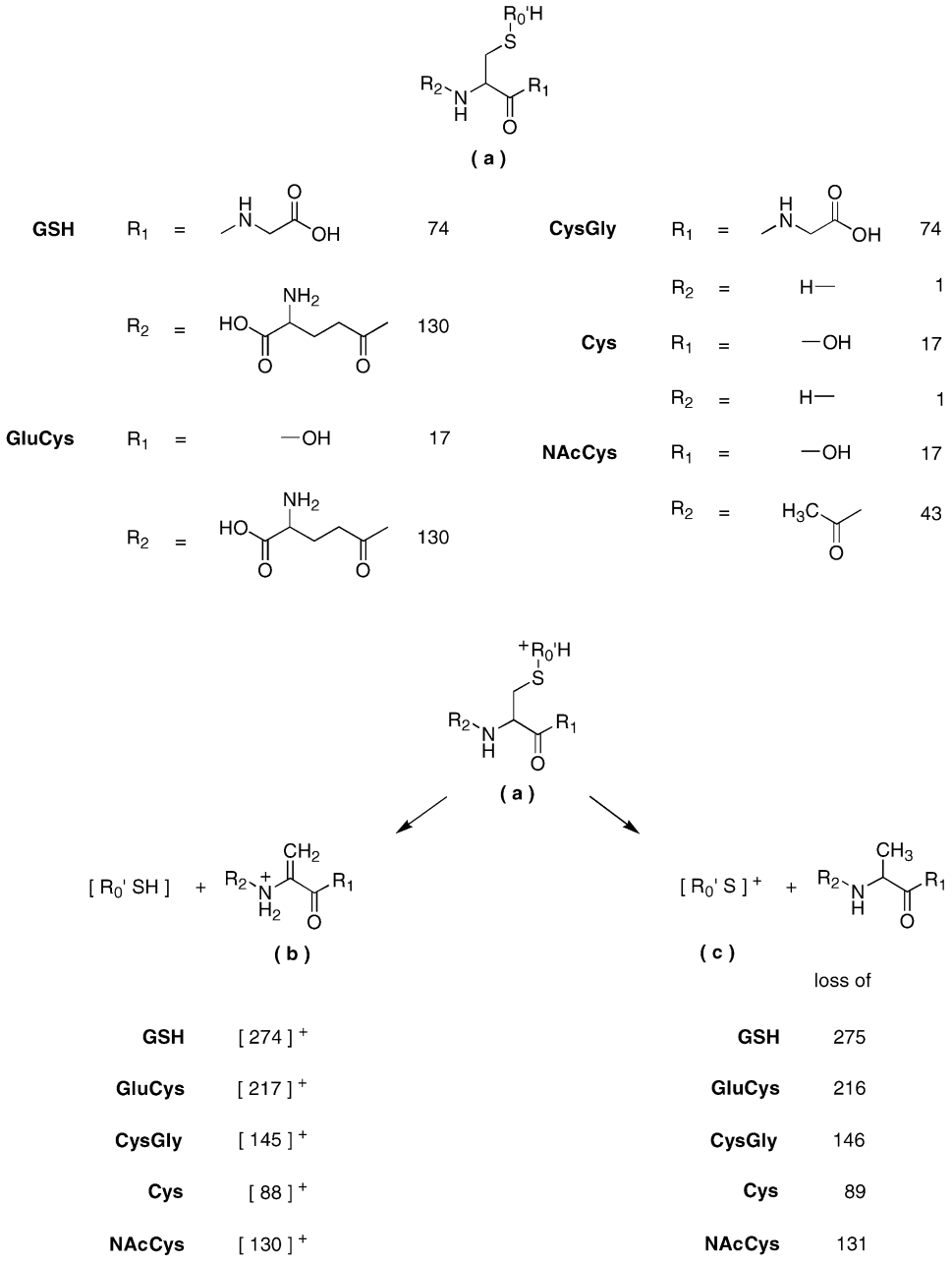


Fig. 4. MS/MS of the protonated glutathione conjugate of boscalid, $[M + H]^+ = 614$ (triple quadrupole, electrospray ionization).

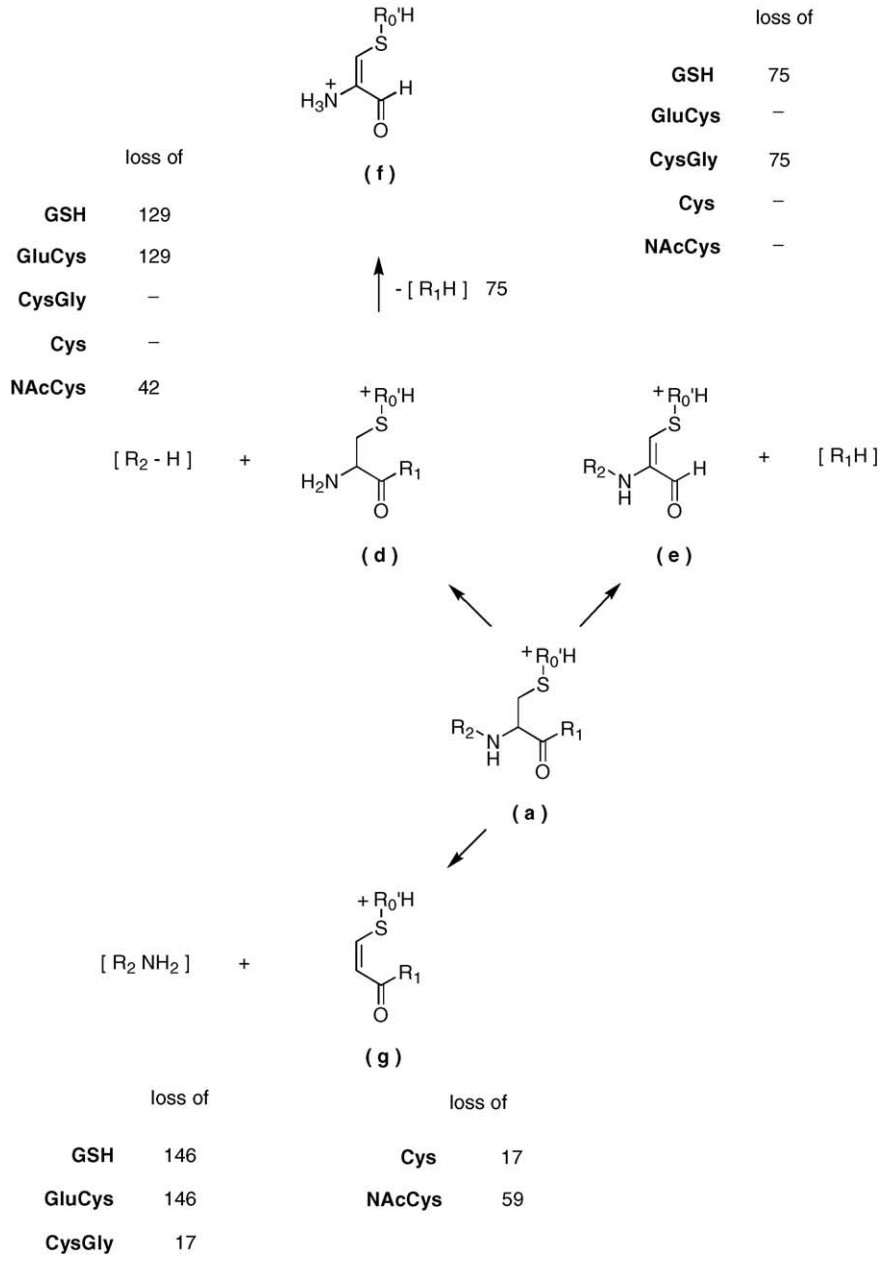
2.8.3. N-Acetylcysteine (NAcCys) conjugates (mercapturic acids)

As mentioned above, the cysteine conjugates can undergo further biotransformation by *N*-acetylation (formation of mercapturic acids), leading to an increase in the *m*/*z* value of the quasi-molecular ion by 42 Da. Upon collision, this NAcCys conjugate exhibits a neutral loss of 42 Da, i.e. a loss of ketene, which allows rapid identification of the acetylated molecule. Ketene loss is usually followed by loss of formic acid. In addition, the NAcCys conjugate may undergo the fragmentations discussed for the Cys conjugate; in particular, cleavage of the CH_2–S bond. The expected *m*/*z* values of these fragments are summarized in Scheme 14.

As an example, the product ion spectrum of the protonated *N*-acetylcysteinyl-boscalid with conjugation of NAcCys to the heteroaromatic ring (see (**27**) in Scheme 9c) is shown in Fig. 5 (triple quadrupole, ESI) [20]. Cleavage of the S–CH_2 bond leads to a dominant neutral loss of 129 Da (giving rise to the fragment ion at *m*/*z* 341) while the corresponding fragment ion is observed at *m*/*z* 130. Loss of 42 Da (CH_2=C=O) leading to *m*/*z* 428 further characterizes this acetate conjugate. After this ketene loss, subsequent cleavage of the S–CH_2 bond leads to a loss of 89 Da. Moreover, a corresponding ion at *m*/*z* 88 is observed. The other fragments seen in the MS/MS spectrum result from bond cleavages within the boscalid moiety.



Scheme 15. General fragmentation scheme of glutathione conjugates and their biotranformation products.



Scheme 15. (*Continued*).

The fragmentation mechanisms describing the main collision-induced dissociations not only of GSH-conjugates, but also of GlyCys-, GluCys-, Cys- and NAcCys-conjugates, are summarized in Scheme 15. The structures of the fragment ions and lost neutrals are tentative.

2.8.4. Further biotransformation of Cys- or NAcCys-conjugates

The further biodegradation of Cys- or NAcCys-conjugates is shown in the general Scheme 8. Upon collisional activation, methyl sulfides lose CH_3SH (48 Da), sulfonic acids fragment under elimination of SO_2 (64 Da) and/or SO_3 (80 Da), and methyl sulfonates undergo collisionally induced loss of CH_3OH (32 Da), SO_2 (64 Da) and/or CH_3OSO_2H (96 Da) [20]. Furthermore, thiols, R′–SH, thioacetic acid derivatives, R′–S–CH_2COOH, as well as the corresponding sulfoxides and sulfones have been observed for various active ingredients [20], as shown in Scheme 8.

2.9. Conjugation with glycine and other amino acids

The amino acid glycine conjugates with aromatic carbonic acids, while such conjugation is rarely observed with other amino acids (see below). This conjugation results in a further increase in water solubility and thus improves excretion.

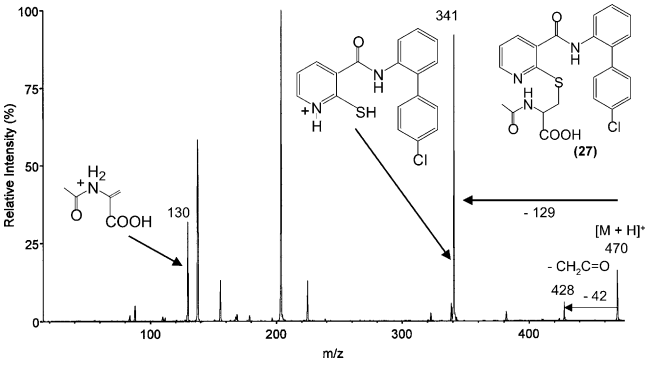


Fig. 5. MS/MS of the protonated *N*-acetylcysteine conjugate of boscalid, $[M+H]^+ = 470$ (triple quadrupole, electrospray ionization).

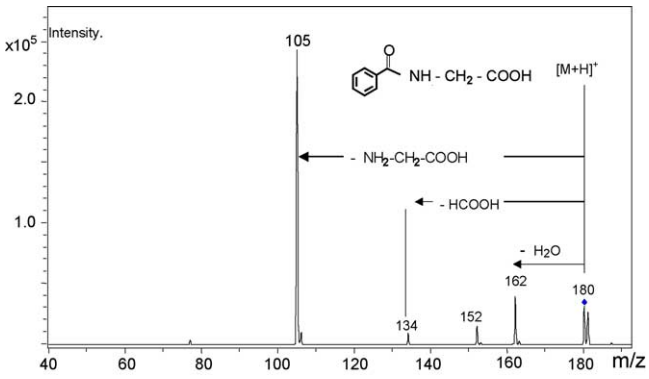


Fig. 6. MS/MS of protonated hippuric acid, $[M+H]^+ = 180$ (ion trap, electrospray ionization).

Scheme 16 shows the conjugation of benzoic acid with glycine, which gives rise to formation of hippuric acid. The MS/MS spectrum of protonated hippuric acid is shown in Fig. 6 [40]. The *m*/*z* value of the quasi-molecular ion in positive ion mode increases by 57 Da as compared to the non-conjugated benzoic acid. Collision-induced dissociation of the $[M+H]^+$ ion is characterized by cleavage of the amide bond with loss of glycine. With lower abundance, consecutive loss of water (18 Da) and CO (28 Da) or vice versa, both of which add up to (formal) loss of formic acid, is also observed, while in negative-ion mode loss of CO_2 (44 Da) is, expectedly, by far the most dominant process.

As mentioned above, other amino acids are less frequently conjugated. Among these other amino acids, in particular ornithine, arginine and glutamine [58] form conjugates with aromatic acids such as benzoic acid. Although only few data can be found in the literature, the collison-induced dissociation is likely to proceed in analogy to that observed with glycine conjugates, i.e. the amide bond will be cleaved preferentially. Furthermore, oxidation of cysteine and decarboxylation leads to formation of the β-amino sulfonic acid taurine which readily conjugates with bile acids such as cholic acid (see also the biotransformation of glutathione, discussed above). The negative-ion MS/MS spectra of taurine conjugates are (as expected) characterized by loss of SO_3 (80 Da) [26,41].

3. Discussion

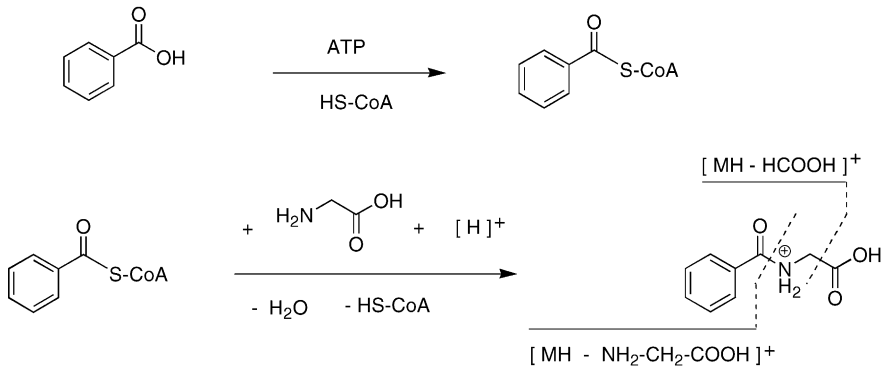
During phase II of their biotransformation xenobiotic compounds, e.g. drugs and pesticides, are conjugated with endogenous agents. Depending on their polarity the conjugates are readily ionized under electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) or atmospheric pressure photo ionization (APPI). Depending on the gas phase acidity or basicity, the conjugates are preferentially detected either as $[M+H]^+$ or as $[M-H]^-$ ions. If the phase I metabolite is known the type of conjugate can be identified by the mass shift of the (quasi-)molecular ion (relative to that of the phase I metabolite) as represented in Table 1. This identification can be corroborated by collision-induced fragments observed in a tandem mass spectrometer, as also summarized in this table.

Collisional activation of protonated conjugates often leads to a preferential cleavage of the bond between the two conjugating partners, e.g. for a metabolite R_o, a conjugating agent A and the leaving group L, the following equations apply:

Conjugation:

$$R_o + A \rightarrow R'_o A' + L$$

(L = small neutral molecule such as H_2O or HCl) (1)



Scheme 16. Conjugation of benzoic acid with glycine and collision-induced dissociation of the protonated molecule.

Ionization (positive ions):

$$R'_oA' + H^+ \rightarrow [R'_oA'H]^+ \quad (2)$$

Dissociation:

$$[R'_oA'H]^+ \rightarrow [R_oH]^+ + A' \quad (3)$$

where $A' = A - L$

If dissociation of protonated conjugates follows this route, the identification of the type of conjugate is straightforward. Such dissociation is observed e.g. with glucuronides, glucosides, malonylglucosides, acetates and conjugates formed by methylation of aromatic amines. In these cases, collision-induced dissociation leads to the protonated phase I metabolite (under positive ion formation) whose structure can be elucidated by recording its MS^3 spectrum in an ion trap. However, the exhaustive structure elucidation of the phase I metabolites by tandem mass spectrometry is significantly more difficult than the identification of the type of the phase II conjugate.

Moreover, also in cases where the collision-induced dissociation of the conjugate cannot be described by the simple process shown in Eq. (3), the fragmentation is usually straightforward. Thus, for glutathione conjugates and their biotransformation products as well as for conjugates formed with amino acids, the fragmentation follows the well-known rules known for peptides. The observation of characteristic neutral losses upon fragmentation of protonated or deprotonated conjugates (as summarized in Table 1) can be used for a rapid screening for such conjugates using the neutral loss scan in a triple quadrupole mass spectrometer. Moreover, they may represent the basis of a computer-aided identification of these conjugates.

In general, the collision-induced dissociation of the even-electron $[M+H]^+$ and $[M-H]^-$ ions of the conjugates formed under ESI, APCI, or APPI conditions follows the even electron rule [42], i.e. dissociation leads to the formation of stable even-electron cations and neutral molecules. An apparent exception from this rule is the loss of a methyl radical from the $[M+H]^+$ of aromatic amines methylated during phase II, e.g. the methyl loss from protonated *N*-methyl aniline discussed above. Obviously no other low-energy decomposition pathway involving loss of a neutral molecule (instead of a radical) is available. Thus, in addition to the discussed methyl loss and formation of ionized aniline, a loss of neutral methylamine and formation of the even-electron phenyl cation $[C_6H_5]^+$, which is in agreement with the even electron rule, is conceivable. However, thermochemical data [43] reveal that the former process is energetically more favorable (ΔH_f $([C_6H_5NH_2]^{+\bullet} + CH_3^\bullet) = 972\,kJ\,mol^{-1}$) than the latter ($\Delta H_f$ $([C_6H_5]^+ + CH_3NH_2) = 1165\,kJ\,mol^{-1}$).

In the case of APPI, depending on the experimental conditions, also $M^{+\bullet}$ radical cations can be formed. Their dissociation follows EI fragmentation rules.

Acknowledgement

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References

[1] A.J.L. Cooper, Enzymology of Cysteine-S-conjugate-β-lyases, in: M.W. Anders, W. Dekant (Eds.), Conjugation-Dependent Carcinogenicity and Toxicity of Foreign Compounds, Advances in Pharmacology, vol. 27, Academic Press, San Diego, USA, 1994, p. 71ff.
[2] J. Borlak, M. Walles, M. Elend, T. Thum, A. Preiss, K. Levsen, Xenobiotica 33 (2003) 655.
[3] M. Barber, R.S. Bordoli, G.J. Elliott, R.D. Sedgewick, A.N. Tyler, Anal. Chem. 54 (1982) 654A.
[4] C.R. Blakley, J.J. Carmody, M.L. Vestal, Adv. Mass Spectrom. 8B (1980) 1616.
[5] D.I. Carroll, I. Dzidic, E.C. Horning, R.N. Stillwell, Appl. Spectrosc. Rev. 17 (1981) 337.
[6] M. Yamashita, J.B. Fenn, J. Phys. Chem. 88 (1984) 4451.
[7] K.L. Bush, G.L. Glish, S.A. McLuckey, Mass Spectrometry/Mass Spectrometry: Techniques and Applications of Tandem Mass Spectrometry, VCH Publisher, New York, USA, 1988.
[8] P.H. Dawson (Ed.), Quadrupole Mass Spectrometry and its Applications, American Institute of Physics, Woodbury, USA, 1995.
[9] R.E. March, R.J. Hughes, Quadrupole Storage MS, Wiley, New York, USA, 1989.
[10] (a) J.W. Hager, Rapid Commun. Mass Spectrom. 16 (2002) 512;
(b) G. Hopfgartner, C. Husser, M. Zell, J. Mass Spectrom. 38 (2003) 138.
[11] (a) H.R. Morris, T. Paxton, A. Dell, J. Langhorne, M. Berg, R.S. Bordoli, J. Hoyes, R.H. Bateman, Rapid Commun. Mass Spectrom. 10 (1996) 889;
(b) I.V. Chernushevich, A.V. Loboda, B.A. Thomson, J. Mass Spectrom. 36 (2001) 849.
[12] (a) R. Harkewics, M.E. Belov, G.A. Anderson, L. Pasa-Tolic, C.D. Masselon, D.C. Prior, H.R. Udseth, R.D. Smith, J. Am. Soc. Mass Spectrom. 12 (2001) 144;
(b) M.E. Belov, E.N. Nikolaev, K. Alving, R.D. Smith, Rapid Commun. Mass Spectrom. 15 (2001) 1172;
(c) J.C. Schwartz, M.W. Senko, J.E.P. Syka, J. Am. Soc. Mass Spectrom. 13 (2002) 659;
(d) http://www.thermo.com/finnigan.
[13] M.V. Buchanan (Ed.), Fourier Transform MS, American Chemical Society, Washington, DC, USA, 1987.
[14] H. Keski-Hynnilä, M. Kurkela, E. Elovaara, L. Antonio, J. Magdalou, L. Luukkanen, J. Taskinen, R. Kostiainen, Anal. Chem. 74 (2002) 3449.
[15] E. Rathahao, A. Hillenweck, A. Paris, L. Debrauwer, Analusis 4 (2000) 273.
[16] G.-Q. Zhang, G. McKay, J.W. Hubbard, K.K. Midha, Xenobiotica 26 (1996) 541.
[17] Y. Yang, W.J. Griffiths, T. Midtvedt, J. Sjövall, J. Rafter, J.-A. Gustafsson, Chem. Res. Toxicol. 12 (1999) 1182.
[18] R. Hempel, H. Schupke, P.J. McNeilly, K. Heinecke, C. Kronbach, C. Grunwald, G. Zimmermann, C. Griesinger, J. Engel, T. Kronbach, Drug Metab. Dispos. 27 (1999) 613.
[19] D.K. Dalvie, N.B. Khosla, K.A. Navetta, K.E. Brighty, Drug Metab. Dispos. 24 (1996) 1231.
[20] B. Behnke, R. Dötzer, W. Dreher, unpublished results.
[21] B. Withopf, E. Richling, R. Roscher, W. Schwab, P. Schreier, J. Agric. Food Chem. 45 (1997) 907.
[22] B. Boss, E. Richling, M. Herderich, P. Schreier, Phytochemistry 50 (1999) 219.

[23] R.W. Reiser, R.F. Dietrich, T.K.S. Djanegara, A.J. Fogiel, W.G. Payne, D.L. Ryan, W.T. Zimmermann, J. Agric. Food Chem. 45 (1997) 2309.
[24] M. Piel, S. Leonhardt, G. Gütter, J. Gerdon, R. Dötzer, W. Dreher, B. Behnke, Proceedings of the 34th Conference of the German Society for Mass Spectrometry, Munich, 4–7 March 2000.
[25] W. Tang, F.S. Abbott, J. Mass Spectrom. 31 (1996) 926.
[26] J.A. Hankin, P. Wheelan, R.C. Murphy, Arch. Biochem. Biophys. 340 (1997) 317.
[27] A.G. Borel, F.S. Abbott, Chem. Res. Toxicol. 8 (1995) 891.
[28] C. Cren-Olivé, S. Déprez, S. Lebrun, B. Coddeville, C. Rolando, Rapid Commun. Mass Spectrom. 14 (2000) 2312.
[29] J.-I. Yamaguchi, M. Ohmichi, M. Hasegawa, H. Yoshida, N. Ogawa, S. Higuchi, Drug Metab. Dispos. 29 (2001) 806.
[30] N.J. Thatcher, S. Murray, Biomed. Chromatogr. 15 (2001) 374.
[31] D. Stöckigt, S. Haebel, Rapid Commun. Mass Spectrom. 12 (1998) 273.
[32] J.C.L. Erve, M.L. Deinzer, D.J. Reed, Chem. Res. Toxicol. 8 (1995) 414.
[33] J.M. Hevko, R.C. Murphy, J. Am. Soc. Mass Spectrom. 12 (2001) 763.
[34] P.G. Pearson, M.D. Threadgill, W.N. Howald, T.A. Baillie, Biomed. Environ. Mass Spectrom. 16 (1988) 51.
[35] P.G. Pearson, W.N. Howald, S.D. Nelson, Anal. Chem. 62 (1990) 1827.
[36] P.E. Haroldsen, M.H. Reilly, H. Hughes, S.J. Gaskell, C.J. Porter, Biomed. Environ. Mass Spectrom. 15 (1988) 615.
[37] T.A. Baillie, M.R. Davis, Biol. Mass Spectrom. 22 (1993) 319.
[38] L.S. Tsuruda, M.W. Lamé, A.D. Jones, Drug Metab. Dispos. 23 (1995) 129.
[39] D.J. Borts, L.D. Bowers, J. Mass Spectrom. 35 (2000) 50.
[40] M. Elend, A. Preiß, Private communication.
[41] Y. Yang, W.J. Griffiths, H. Nazer, J. Sjövall, Biomed. Chromatogr. 11 (1997) 240.
[42] M. Karni, A. Mandelbaum, Org. Mass Spectrom. 15 (1980) 53.
[43] H.M. Rosenstock, K. Draxl, B.W. Steiner, J.T. Herron, Energetics of Gaseons Ions vol. 6 (Suppl. 1) (1977), 783 p.
[44] Q.-G. Dong, J.-K. Gu, D.-F. Zhong, J.P. Fawcett, H. Zhou, Acta Pharmocol. Sin. (2003) 576.
[45] T. Kuuranne, T. Kotiaho, S. Pedersen-Bjergaard, K.E. Rasmussen, A. Leinonen, S. Westwood, R. Kostiainen, J. Mass Spectrom. 38 (2003) 16.
[46] D. Thomassen, P-G. Pearson, J.T. Slattery, S.D. Nelson, Drug Metab. Dispos. 19 (1991) 997.
[47] J.Y.A..J. Wang, G.J. Sun, Y.C. Gu, M.S. Wu, J.H. Liu, Eur. J. Drug Metab. Pharmacokinet. 28 (2003) 265.
[48] L.J. Meng, W.J. Griffiths, J. Sjövall, J. Steroid Biochem. Mol. Biol. 58 (1996) 585.
[49] K.D. Ballard, M.J. Raftery, H. Jaeschke, S.J. Gaskell, J. Am. Soc. Mass Spectrom. 2 (1991) 55.
[50] M.R. Davis, T.A. Ballie, J. Mass Spectrom. 30 (1995) 57.
[51] L. Jin, M.R. Davis, P. Hu, T.A. Ballie, Chem. Res. Toxicol. 7 (1994) 526.
[52] C.M. Murphy, C. Fenselau, P.L. Gutierrez, J. Am. Soc. Mass Spectrom. 3 (1992) 815.
[53] G. Aldini, P. Granata, M. Orioli, E. Santaniello, M. Carini, J. Mass Spectrom. 38 (2003) 1160.
[54] W. Yin, G.A. Doss, R.A. Stearns, S. Kumar, Drug Metab. Dispos. 32 (2004) 43.
[55] M. Zhu, A.P. DeCaprio, C.R. Hauer, D.C. Spink, J. Chromatogr. B 688 (1997) 187.
[56] C.H. Oberth, J.D. Jones, J. Am. Soc. Mass Spectrom. 8 (1997) 729.
[57] G.K. Poon, Q. Chen, Y. Teffera, J.S. Ngui, P.R. Griffin, M.P. Braun, G.A. Doss, C. Freeden, R.A. Stearns, D.C. Evans, T.A. Baillie, W. Tang, Drug Metab. Dispos. 29 (2001) 1608.
[58] N.M. Barratt, W. Dong, D.A. Gage, V. Magnus, C.D. Town, Physiol. Plant. 105 (1999) 207.