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Toxicokinetics of Emerging Drugs of Abuse: In vivo and in vitro studies on the metabolic fate of the cocaine-derived designer drug dimethocaine

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Für meine Eltern

„Ich habe eine natürliche Besessenheit, die ist angeboren.

Aber ohne Leidenschaft und Ehrgeiz geht es nicht.“

Christiane Nüsslein-Volhard

deutsche Nobelpreisträgerin für Medizin / Physiologie 1995

Inhaltsverzeichnis

1 ZUSAMMENFASSUNG 2
2 SUMMARY 3
3 INTRODUCTION 4
3.1 Pharmacology and toxicology of dimethocaine 5
3.2 Role of the *N*-acetyltransferases NAT1&2 and their genetic polymorphism 6
4 AIM OF THE STUDY 8
5 EXPERIMENTAL PROCEDURES 9
5.1 Chemicals and reagents 9
5.2 Urine samples 9
5.3 Sample preparation for the identification of the phase I metabolites by LC-HR-MS^n 10
5.4 Sample preparation for identification of phase II metabolites by LC-HR-MS^n 10
5.5 Incubations with NAT1 and NAT2 10
5.6 LC-HR-MS^n apparatus for identification of phase I and II metabolites 12
5.7 LC-MS apparatus for analysis of NAT incubations 12
6 RESULTS AND DISCUSSION 15
6.1 Identification of the phase I and II metabolites by LC-HR-MS^n 15
6.2 NAT 1 and 2 kinetic studies with dimethocaine and sulfamethazine 34
7 REFERENCES 36
8 ABBREVIATIONS 38
9 DANKSAGUNG 39
10 CURRICULUM VITAE 40

1 ZUSAMMENFASSUNG

Unter den verschiedenen Substanzklassen die in den letzten Jahren auf dem weltweiten Drogenmarkt erschienen, waren auch einige synthetische Cocain Analoga zu finden. Eines davon ist Dimethocaine, (DMC, larocaine, 3-diethylamino-2,2-dimethylpropyl)-4-aminobenzoate) welches bereits in den 1930er Jahren als Lokalanästhetikum vermarktet wurde, jedoch psychoaktive Nebenwirkungen sowie ein hohes Abhängigkeitspotential zeigte. Heutzutage wird DMC als Cocain Ersatz verkauft und konsumiert. Das Ziel dieser Arbeit war es den *in vivo* und *in vitro* Metabolismus von DMC mittels Flüssigchromatographie und hochauflösender Massenspektrometrie (LC-HR-MS^n) aufzuklären. DMC wurde dazu männlichen Wistar Ratten verabreicht und deren Urin über 24 Stunden gesammelt. Die Urinaufbereitung erfolgte durch enzymatische Konjugatspaltung mit anschließender Extraktion mittels Proteinfällung oder Festphasenextraktion. Die so gewonnenen Extrakte wurden dann per LC-HR-MS^n analysiert. Darüber hinaus benutzten wir humane *N*-Acetyltransferasen 1 (NAT1) und NAT2 um die beobachteten *N*-Acetylierungen genauer charakterisieren zu können. Als Hauptreaktionen des Phase I und II Metabolismus konnten die Esterhydrolyse, die Deethylierung, die Hydroxylierung, die *N*-Acetylierung sowie Kombinationen von diesen identifiziert werden. Für die *N*- Acetylierung von DMC stellte sich die NAT2 als wichtigstes Enzym dar.

2 SUMMARY

Among the novel substance classes, which appeared on the drugs of abuse market in the last years, also synthetic cocaine analogs were identified. One of them, dimethocaine (DMC, larocaine, 3-diethylamino-2,2-dimethylpropyl)-4-aminobenzoate) was already marketed as local anesthetic in the 1930s showing also psychoactive effects and risk of addiction. Nowadays, DMC is sold as cocaine replacement. The aim of this work was to study its *in vivo* and *in vitro* metabolism by means of liquid chromatography-(high resolution)-mass spectrometry (LC-HR-MS^n) techniques. DMC was administered to male Wistar rats and pooled urine samples were collected for 24h. The urine was then prepared by enzymatic cleavage, solid phase extraction or protein precipitation. The extracts were then analyzed by LC-HR-MS^n. Furthermore, human *N*-acetyltransferase 1 (NAT1) and NAT2 were used for characterizing the N-acetylation. The main phase I and II reactions observed were ester hydrolysis, deethylation, hydroxylation, *N*-acetylation, and combination of them. NAT2 was identified to be the most relevant enzyme for DMC *N*-acetylation.

3 INTRODUCTION

In the last years, numerous compounds such as synthetic cocaine analogs appeared on the drugs of abuse market mainly to overcome legislation issues. Dimethocaine, already marketed as local anesthetic in the 1930s (Figure 1, DMC, larocaine, 3-diethylamino-2,2-dimethylpropyl)-4-aminobenzoate), was one of them. However, only a few years after introduction into the market, DMC was removed due to this psychoactive effects and risk of addiction. Nowadays, DMC caught the attention of the world wide drugs of abuse market and it is sold as cocaine replacement (http://research-chemicals-direct.com/acatalog/Dimethocaine__Larocaine_.html 2013 Nov 07).

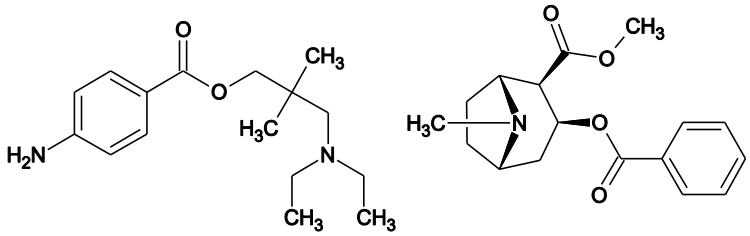


Figure 1: Chemical structures of DMC and cocaine

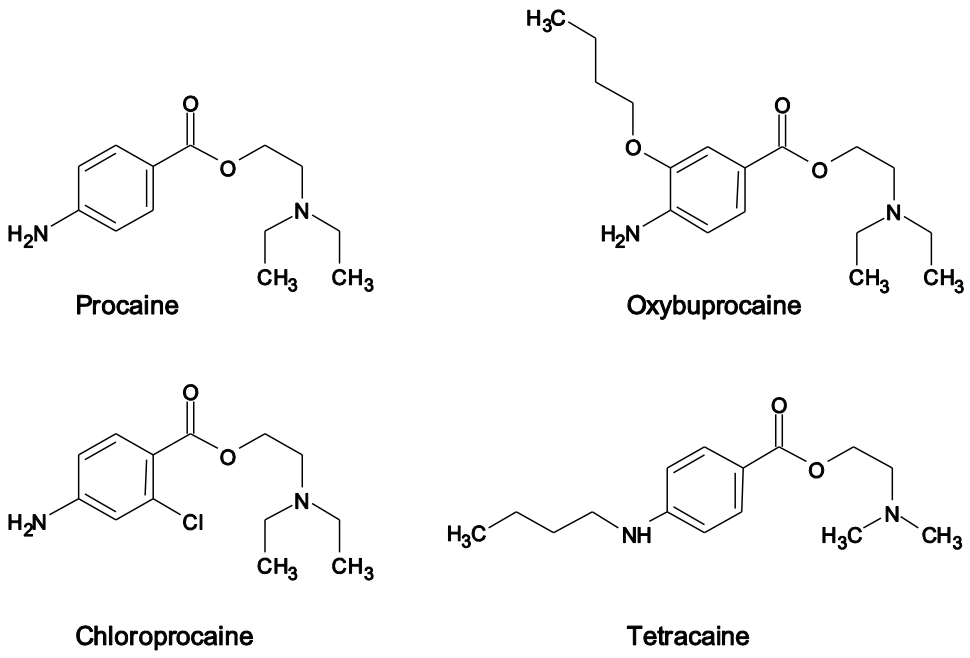


Figure 2: Chemical structure different local anesthetics of amino-ester-type.

3.1 PHARMACOLOGY AND TOXICOLOGY OF DIMETHOCAINE

As expected for a central nervous system (CNS) stimulant, DMC acts mainly as a dopamine-reuptake-inhibitor [1-4]. Like cocaine, DMC is snorted since oral ingestion would lead to rapid hydrolysis of DMC [5]. After consumption of DMC, consumers describe feelings of euphoria but also dyspnea and complaints of angina pectoris (www.eve-rave.ch/Forum/viewtopic.php?f=38&t=15752, 2013 Nov 07). The intravenous abuse is described to produce an initial flush and an alert or sometimes even relaxed feeling in the first minutes after injection accompanied with side effect such as a strong hangover and fatigue (www.land-der-traeume.de, 2013 Nov 07). All in all, DMC is reported to have similar effects like cocaine (www.salvia-community.net, 2013 Nov 07). Graham and coworkers recently compared the pharmacological properties of DMC and cocaine in the rat via intraperitoneal injection [3]. Afterwards, levels of DA and its metabolites were measured 10, 25, and 40 min after application. DMC was shown to have high affinity for the DA transporter mainly in the nucleus accumbens stimulating the reward system. Woodward *et al.* described DMC to be similarly potent as cocaine concerning the DA-reuptake-inhibitor efficiency [4]. Comparison of the pharmacological potencies of different local anesthetics revealed the following potency order cocaine > DMC > tetracaine > procaine > chloroprocaine [1]. However, nothing is known so far about its toxicokinetics such as metabolism and elimination.

Figure 3: Advertisement of DMC as a “research chemical”

3.2 ROLE OF THE *N*-ACETYLTRANSFERASES NAT1&2 AND THEIR GENETIC POLYMORPHISM

Arylamine N-acetyltransferases (NATs) belong to a special family of enzymes that are involved in the activation and detoxification of aromatic amines [6;7]. Two isoforms of the NAT enzymes are known, NAT1 and NAT2. They acetylate amino-, hydroxyl-, and sulfhydryl-groups in phase II metabolism of different xenobiotics and carcinogens. The typical chemical reactions of the NAT enzymes are shown in Figure 4.

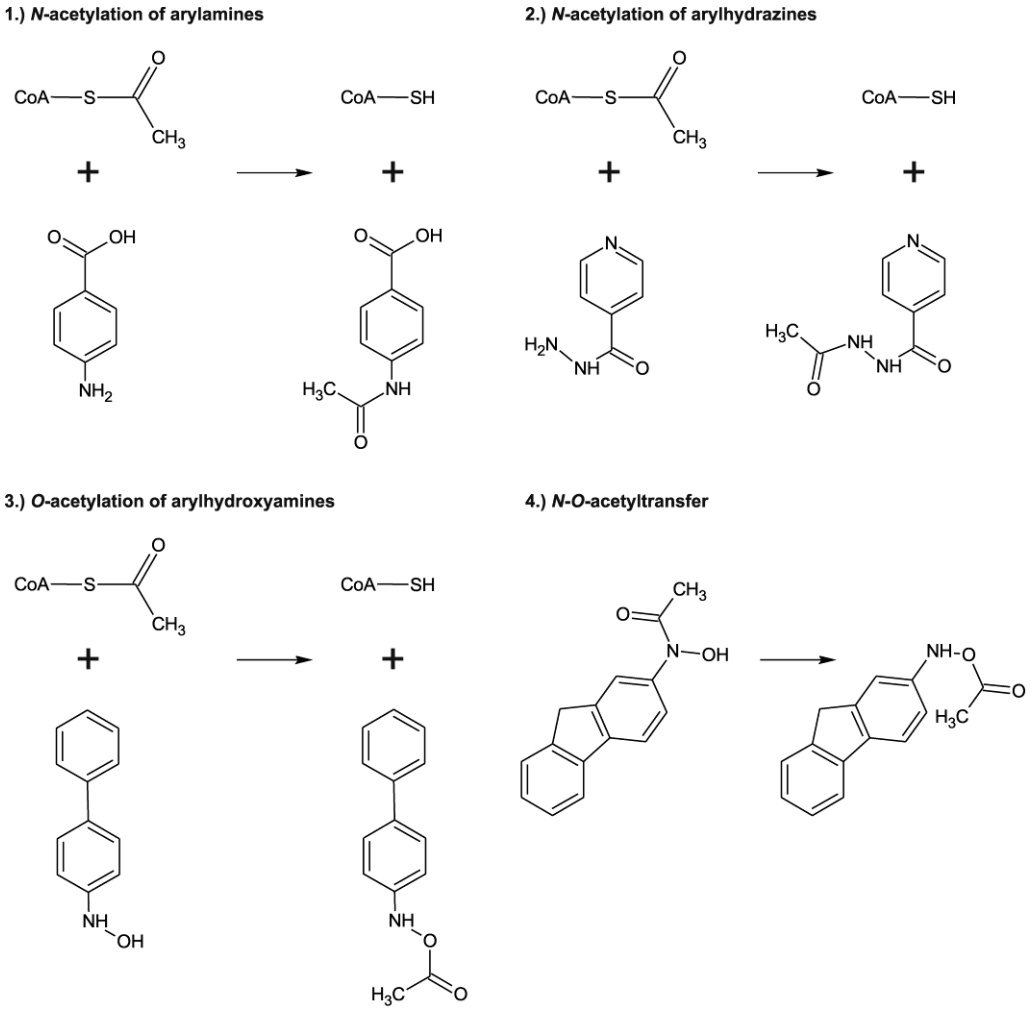


Figure 4: Reactions catalyzed by NATs. *N*-acetylation from acetyl-CoA of arylamine (para-aminobenzoic acid is shown) (A), arylhydrazines (isoniazid is shown) (B), *O*-acetylation of *N*-arylhydroxylamine (the carcinogen N-hydroxy-4- aminobiphenyl is shown) (C). In (D) *N*,*O* acetyl transfer of an *N*-hydroxamic acid is shown (the carcinogen N-hydroxy-2- acetylamino)-fluorene) [8].

The *N*-acetylation reactions of (A) and (B) use acetyl-CoA as cofactor and leading generally to inactivation of carcinogens or drugs. The *O*-acetyl transferase reaction (C) also uses acetyl-CoA as cofactor but the *N,O*-transfer reaction (D) occurs without acetyl-CoA as a cofactor. The both last reactions are activating reactions and generate *N*-acetoxy esters, which lead to the production of carcinogenic nitrenes [8]. The *N*-acetylation is a typical detoxifying reaction and is generally catalyzed by NAT2. In *O*-acetylation of *N*-hydroxyl aromatic amines NAT1 has a major role and leads to the activation of this metabolites [9].

Genes for human NAT1 and NAT2 are localized on chromosome 8. Both enzymes NAT1 and NAT2 are highly polymorphic and can create individual variations in the biotransformation of aromatic amines [9]. The NAT polymorphism was one of the first examples of pharmacogenetic variations, which were described. Popular is the example of the antitubercular drug isoniazid, which was the reason for further investigation on the NAT metabolism and its polymorphism [8]. This polymorphism is caused by a switch of nucleotides in the DNA sequence of the NAT genes (single nucleotide polymorphism, SNP) [10]. Because of combination of these switches of nucleotides, it can be possible that one or more amino acids in the NAT proteins are different. As a result of this exchange of amino acids for example the NAT2 enzyme cannot work in the same way a normal NAT2 enzyme could, or it can work even better, depending on the way the molecular structure of the protein changed. Because of this polymorphism we can divide populations in slow or poor acetylation phenotype, intermediate acetylation phenotype and rapid acetylation phenotype. Depending on the NAT polymorphism, persons of the rapid acetylation group catalyze and inactivate administered drugs faster than persons of the slow acetylation group. This is important to know and explains why some persons need higher doses of drugs than others and why on the other hand slow acetylators have more negative side effects or a higher risk of specific cancers, for example bladder cancer, because many cancer-causing substances were

metabolized by the NAT isoenzymes [8]. But also an overexpression of the NAT enzymes is possible and was detected in a special subpopulation of breast cancer cells [11]. This could be explained by the activating properties of the NAT, and because of this it could be possible that some special carcinogens are formed faster. A typical substrate for NAT1 is for example para-aminobenzoic acid and for NAT2 sulfamethazine [6;12]. However, a lot of drugs and cancer-causing substances, such as 2-aminofluoren, cannot be allocated to one of the two isoenzymes, because they are substrate of both.

4 AIM OF THE STUDY

The aim of the presented study was to examine the dimethocaine *in vivo* metabolism by the rat and the involvement of *N*-acetyltransferase (NAT) isozymes in the main metabolic steps to clarify whether a higher risk of increased toxic side effects in poor metabolizer subjects and of drug-drug or drug-food interactions with this emerging drug of abuse can be expected.

5 EXPERIMENTAL PROCEDURES

5.1 CHEMICALS AND REAGENTS

DMC was obtained from LGC (Teddington, UK), Isolute HCX cartridges (130 mg, 3 mL) from Biotage (Uppsala, Sweden), $NADP^+$ from Biomol (Hamburg, Germany), and isocitrate, isocitrate dehydrogenase, carnitin-acetyl-transferase (from pigeon breast muscle), and acetyl-d,l-carnitine from Sigma-Aldrich (Taufkirchen, Germany). All other chemicals and reagents were from VWR (Darmstadt, Germany) and of analytical grade. Baculovirus-infected insect cell-expressed NAT1 (human arylamine *N*-acetyltransferase 1*4, wild-type allele) and NAT2 (human arylamine *N*-acetyltransferase 2*4, wild-type allele) as well as control cell cytosol were from BD Biosciences (Heidelberg, Germany). After delivery, the cytosols were thawed at 37°C, aliquoted, snap-frozen in liquid nitrogen, and stored at -80°C until use.

5.2 URINE SAMPLES

The *in vivo* metabolism studies were performed using urine of male rats (Wistar, Charles River, Sulzfeld, Germany) for toxicological diagnostic reasons corresponding to the German law (http://www.gesetze-im-internet.de/tierschg/BJNR012770972.html, 2013 Nov 07). They were administered a single (high) dose of 20 mg/kg body mass dose of DMC in aqueous suspension by gastric intubation for identification of metabolites. The rats were placed in metabolism cages for 24 h, having water *ad libitum*. Urine was collected as a pooled sample separately from the feces over a 24-h period. All samples were directly analyzed and then stored at -20°C. Blank urine samples were collected before drug application to check if samples were free of interfering compounds.

5.3 SAMPLE PREPARATION FOR THE IDENTIFICATION OF THE PHASE I METABOLITES BY LC-HR-MS^n

According to Welter et al. [13], a 2.5 mL portion of urine was adjusted to pH 5.2 with acetic acid (1 M, approximately 50 µL) and incubated at 56°C for 1.5 h with 50 µL of a mixture (100,000 Fishman units/mL) of glucuronidase (EC No. 3.2.1.31, E. Merck, Darmstadt, Germany) and arylsulfatase (EC No. 3.1.6.1, E. Merck, Darmstadt, Germany), from *Helix Pomatia* L. The urine sample was then diluted with 2.5 mL of water and loaded on a HCX cartridge, previously conditioned with 1 mL of methanol and 1 mL of water. After passage of the sample, the cartridge was washed with 1 mL of water, 1 mL of 0.01 M hydrochloric acid, and again with 1 mL of water. The retained non-basic compounds were first eluted into a 1.5 mL reaction vial with 1 mL of methanol (fraction 1), whereas the basic compounds were eluted in a second step into a different vial with 1 mL of a freshly prepared mixture of methanol/aqueous ammonia 32% (98:2 v/v, fraction 2). The eluates were gently evaporated to dryness under a stream of nitrogen at 56°C and reconstituted in mobile phase A/B (1/1).

5.4 SAMPLE PREPARATION FOR IDENTIFICATION OF PHASE II METABOLITES BY LC-HR-MS^n

Formation of glucuronides and sulfates was elucidated as described previously [13;14]. Briefly, 200 µL of urine was mixed with 200 µL of acetonitrile, centrifuged at *14,000g* for 5 min, and 10 µL of the supernatant were injected onto the LC-HR-MS system.

5.5 INCUBATIONS WITH NAT1 AND NAT2

Incubations were performed at 37°C with 50 µM DMC or the PS sulfamethazine using human NAT1, human NAT2, and control cytosol for 20 min. Besides enzymes and substrate,

incubation mixtures (final volume: 100 µL) consisted of TEA-Buffer pH 7.5 (triethanolamine 100 mM, ethylenediaminetetraacetic acid 500 mM, and dithiotreitol 50 mM) and CoA system (acetyl-CoA 1 mM, acetyl-carnitin 23 mM, and carnitin-acetyltransferase 0.08 U/µL). The CoA system was preincubated for 10 min and TEA-Buffer for 5 min at 37°C. Reactions were started by addition of the pre-warmed cytosol and stopped with 100 µL of an ice-cold mixture of methanol, containing the internal standard (diphenhydramine, 5µM). The solution was centrifuged for 2 min at 14,000*g*, 50 µL of the supernatant phase was transferred to an autosampler vial, and injected onto the LC apparatus for analysis.

For initial screening studies, incubations were performed with 50 µM of DMC or sulfamethazine and 0.05 mg/mL NAT1, NAT2, or control cytosol for 20 min.

Kinetic data of *N*-acetylation were deduced from incubations with an incubation time of 10 min and 0.05 mg/mL (NATs) protein concentration. Incubation time and enzyme concentration were chosen to be within a linear range of metabolite formation. Sulfamethazine and DMC were incubated using substrate concentrations ranging from 0.5 to 2000 µM (Table 1). Values were estimated by non-linear curve-fitting using GraphPad Prism 5.00 software (San Diego, CA). The Michaelis-Menten equation (Eqn (1)) was used to calculate apparent K_m and V_{max} values for single-enzyme systems.

$$(1)\ V = V_{max} \times [S] / K_m + [S]$$

Additionally a modified equation was used (Eqn (2)) to calculate estimated K_i representing the inhibition constant considering the total substrate concentration range.

$$(2)\ V = V_{max} \times [S] / K_m + [S] \times (1 + S/K_i)$$

The best kinetic model was selected, considering the randomness of the residuals, the standard errors of the estimates and the correlation coefficients.

5.6 LC-HR-MS^n APPARATUS FOR IDENTIFICATION OF PHASE I AND II METABOLITES

According to Welter et al. [13], the extracts were analyzed using a Dionex UltiMate 3000 RS pump (ThermoFisher Scientific, TF, Dreieich, Germany) consisting of a degasser, a quaternary pump and an UltiMate 3000 RS autosampler, coupled to a TF Velos Orbitrap Pro equipped with a heated electrospray ionization (HESI) II source. The LC column was a TF Hypersil Gold (150 x 2.1 mm, 1.9 µm) with gradient elution with 10 mM aqueous ammonium formate buffer containing 0.1 % (v/v) formic acid as mobile phase A and acetonitrile containing 0.1 % (v/v) formic acid as mobile phase B. The gradient and flow rates were programmed from 98 % to 0 % A at 500 µL/min within 21 min. Injection volume was 10 µL. The MS conditions for the OT were as follows: ESI, positive mode; sheath nitrogen gas flow rate of 40 AU; auxiliary gas, 20 AU; source voltage, 4 kV; source heater temperature, 400°C; ion transfer capillary temperature, 300°C; capillary voltage, 4 V; CID-MS/MS experiments were performed in a data-dependent scan mode (*m/z* 100-800). Other settings were as follows: normalized collision energies, 35%; minimum signal threshold: 100 counts; with a resolution of 30,000; isolation width, 1.5 u; activation Q, 0.25; activation time, 30 ms; dynamic exclusion mode, repeat counts 2, repeat duration 15 s, exclusion duration 15 s. The TF calibration mixture was used for daily mass calibration.

5.7 LC-MS APPARATUS FOR ANALYSIS OF NAT INCUBATIONS

The NAT incubation extracts were separated and quantified using an Agilent Technologies (AT, Waldbronn, Germany) AT 1100 series atmospheric pressure chemical ionization (APCI) electrospray LC-MSD, SL version and a LC-MSD ChemStation using the A.08.03 software. The general conditions were according to Peters et al. [15]. Briefly, gradient elution was achieved on a Merck LiChroCART column (125 x 2 mm I.D.) with Superspher 60 RP Select

B as stationary phase and a LiChroCART 10-2 Superspher 60 RP Select B guard column. The mobile phase consisted of 50 mM ammonium formate adjusted to pH 3 with formic acid (eluent A) and acetonitrile containing 1 mL/L formic acid (eluent B). The MS operated in full-scan mode (m/z 100-600). For quantification, the peak area ratios of the respective target ions ($M+H^+$) of DMC, acetylated DMC, and the internal standard diphenhydramine were used.

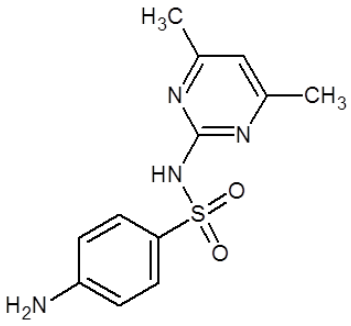


Figure 5: Probe substrate sulfamethazine

Table 1. Substrate concentrations used for NAT incubations [μM]

Sulfamethazine		**Dimethocaine**	
NAT1	NAT2	NAT1	NAT2
0.5	5	0.5	0.5
1	10	1	1
2.5	15	2.5	2.5
5	30	5	5
10	80	10	10
25	100	25	25
50	200	50	50
80	400	80	80
100	500	100	100
200	1000	200	200
400		400	400
500		500	500
1000		1000	1000
2000		2000	2000

6 RESULTS AND DISCUSSION

6.1 IDENTIFICATION OF THE PHASE I AND II METABOLITES BY LC-HR-MS^n

Via interpretation of the HR-MS^n spectra and fragmentation patterns depicted in Figure 6/7 and Figure 8/9, the following phase I and phase II reactions could be elucidated, respectively: ester cleavage (spectrum no. 2), N-deethylation (9), hydroxylation (8), N-deethylation and hydroxylation (5,7), bis hydroxylation (3), and N-bis deethylation and hydroxylation (4,6). *N*-acetylation (17, 19, 20, 21, 22, 23, 24), glucuronidation (10, 11, 12, 13, 14, 15, 16) and combination of both (18). The metabolic reactions are also summarized in Figure 10.

The urinary metabolites of DMC were identified by full-scan HR-MS^n after LC separation of rat urine extracts. The postulated structures of the metabolites were deduced from the fragments detected in the different MS stages, which were interpreted in correlation to those of the parent compound. LC-HR-MS^n allowed thorough identification of the metabolites based on their accurate masses. The numbers of the corresponding mass spectra in Figure 6 to 9 are given in brackets. The calculated and the measured masses and the delta values in ppm of all phase I and phase II metabolites are listed in Table 2. Fragments of protonated DMC (*m/z* 279.2071, spectrum 1 in Figure 6) could be interpreted as follows: cleavage next to the ester bond (*m/z* 120.0446, *p*-aminobenzoic part) and loss of water at the side chain (*m/z* 142.1593). Another pattern is cleavage of the side chain amine part (*m/z* 206.1181, loss of diethylamino part). Further fragmentation of the most abundant ion in the MS^2 (*m/z* at 142.1593) and in the stage of MS^3 led to the fragment ion at *m/z* 86.0964.

Table 2. Identified phase I and phase II metabolites with their measured and calculated masses and the delta values in ppm sorted according to increasing *m/z*.

Compound	**Accurate mass** ***m/z***	**Exact mass** ***m/z***	**delta** **ppm**
DMC	*279.2071*	*279.2067*	*1.49*
3-(Diethylamino)-2,2-dimethylpropanol	160.1695	160.1695	-0.79
DMC-HO-bis-deethyl isomer 1	239.1391	239.1390	0.52
DMC-HO-bis-deethyl isomer 2	239.1392	239.1390	0.65
DMC-deethyl	251.1754	251.1754	0.04
DMC-bis-deethyl *N*-acetyl	265.1547	265.1546	-0.02
DMC-HO-deethyl isomer 1	267.1703	267.1703	-0.11
DMC-HO-deethyl isomer2	267.1703	267.1703	-0.11
DMC-HO-bis-deethyl *N*-acetyl	281.1498	281.1495	0.91
DMC-deethyl *N*-acetyl	293.1858	293.1859	-0.50
DMC-HO	295.2015	295.2016	-0.27
DMC-HO-deethyl *N*-acetyl	309.1805	309.1808	-1.30
DMC-di-HO	311.1961	311.1965	-1.37
DMC-*N*-acetyl	321.2177	321.2172	1.37
3-(Diethylamino)-2,2-dimethylpropanol glucuronide	336.2019	336.2010	0.67
DMC-HO *N*-acetyl isomer 1	337.2128	337.2121	1.73
DMC-HO *N*-acetyl isomer 2	337.2728	337.2121	1.91
DMC-HO-bis-deethyl glucuronide	415.1710	415.1711	-0.35
DMC-HO-deethyl glucuronide	443.2012	443.2024	-2.65
DMC-deethyl-*N*-*O* glucuronide	443.2015	443.2024	-2.20
DMC-HO glucuronide	471.2332	471.2337	-1.09
DMC-*N*-*O* glucuronide	471.2332	471.2337	-1.11
DMC-di-HO glucuronide	487.2296	487.2286	-0.77
DMC-HO-*N*-acetyl glucuronide	513.2445	513.2442	0.45

Metabolites of DMC could be identified by comparing the different MS^n spectra considering the mass shifts caused by metabolic reactions and different elemental compositions. Deethylation of the tertiary amine led to the respective nor metabolite (*m/z* 251.1754, 9). A change of 28 u in the fragment ions of the side chain referred to the deethylation (*m/z* 142.1593 to *m/z* 114.1279 in MS^2 and *m/z* 86.0964 to *m/z* 58.0650 in MS^3). Hydroxylation of the aromatic ring system in spectra 8 lead to the protonated molecule at *m/z* 295.2015 in MS^1 and a shift of 16 u in the fragment ion corresponding to the *p*-aminobenzoic acid part (*m/z* 120.0446 to *m/z* 136.0395 and *m/z* 206.1181 to *m/z* 222.1129) in MS^2. Compounds represented by spectra nos. 5 and 7 are two isomers after deethylation and hydroxylation of the aromatic ring system. Compounds represented by spectra nos. 4 and 6 are isomers after bis-deethylation and hydroxylation of the aromatic ring system.

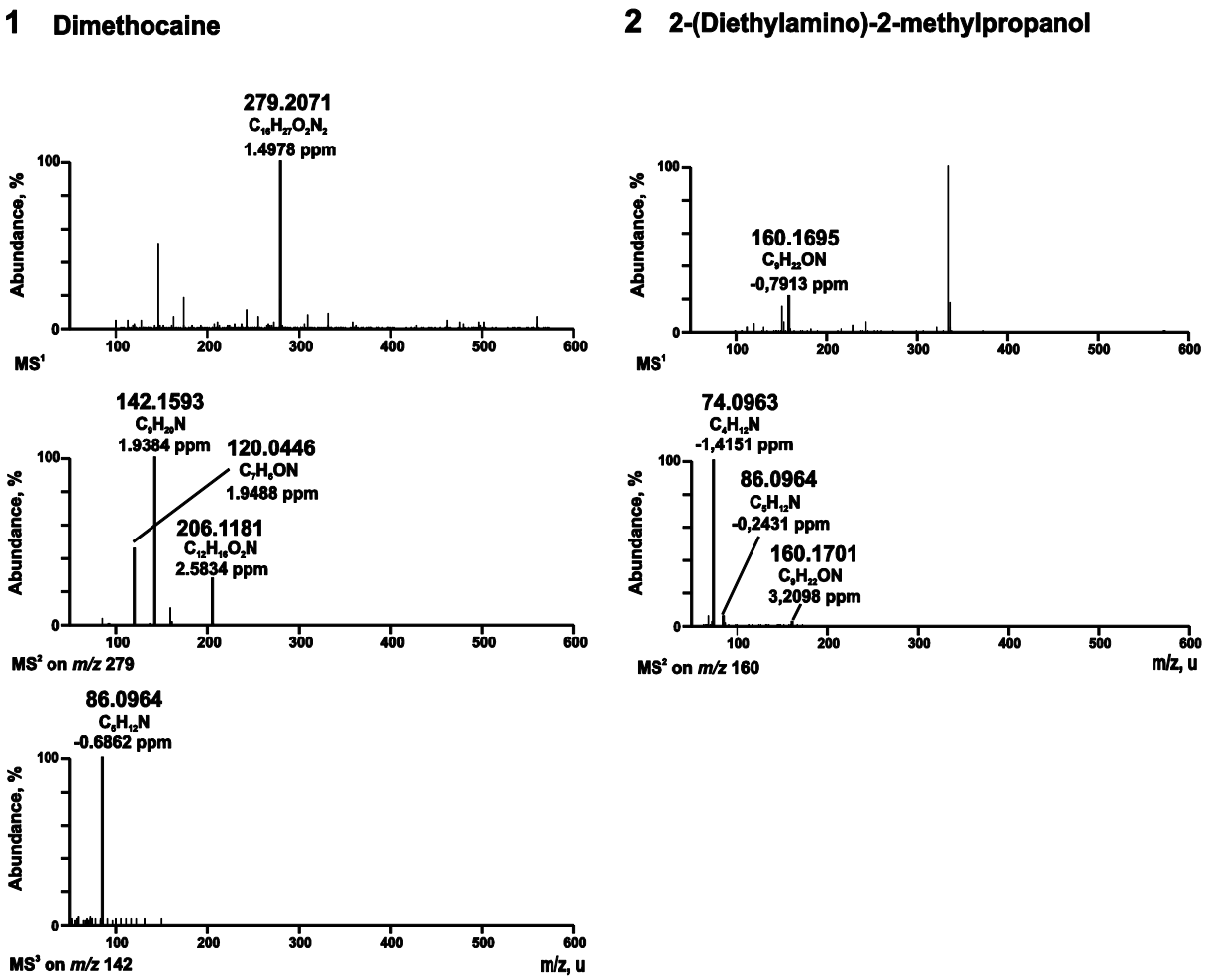


Figure 6: ESI HR-MS^n mass spectra of DMC and its phase I metabolites arranged according to their elution order.

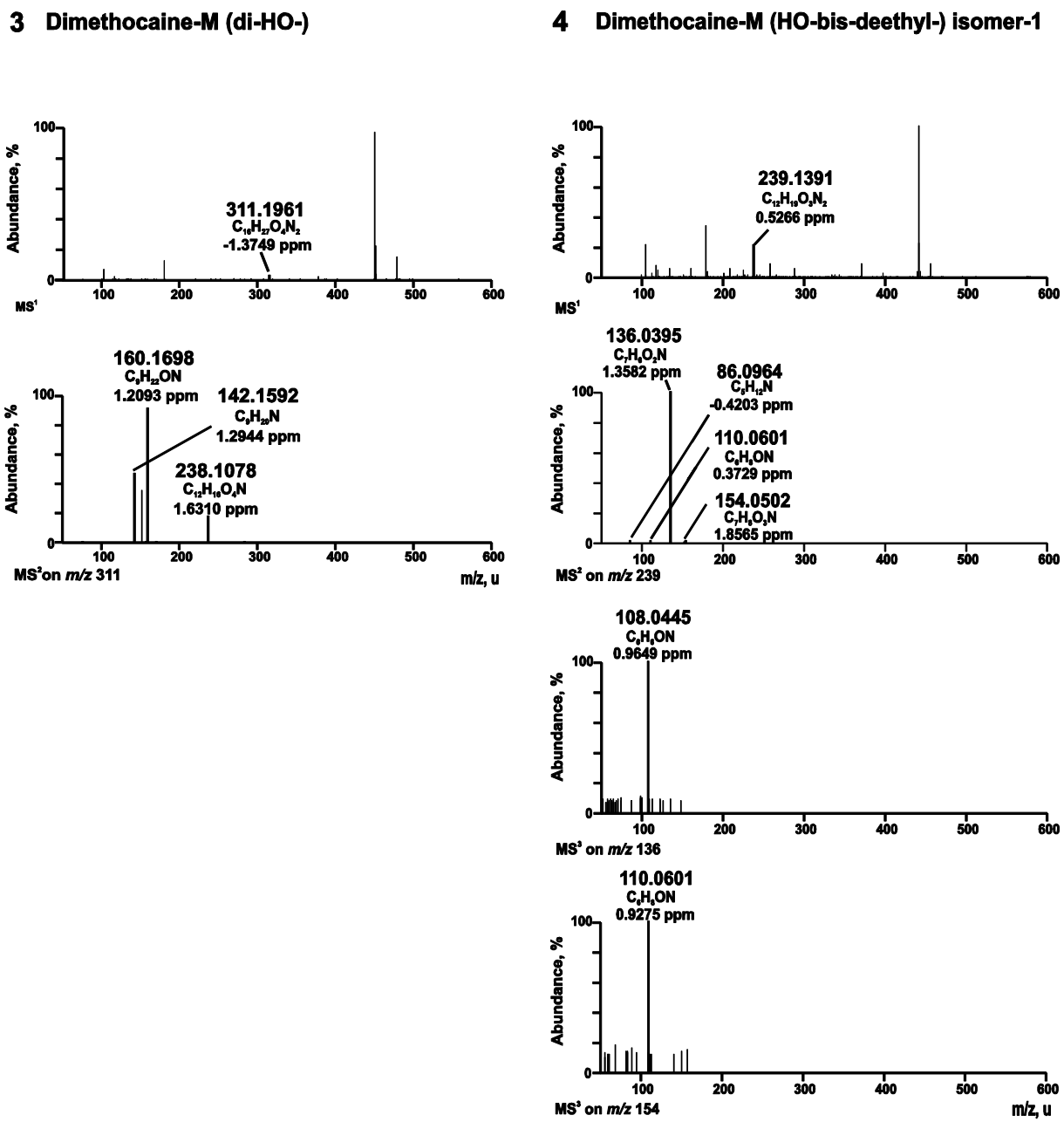


Figure 6 continued

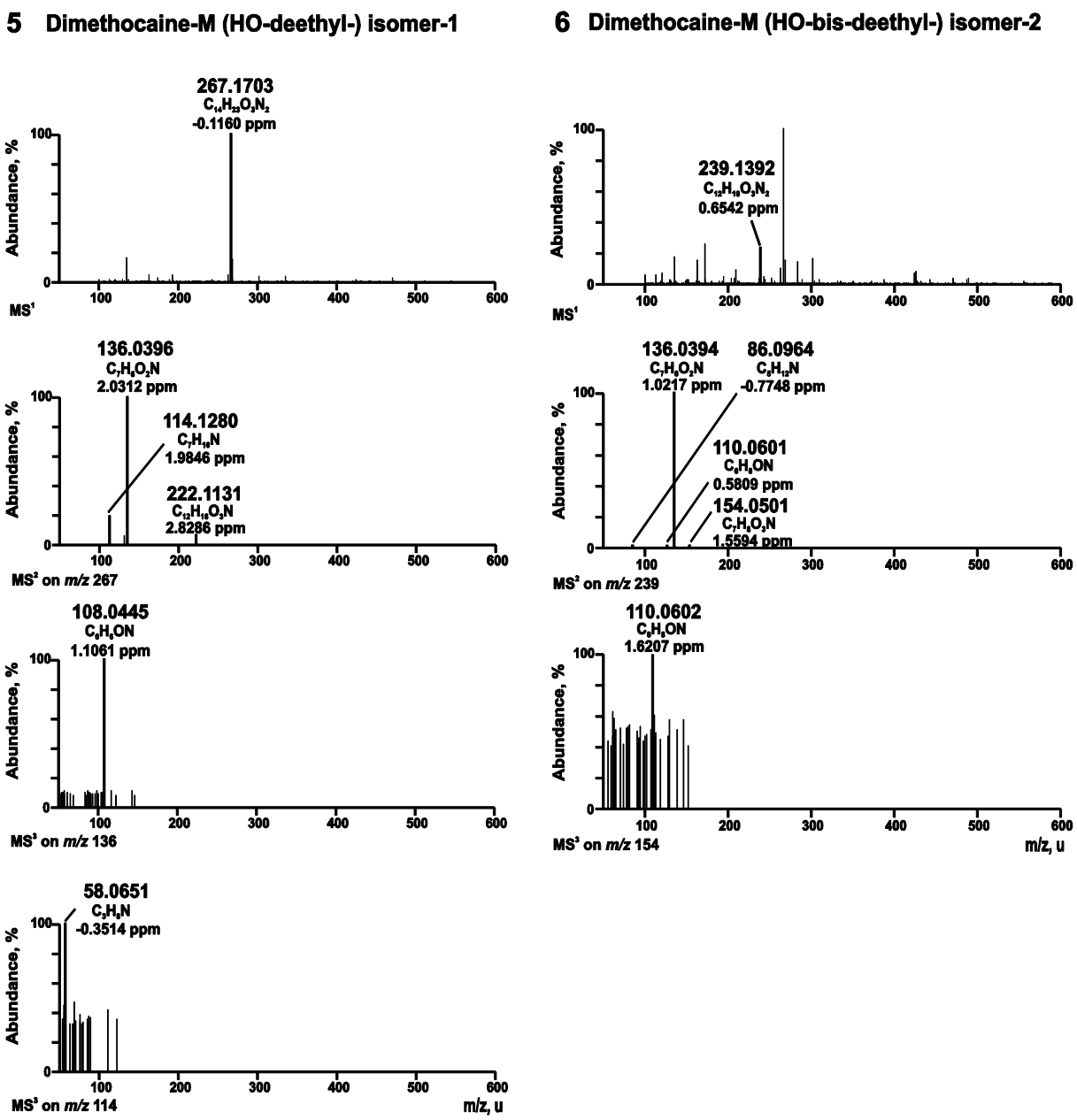


Figure 6 continued

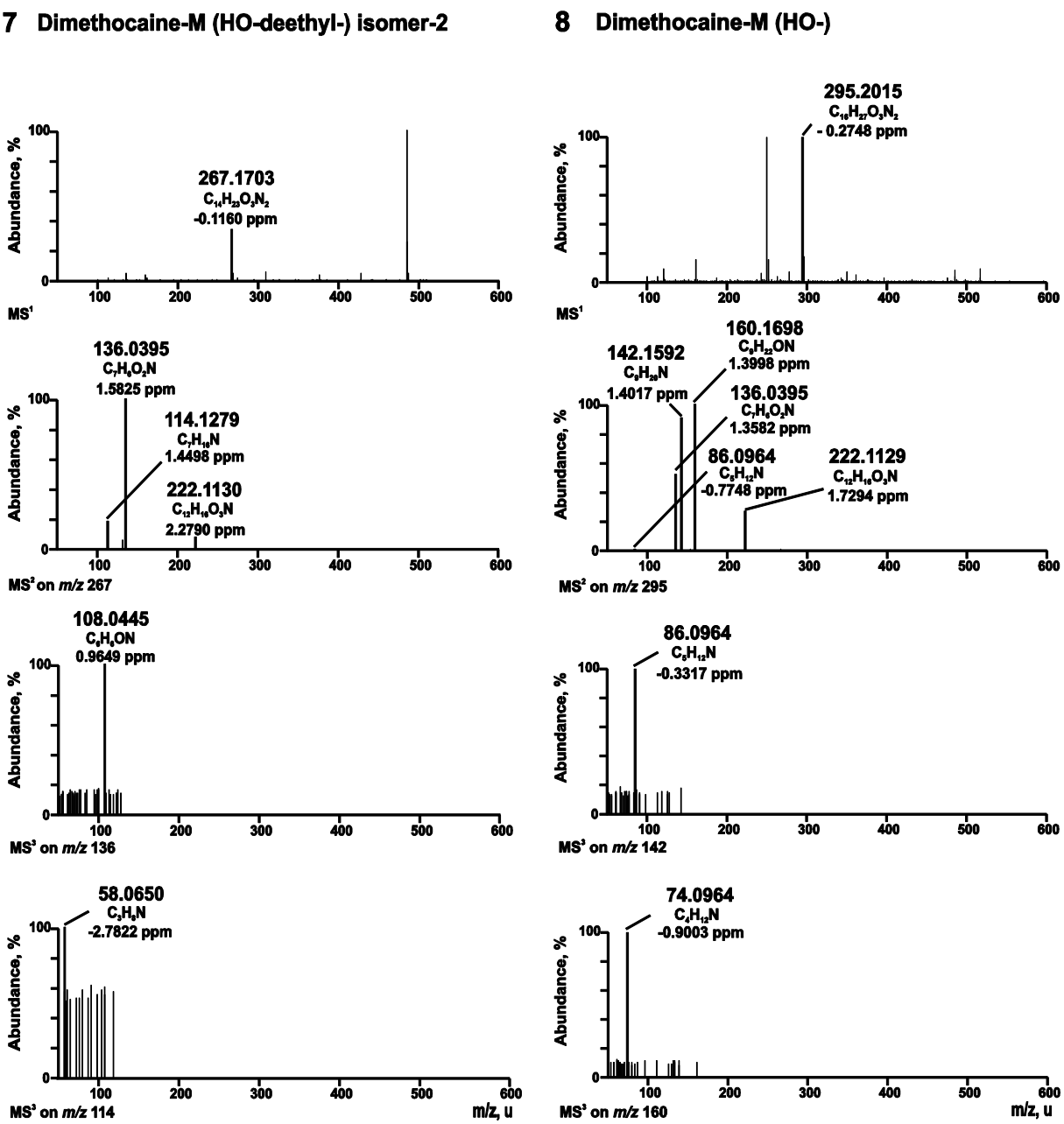


Figure 6 continued

9 Dimethocaine-M (deethyl-)

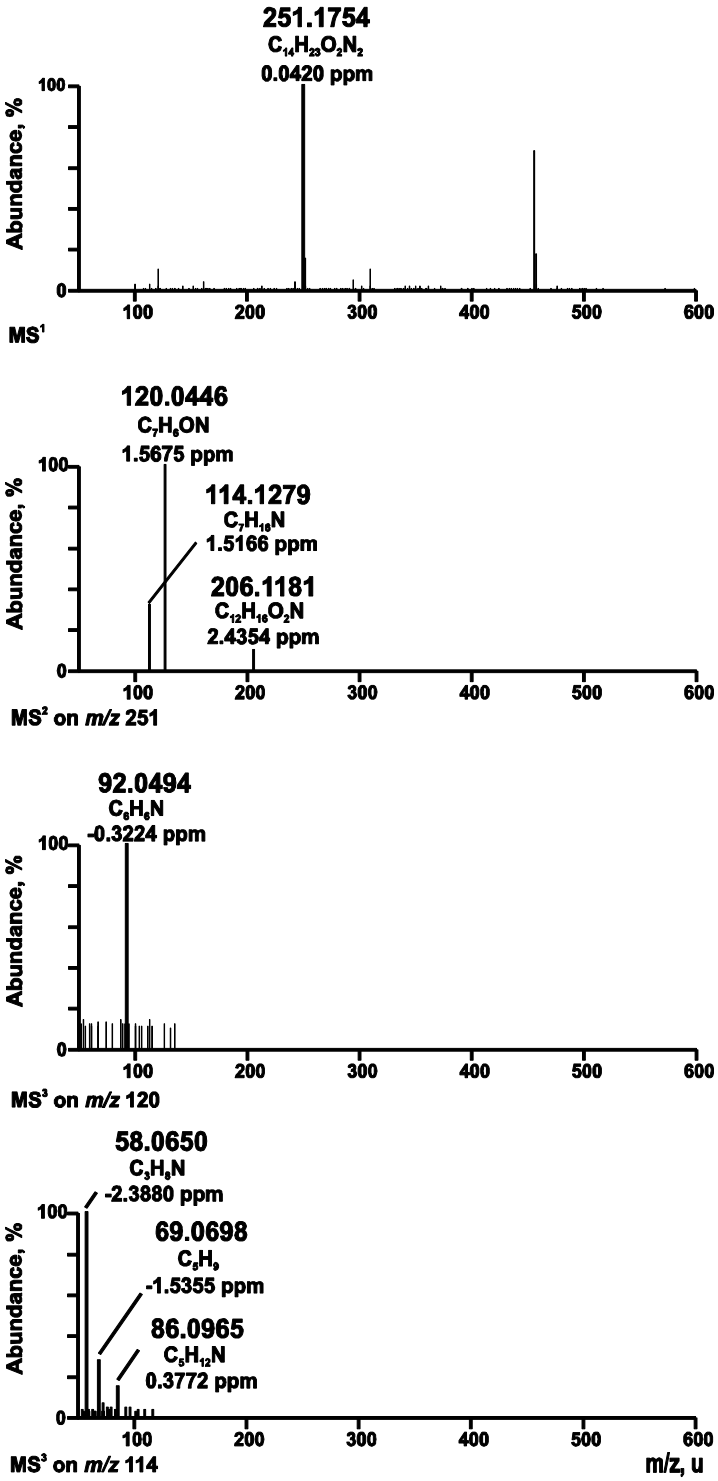


Figure 6 continued

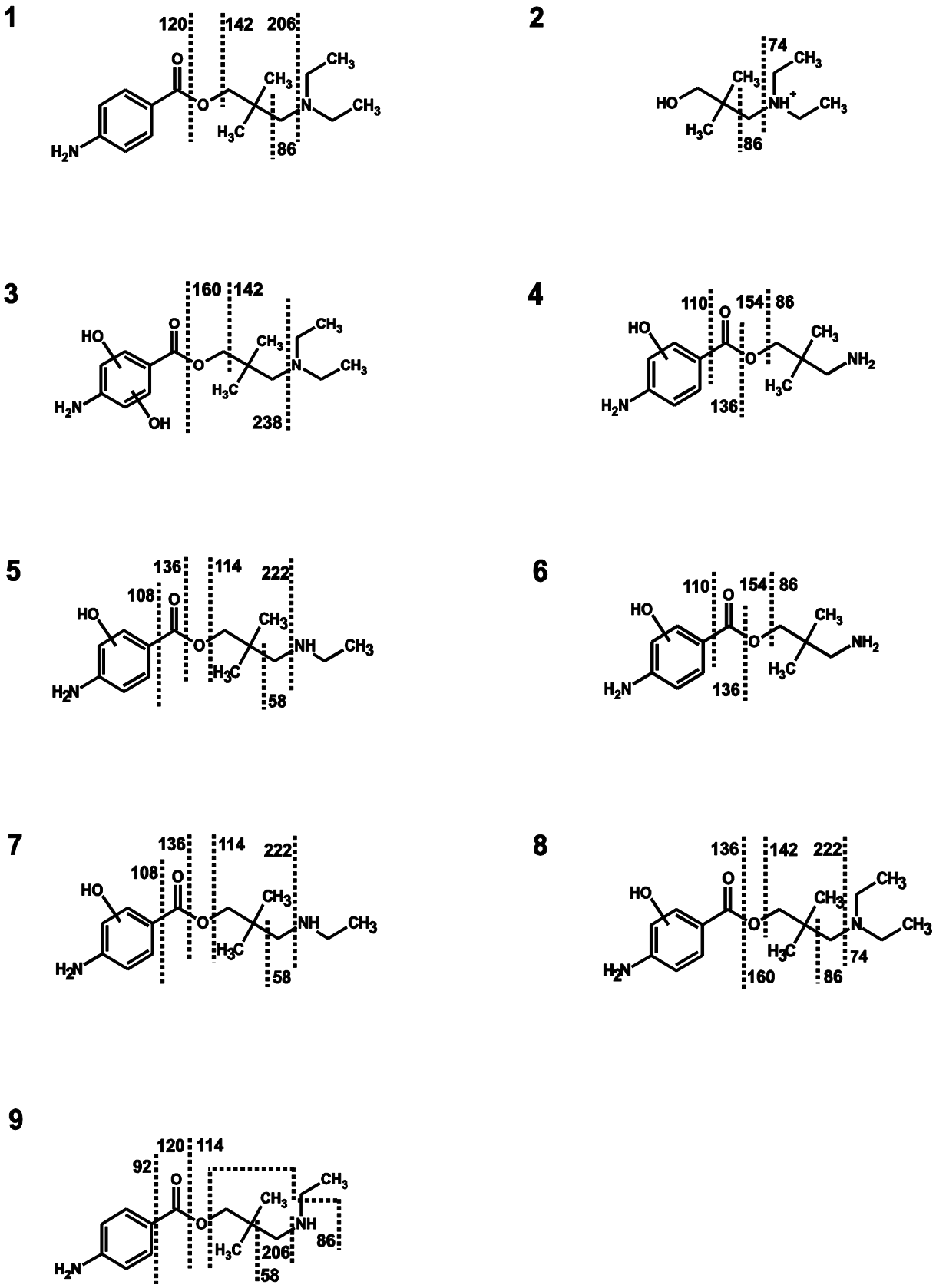


Figure 7: Proposed structures and predominant fragmentation patterns of DMC and its phase I metabolites. The numbers correspond to the ones in Figure 6.

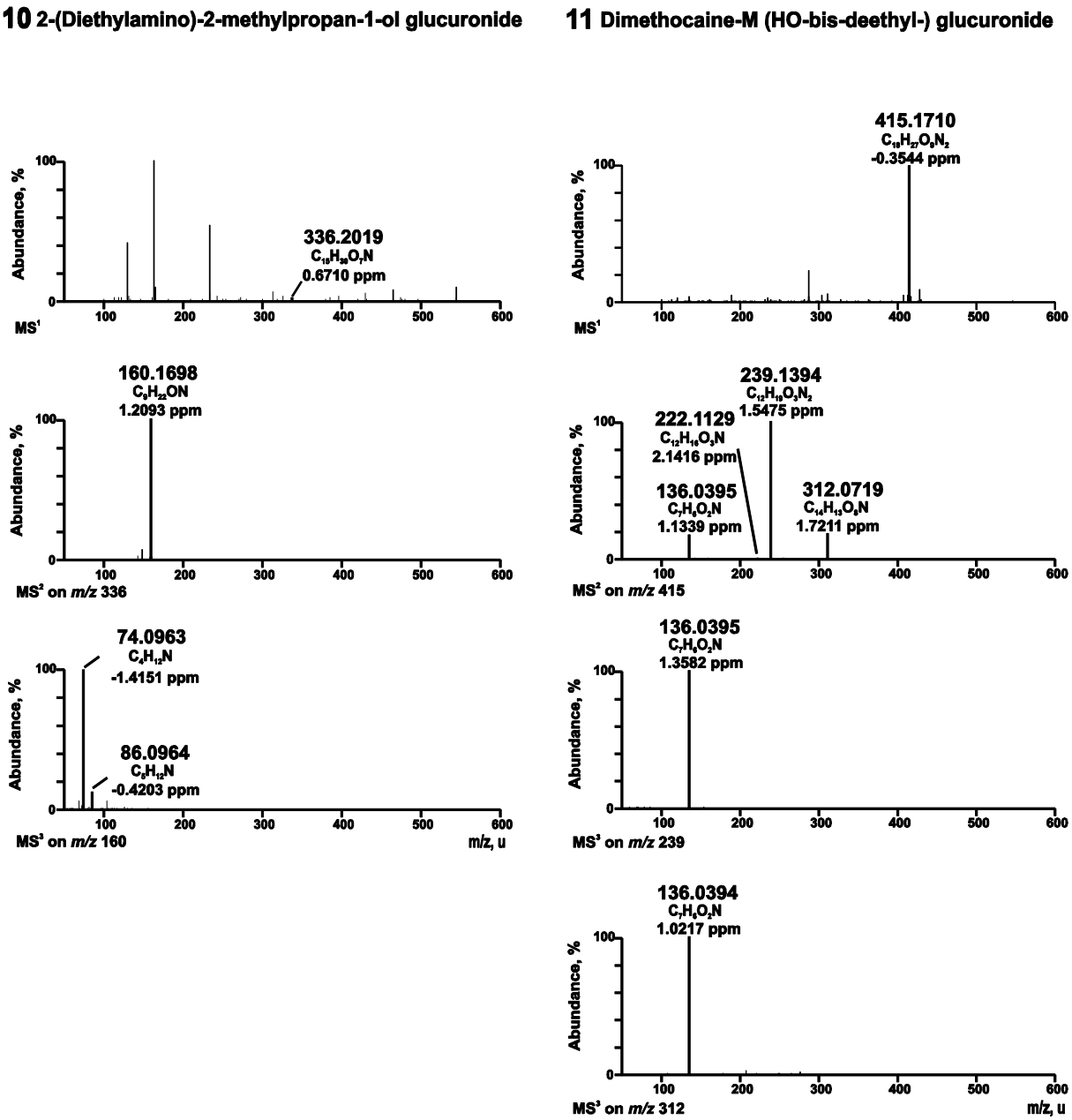
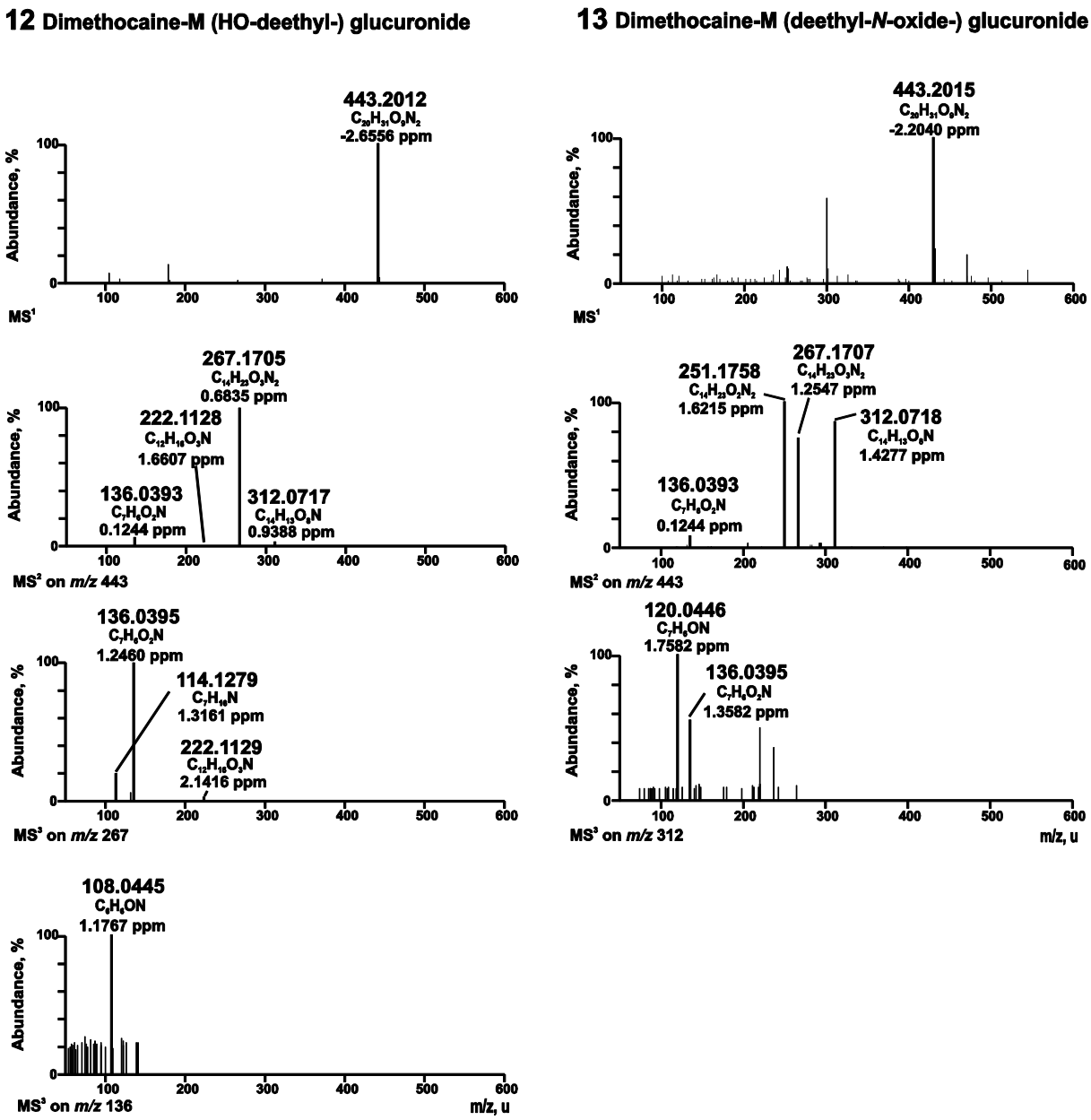


Figure 8: ESI HR-MS^n mass spectra of DMC and its phase II metabolites arranged according to their elution order.



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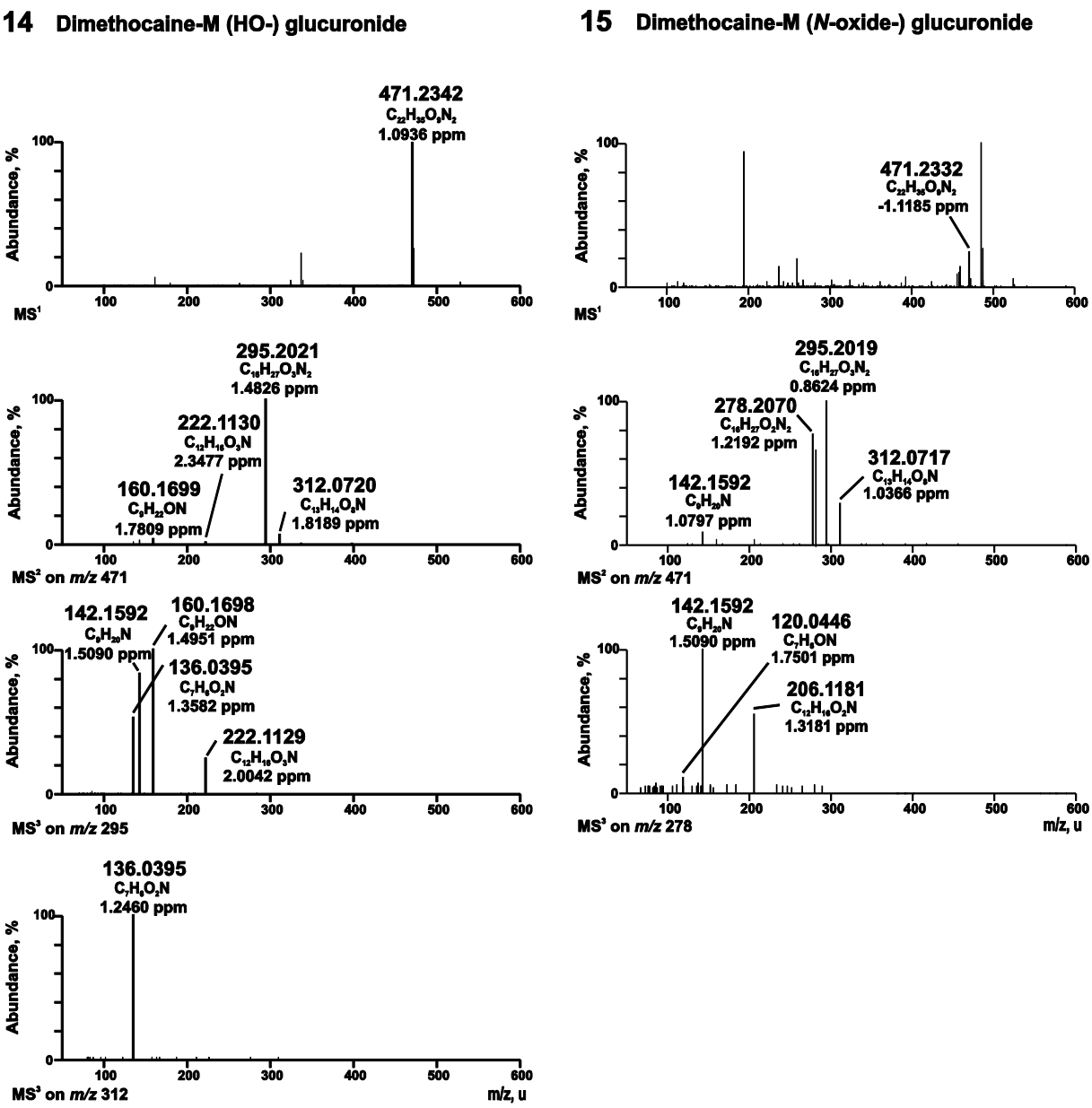

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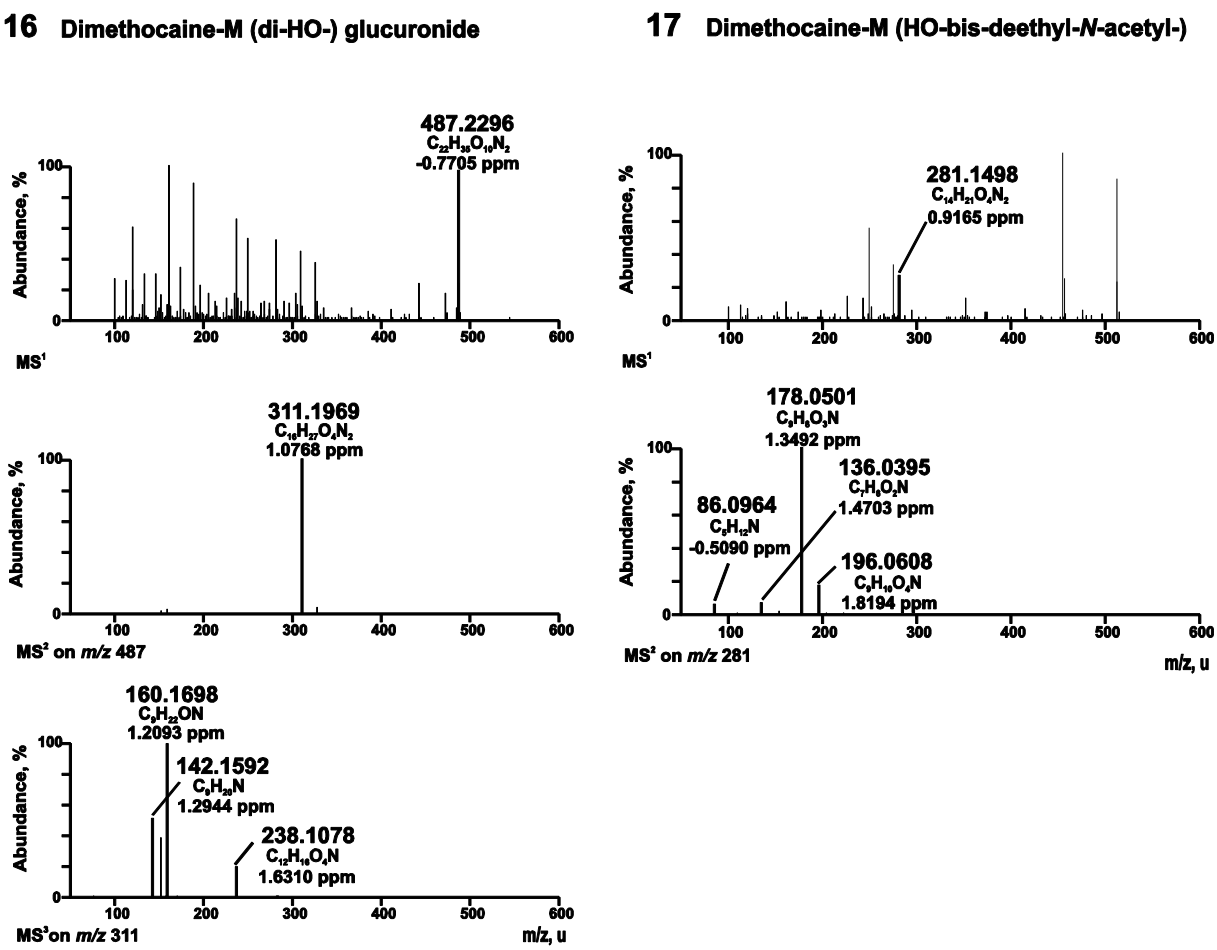

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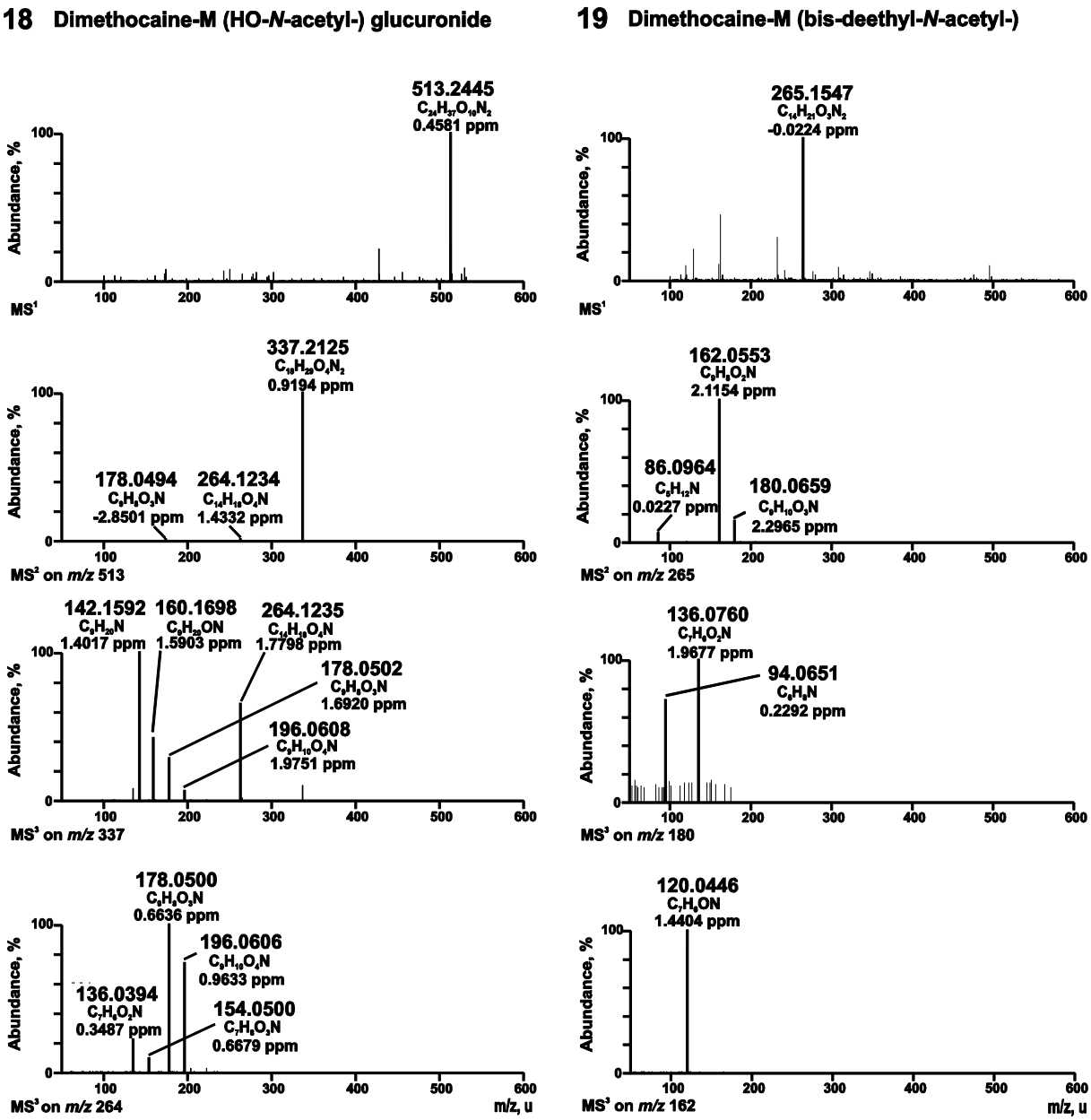

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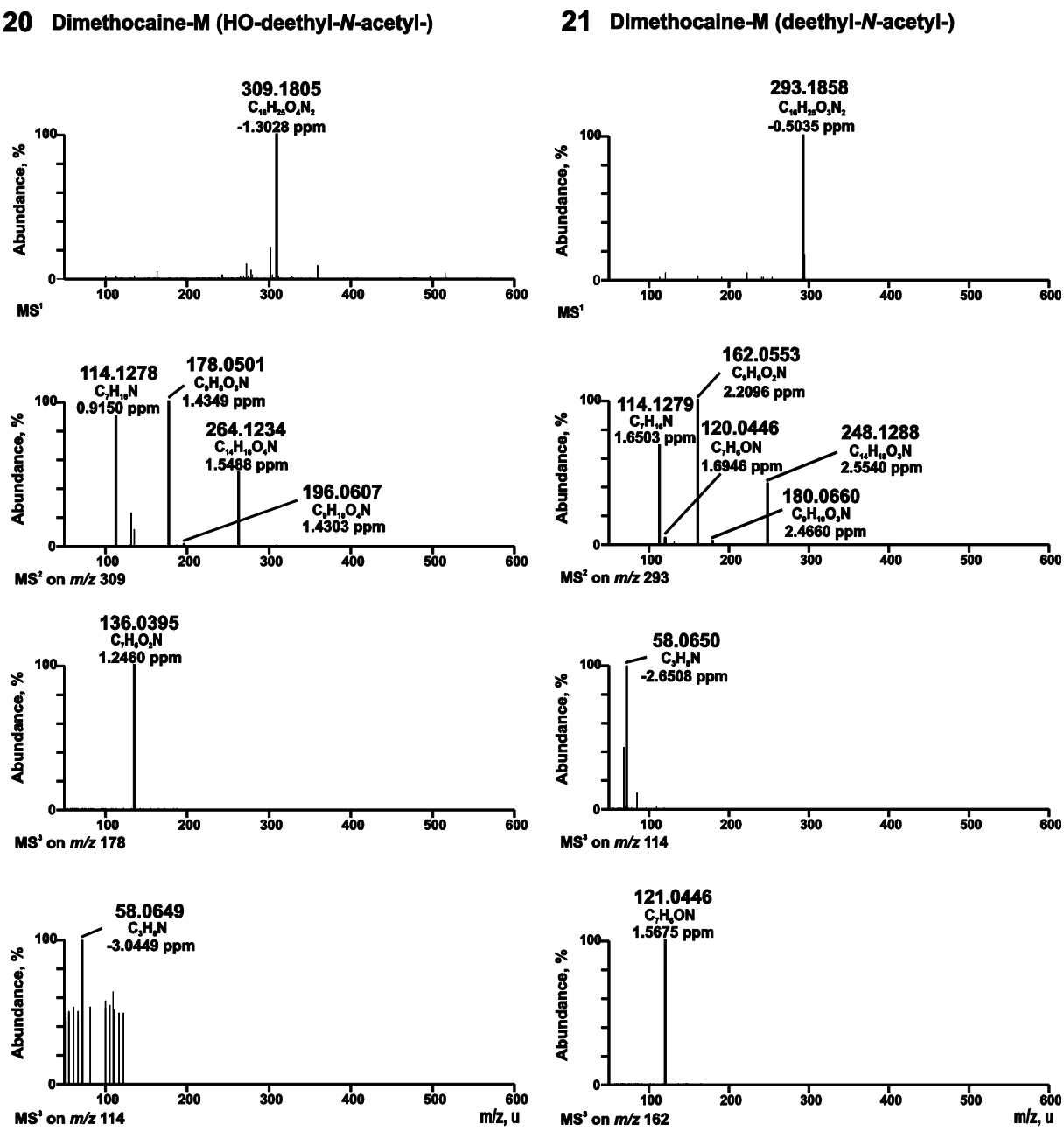

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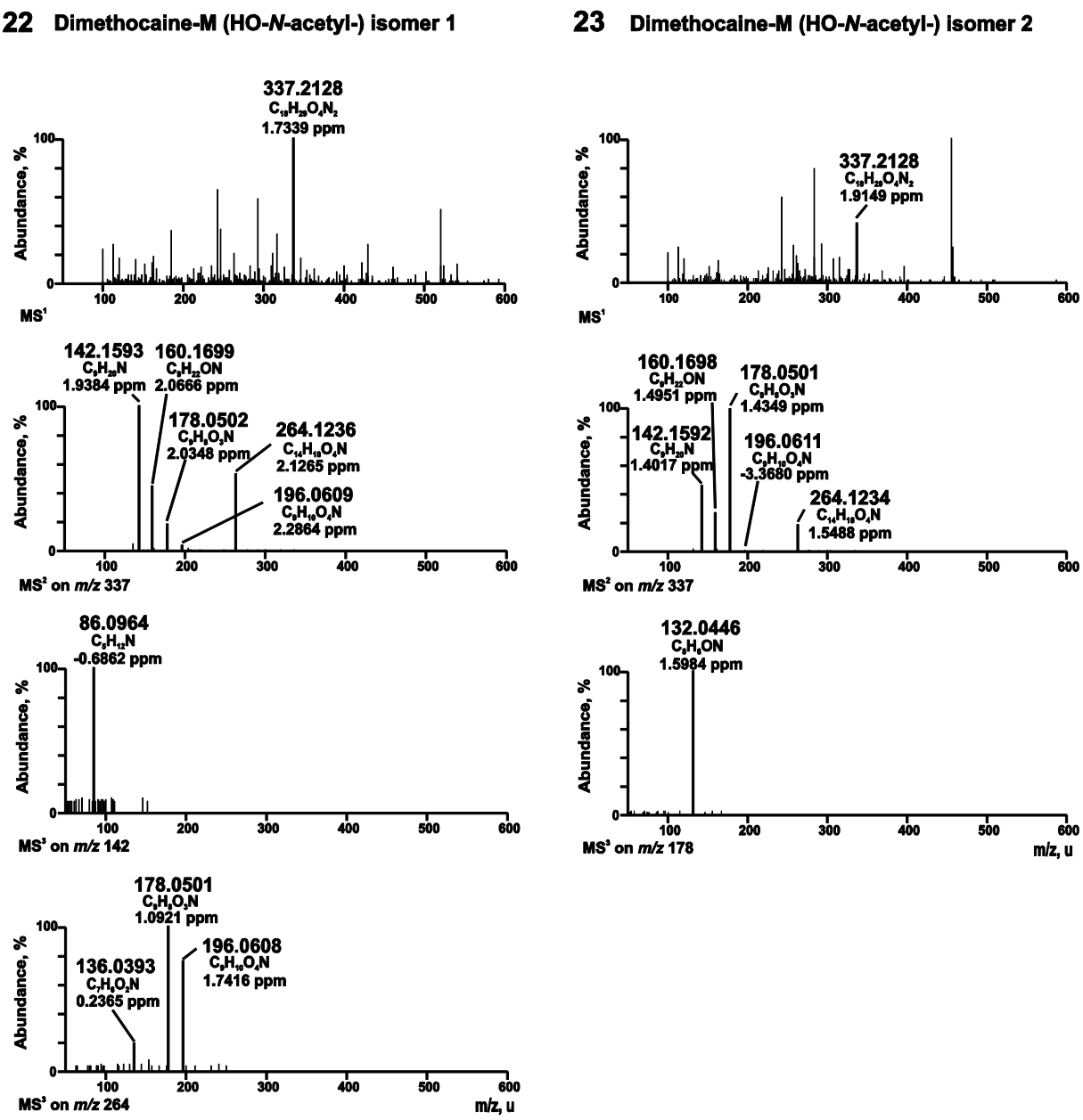
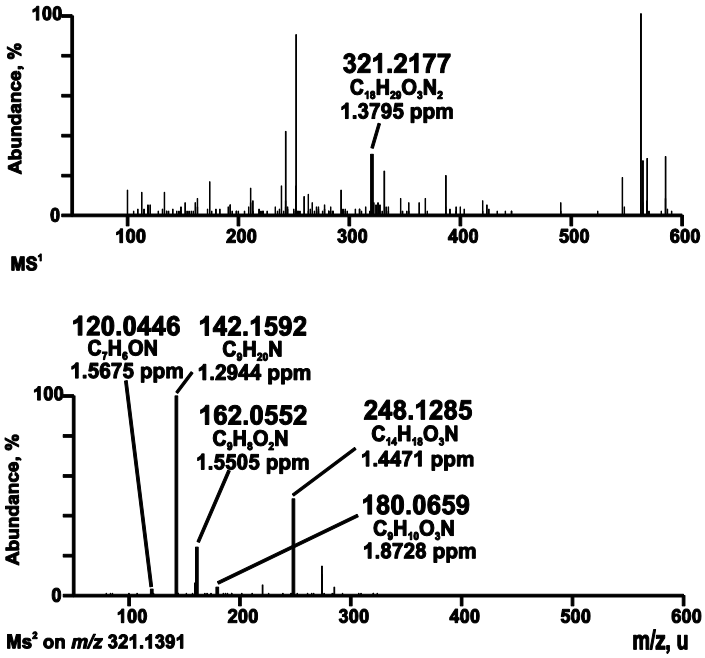


Figure 8 continued

24 Dimethocaine-M (*N*-acetyl-)



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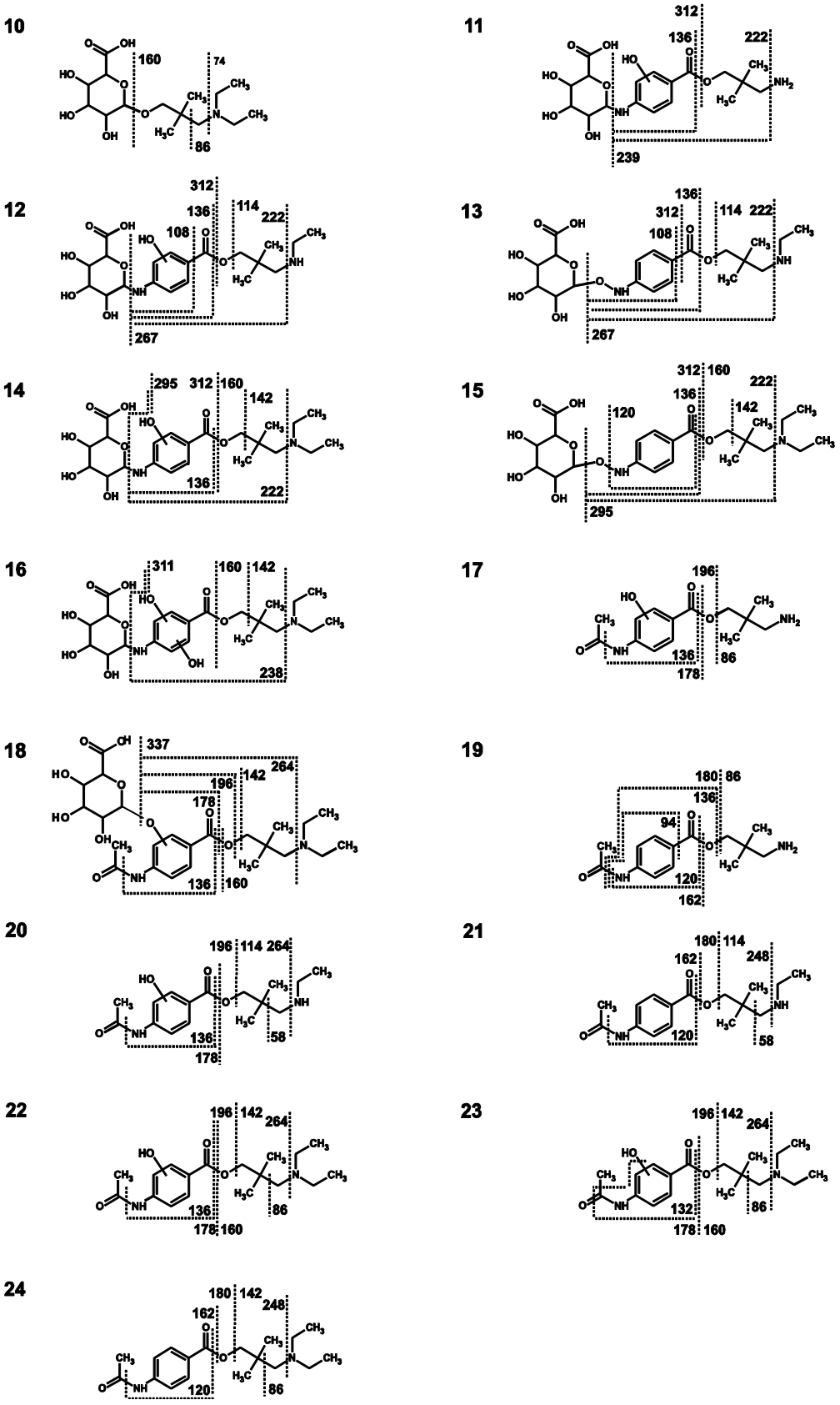

Figure 9: Proposed structures and predominant fragmentation patterns of DMC and its phase II metabolites. The numbers correspond to the ones in Figure 8.

N-acetylation was the most abundant step in phase II. Fragmentation protonated and acetylated DMC (*m/z* 321.2177, spectrum 24) could be interpreted as follows: cleavage next to the ester bond lead to the acetylated *p*-aminobenzoic acid part at *m/z* 162.0552 (*m/z* 120.0446 shift of 42 units). This shift is also present in spectra nos. 19 and 21. Beta-cleavage of the side chain led to the fragment ions at *m/z* 142.0446 and *m/z* 180.0659. Based on this fragmentation patterns, the acetylated metabolites of DMC could be identified by comparing the different MS^n spectra of the acetylated metabolites considering the mass shifts according to the different elemental compositions. For example in case of a hydroxylated aromatic ring system, the additional acetyl-group led to a shift from *m/z* 136.0395 to *m/z* 178.0502 (17, 18, 20, 22, and 23). The glucuronides were identified based on an accurate mass shift of *m/z* 176.0321 due to addition of the glucuronic acid part and interpretation of their MS^3 and MS^4 mass spectra.

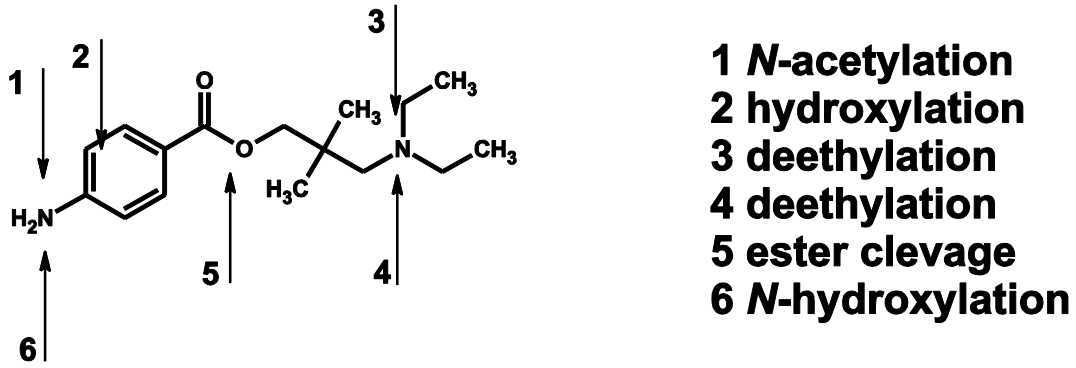

Figure 10: Structure of DMC with arrows indicating the described metabolic reactions.

6.2 NAT 1 AND 2 KINETIC STUDIES WITH DIMETHOCAINE AND SULFAMETHAZINE

Initial activity screening revealed that only NAT2 was capable catalyzing the *N*-acetylation of DMC. The kinetic profile of DMC acetylation by NAT2 fitted best into Michaelis-Menten kinetic. The K_m value was determined to be 102 µM, the V_{max} value to be 1.1 units/min/pmol. The kinetic profile of sulfamethazine *N*-acetylation followed best a substrate inhibition equation with K_m = 588 µM, V_{max} = 24 units/min/pmol, and K_i = 12, which fitted well to previously published data [6;16;17](Table 3).

Table 3. K_m (µM) and V_{max} values (dimensionless PAR/min/mg protein.) for acetylation of DMC and sulfamethazine by NAT2

DMC		**sulfamethazine**	
K_m	**V_{max}**	**K_m**	**V_{max}**
102 +/- 19	1.1 +/- 0.06	588 +/- 40	24 +/- 1.6

Besides the extensive *N*-acetylation of the *p*-aminobenzoic acid part of the parent compound, most of the phase I metabolites were excreted as *N*-acetyl derivatives. Therefore, DMC *N*-acetylation was investigated by incubation studies with human NAT1 and NAT2 to evaluate their ability to catalyze this most dominant metabolic step of DMC. The initial incubation conditions chosen were adequate to make a statement on the general involvement of the NAT isozymes. Sulfamethazine was used as suitable probe substrate with K_m values available for both NAT enzymes [6;16]. It seemed likely that isozyme NAT1 should be of importance for DMC *N*-acetylation because of its *p*-aminobenzoic acid structure, which was described to be a substrate of this enzyme [18]. However, initial activity studies showed that NAT2 played a more important role in the DMC metabolism than NAT1. This was further supported by

enzyme kinetic studies, where data could only be acquired for NAT2 due to a very low product formation in NAT1 incubations. The respective K_m and V_{max} values can be found in Table 3. Endogenous *N*-acetylation of aromatic or heterocyclic amines by NAT1 or NAT2 is an important metabolic part of many drugs. As both enzymes, NAT1 and NAT2, are highly polymorphic, individual variations in the biotransformation of aromatic amines may occur [9]. It is obvious that there might be individual pharmacokinetic differences of the endogenous *N*-acetylation of DMC, which may lead to increased negative side effects such as cardiotoxicity. Such interactions may be much more important for people with a slow acetylation phenotype, in fact 50% of all Europeans have the slow or intermediary acetylation phenotype [19]. To what extent these points affect the plasma concentrations of DMC and also the concentration of acetylated DMC metabolites in urine should be target of further studies.

In conclusion, the presented metabolism study demonstrated the extensive metabolism of DMC by the rat mainly via hydroxylation and deethylation as well as acetylation and glucuronidation. The endogenous *N*-acetylation as main part of DMC metabolism was catalyzed only by the NAT2 isozyme. Due to the enormous increase of new designer drugs and the corresponding health risks, it is an important issue to identify and to study new emerged substances. This study could therefore contribute to identification and detection of DMC by elucidating its metabolic pathways. Supposing similar kinetic processes in rats and humans, this study could serve as a basis for developing suitable screening strategies for detection of a DMC intake. This will be investigated in a further study.

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8 ABBREVIATIONS

3-MT	3-methoxy-tyramine
acetyl-CoA	Acetyl-coenzyme-A
AT	Agilent Technologies
AU	Arbitrary units
COMT	Catechol-O-methyltransferase
CNS	Central nervous system
Da	Dalton
DA	Dopamine
DMC	Dimethocaine
DOPAC	Dihydroxyphenylacetic acid
HCl	Hydrochloride
HR	High resolution
IS	Internal standard
LC	Liquid chromatography
NAT	(arylamine) *N*-acetyltransferase
MS	Mass spectrometry
PS	Probe substrate
SNP	Single nucleotide polymorphism
SPE	Solid phase extraction

9 DANKSAGUNG

„Vergiß den Anfang nicht, den Dank!“ *Albert Schweitzer*

Als ich anfing nach einer passenden Doktorarbeit zu suchen, war es mir vor allem wichtig, einen guten Betreuer zu finden. Jemanden der einem nicht nur fachlich mit Rat und Tat zur Seite steht, sondern der einen auch auf menschlicher Ebene bei allen Erfahrungen, Rückschlägen und Erfolgen begleiten kann. Genauso wichtig ist es, einen Doktorvater zu haben, der durch Kompetenz und uneingeschränktes Fachwissen, aber auch durch Offenheit und Freundlichkeit überzeugt, der einen Arbeitskreis leitet, in dem Kollegen mehr als nur die gemeinsame Arbeit verbindet, wo Freundschaften entstehen und trotzdem noch ausreichend Raum für Diskussionen ist.

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