Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 1047–1058

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Fenton's oxidation: A tool for the investigation of potential drug metabolites

Eva Kugelmann[a], Christian R. Albert[b], Gerhard Bringmann[b], Ulrike Holzgrabe[a,*]

[a] Institute of Pharmacy and Food Chemistry, University of Würzburg, Am Hubland, 97074 Würzburg, Germany
[b] Institute of Organic Chemistry, University of Würzburg, Am Hubland, Würzburg, Germany

ARTICLE INFO

Article history:
Received 29 September 2010
Received in revised form 9 December 2010
Accepted 11 December 2010
Available online 21 December 2010

Keywords:
Antibiotics
HPLC
Metabolite identification
Structure elucidation
LC–MS/MS

ABSTRACT

Within the scope of searching for new lead structures in the field of anti-infectives, we ascertained Fenton's reagent to be an easy-to-handle and non-expensive tool for screening the metabolic profile of new bioactive compounds. The underlying chemistry of the Fenton's one-electron oxidation is comparable to that of cytochrome P450, which is the main metabolism enzyme. To study the metabolic screening capability, we subjected different antibiotics and the antiplasmodial naphthylisoquinoline alkaloid dioncophylline A to Fenton's reagent and examined the obtained compound libraries by liquid chromatography/tandem mass spectrometry (LC–MS/MS). For ciprofloxacin and linezolid about half of literature-known metabolites were identified as products of Fenton's oxidation. For dioncophylline A six new possible metabolites were discovered.

1. Introduction

The discovery of new lead structures for the development of a new drug is still a challenge even though a multitude of approaches are available. Thereby, the concept of so-called "re-combinatorial or random chemistry" is a relatively new and unusual way of creating new lead compounds, which already proved to be valuable for the development of new inhibitors of the acetylcholinesterase [1] as well as antiplasmodial and antiviral compounds [2,3]. Initially, the methodology was based on the utilization of high-energy γ-irradiation as an initiator of random free-radical re-combinations of drugable compounds in aqueous solutions. The earlier studies showed as well that most of the new compounds formed by γ-irradiation are products of radical chemistry. Thus, the idea arose to replace the gainless γ-irradiation by cheaper and easier-to-handle radical starters. As the use of Fenton's reagent is an easy, inexpensive and fast-to-handle strategy, we decided to optimize the reaction conditions. Comparison of Fenton's reagent application with the results of the γ-irradiation revealed similar compound libraries [4].

In search for new lead structures we added Fenton's reagent to a number of fluoroquinolones, like norfloxacin, ciprofloxacin or an analog of sarafloxacin, all used for treatment of infections, as well as to tacrine [1], an acetylcholinesterase inhibitor and examined the obtained compound libraries. In addition, we applied the method to gain insight into the metabolic profile of naphthylisoquinoline alkaloids. By screening the structures of the new substances formed in each of the quinolone-derived compound libraries, we realized that a great part of the produced substances resemble known metabolites of the aforementioned compounds; e.g. hydroxytacrine was identified as a tacrine metabolite [1].

Hence, we decided to check whether the conversion with Fenton's reagent may generally serve as a screening tool for the metabolic profile of a new biologically active compound. For this purpose, we chose three structurally different antibiotics, ciprofloxacin (**1**), linezolid (**2**), and cinoxacin (**3**) and, as an example of antiplasmodial compounds, the alkaloid dioncophylline A (**4**) isolated from *Triphyophyllum peltatum* [5–7] (see Fig. 1) for treatment with Fenton's reagent. The oxidation products obtained by the Fenton reaction were compared to the metabolites of the drugs, reported in the literature.

2. Experimental

2.1. Materials

Solutions of the antibiotics and dioncophylline A were prepared in solvents of analytical grade and solvents used for LC, LC/MS, and LC/MS/MS measurements were HPLC grade throughout. HPLC and analytical grade methanol and acetonitrile as well as silica gel were purchased from Fisher Scientific (Schwerte, Germany). Ferrous sulfate, formic acid and hydrogen peroxide were from Fluka (Taufkirchen, Germany). HPLC grade water prepared by means of the Milli-Q purifi-

* Corresponding author. Tel.: +49 931 318 5460.
E-mail addresses: u.holzgrabe@pzlc.uni-wuerzburg.de, u.holzgrabe@pharmazie.uni-wuerzburg.de (U. Holzgrabe).

0731-7085/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.12.016

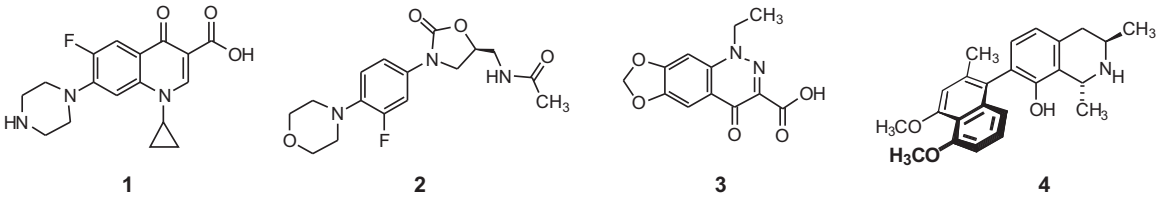

Fig. 1. Structural formulas of the antibiotics used.

cation system (Millipore, Eschborn, Germany) was used throughout.

2.2. Conversion with Fenton's reagent

To a solution of linezolid (1 mM) in ethanol, an aqueous solution of ferrous sulfate (1.7 mM) and an aqueous hydrogen peroxide solution (0.17 M) were added and the mixture was stirred for 2 h at room temperature. After evaporation of the solvent the residue was purified by column chromatography on silica gel using ethanol:NH_3 (9:1) as the mobile phase and analyzed subsequently by LC/MS and LC/MS/MS measurements.

Ciprofloxacin (0.25 mM) was dissolved in a dimethylsulfoxide/methanol mixture (14:1) that was acidified with 100 μl of concentrated sulfuric acid. An aqueous solution of ferrous sulfate (0.43 mM) and an aqueous hydrogen peroxide solution (43.0 mM) were added and the mixture was stirred for 2 h at room temperature. Column chromatography was performed using a mixture of water:diethylamine:toluol:chloroform:methanol = 8:14:20:40:40 as the eluent, as described for norfloxacin in Ph. Eur. [8].

Cinoxacin, however is insoluble in water or any organic solvent [9]. Thus, it was converted into its ammonium salt [10], which was dissolved in water and transformed as described above. The mobile phase for column chromatography was acetonitrile:water:NH_3 = 10:3.5:0.8 as described in HagerROM [9].

Dioncophylline A (37.7 μM) was dissolved in a dimethylsulfoxide/methanol mixture (10:1). Aqueous solution of ferrous sulfate (64.1 μM) and aqueous hydrogen peroxide solution (6.41 mM) were added and stirred for 2 h at room temperature. Column chromatography was performed on deactivated silica using methanol:dichloromethane (1:10).

2.3. LC–ESI–MS/MS analysis

All analyses were performed using an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) equipped with an electrospray ionization (ESI) source consisting of an orifice with nitrogen curtain gas and a capillary interface. The MS detection was performed in positive ion mode. The conditions of the spray chamber were as follows: ion polarity, positive; capillary voltage, 3500 V; drying gas temperature, 350 °C; nebulizer pressure, 50 psi; drying gas flow, 12.00 l/min for the quinolones, 10.00 l/min for dioncophylline A. LC/MS/MS analyses were performed using multiple reaction monitoring (MRM) mode.

2.3.1. Linezolid

The analyses were done using a NUCLEOSIL column (Macherey-Nagel, 250 mm × 4.0 mm i.d., 5 μm particle size, Düren, Germany) at 25 °C and a flow rate of 1 ml/min. According to Boak et al. [11] and Borner et al. [12] the mobile phase consisted of 20% acetonitrile and 80% water containing 0.2% formic acid. Runtime was 30 min.

2.3.2. Cinoxacin

The analyses were carried out by means of a NUCLEOSIL column (Macherey-Nagel, 250 mm × 4.0 mm i.d., 5 μm particle size, Düren, Germany). The mobile phase was composed of acetonitrile and water containing 0.2% formic acid. Gradient elution was performed as follows: 0–5 min 10–70% acetonitrile, 5–10 min 70% acetonitrile at a flow rate of 1.0 ml/min [13].

2.3.3. Ciprofloxacin

Analyses were done using a SYNERGY Max-RP column (Phenomenex, 150 mm × 4.6 mm i.d., 4 μm particle size, Aschaffenburg, Germany) at 25 °C with a flow rate of 1 ml/min. The mobile phase consisted of methanol and water containing 0.2% formic acid. Gradient elution was performed as follows: 0–20 min 20–30% methanol.

2.3.4. Dioncophylline A

For the analysis of the reaction products of dioncophylline A, a SYMMETRY C_{18} column (Waters, 250 mm × 4.6 mm, 5 μm particle size) was used and the mobile phase was composed of acetonitrile (0.2% formic acid) (A) and water (0.2% formic acid) (B). The gradient elution was done as follows: 0–30 min 10–70% A, 30–35 min 70–100% A, 35–40 min 100% A, 40–41 min 100–5% A, 41–46 min 5% A at a flow rate of 0.8 ml/min. Accurate electrospray ionization (ESI) and ISD–ESI (in-source decay) mass spectra were obtained by a Bruker Daltonics micrOTOF focus. For the exact ESI analyses a SYMMETRY C_{18} column (Waters, 250 mm × 4.6 mm, 5 μm particle size) was used. The mobile phase was composed of acetonitrile (0.2% formic acid) (A) and water (0.2% formic acid) (B). Gradient elution was performed as follows: 0–5 min 10–30% A, 5–50 min 30–70% A, 50–70 min 70–100% A, 70–75 min 100% A, 75–76 min 100–10% A, 76–80 min 10% A at a flow rate of 0.1 ml/min. ESI and ISD–ESI spectra were measured in the positive ion electrospray mode with a mass accuracy better than 3 ppm. For ISD–ESI measurements a capillary exit of 200 V and a voltage of 25.0 V were used for skimmer 1.

3. Results and discussion

Former experiments [4] exploring limits and possibilities of reaction conditions for the conversion with Fenton's reagent in order to create a compound library of greatest diversity revealed the following conditions. These experiments were mainly performed for the conversion of tacrine, an inhibitor of the acetylcholinesterase as model compound, but can be translated with just minor changes into the conversion of antibiotics. The main influence on product composition showed the constitution of used solvent and the variation of pH. To guarantee a satisfying conversion, a sufficient solubility has to be ensured [1] for both the compounds to be converted and the ferrous ions. Therefore the pH should not be too high, to avoid the precipitation of ferrous hydroxide. Since a constant part of Fenton's reagent always reacts with the solvent, a 150–200-fold excess of hydrogen peroxide was found to be necessary to provide a satisfying conversion of the starting compound within reaction time of 2 h. The reaction of generated hydroxyl radicals with solvent in this case is an undesirable side reaction, because improbable metabolites are generated. The addi-

tion of EDTA did not show an effect on any yields although an increase of yields is often described in literature in similar cases. These findings led to the reaction conditions applied here: compounds were dissolved due to their best solubility in alcohol or acidic alcohol/dimethylsulfoxide mixtures and converted with a 1.7-fold excess of ferrous sulfate and a 170-fold excess of hydrogen peroxide for 2 h at room temperature.

These oxidation conditions are not physiological. However, this is not necessary, as this is a way of finding metabolites by methods typical for organic chemistry.

3.1. Oxidation of cinoxacin by Fenton's reagent

Problems occurred with cinoxacin due to the limited solubility of Fenton's reagent in organic solvents on the one hand and the exclusive solubility of cinoxacin in aqueous alkaline environment on the other hand [9]. In alkaline solutions iron hydroxide precipitates and the reaction is stopped. Therefore, almost no conversion of cinoxacin could be observed.

3.2. Oxidation of ciprofloxacin by Fenton's reagent

Ciprofloxacin shows a high metabolic stability as about one-third of ciprofloxacin remains unchanged after oral administration and can be recovered in urinary excretion [14]. Anyway, a broad variety of metabolites of ciprofloxacin are described in literature [15–19]. These can be subdivided into different groups (see Fig. 2): (1) decarboxylated metabolites of type **C1**, (2) metabolites with a degraded piperazine moiety, **C2**, (3) one metabolite **C3** where the quinolone structure is constrained to an indoledione system and (4) hydroxylated or oxidated metabolites of class **C4**.

When ciprofloxacin was subjected to chemical oxidation by Fenton's reagent, a large portion remained unchanged which is in agreement with the situation after oral administration of the drug. However, also a great part of the metabolites known in literature were generated and identified by LC/MS analysis (framed compounds in Fig. 2); the respective MS^n fragmentation experiments are indicated in Table 1.

In general, the loss of the carboxyl moiety $[M+H-44]^+$ and the loss of the fluoride $[M+H-20]^+$ are characteristic fragmentation pathways for fluoroquinolones. Moreover ciprofloxacin in particular shows a typical loss of the cyclopropyl group $[M+H-41]^+$ and typical degradation steps of the piperazinyl moiety $[M+H-42]^+$ and $[M+H-84]^+$ [15,18]. Thus, the appearance of these fragmentations in the MS^n spectra of the Fenton-generated compounds gives hints, for example, at the existence of an intact carboxyl moiety and an intact piperazinyl moiety, respectively.

1) The compound with m/z 304 could theoretically represent two known metabolites of ciprofloxacin, **C1d** or **C2b** (see Fig. 2). The MS/MS spectrum of this compound with m/z 304 is shown in Fig. 3a, evidencing the degradation of the piperazinyl moiety $[M+H-42]^+$, which leads to m/z 262, a loss of water giving m/z 286, a loss of the entire piperazinyl moiety $[M+H-85]^+$ resulting in m/z 219, and a combined loss of the piperazinyl and the cyclopropyl moiety $[M+H-84-41]$ leading to m/z 179 (see Fig. 3a and b). As no signal for the cleavage of a carboxy group was observed the compound with m/z 304 can be assigned to the decarboxylated metabolite **C1d**, possessing an intact piperazinyl moiety, no carboxy group but a hydroxy group in position 3.
2) The compounds with m/z 318 and 320 were accordingly assigned to the metabolites **C1a** and **C1c** (see Fig. 2), as both structures showed no decarboxylation fragment $[M+H-44]^+$ in the MS^2 spectra. Furthermore the compound with m/z 318 lacked the typical fragment of $[M+H-20]^+$ representative for the loss of fluoride. Hence the compound with m/z 318 has to be a substance without a fluoride ion and without a carboxy group, which is in agreement with the known metabolite **C1a**. The MS^2 spectrum of the compound with m/z 320 is characterized by a loss of fluoride, several losses of H_2O and the loss of the cyclopropyl moiety $[M+H-41]^+$, which supports the assignment to metabolite **C1c** (see Table 1). Taken together, in all decarboxylated metabolites the carboxy group is replaced with a hydroxy group.
3) The substance with m/z 306 possessed the fragment ions of the decarboxylation $[M+H-44]^+$, of the loss of water $[M+H-18]^+$, and of the loss of fluoride $[M+H-20]^+$ but lacked the typical piperazinyl moiety fragment ions $[M+H-42]^+$ or $[M+H-84]^+$. However MS^3 measurements showed the loss of a $[M+H-59]^+$ ion, which is equivalent to the loss of an ethylenediamine unit. Hence, the compound with m/z 306 was assigned to metabolite **C2c** (see Fig. 2). Volmer et al. [15] described corresponding product ions for metabolite **C2c**, i.e., the loss of water leading to m/z 288, the combined loss of water and fluoride leading to m/z 268 and the combined loss of ethylenediamine and water leading to m/z 227 (compare to Table 1). For metabolite **C2c** Gau et al. [14] furthermore described the occurrence of the product ion m/z 262 representing the loss of the carboxy group, which is in agreement with our observations.
4) The compound with m/z 322 showed nearly the same fragment ions as metabolite **C2c**, which points to a similar structure. Additionally, a loss of H_2O was observed. Thus, compound m/z 322 was assigned to metabolite **C2a** (see Fig. 2), which possesses an additional hydroxy group in position 8.
5) The compound with m/z 263 displayed all typical product ions of ciprofloxacin except for the degradation of the piperazinyl moiety. Consequently, it reflects a ciprofloxacin derivative with a small substituent in position 7 as is the case for metabolite **C2d** (see Fig. 2).
6) The compound with m/z 346 could again reflect two known metabolites of ciprofloxacin **C4d** or **C4f**. MS^n measurements showed typical fragmentation pathways such as the losses of H_2O leading to m/z 328 and the loss of the carboxy group resulting in m/z 302. These fragments do not allow a differentiation between the metabolites **C4d** and **C4f**, but the combined loss of cyclopropyl and fluoride yielding m/z 285 and the loss of $[M+H-101]^+$ leading to m/z 245, which reflects the loss of a piperazinone moiety, indicates that compound m/z 346 can be assigned the structure of metabolite **C4f** (see Fig. 2). These fragmentations are in agreement with the product ions m/z 245, m/z 285 and m/z 328 observed by Volmer et al. [15] for **C4f**. Moreover, Gau et al. [14] described the product ion m/z 302 as a fragment of the metabolite **C4f** (Fig. 4).

In addition to the identified metabolites **C1a**, **c**, **d**, **C2a**, **c**, **d**, **C4b**, and **f**, the conversion with Fenton's reagent generated a multiplicity of further substances (see Fig. 5), among them, for example, two compounds with m/z 334 and m/z 348, which could theoretically represent the metabolites **C2e** and **C4a** of ciprofloxacin (see Fig. 2), but MS/MS experiments did not allow a definitive assignment to these structures.

Main metabolites of ciprofloxacin found in body fluids are sulfociprofloxacin, oxociprofloxacin, formylciprofloxacin and desethyleneciprofloxacin [20]. Sulfociprofloxacin cannot be detected by this assay and is therefore not listed in Fig. 2. However, the other three metabolites are found as compounds **C2c** (desethyleneciprofloxacin), **C4b** (formylciprofloxacin) and **C4f** (oxocipro-floxacin) [14,15].

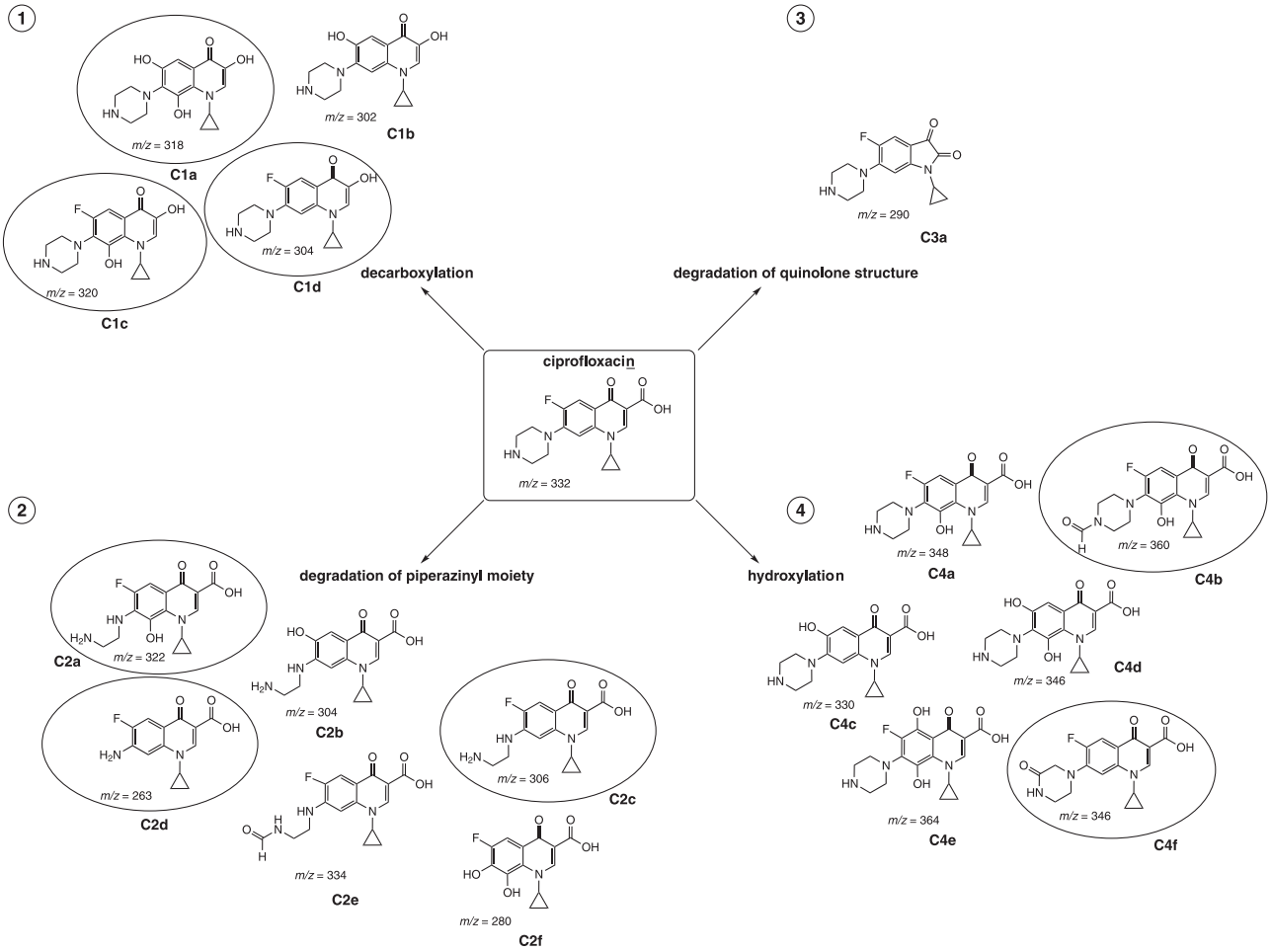

Fig. 2. Metabolic pathways of ciprofloxacin; metabolites found in this study by means of Fenton's reagent are put in ellipses.

3.3. Oxidation of linezolid by Fenton's reagent

After oral administration 35% of linezolid are deposited unchanged in urinary excretion. The rest of the drug is converted into eleven, currently known, metabolites [21]; these metabolites are produced by four reactions: (1) degradation of the acetamide group (**L1**), (2) hydroxylation or oxidation (**L2**), (3) degradation of the morpholine moiety (**L3**) and (4) defluorination (**L4**). The conversion with Fenton's reagent again reflects the situation in body. Linezolid showed a great stability as a high portion remained unchanged. Nevertheless a small-compound library was generated in which five known metabolites were identified by LC/MS^n measurements (see framed compounds in Fig. 6).

Linezolid showed characteristic LC/MS^n fragmentation pathways that mainly include the loss of the acetamide $[M+H-58]^+$ or the acetaldehyde group $[M+H-42]^+$, and typical degradation steps of the morpholine moiety, $[M+H-44]^+$ and $[M+H-86]^+$. Thus, main product ions described in the literature are the product ion *m/z*

Table 1
List of $[M+H]^+$ and fragments originating from MS and MS^n spectra obtained from ciprofloxacin and its metabolites.

Precursor ion	MS^2	MS^n
263	169 (–HF, –H_2O, –NH_2, –C_3H_4) 204 (–F, –C_3H_4) 219 (–COO) 245 (–H_2O)	
304	179 (–$N(CH_2CH_2)_2N$, –C_3H_5) 185 (–HF, –H_2O, –NCH_2CH_2, –C_3H_4) 219 (–$N(CH_2CH_2)_2NH$) 241 (–HF, –$HNCH_2CH_2$) 262 (–NCH_2CH_2) 286 (–H_2O)	
306	262 (–COO) 286 (–HF) 288 (–H_2O)	227 (–$H_2NCH_2CH_2NH$), 268 (–H_2O) (→240 (–CO))
318	199 (–H_2O, –H_2O, –$HNCH_2CH_2$, –C_3H_4) 219 (–NCH_2CH_2, –OH, –C_3H_4) 276 (–NCH_2CH_2) 301 (–OH)	177 (–NCH_2CH_2), 172 (–CH_2CH_3, –H_2O) 261 (–CH_3), 172 (–CH_2CH_2, –H_2O, –H_2O, –C_3H_4)
320	241 (–HF, –H_2O, –C_3H_5) 262 (–H_2O, –C_3H_4) 263 (–$CH_2HNCH_2CH_2$) 282 (–HF, –H_2O) 300 (–HF)	
322	262 (–HF, –C_3H_4) 278 (–COO) 304 (–H_2O)	203 (–$H_2NCH_2CH_2NH$)
346	204 (–COO, –CH_2NHCO, –C_3H_5) 245 (–$NCH_2CONHCH_2CH_2$) 285 (–HF, –C_3H_5) 302 (–COO) 328 (–H_2O)	

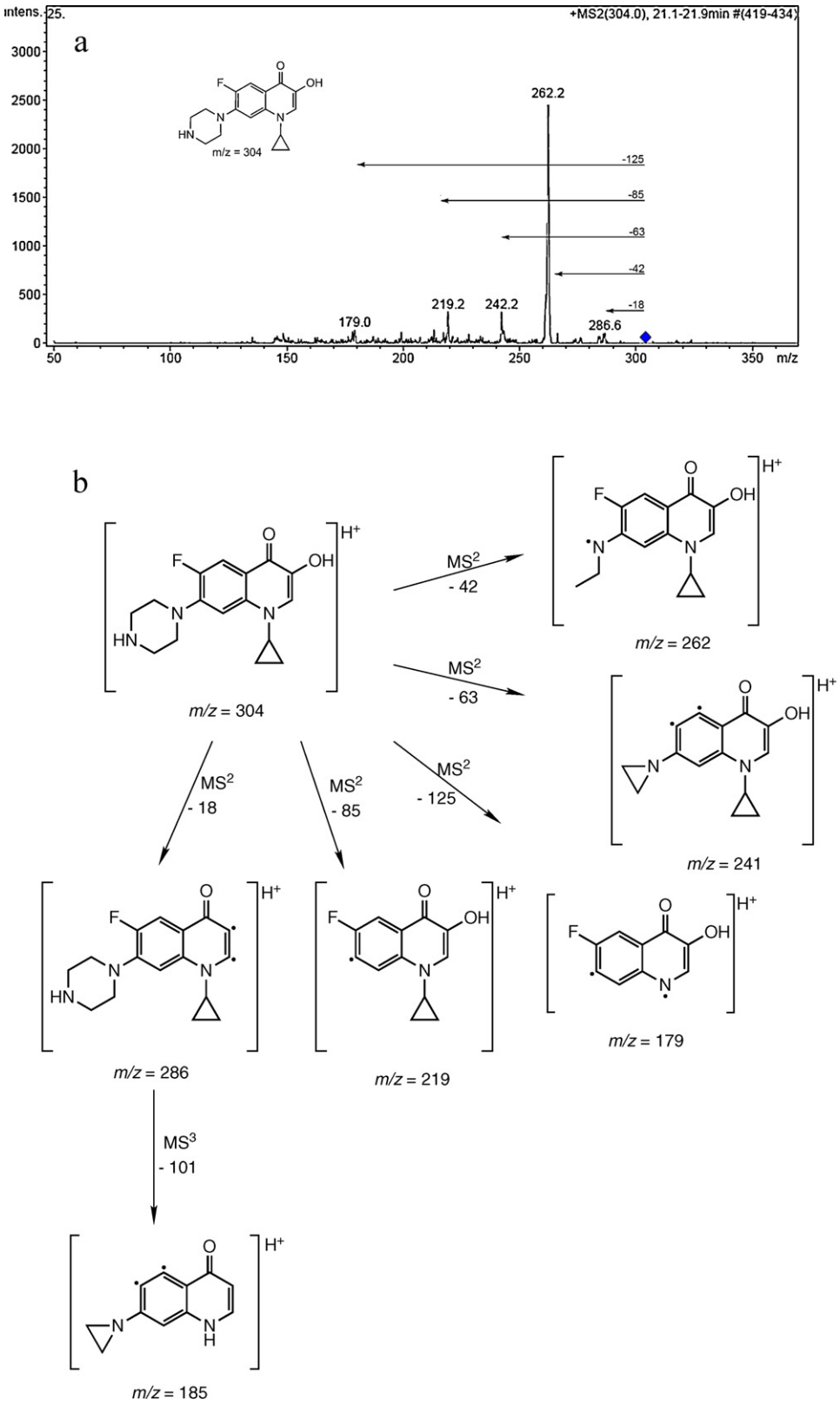

Fig. 3. (a) Daughter-ion spectrum of the fluoroquinolones metabolite **C1d** with *m*/*z* 304. (b) Fragmentation pathways of the fluoroquinolones metabolite **C1d** with *m*/*z* 304.

296 [22,23], which reflects the loss of the acetaldehyde group and the product ion *m*/*z* 195, which reflects the combined loss of the acetamide group and the morpholine group [24,25]. In the cases where these fragmentation steps are found in MS^n spectra of the inspected compounds generated by Fenton's reagent, this indicates the existence of an intact acetamide and morpholine moiety, respectively.

1) The compound with *m*/*z* 296 was assigned to metabolite **L1a**, as the loss of 42 was absent (see Table 2), indicating a lack of

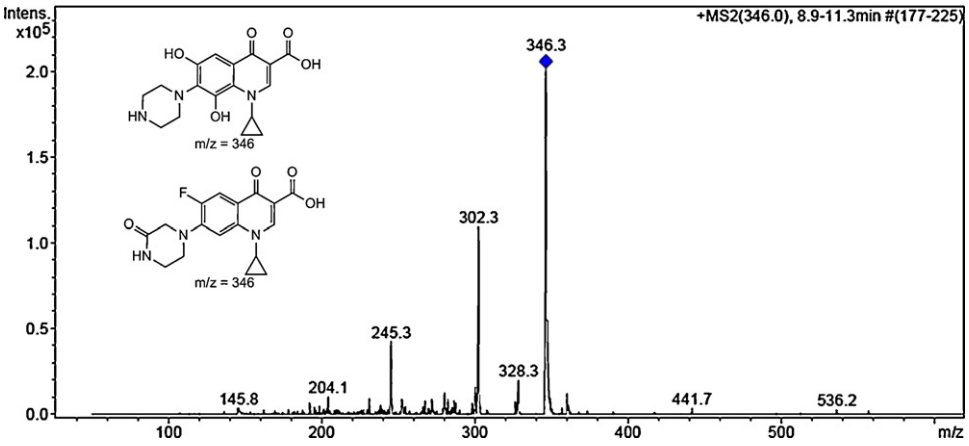

Fig. 4. Daughter-ion spectrum of the fluoroquinolones metabolite **C4f** with *m*/*z* 346.

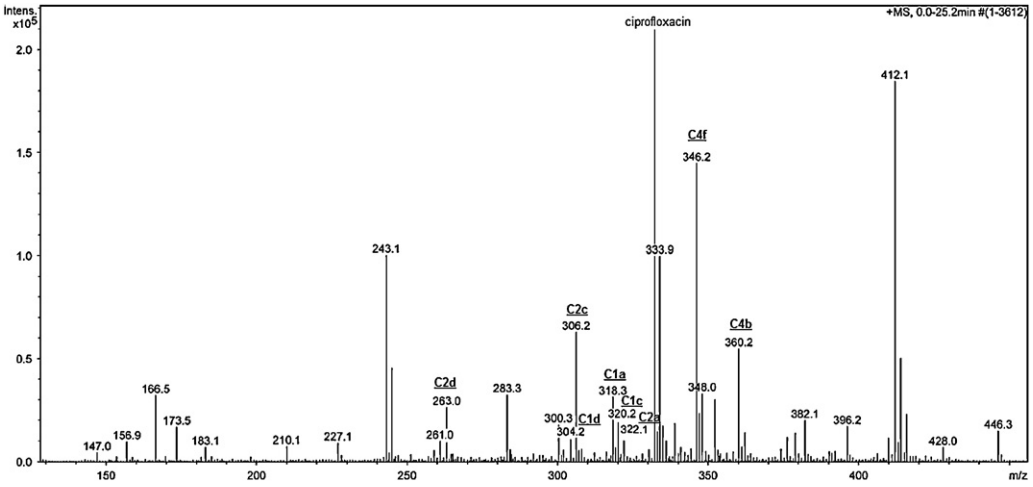

Fig. 5. TIC of the compound library of ciprofloxacin.

the acetaldehyde group. Furthermore, fragmentation steps were monitored showing the degradation of the morpholine moiety $[M+H-44]^+$ leading to *m*/*z* 252 and the degradation of the 5-(aminomethyl)oxazolidin-2-one moiety $[M+H-101]^+$ giving *m*/*z* 195.

2) a) The compounds with *m*/*z* 352 and *m*/*z* 354 both gave characteristic fragmentation steps for linezolid derivatives as the loss of the 42 u indicating the cleavage of an acetaldehyde group and a typical degradation step for the morpholine ring $[M+H-44]^+$. However a product ion of $[M+H-86]^+$ indicating the loss of the complete morpholine ring were not observed. For the compound with *m*/*z* 352 MS^3 measurements showed the loss of 100 u (see Table 2). Consequently instead of the morpholine ring a morpholinone moiety is arranged at the benzene ring. Thus, the compound with *m*/*z* 352 was assigned to metabolite **L2a**. However, it could not be decided whether the carbonyl group was located at C-2 or at C-3 of the morpholine ring (see Fig. 6).

b) The compound with *m*/*z* 354 was clearly distinguishable from the compound with *m*/*z* 352, as on the one hand a distinct loss of H_2O was observed and on the other hand MS^3 measurements showed the loss of $[M+H-102]^+$ indicating the elimination of a hydroxymorpholine moiety, permitting, in turn, an assignment to structure **L2b**. Again, fragmentation steps did not allow to locate the position of the hydroxy group at the morpholine ring (see Table 2).

Table 2
List of $[M+H]^+$ and fragments originating from MS and MS^n spectra obtained from linezolid and its metabolites.

Precursor ion	MS^2	MS^n
296	195 ($-NH_2CH_2CHCH_2OCO$) 252 ($-COO$) 278 ($-H_2O$)	151 ($-CH_2CH_2O$), 138 ($-CH_2CH_2OCH$) 221 ($-H_2NCH_2CHCH_2$), 205 ($-H_2NCH_2CHCH_2O$), 235 ($-H_2NCH_2CH$)
312	209 ($-CH_2CO$, $-HOCH_2CH_2NH$) 270 ($-CH_2CO$)	189 ($-HF$) 252 ($-H_2O$), 225 ($-HOCH_2CH_2$), 169 ($-NH_2CH_2CHCH_2OCO(\rightarrow 151\ -H_2O)$), 209 ($-HOCH_2CH_2NH_2$)
320	177 ($-CH_2CH_2OCH_2CH_2N$, $-CH_2CONH$) 276 ($-CH_2CH_2O$) 278 ($-CH_2CO$)	217 ($-H_3CCONH_2$)
352	308 ($-CH_2CH_2O$) 310 ($-CH_2CO$)	207 ($-H_3CCONHCH_2CHO$), 252 ($-NCH_2CO$)
354	310 ($-CH_2CH_2O$)312 ($-CH_2CO$) 336 ($-H_2O$)	209 ($-H_3CCONHCH_2CHO$) 252 ($-CH_2CHOHO$), 293 ($-F$)

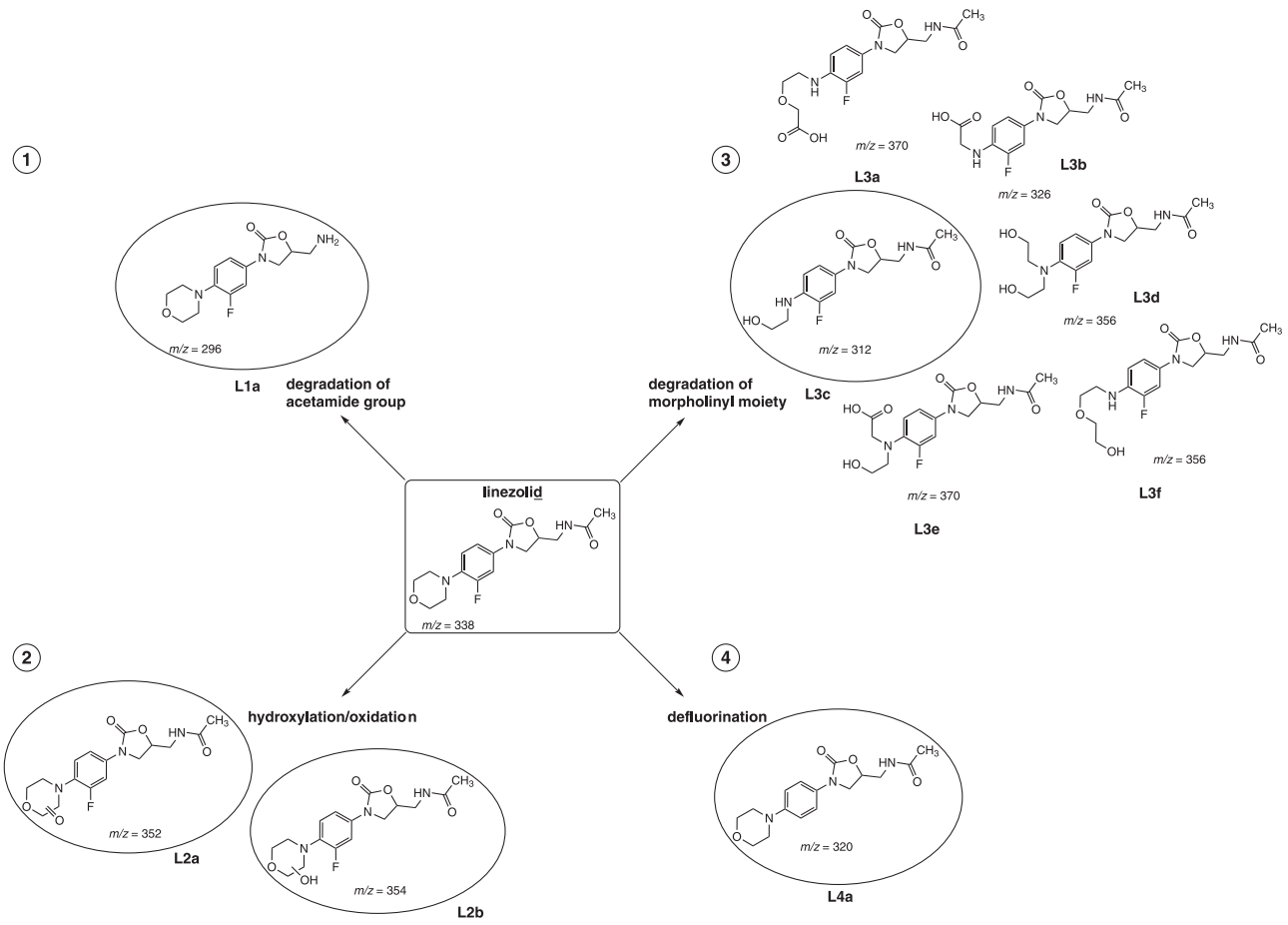

Fig. 6. Metabolic pathways of linezolid; metabolites found in this study by means of Fenton's reagent are put in ellipses.

3) The lack of the fragmentation step $[M+H-44]^+$ of the compound with *m/z* 312 alluded to a structure with no or a partially degraded morpholine moiety. However, the loss of the acetaldehyde group $[M+H-42]^+$ was observed, leading to the first-generation product ion *m/z* 270. Furthermore second-generation product ions showed the degradation of a 2-aminoethanol residue by the loss of 18 u leading to *m/z* 252, the loss of 45 u providing *m/z* 225 and the loss of 61 u leading to *m/z* 209 (see Table 2). The product ion *m/z* 209 was observed both, in MS^2 and in MS^3 experiments of the product ion *m/z* 270, evidencing that the fragmentation of the acetaldehyde group and the 2-aminoethanol residue occurred in one or in two steps (see Fig. 7). Further fragmentation of the product ion *m/z* 209 yielded the elimination of fluoride generating the product ion *m/z* 189. Hence, the compound with *m/z* 312 possessed a fluoride ion, the acetamide group and a 2-aminoethanol residue instead of the morpholine moiety. Due to these findings the compound with *m/z* 312 was assigned to metabolite **L3c** (see Fig. 7).
4) MS^n measurements of the compound with *m/z* 320 showed all characteristic fragmentation steps for linezolid suggesting in turn that the acetamide and the morpholine groups were still present. Furthermore, the appearance of the product ion *m/z* 177 (see Table 2) reflected the simultaneous loss of the acetamide and morpholine groups. This and the fact that the molecular weight was lower than that for linezolid by 18 u, confirmed the assignment to the defluorinated analog of linezolid, metabolite **L4a**.

Furthermore a few more compounds were generated by Fenton's reagent that could not be assigned to any known metabolite. According to an *in vivo* study [26] the major and most relevant metabolites in human urine and feces are compounds labeled **L3a** and **L3e**. Both were not detected by this assay. The main metabolite in our studies was compound **L3c**, resembling a further degraded structure of **L3a** and **L3e**. Already Slatter et al. [26] described an almost complete decomposition of **L3e** into **L3c** by an irreversible *N*-dealkylation during storage in acid media. As our studies are performed in acid media, the discovery of only **L3c** is in line with Slatter's observation and thus expected.

3.4. Oxidation of dioncophylline A by Fenton's reagent

As a further substance for oxidative conversions induced by Fenton's reagent, the naphthylisoquinoline alkaloid dioncophylline A was chosen, as a representative of a new, emerging class of promising anti-infectives. For this type of alkaloids only two studies on the metabolism had been described in the literature, of which, after preliminary GC–MS experiments on dioncophylline A [27], a more in-depth study reported on the biotransformation of this alkaloid by liver microsomes and its pharmacokinetics [28]. This study led to the identification of 5′-*O*-demethyldioncophylline A as the major metabolite, which was unambiguously identified by NMR and coelution experiments. In addition, a second, minor metabolite was found, whose mass corresponded to that of a 4-hydroxydioncophylline A.

When dioncophylline A was exposed to chemical oxidation by Fenton's reagent, most of the material remained unchanged, which, in agreement with earlier observations [28], suggested a high metabolic stability of the compound under these conditions. Nevertheless several metabolites were identified by LC/MS^n

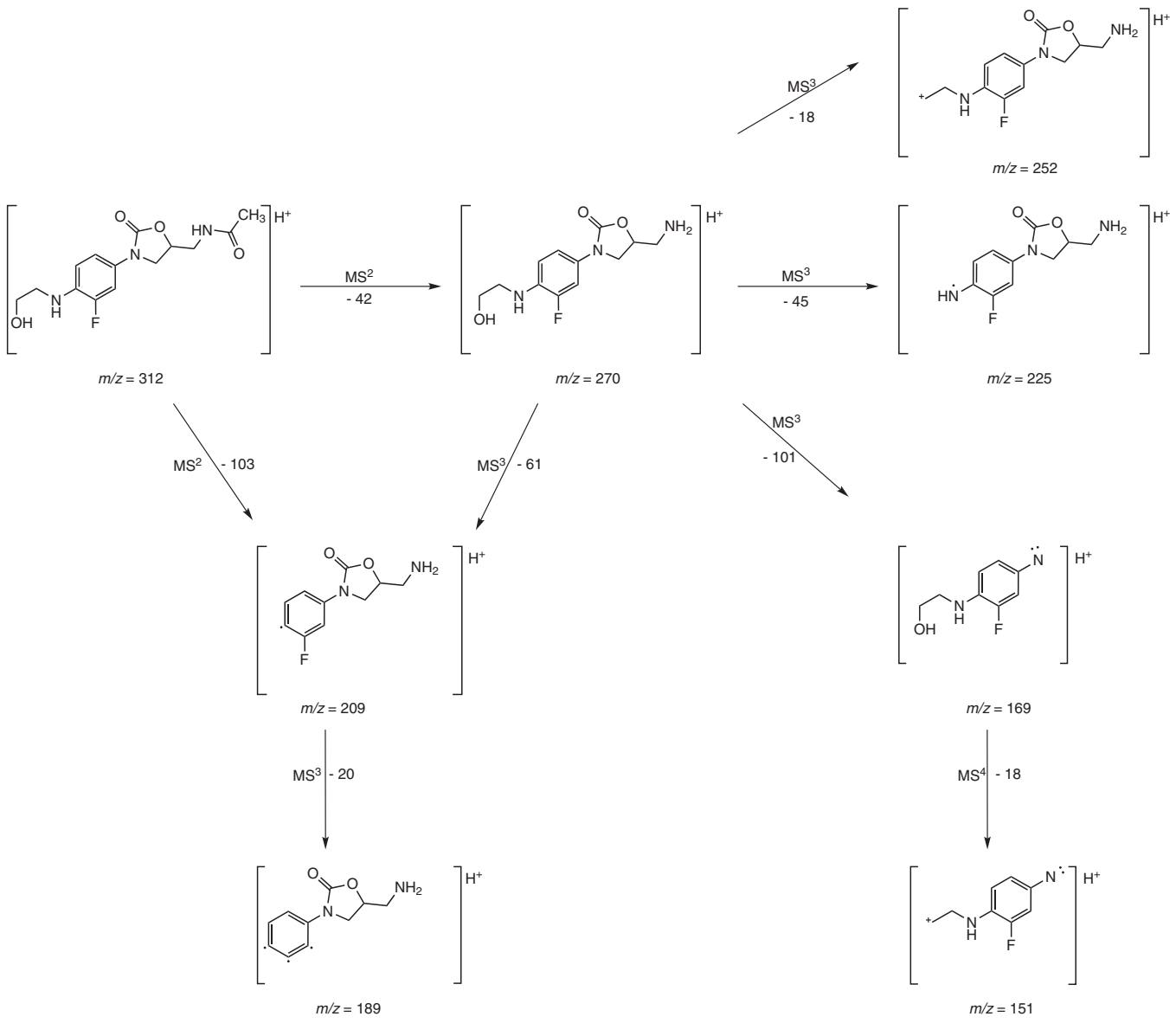

Fig. 7. Fragmentation pathways of the linezolid metabolite **L3c** with *m*/*z* 312.

measurements with *m*/*z* 392, 394, 408, and 410 (Fig. 8). The corresponding MS/MS fragmentation experiments are shown in Table 3.

For a better understanding of the fragmentation pathway of this type of naphthylisoquinoline alkaloids, an ESI–ISD experiment was performed on pure dioncophylline A (Fig. 9). The neutral loss of an ammonia unit $[M+H-17]^+$, also combined with the elimination of methyl a group $[M+H-17-15]^+$, a retro-Diels–Alder fragmentation (neutral loss of 43 u) in the nitrogen-containing heterocycle resulting in $[M+H-C_2H_5N]^+$ ions, and a cleavage of the biaryl axis leading to *m*/*z* 202 for the intact naphthalene moiety were observed. These experiments led to a better interpretation of the six dioncophylline A metabolites:

Table 3
List of $[M+H]^+$ and fragments originating from MS/MS spectra obtained from dioncophylline A and its metabolites.

Precursor ion	Substance	MS^2
378	Dioncophylline A (**4**)	361 ($-NH_3$) 346 ($-NH_3$, $-CH_3$) 335 ($-C_2H_5N$) 329 ($-OCH_3$, $-NH_3$) 314 ($-OCH_3$, $-CH_3$, $-NH_3$) 288 ($-C_2H_5N$, $-OCH_3$, $-CH_3$) 202 ($-C_{11}H_{14}NO$)
392	**D1** (*N*-methyldioncophylline A)	376 ($-CH_4$) 361 ($-H_2NCH_3$) 346 ($-H_2NCH_3$, $-CH_3$) 335 ($-C_2H_4NCH_3$) 202 ($-C_{12}H_{17}NO$)
394	**D2**	376 ($-H_2O$) 359 ($-H_2O$, $-NH_3$) 348 ($-OCH_3$, $-CH_3$) 333 ($-H_2O$, $-C_2H_5N$) 305 ($-C_2H_5N$, $-OCH_3$, $-CH_3$)
394	**D3**	377 ($-NH_3$) 376 ($-H_2O$) 363 ($-OCH_3$) 348 ($-OCH_3$, $-CH_3$) 202 ($-C_{11}H_{14}NO_2$)
394	**D4** (habropetaline A)	376 ($-H_2O$) 359 ($-H_2O$, $-NH_3$) 333 ($-H_2O$, $-C_2H_5N$) 320 ($-C_2H_5N$, $-OCH_3$)
408	**D5**	390 ($-H_2O$) 376 ($-NH_3$, $-CH_3$) 362 ($-OCH_3$, $-CH_3$) 348 ($-C_2H_5N$, $-OH$) 333 ($-C_2H_5N$, $-OH$, $-CH_3$)
410	**D6**	392 ($-H_2O$) 378 ($-NH_3$, $-CH_3$) 360 ($-H_2O$, $-NH_3$, $-CH_3$) 349 ($-C_2H_5N$, $-H_2O$)

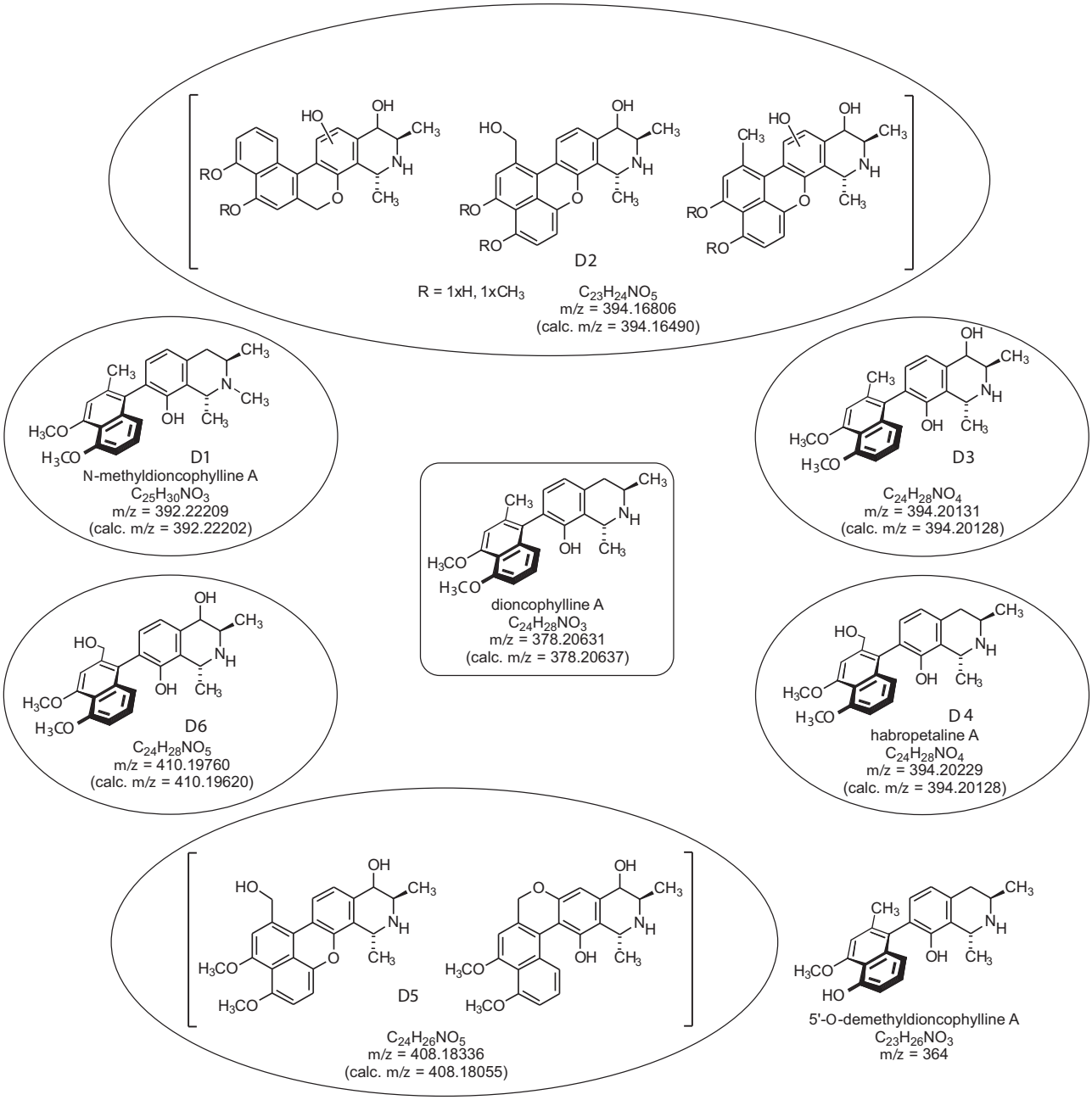

Fig. 8. Metabolites of dioncophylline A identified after treatment with Fenton's reagent.

1) Among these products, the compound with *m*/*z* 392 and a molecular formula of $C_{25}H_{30}NO_3$ from accurate LC–MS experiments, suggesting the presence of an additional methyl group compared to dioncophylline A, showed fragment ions for the loss of methane $[M+H-16]^+$, the elimination of methylamine $[M+H-31]^+$, also combined with the loss of a methyl group leading to *m*/*z* 346. In addition, a fragment ion $[M+H-C_2H_4NCH_3]^+$ at *m*/*z* 335 hinting at an *N*-methylated isoquinoline and an intense signal in the mass spectrum with *m*/*z* 202 for the intact naphthyl moiety compared to the parent compound **4** were observed. The fragmentation steps and the lack of the fragment ion for the loss of an ammonia unit $[M+H-17]^+$ supported the assignment of the compound to metabolite **D1** (see Fig. 8) with an additional methyl group on the isoquinoline nitrogen. Metabolite **D1** is, itself, a known natural product, named *N*-methyldioncophylline A [29]; its identity was confirmed by comparison of the mass spectra and a coelution experiment with authentic isolated material. Likewise by ESI–ISD comparison with the authentic alkaloid a correct attribution of the nitrogen-containing fragments of this *N*-methylated of naphthylisoquinoline alkaloid was achieved.
2) a) The compound with *m*/*z* 394 showed a typical fragmentation step, the loss of water $[M+H-18]^+$ for hydroxylated naphthylisoquinoline alkaloids in benzylic positions and a combined loss of a methoxy function and a methyl group leading to *m*/*z* 348. Further fragmentation of the isoquinoline moiety $[M-H-43]^+$, together with the loss of water $[M-H-43-18]^+$ and combined with the elimination of methoxy and methyl groups $[M-H-43-31-15]^+$ led to *m*/*z* 333 and *m*/*z* 305. In this case, no signal for the cleavage of the biaryl axis was observed, suggesting a further stabilization of this axis by the presence of an additional ether bridge

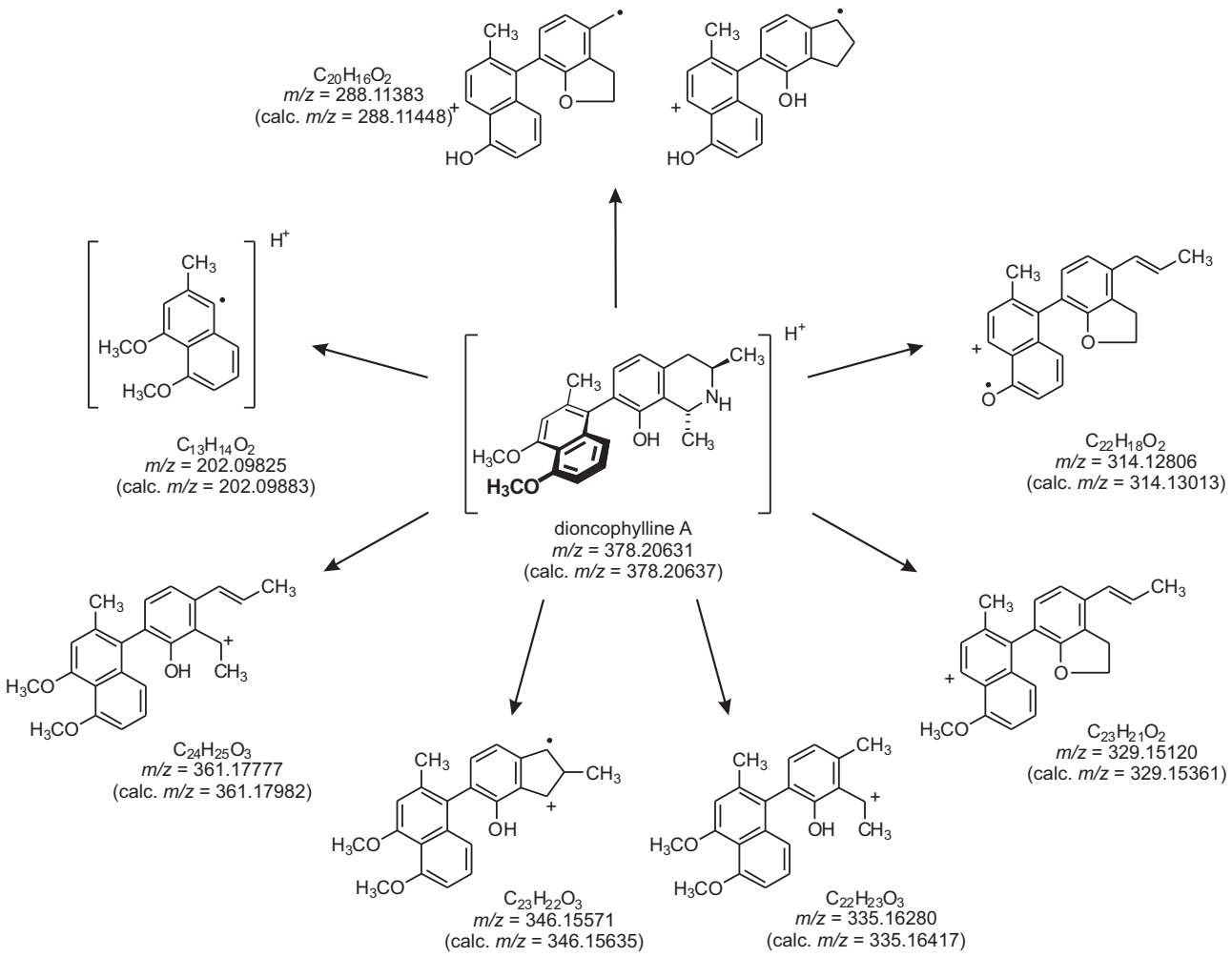

Fig. 9. Fragmentation of dioncophylline A with exact masses obtained from ESI–ISD.

between the naphthalene and the isoquinoline. This, combined with the accurate LC–MS analysis, which evidenced a molecular formula of $C_{23}H_{24}NO_5$, led to metabolite **D2** (see Fig. 8), for which three possible structures remained imaginable, between which the MS/MS experiments could not permit a definitive distinction.

b) A second compound with *m/z* 394 showed product ions for the loss of an ammonia unit $[M+H-17]^+$, the elimination of water $[M+H-18]^+$, the loss of a methoxy group $[M+H-31]^+$ and a combined cleavage of a methoxy function and a methyl group leading to *m/z* 348. The molecular formula of $C_{24}H_{28}NO_4$, from accurate LC–MS analysis and an intense signal with *m/z* 202 in the mass spectrum, indicated that the naphthyl moiety of the compound remained unaltered compared to that of the parent compound **4**, suggesting the addition of an oxygen to the isoquinoline moiety of the molecule. Taking into account that the benzylic position was favored for oxidations and that this oxygenation had already been proposed in literature [28], structure **D3** seemed probable for this metabolite (see Fig. 8).

c) A third compound with *m/z* 394 generated fragment ions corresponding to the loss of water $[M+H-18]^+$ and a combined elimination of water and an ammonia unit $[M+H-18-17]^+$. In addition, the spectrum showed a fragmentation of the isoquinoline moiety together with the loss of water and combined with the cleavage of a methoxy group leading to *m/z* 333 and *m/z* 320. The LC/MS^n analysis and accurate LC–MS evidencing a molecular formula of $C_{24}H_{28}NO_4$ matched the possible structure of the known natural habropetaline A. The metabolite gave a mass spectrum identical to that of isolated reference material of habropetaline A, which was presented from previous isolation work [30]. Coelution of the metabolite with habropetaline A further confirmed the assignment of the metabolite **D4** to be habropetaline A (see Fig. 8). This alkaloid – and now probable metabolite – is known to possess an even higher antimalarial activity as compared to that of dioncophylline A [30].

3) For the compound with *m/z* 408, which had a molecular formula of $C_{24}H_{26}NO_5$, suggesting the presence of two additional hydroxy functions as compared to dioncophylline A and the loss of two hydrogens, several structures seemed possible. The compound possessed fragment ions corresponding to the cleavage of water $[M+H-18]^+$, the loss and an ammonia unit combined with the elimination of a methyl group $[M+H-17-15]^+$, which indicated that the oxidation had not occurred in the isoquinoline moiety resulting, e.g. in a dihydroisoquinoline structure. Further fragmentation of the isoquinoline moiety $[M-H-43]^+$ combined with a hydroxy group $[M-H-43-17]^+$ and together with a methyl group $[M-H-43-17-15]^+$ yielded *m/z* 348 and *m/z* 333. As no signal for the cleavage of the biaryl axis was observed, suggesting the presence of an additional ether bridge between the naphthalene and the isoquinoline portions, the compound was tentatively assigned to be the metabolite **D5** (see

Fig. 8), for which two structures seemed possible, but MS/MS experiments did not permit a definitive structural elucidation.

4) The compound with m/z 410 showed two intensive signals in the mass spectrum. It possessed the fragment ions corresponding to the cleavage of water $[M+H-18]^+$ exclusively, combined with the loss of 43 u due to a retro-Diels–Alder fragmentation resulting in $[M+H-H_2O-C_2H_5N]^+$ at m/z 349, but lacked the typical signal of m/z 202 for the naphthyl moiety. Further minor product ions occurred, resulting from the elimination of an ammonia unit together with a methyl group $[M+H-17-15]^+$ and combined with the loss of water leading to m/z 360. Together with the calculated molecular formula of $C_{24}H_{28}NO_5$ this supported the assignment to metabolite **D6** (see Fig. 8).

In addition, the Fenton reaction produced several further minor substances which could not be structurally elucidated.

4. Conclusions

For linezolid and ciprofloxacin, about half of the metabolites described in the literature could be generated by the use of Fenton's reagent. This holds especially true for metabolites which are formed by the cytochrome enzyme complex. For dioncophylline A, six metabolites were identified, among them the known alkaloid habropetaline A, which has an even better antiplasmodial activity than the parent compound dioncophylline A.

Even though no statement can be made which CYP isoenzyme is responsible for the metabolism or if there are any CYP isoenzyme-related interactions there are several advantages of Fenton approach. Alternative procedures for *in vitro* investigation of the metabolic profile of a potential drug such as the incubation with liver microsomes, isolated CYP enzymes or intact cells [31] always demand liver tissues. Because of the inability of differentiated hepatocytes or liver slices to grow efficiently *in vitro*, cell cultures need to be prepared freshly each time from liver samples. This is a problem because of the restricted accessibility to suitable liver tissues. Microsomes on the other hand can be stored at −80 °C for years with little or no loss of CYP enzyme activities, but incubation can be performed only for a short time (usually less than 1 h) so that for drugs, which are metabolically stable, metabolites can be hardly detected [31,32]. Moreover, growing cell cultures need special equipment and the reagents used are often expensive.

For the prediction of metabolites by means of the Fenton approach, obviously no human liver tissues are needed. The method turns out to be easy, stable, fast-to-handle and reveals reproducible results. Additionally the instrumentation for organic reactions is inexpensive.

A major limiting factor for all *in vitro* investigations is the solubility of organic compounds and potential drugs in aqueous solutions, even though newly synthesized drugs should exhibit a satisfying water solubility to ensure sufficient bioavailability. In contrast to the application of enzymes and cell cultures, Fenton's reagent enables the use of solvent mixtures, e.g. with substantial amounts of DMSO or others, and thus allows the investigation of more possible drug candidates.

Taken together, the Fenton approach is not able to completely replace the classical biotransformation studies but provides a fast and cheap alternative for metabolite screening.

Acknowledgements

Thanks are due to the Deutsche Forschungsgemeinschaft (SFB 630 "Recognition, Preparation and Functional Analysis of Agents against Infectious Diseases") for financial support.

References

[1] P. Kapkovà, E. Heller, M. Unger, G. Folkers, U. Holzgrabe, Random chemistry as a new tool for the generation of small compound libraries: development of a new acetylcholinesterase inhibitor, J. Med. Chem. 48 (2005) 7496–7499.
[2] G. Folkers, U. Kessler, Random chemistry: look for the unexpected, Curr. Opin. Drug Discov. Devel. 1 (2003) 1–4.
[3] P. Kapkovà, E. Heller, E. Kugelmann, J. Faber, G. Bringmann, U. Kessler, G. Folkers, U. Holzgrabe, Random chemistry as a new tool for the generation of small-compound libraries, Arch. Pharm. (Weinheim) 339 (2006) 489–497.
[4] E. Kugelmann, U. Holzgrabe, Generation of small-compound libraries via random chemistry by Fenton's reagent, Arkivoc 11 (2008) 247–255.
[5] G. Bringmann, M. Rübenacker, J.R. Jansen, D. Scheutzow, On the structure of the dioncophyllaceae alkaloids dioncophylline A "triphyophylline" and "O-methyltriphyophylline", Tetrahedron Lett. 31 (1990) 639–642.
[6] G. Francois, G. Bringmann, J.D. Phillipson, L. Aké Assi, C. Dochez, M. Rübenacker, C. Schneider, M. Wéry, D.C. Warhust, G.C. Kirby, Activity of extracts and naphthylisoquinoline alkaloids from *Triphyophyllum peltatum*, *Ancistrocladus abbreviatus* and *A. barteri* against *Plasmodium falciparum* in vitro, Phytochemistry 35 (1994) 1461–1464.
[7] G. Francois, G. Timperman, W. Eling, L. Aké Assi, J. Holenz, G. Bringmann, Naphthylisoquinoline alkaloids against malaria: evaluation of the curative potentials of dioncophylline C and dioncopeltine A against *Plasmodium berghei* in vivo, Antimicrob. Agents Chemother. 41 (1997) 2533–2539.
[8] European Directorate for Quality of Medicines, in: C.o. Europe (Ed.), European Pharmacopoeia, Council of Europe, Strasbourg, 2008.
[9] W. Blaschek, S. Ebel, E. Hackenthal, U. Holzgrabe, K. Keller, J. Reichling, V. Schulz, Hagers Handbuch der Pharmazeutischen Praxis, Springer Medizin Verlag, Heidelberg, 2009.
[10] G. Kammann, G. Dietz, H. Schmidt, J. Franke, R. Scheffler, H. Toenjes, Purification of 1-ethyl-6,7-methylenedioxy-4(1H)-oxocinnoline-3-carboxylic acid, a urinary tract antibacterial, DD 1983-249852 ed., Deutschland, 1987.
[11] L.M. Boak, J. Li, R.L. Nation, C.R. Rayner, High-performance liquid chromatographic method for simple and rapid determination of linezolid in human plasma, Biomed. Chromatogr. 20 (2006) 782–786.
[12] K. Borner, E. Borner, H. Lode, Determination of linezolid in human serum and urine by high-performance liquid chromatography, Int. J. Antimicrob. Agents 18 (2001) 253–258.
[13] G. van Vyncht, A. Jànosi, G. Bordin, B. Toussaint, G. Maghuin-Rogister, E. De Pauw, A.R. Rodriguez, Multiresidue determination of (fluoro)quinolone antibiotics in swine kidney using liquid chromatography–tandem mass spectrometry, J. Chromatogr. A 952 (2002) 121–129.
[14] W. Gau, J. Kurz, U. Petersen, H.J. Ploschke, C. Wuensche, Isolation and structural elucidation of urinary metabolites of ciprofloxacin, Arzneim. Forsch. 36 (1986) 1545–1549.
[15] D.A. Volmer, B. Mansoori, S.J. Locke, Study of 4-quinolone antibiotics in biological samples by short-column liquid chromatography coupled with electrospray ionization tandem mass spectrometry, Anal. Chem. 69 (1997) 4143–4155.
[16] G.J. Krol, G.W. Beck, T. Benham, HPLC analysis of ciprofloxacin and ciprofloxacin metabolites in body fluids, J. Pharm. Biomed. Anal. 14 (1995) 181–190.
[17] A. Anadón, M.R. Martinez-Larranga, J. Iturbe, M.A. Martinez, M.J. Diaz, M.T. Frejo, M. Martinez, Pharmacokinetics and residues of ciprofloxacin and its metabolites in broiler chickens, Res. Vet. Sci. 71 (2001) 101–109.
[18] P. Calza, C. Medana, F. Carbone, V. Giancotti, C. Baiocchi, Characterization of intermediate compounds formed upon photoinduced degradation of quinolones by high-performance liquid chromatography/high-resolution multiple-stage mass spectrometry, Rapid Commun. Mass Spectrom. 22 (2008) 1533–1552.
[19] H.G. Wetzstein, M. Stadler, H.V. Tichy, A. Dalhoff, W. Karl, Degradation of ciprofloxacin by basidiomycetes and identification of metabolites generated by the brown rot fungus *Gloeophyllum striatum*, Appl. Environ. Microbiol. 65 (1999) 1556–1563.
[20] P.M. Shah, Ciprofloxacin, Int. J. Antimicrob. Agents 1 (1991) 75–96.
[21] F. Sörgel, J. Bulitta, M. Kinzig-Schippers, Chemie pharmakokinetik und pharmakodynamik von linezolid, in: C. von Eiff (Ed.), Oxazolidinone–Aktuelle Erkenntnisse zu einer neuen Klasse von Antibiotika, Socio-medico Verlag, Wessobrunn, 2001, pp. 59–96.
[22] O.A. Phillips, M. Abdel-Hamid, N.A. Al-Hassawi, Determination of linezolid in human plasma by LC–MS–MS, Analyst 126 (2001) 609–614.
[23] H.Y. Ji, H.W. Lee, S.G. Chang, J.J. Lee, J.K. Rhee, W.B. Kim, H.S. Lee, Liquid chromatography–tandem mass spectrometry for the determination of a new oxazolidinone antibiotic DA-7867 in human plasma, Biomed. Chromatogr. 18 (2004) 86–89.
[24] W.Y. Song, N.J. Kim, S.Y. Kim, H.S. Lee, Liquid chromatography–tandem mass spectrometry for the determination of jaceosidin in rat plasma, J. Pharm. Biomed. Anal. 49 (2009) 381–386.
[25] Y.H. Kim, H.Y. Ji, S. Lee, K.Y. Yi, Y.S. Kim, K.H. Lee, H.S. Lee, Determination of a selective Na+/H+ exchanger inhibitor, 4-cyano(benzo[b]thiophene-2-carbonyl)guanidine (KR-33028) in rat plasma by liquid chromatography–tandem mass spectrometry, Biomed. Chromatogr. 21 (2007) 810–815.
[26] J.G. Slatter, D.J. Stalker, K.L. Feenstra, I.R. Welshman, J.B. Bruss, J.P. Sams, M.G. Johnson, P.E. Sanders, M.J. Hauer, P.E. Fagerness, R.P. Stryd, G.W. Peng, E.M. Shobe, Pharmakokinetics, metabolism, and excretion of linezolid following an oral dose of [^{14}C]linezolid to healthy human subjects, Drug Metab. Dispos. 29 (2001) 1136–1145.

[27] G Bringmann, S. Gramatzki, R. God, P. Proksch, Enzymatic biotransformation of naphthylisoquinoline alkaloids: first hints at the metabolism of dioncophylline A, Planta Med. 58 (1992) 577–578.
[28] M. Sieber, W. Dekant, J.H. Faber, G. Bringmann, Biotransformation and pharmacokinetics of the antiplasmodial naphthylisoquinoline alkaloid dioncophylline A, Xenobiotica 36 (2006) 750–762.
[29] G. Bringmann, D. Lisch, H. Reuscher, L. Aké Assi, K. Günther, Atrop-diastereomer separation by racemate resolution techniques: N-methyl-dioncophylline A and its 7-epimer from *Ancistrocladus abbreviatus*, Phytochemistry 30 (1991) 1307–1310.
[30] G. Bringmann, K. Messer, B. Schwöbel, R. Brun, L. Aké Assi, Habropetaline A, an antimalarial naphthylisoquinoline alkaloid from *Triphyophyllum peltatum*, Phytochemistry 62 (2003) 345–349.
[31] M.T Donato, J.V. Castell, Strategies and molecular probes to investigate the role of cytochrome P450 in drug metabolism, Clin. Pharmacokinet. 42 (2003) 153–178.
[32] S. Härtter, In vitro methoden zur untersuchung des phase-I metabolismus, Pharm. Unserer Zeit 29 (2000) 365–371.