Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

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Options for the Phytoremediation of Polar Pharmaceuticals:

Uptake and Removal of Metformin and Iopromide by *Typha latifolia* L.

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Zusammenfassung

Im vergangenen Jahrzehnt ließen die zunehmende Verschreibung und der Gebrauch von Arzneimitteln und deren darauffolgende Freisetzung in die Umwelt Bedenken hinsichtlich des Schicksals der Ausgangsstoffe wie auch potentieller Rückstände aufkommen. Obwohl Pharmaka aus unserem täglichen Leben nicht mehr wegzudenken sind, ist deren unkontrolliertes Vorkommen in der Umwelt zu einer ernstzunehmenden Bedrohung für die menschliche Gesundheit und auch für die Integrität von Ökosystemen geworden. Einen entscheidenden Aspekt in diesem Zusammenhang stellt die unvollständige Elimination vieler Arzneistoffe durch traditionelle Kläranlagen dar, die deswegen sehr häufig im aufbereiteten Effluenten und auch bereits in natürlichen Gewässern nachweisbar sind. Es ist heute wichtiger denn je, den Gefahren einer Freisetzung von Arzneimitteln, inkl. deren Abbauprodukte, für aquatische Ökosysteme und ebenso für die Qualität unseres Trinkwassers zuvorzukommen.

In dieser Studie untersuchten wir die Aufnahme, Translokation und Transformation von Metformin und Iopromid im breitblättrigen Rohrkolben (*Typha latifolia* L.). Beide dieser polaren Arzneistoffe sind weitverbreitete Präparate in der menschlichen Medizin. Die Pflanzen wurden in einer modifizierten Nährstofflösung nach Hoagland unter Gewächshausbedingungen kultiviert. Nach einer Etablierung hydroponischer Experimente wurde jeweils das Schicksal beider Verbindungen in *T. latifolia* über einen Zeitraum von 28 Tagen hinweg analysiert. Dazu wurden nach der Behandlung mit Metformin (50, 150 und 250 $\mu mol \cdot L^{-1}$) bzw. Iopromid (20 $\mu mol \cdot L^{-1}$) sowohl die Nährstofflösung als auch das Pflanzenmaterial nach 1, 3, 7, 14 und 28 Tagen beprobt.

Hochdruck-Flüssigchromatographie mit Tandem-Massenspektrometrie (LC-MS/MS) diente zur Detektion von Metformin, Iopromid und deren Transformationsprodukten im Pflanzengewebe.

Wir wiesen nach, dass Metformin durch die Wurzeln von *T. latifolia* aufgenommen werden kann. Dies geschah unabhängig von der Ausgangskonzentration. Chinidin, ein Inhibitor für Transporter organischer Kationen, kann die Aufnahme von Metformin durch *T. latifolia* maßgeblich inhibieren. Dies weist auf die hohe Bedeutung dieser membrangebundenen Ionen-Transporter bei der Aufnahme von Metformin in den Symplasten der Pflanze hin. Insgesamt war das Maß der Bioakkumulation von Metformin in Wurzeln viel höher verglichen mit Blättern und Rhizomen. Methylbiguanidin wurde in den Pflanzen als einziges Abbauprodukt von Metformin mit einem Konzentrationsniveau im Bereich von $\mathrm{nmol \cdot g^{-1}}$ (FW) nachgewiesen.

Auch Iopromid kann durch *T. latifolia* aus der Lösung entfernt werden. Dieser Prozess folgt einer Kinetik erster Ordnung mit einem Bestimmtheitsmaß von $R^2 = 0{,}983$. Die Konzentration von Iopromid war in Wurzeln und Rhizomen höher als in Blättern. In *T. latifolia* konnte eine Gesamtzahl von acht Abbauprodukten (23 Isomere) von Iopromid nachgewiesen werden, von denen vier (13 Isomere) hier zum ersten Mal identifiziert wurden. Vier verschiedene Klassen von Abbauprodukten wurden in den Pflanzen gefunden und nach dem Reaktionstyp des Abbaus klassifiziert: 1) Abbau zu Aldehyden und Ketonen, 2) Abbau zu Carbonsäuren, 3) Abbau durch Decarboxylierung, 4) Abbau durch Eliminierung von Iod. Alle Transformationsprodukte in den Pflanzen wurden zum Zwecke einer Vorhersage der möglichen Stoffwechselwege *in planta* zu verschiedenen Zeitpunkten untersucht. Die Ergebnisse der vorliegenden Studie tragen zu einem besseren Verständnis des Schicksals polarer Pharmaka in Pflanzen bei und werden bei der Verbesserung von Technologien für die Phytoremediation solcher Verbindungen von Nutzen sein.

Summary

The increasing prescription and use of pharmaceuticals and their subsequent release into the environment has raised concerns about the fate of the parent compounds as well as potential residues in the environment since the last decade. Although pharmaceuticals have become an indispensable part of our daily life in health and disease, their uncontrolled occurrence in the environment let them become a very important issue for human health and the integrity of ecosystems. A crucial fact in this respect is the incomplete removal of many pharmaceuticals in traditional wastewater treatment plants (WWTPs), causing their widespread detectability in WWTP effluents and natural water bodies. It is ever so important to prevent that pharmaceuticals and their transformation products become major threats to aquatic ecosystems or even to drinking water quality.

In this study, we have investigated the uptake, translocation and transformation of metformin and iopromide in common cattail (*Typha latifolia* L.). Both polar pharmaceuticals are widely used human medicines. The plants were grown in modified Hoagland nutrient solution under greenhouse conditions. Hydroponic experiments were established to assess the fate of both compounds in *T. latifolia* over a period of 28 days. After treatment with metformin (50, 150 and 250 $\mu mol \cdot L^{-1}$) and iopromide (20 $\mu mol \cdot L^{-1}$), nutrient solutions and plant samples were collected after 1, 3, 7, 14 and 28 days. Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to detected metformin, iopromide and their transformation products in plant tissues.

We demonstrated that metformin can be taken up by *T. latifolia* roots, and the uptake processes were independent of initial concentrations. Inhibitors of membrane transporters for

organic cations (e.g. quinidine) can significantly affect uptake of metformin by *T. latifolia* roots. This indicates that organic cation transporters could be one of the important pathways for metformin uptake into the plant´s symplast. The bioaccumulation level of metformin in roots was much higher than in leaves and rhizomes. Methylbiguanide was detected as the only transformation product of metformin in plants with a concentration level of $nmol \cdot g^{-1}$.

Iopromide can be removed by *T. latifolia* and the removal processes followed first order kinetics with a linear regression R^2 value of 0.983. The iopromide concentration in roots and rhizomes was higher than in leaves. A total of 8 transformation products (23 isomers) of iopromide was detected in *T. latifolia*, especially four of them (13 isomers) were identified for the first time. Four different kinds of transformation products were shown in plants and could be classified by the type of reaction: 1) aldehyde and ketone transformation products; 2) carboxylic transformation products; 3) decarboxylated transformation products; 4) deiodinated transformation products. All the transformation products in plants were investigated with the purpose of prediction of the possible transformation pathway *in planta* at different time periods.

The findings of the present study provide a better understanding of the fate of polar pharmaceuticals in plants and will be useful to improve phytoremediation technologies of such kind of compounds.

Chapter 1

Introduction

1.1 Overview

The paramount importance of high quality water resources to human existence and development should not be underestimated. Over the last decades, the quality of water resources has been significantly improved through various governmental interventions. According to the European Commission Water Framework Directive article 4, all community waters including surface and ground are supposed to be in good status by 2015 (Belgiorno et al. 2007). However, due to the increasing human population and less thoroughgoing approaches in wastewater treatment, the security of our water resources is more and more being threatened by many anthropogenic inputs and we are far away from the aforementioned "good status". With the rapid advancement of detection techniques, many emerging contaminants have been found in the environment which provoked increasing concern, such as pharmaceuticals.

Pharmaceutical compounds have been recognized as environmental contaminants and became a widespread concern in the last fifteen years because of their presence and possible harmful effects in the environment (Daughton & Ternes, 1999; Ternes, 1998). Pharmaceuticals include various synthetic chemicals, such as antibiotics, steroid hormones, anti-inflammatorics, anti-diabetics, sedatives, X-ray contrast media, antiepileptics, analgesics, antihypertensive agents, and a lot of other compounds. They are considered a group of the most important emerging pollutants in waterbodies, particularly as no legal requirements have been set yet (Bu et al. 2013; Rivera-Utrillaa et al. 2013; Ternes et al. 2002). Table 1.1 lists pharmaceuticals frequently found in the environment. Although many pharmaceuticals are not long-lived in the biota, these kind of compounds are largely and frequently used for human and livestock health and as such permanently fed into the sewage stream, by this ending up in all kinds of receiving water bodies such as lake, river, wetland, groundwater, coastal and marine environments (Gaw et al. 2014; Li et al. 2012; López-Serna et al. 2013). A special complication arises since a considerable number of these pharmaceuticals is not metabolized or only partly metabolized in the human body. Hence, the parent compounds and their metabolites could be discharged to the environment which may lead to interference in the normal physiological action of the biota (Boxall et al. 2003). Although at present, pharmaceuticals are mostly detected at low concentrations in the environment ($ng \cdot L^{-1}$), the effects of water quality and human health still must not be ignored (Kolpin et al. 2002; Yuan et al. 2009). The impact of increasing numbers of pharmaceuticals on future public health has been on the horizon.

There are already pharmaceuticals in the aquatic environment within the range of concentrations known to cause acute or chronic toxicity (Aus der Beek et al. 2016). Exposure to pharmaceuticals including caffeine, ibuprofen, naproxen, oxytetracycline, carbamazepine, trimethoprim, etc., may lead in many cases to decreased cell viability. It has been shown that redox properties of pharmaceuticals could affect oxidative metabolism in liver cells, causing oxidative damage of rainbow trout hepatocytes (Gagné et al. 2006a). The feminization of fish under the influence of pharmaceuticals in the aquatic environment has already been reported for many countries worldwide (Harris et al. 2011). Gagné et al. (2006b) found that mussels exposed to wastewater containing pharmaceuticals had decreased lipid peroxidation, intra-cellular esterase and phagocytic activity. Their results indicate that these compounds could disrupt the cytokine signalling network in the nitric oxide pathway and lead to adverse effects on the immune system. Exposure to diclofenac, one frequently applied anti-inflammatory drug, almost led to extinction of vultures on the Indian subcontinent (Oaks et al. 2004).

Table 1.1 Frequently found pharmaceuticals in the environment

Compound	Concentration ($ng \cdot L^{-1}$)	Aquatic environment	Reference
Antidiabetics			
Metformin	1220~26000	wastewater effluent	Oosterhuis et al., 2013; Scheurer et al., 2012
	60~3100	surface water	Vulliet & Cren-Olivé, 2011; Scheurer et al., 2012
Glibenclamide	27~110	wastewater effluent	Al Aukidy et al., 2012; Verlicchi et al., 2012
	0.2~3	surface water	Al-Odaini et al., 2013
X-ray contrast media			
Iopromide	3400	wastewater effluent	Kormos et al., 2011
	43~120	surface water	Kormos et al., 2011
Iomeprol	13000	wastewater effluent	Kormos et al., 2011
	100~1450	surface water	Kormos et al., 2011
Iohexol	1200	wastewater effluent	Kormos et al., 2011
	25~57	surface water	Kormos et al., 2011
Iopamidol	16000	wastewater effluent	Kormos et al., 2011
	91~1230	surface water	Kormos et al., 2011
Analgesics			
Diclofenac	210~620	wastewater effluent	Gracia-Lor et al., 2012
	32~156	surface water	Matamoros et al., 2012
Ibuprofen	112~2129	wastewater effluent	De la Cruz et al., 2012; Loos et al., 2013
	8~32	surface water	Matamoros et al., 2012
Psychiatric drugs			
Carbamazepine	216~265	wastewater effluent	Al Aukidy et al., 2012
	12~95	surface water	Matamoros et al., 2012
Lorazepam	46~82	wastewater effluent	Al Aukidy et al., 2012
	26~117	surface water	Moreno-González et al., 2014
Antibiotics			
Sulfamethoxazole	35~185	wastewater effluent	Al Aukidy et al., 2012
	2~65	surface water	Moreno-González et al., 2014

1.2 Occurrence, source and fate of pharmaceuticals in the environment

The occurrence and level of pharmaceuticals in the natural environment depends on many factors, including their consumption and use, metabolism in human body and removal efficiency in wastewater treatment plants (WWTPs). Due to the fact that pharmaceuticals are constantly entering the environment and are extremely resistant to biological and chemical degradation, it is not surprising to detect these compounds in the water bodies with concentration levels ranging from $\mu g \cdot L^{-1}$~$ng \cdot L^{-1}$, while several of them have imposed great threats to the health and security of aquatic ecosystems.

It is already well-established that pharmaceuticals are introduced into the environment mainly through municipal wastewater effluents, hospital effluents, landfills, household disposal and livestock activities (Bound & Voulvoulis, 2005; Fent et al. 2006; Halling-Sorensen et al. 1998; Kümmerer & Al-Ahmad, 2010; Kümmerer & Helmers, 2000; Ternes, 1998). The domestic pathway through municipal wastewater plants has been recognized as the main source of human pharmaceuticals to the aquatic environment, preceded by incomplete metabolism in the human body and excretion or the disposal of medicine via toilets (Kümmerer, 2009; Ternes, 1998; Ternes, 2002). Incompletely eliminated pharmaceuticals and their metabolites are then discharged into natural water bodies. Some recent studies showed that using reclaimed wastewater for irrigation leads to the accumulation of many pharmaceuticals in the soil (Malchi et al. 2014; Paltiel et al. 2016; Prosser et al. 2015). The pharmaceuticals can then leach into the groundwater and are taken up into agricultural crops. The accumulation in edible plant parts will probably continue to imply more risk exposure (Wu et al. 2014; Zemann et al. 2016).

The fate of pharmaceuticals is dependent on a range of impact factors, including their physicochemical properties, such as polarity and pH value; biological activities, such as degradation and natural attenuation (Pal et al. 2010). Generally, hydrophilic compounds have lower sorption coefficients compared to hydrophobic compounds. Pharmaceuticals with low logP < 2.5 are not easily sorbed to sediments and/or soils, while those of high logP of > 5 have high sorption potential and could be sequestered within the riparian zone (Mompelat et al. 2009; Pal et al. 2010).

The present thesis investigates the fate of two contrasting pharmaceuticals of extremely high impact for human health with view to options in phytoremediation. The currently most prescribed antidiabetes drug (metformin) as well as the most frequently applied x-ray contrast compound (iopromide) are taken as examples.

1.3 Metformin

Metformin is the first-line type II antidiabetic agent which is used now worldwide. It can down-regulate hepatic gluconeogenesis, which may be the primary mechanism in managing or preventing diabetes (Kirpichnikov et al. 2002). Metformin exists largely as the hydrophilic cationic species at physiological pH and less than 0.01% as its nonionized form in the blood. The logP value (-2.31) of the nonionized metformin shows its low lipophilicity. Therefore, passive diffusion of metformin through cell membranes is unlikely. This is also confirmed by pharmacokinetic data indicating that metformin interacts with membrane transporters for intercellular distribution. Furthermore, metformin is not metabolized in the human body and excreted unchanged in the urine (Robert et al. 2003).

Many studies have shown that metformin can reach the environment. The concentration of metformin in WWTP influents and effluents ranges from 20 to 129 $\mu g \cdot L^{-1}$ and 2.2 to 47 $\mu g \cdot L^{-1}$, respectively (Blair et al. 2013a, 2013b; Scheurer et al. 2009; Trautwein & Kümmerer, 2011; van Nuijs et al. 2010). In Germany, metformin was detected in Lake Constance， rivers Elbe, Weser and in the North Sea at average concentrations of 102 $ng \cdot L^{-1}$, 472 $ng \cdot L^{-1}$, 349 $ng \cdot L^{-1}$ and 13 $ng \cdot L^{-1}$, respectively (Trautwein et al. 2014). Hence, these observations indicated that the conventional method of wastewater treatment may not be sufficient for the removal of metformin. Moreover, a recent study showed that metformin even occurred in tap water in $ng \cdot L^{-1}$ level (Trautwein et al. 2014).

Whilst metformin could not be thoroughly degraded in conventional wastewater treatment systems, Trautwein & Kümmerer (2011) demonstrated that it can be biodegraded to guanylurea by anaerobic bacteria, but this compound itself remains a recalcitrant transformation product in WWTPs. The mechanism for formation of guanylurea has been recognized as N-dealkylation and oxidative deamination (Eggen & Lillo, 2012; Trautwein & Kümmerer, 2011). Guanylurea has been detected in wastewater effluents in a concentration range of 0.63~99 $\mu g \cdot L^{-1}$, and in surface water in a concentration range of 0.01~28 $\mu g \cdot L^{-1}$ (Kosma et al. 2015; Scheurer et al. 2012; Trautwein et al. 2014).

Despite this work, there is little knowledge about the toxic effects of metformin on non-target organisms. Several recent studies showed that metformin may cause endocrine disruption and affect reproductive capacity in fish (Niemuth et al. 2015; Niemuth & Klaper, 2015). Niemuth et al. (2015) indicated that an exposure at an environmentally relevant concentration of metformin can significantly induce transcription of the mRNA for vitellogenin in male fathead minnow (*Pimephales promelas*). In their further experiment, impacts on reproduction and gonad histology of metformin exposure were revealed. Metformin exposure can induce the development of intersex gonads and the production of significantly fewer clutches in male fish (Niemuth & Klaper, 2015). Furthermore, Niemuth et al. (2015) suggested that metformin may have impacts on endocrine pathways by affecting insulin signaling.

1.4 Iopromide

Iopromide is a low osmolar, non-ionic X-ray contrast medium used for medical imaging. This organic iodine compound can block X-rays and makes the vascular system appear opaque in its path of flow, thereby allowing visualization of the body structures. In Germany, approximately 130 tons per year of iopromide are applied, and it is excreted mainly in its nonmetabolized form (>95%) in the urine (Steger-Hartmann et al. 1999; Schulz et al. 2008). Because of its high usage and rapid excretion, iopromide has frequently been detected in the environment, especially in water bodies (Kormos et al. 2011; Pérez & Barceló, 2007; Ternes

& Hirsch, 2000).

The concentration of iopromide in WWTP influents ranges from 60 to 176.6 $\mu g \cdot L^{-1}$, and in WWTP effluents ranges from 2.6 to 3.4 $\mu g \cdot L^{-1}$, respectively (Kormos et al. 2011; Kovalova et al. 2012). Kormos et al. (2011) reported that iopromide has been detected in a concentration range of 43~120 $ng \cdot L^{-1}$ in Germany´s aquatic environment, while it also has been detected at a concentration of 134 $ng \cdot L^{-1}$ in South Korea (Kim et al. 2007).

Iopromide cannot be completely degraded in traditional WWTPs, but it can form many related TPs. The ecotoxicological effects of these TPs may not be ignored because many of these iodine containing compounds were shown to be even more toxic than iopromide (Richardson et al. 2008). Recent studies indicate that highly toxic iodinated iopromide TPs can form during drinking water disinfection (Duirk et al. 2011). Thus, for wastewater re-use, care must be taken to avoid the formation of these TPs or to remove them from the water.

1.5 Phytoremediation

Phytoremediation is an efficient clean up technology for a great variety of organic pollutants. It is worth mentioning that phytoremediation might be especially important for those organic compounds which are recalcitrant to degradation in the environment. Phytoremediation is also a recommended approach as ternary stage for older and small scale treatment facilities considering its powerful adaptability, low cost and easy maintenance (Schröder et al. 2007a). But although the technology is frequently being used, there is little information with regards to the ability of plants to assimilate and translocate pharmaceuticals, and the only available data is limited to a few types of compounds and plant species (Zhang et al. 2014).

Uptake, translocation and degradation of contaminants by plants have been considered as important mechanisms for phytoremediation (Burken & Schnoor, 1998; Collins et al. 2006). The most widely used approaches for evaluation of plant uptake and translocation potential of such compounds are based on their hydrophobicity (Briggs et al. 1982; Topp et al. 1986). Although earlier studies have indicated that the uptake of many chemicals into plant roots was proportional to their logP, recent studies indicate that plant uptake might be independent or not linearly dependent of logP in aquatic plant systems (Briggs et al. 1982; Dettenmaier et al. 2009; Reinhold et al. 2010). Limmer & Burken (2014) use weighted desirability functions of several molecular descriptors, such as hydrophobicity, molecular mass, hydrogen bonding, polar surface area, and rotatable bonds identified chemicals amenable to translocation by plants.

Following plant uptake and translocation, contaminants may be transformed to less toxic compounds and may then be bound in plant tissues to yield unavailable endproducts. It seems a common feature of higher aquatic plants to have the potential for transformation of organic contaminants to nontoxic products through phytodegradation (Zhang et al. 2014). In all higher organisms, including plants, organic chemicals generally degrade via three phases: Phase I) chemical modification (e.g. oxidations, reductions); Phase II) conjugation (e.g. with glutathione, glucoside, amino acids); Phase III) excretion/compartmentation (e.g. vacuoles, cell walls) (Shimabukuro, 1975; Schröder & Collins, 2002). Due to the lack of excretion organs, the term “storage excretion” has been coined for phase III in plants (Theodoulou 2000).

The enzymatic transformation of organic contaminants can occur in both, plant root and shoot and is generally attributed to the living plant cells. However, in some cases also endophytic microorganisms may be involved (Barac et al. 2004). Of the enzyme classes involved in transformation, the most important are dehalogenases, monooxygenases, peroxidases, nitrate reductase, glutathione-S-transferases, glucosyltransferases, etc. The transformation products (TPs) of contaminants will later accumulate in vacuoles or cell walls (Pilon-Smits, 2005).

1.6 Aims of the thesis

The overall aim of this work is to understand the mechanisms involved in the phytoremediation of contaminated water bodies. Since pharmaceuticals and their TPs are recognized as relevant environmental contaminants, the removal, uptake, translocation and transformation of selected pharmaceuticals in *T. latifolia* have been investigated. The investigated pharmaceuticals are metformin and iopromide, which are two frequently detected compounds in water bodies.

The main aims of the thesis are as follow:

I) Investigation of the uptake and translocation of metformin and iopromide in plants.

II) Investigation of the function of organic cation transporters (OCTs) during the transport process in plants.

III) Identification of the TPs of metformin and iopromide in plants.

IV) Exploration of the transformation pathway of metformin and iopromide in plants.

Chapter 2

Materials and Methods

2.1 Plant material

Typha latifolia, commonly known as broadleaf cattail, is a cosmopolitan perennial hydrophyte found in North America, Europe and Asia. The plants are 1~3 metres high and have 1.5~2.5 cm broad leaves. They form underground thick starchy rhizomes, which are 0.5~3 cm in diameter (Grace & Harris, 1986). Roots are produced from the rhizomes at the base of the leaves. The brown continuous spike includes a pistillate portion below the staminate portion, which form at the top of the stem (Krattinger, 1975; Grace & Harris, 1986).

T. latifolia is an "obligate wetland" species, which generally grows in a variety of freshwater systems or even slightly brackish marshes with water depths ranging from 0.2 to 0.95 meters (Grace, 1989; Grace & Harrison, 1986). The plant is well adapted to such anoxic habitats, notably through the development of aerenchyma which can facilitate diffusion of enough oxygen to the rhizomes and roots (Sale & Wetzel, 1983).

Phragmites australis, known as the common reed, is a wetland plant with an extensive range throughout the world. It is a stout grass that measures 2~6 m in height. The stems and leaves are smooth and glabrous, up to 70 cm long and 1~5 cm wide. The dull purple to yellow inflorescence is a terminal panicle, approximately 30 cm long (Mal & Narine, 2004).

P. australis rhizomes have extensive aerenchymatous tissues (Mal & Narine, 2004). Thus, it is in tolerance of water depth, which can be up to 2m (Hocking et al. 1998). *P. australis* tolerates moderate levels of salt, so is also often found in wetlands which are occasionally inundated by the sea (Hocking et al. 1998).

Both *T. latifolia* and *P. australis* are the most frequently used plants in constructed wetlands. These plants are used to purify raw water by absorption, transformation and filtration through their extended root systems. Due to their great tolerance and capacity to toxicity many pollutants, the plants have advantages in sewage disposal, especially for some recalcitrant emerging contaminants.

Table 2.1 Taxonomic classification of *Typha latifolia*.

Kingdom	Plantae
Division	Angiospermae
Class	Monocotyledonae
Subclass	Commelinids
Order	Poales
Family	Typhaceae
Genus	*Typha*

Table 2.2 Taxonomic classification of *Phragmites australis*.

Kingdom	Plantae
Division	Angiospermae
Class	Monocotyledonae
Subclass	Commelinids
Order	Poales
Family	Poaceae
Genus	*Phragmites*

2.2 Experimental setup

2.2.1 Plant growth conditions

Typha latifolia and *Phragmites australis* plants were purchased from a local nursery (Jörg Petrowsky, Eschede, Germany). Rhizomes were thoroughly washed in tap water. Plants were grown in 5 L vessels filled with perlite and grown under the following greenhouse conditions: light/darkness, 12/12h; diurnal temperature, 23/18°C; humidity, 65%. Nutrients were provided in a modified Hoagland nutrient solution: 2.5 $mmol\cdot L^{-1}$ K^+, 2.0 $mmol\cdot L^{-1}$ Mg^{2+}, 2.0 $mmol\cdot L^{-1}$ Ca^{2+}, 2.0 $mmol\cdot L^{-1}$ SO_4^{2-} , 6.0 $mmol\cdot L^{-1}$ NO_3^-, 0.5 $mmol\cdot L^{-1}$ $H_2PO_4^-$,50 $\mu mol\cdot L^{-1}$ Fe^{2+}, 50 $\mu mol\cdot L^{-1}$ BO_3^{3-}, 1$\mu mol\cdot L^{-1}$ Mn^{2+}, 0.5 $\mu mol\cdot L^{-1}$ Cu^{2+}, 0.5 $\mu mol\cdot L^{-1}$ Zn^{2+}, 0.1 $\mu mol\cdot L^{-1}$ MoO_4^{2-} and the pH was adjusted to 6.0. Plants were acclimated to greenhouse conditions at least two months before they were used in experiments.

2.2.2 Pitman chamber experiments

The incubation chambers used in this study had been shown in previous studies (Pitman, 1971; Schröder et al. 2007b). Five unbroken roots (approximate 10 cm long) were carefully cut from selected plants then inserted into the chamber immediately. Each chamber is divided into three independent compartments by baffles, the compartments named A, B and C from left to right. Root tip sections were oriented towards compartment A, whereas the root base stretched into compartment C. Vaseline was applied to prevent the exchange of solutes between compartments by filling all the gaps in the baffles.

For comparison with metformin, carbamazepine and caffeine were individually spiked into compartments A at an initial concentration of 0.5 $mmol\cdot L^{-1}$. Roots of two plant species (*T. latifolia* and *P. australis*) were used to determine metformin transport rates and to investigate the difference of transport between plant species. Metformin was spiked into compartment A at three different concentrations of 0.5, 1.0 and 2.0 $mmol\cdot L^{-1}$. In a third study, only *T. latifolia* was used to investigate the inhibition of OCTs in the root tissue. An initial metformin concentration of 0.5 mmol·L^{-1} and an OCT inhibitor (quinidine, 0.5 mmol·L^{-1}) were spiked into compartment A, while in the absence of OCT inhibitor chambers containing only metformin were used as control.

Compartments B and C were filled with sucrose solution at concentrations of 0.03 mol·L^{-1} in order to mimic an appropriate osmotic pressure in the roots (Tazawa et al. 2001). A control receiving an equivalent amount of metformin and sucrose solution without roots was used to investigate degradation of metformin during the experiment. Water samples were collected from both compartments A and C after 10, 20, 30, 45, 60, 75 and 90 mins, and metformin concentrations were analysed by LC/MS.

2.2.3 Hydroponic experiment with metformin and iopromide

Plants of uniform size were selected for the experiments. Plant roots were carefully rinsed in distilled water and transferred to 2.5 L pots which contained 1.5 L perlite. Each pot contained 1 L nutrient solution that was spiked with the respective pharmaceutical (metformin or iopromide). The mock plants were grown under the same condition in absence of the pharmaceutical to ensure plants could not get into contact with the pharmaceuticals from other sources. Controls received equivalent amounts of the pharmaceuticals in absence of plants.

Samples were collected at each exposure period, i.e., after 1, 3, 7, 14 and 28 days. For each of the three different concentrations, three parallel treatments were set up corresponding to each exposure period studied. Three replicates were treated for each assay and all assays were performed during the same period of time. Three replicates were set up for the control throughout the experimental period. For each exposure time, one replicate was harvested. Root, leaf and rhizome, respectively, were collected at the same time, frozen in liquid nitrogen and then stored at -80°C for later analysis. The nutrient solutions remaining in each pot were also collected at each exposure period and were analyzed later.

The three different initial concentrations of metformin were: 50, 150 and 250 μmol·L^{-1}.

The three different initial concentrations of iopromide were: 5, 10 and 20 µmol·L^{-1}.

2.3 Extraction of the pharmaceuticals and their TPs

2.3.1 Metformin and its TPs

The extraction of metformin and its TPs from plant tissues followed a recently published method (Hormazábal & Østensvik, 2010). A mixture of 0.5 mol·L^{-1} ammonium acetate/formic acid (90/10, v/v) was used as extraction solution. 0.2 g fresh plant material was ground under liquid nitrogen and extracted with 1.8 ml extraction solution. The mixture was vortexed for 30 sec, ultrasonicated for 15 mins and centrifuged at 16,000 g and 4°C for 5 mins. The supernatant was filtrated (0.45 µm, nylon) then transferred to a solid phase extraction (SPE) cartridge (Bond Elut LMS, Agilent, Germany). The eluate was slowly collected at a flow rate of 2 mL·min^{-1} and then injected into the LC-MS/MS system.

2.3.2 Iopromide and its TPs

The extraction method of iopromide and its TPs from plant tissues may be described as follows. 0.5 g fresh plant material was ground under liquid nitrogen and extracted with 4.5 ml extraction solution (ultrapure water with 0.1% formic acid). The mixture was homogenized for 30sec, ultrasonicated for 10 mins and then centrifuged at 10,000 g and 4°C for 5 mins. The supernatant was filtrated through a 0.45 µm Nylon filter, and then transferred to SPE cartridges (Oasis HLB, Waters, Germany). The SPE cartridge had been preconditioned with 3×1 mL methanol followed by 3×1 mL ultrapure water. Plant extracts were percolated through the cartridge with 5 mL, following 3×1 mL of ultrapure water with 5% methanol for washing the cartridge. Finally, the analytes were slowly eluted using 1 mL methanol/acetonitrile solution (50/50, v/v) and injected into the LC-MS/MS system.

2.4 Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of the pharmaceuticals

2.4.1 Metformin and its TPs

The analysis for determination of metformin and its TPs followed published methods (Cui & Schröder, 2016). Briefly, the HPLC system (Varian ProStar 210, Darmstadt, Germany) was coupled to an ion trap mass spectrometer (Varian 500-MS, Darmstadt, Germany) with an electrospray ionization (ESI) source. The electrospray interface operated in the positive ion mode. Separation was achieved on a Synergi Polar-RP 80a column (150 mm × 2 mm, 4 µm, Phenomenex, Germany). Gradient elution was carried out with 0.1% formic acid in water-acetonitrile. The injection volume was 15 µL and the flow rate 0.3 mL·min^{-1}. The gradient elution of metformin and its TPs are displayed in Table 2.3. For the determination of metformin and its TPs, the precursor ion and the characteristic fragment ions are shown in Table 2.4.

Table 2.3 The LC-MS/MS solvent gradient program for metformin identification

Time / min	Solvent A (H_2O + 0.1% HCOOH) / %	Solvent B (CH_3CN + 0.1% HCOOH) / %
0	97	3
2.5	97	3
7.5	5	95
10.0	5	95
11.0	97	3
13.0	97	3

Table 2.4 Characteristic MS/MS fragmentation of metformin and methylbiguanide

Compound	Precursor ion *m/z* (M+H)	Fragment ions *m/z* (M+H)
Metformin	130.2	113.1, 71.1, 60.1
Methylbiguanide	116.2	60.1, 74.1, 99.0

2.4.2 Iopromide and its TPs

Iopromide and its TPs were analyzed using the method described by Schulz et al., (2008). In short, the same LC-MS/MS system which was described in chapter 2.4.1 was used for iopromide analysis. The gradient elution for iopromide and its TPs is summarized in Table 2.5, and the precursor ion and fragment ions of iopromide and its TPs are displayed in Table 2.6.

Table 2.5 The LC-MS/MS gradient program for iopromide metabolism studies

Time / min	Solvent A (H_2O + 0.1% HCOOH) / %	Solvent B (CH_3CN + 0.1% HCOOH) / %
0	90	10
2.5	90	10
12.5	5	95
16.0	5	95
17.5	90	10
19.0	90	10

Table 2.6 Characteristic MS/MS fragmentation of iopromide and its TPs

Compound	Precursor ion *m/z* (M+H)	Fragment ions *m/z* (M+H)
Iopromide	791.8	773.8, 572.9, 558.9
TP789A1-2	789.8	698.9, 686.8, 559.1
TP789B1-2	789.8	701.0, 699.9, 572.9
TP787A	787.8	718.1, 686.9, 559.2
TP787B	787.8	701.3, 700.0, 572.9
TP787C1-4	787.8	716.0, 698.9, 571.2
TP805A	805.8	714.9, 587.2, 559.1
TP805B	805.8	760.2, 700.7, 573.1
TP803A1-2	803.8	715.0, 684.9, 557.3
TP803B1-2	803.8	700.8, 698.9, 571.2
TP819	819.8	715.1, 700.8, 587.2
TP817A	817.8	713.0, 700.9, 573.1
TP817B	817.8	714.9, 699.0, 587.1
TP815	815.8	712.9, 670.0, 585.0
TP787D1	787.8	712.9, 671.0, 542.8
TP787D2	787.8	699.0, 684.8, 557.0
TP665	665.8	575.0, 561.0, 520.0

2.5 High performance liquid chromatography (HPLC) analysis of metformin

Methods for metformin determination in water samples have been described in a previous publication (Cui et al. 2015). In short, samples were filtrated by 0.45 μm Nylon filter then injected into the HPLC (Varian ProStar 210, Darmstadt, Germany) via an auto sampler (Varian ProStar 410, Darmstadt, Germany). Separation was achieved on a reverse-phase XDB C18 column (4.6 × 150 mm, Agilent, Germany) with an injection volume of 25 μL. Detection of metformin was carried out using an UV detector (Shimadzu SPD-20, Germany) at a wavelength of 226 nm. A mixture of the aqueous buffer and acetonitrile (68:32, v/v) was used for the isocratic elution at a flow rate of 1.0 $mL \cdot min^{-1}$. The aqueous buffer consisted of 10 $mmol \cdot L^{-1}$ disodium hydrogen phosphate and 10 $mmol \cdot L^{-1}$ sodium dodecyl sulfate (SDS) and its pH value adjusted to 7.5.

2.6 Statistical analyses

Data were analyzed for normal distribution. One-way ANOVA and Tukey's tests at $p<0.05$ were used to determine significant differences between groups. These analyses were performed using SPSS v16.0 (SPSS, Chicago, IL, USA).

Chapter 3

Manuscript overview

This thesis contains four manuscripts, for which a brief summary, the publication status and the contribution of the authors is given.

Manuscript 1: Cui, H., Hense, B. A., Müller, J., Schröder, P., 2015. Short term uptake and transport process for metformin in roots of *Phragmites australis* and *Typha latifolia*. Chemosphere 134: 307-312.

Manuscript 2: Cui, H & Schröder, P., 2016. Uptake, translocation and possible biodegradation of the antidiabetic agent metformin by hydroponically grown *Typha latifolia*. Journal of hazardous materials 308: 355-361.

Manuscript 3: Cui, H., Sauvêtre, A., Schröder, P. Identification of X-ray contrast medium iopromide and its transformation products in *Typha latifolia* L.: Side chain oxidation and deiodination. Under revision with Environmental Science & Technology.

Manuscript 4: Cui, H., Hrabé de Angelis, M., Schröder, P. Iopromide exposure in *Typha latifolia* L.: Evaluation of uptake, translocation and possible transformation pathway in planta. In preparation.

Manuscript 1: Short term uptake and transport process for metformin in roots of *Phragmites australis* and *Typha latifolia*

Hao Cui, Burkhard A. Hense, Johannes Müller, Peter Schröder

Published 2015 in Chemosphere 134: 307-312. doi:10.1016/j.chemosphere.2015.04.072.

This manuscript investigates uptake and transport of metformin in roots of *Phragmites australis* and *Typha latifolia*. Metformin as an emerging contaminant has been detected in surface water and wastewater in numerous countries, due to insufficient retention in classical waste water treatment plants. In order to characterize the uptake of the compound during phytotreatment of waste water, a short term pitman chamber experiment was carried out to assess the characteristics of metformin uptake and transport by roots. Three different concentrations (0.5, 1.0 and 2.0 $\mathrm{mmol \cdot L^{-1}}$) were applied to cattail (*Typha latifolia*) and reed (*Phragmites australis*) roots which were used to investigate the uptake mechanism because they are frequently utilized in phytoremediation. In addition, quinidine was used as an inhibitor to assess the role of OCTs in the uptake of metformin by *T. latifolia*. The transport process of metformin is different from carbamazepine and caffeine. In both *T. latifolia* and *P. australis*, the uptake processes were independent of initial concentrations. Quinidine, a known inhibitor of organic cation transporters, can significantly affect metformin uptake by *T. latifolia* roots with inhibition ratios of 70~74%. Uptake into the root could be characterized by a linear model with R^2 values in the range of 0.881~0.999. Overall, the present study provides evidence that metformin is taken up by plant roots and has the potential for subsequent translocation. OCTs could be one of the important pathways for metformin uptake into the plant. The difference in transport process in roots between ionic organic chemicals and non-ionic organic chemicals suggested that membrane proteins may considered as the potential conduit for xenobiotic transport.

HC & PS conceived the idea for the manuscript. HC performed all the experiments and analyzed the data. BAH & JM were involved in setting up the mathematic model. HC wrote the first manuscript draft. PS commented on all manuscript versions.

Manuscript 2: Uptake, translocation and possible biodegradation of the antidiabetic agent metformin by hydroponically grown *Typha latifolia*

Hao Cui & Peter Schröder

Published 2016 in Journal of Hazardous Materials 308: 355-361.

doi:10.1016/j.jhazmat.2016.01.05.

This manuscript investigates the fate of metformin in *Typha latifolia*. The increasing load of pharmaceutical compounds has raised concerns about their potential residues in aquatic environments and ecotoxicity. Metformin, a widely prescribed antidiabetic II medicine, has been detected in high concentration in sewage and in wastewater treatment effluents. An uptake and translocation study was carried out to assess the ultimate fate of metformin in phytoremediation. Metformin was removed from media by *T. latifolia*, and the removal processes followed first order kinetics. After 28 days, the removal efficiencies were in a range of 74.0±4.1~81.1±3.3%. In roots, metformin concentration was increasing during the first two weeks of the experiment but thereafter decreasing. In contrast, metformin concentration was continuously increasing in rhizomes and leaves. Bioaccumulation of metformin in roots was much higher than in leaves and rhizomes. As degradation product of metformin in the plant, methylbiguanide was detected whereas guanylurea was undetectable. Moreover, methylbiguanide concentration in roots increased with exposure time. An enzymatic degradation experiment showed the degradation rate followed the order of metformin < methylbiguanide << guanylurea. This may explain the low concentration of methylbiguanide in the plant tissue. The findings of this study contribute to understand and evaluate the potential for phytoremediation of such kind of contaminants. The results suggest that metformin can be taken up and then further degraded by plants.

HC & PS conceived the idea for the manuscript. HC performed all the experiments and analyzed the data. HC wrote the first manuscript draft. PS commented on all manuscript versions.

Manuscript 3: Identification of X-ray contrast medium iopromide and its transformation products in *Typha latifolia* L.: Side chain oxidation and deiodination

Hao Cui, Andrés Sauvêtre, Peter Schröder

Under revision with Environmental Science & Technology

This manuscript investigates the transformation products (TPs) of iopromide in *Typha latifolia*. The TPs of environmental contaminants have to be clearly identified to predict biodegradation processes and the efficiency of treatment processes. A hydroponic experiment was conducted to assess the fate of iopromide in *T. latifolia* over a period of 14 days. Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to identify the structure of iopromide TPs. A total of 8 TPs (23 isomers) were detected in *T. latifolia* after the iopromide treatment, especially four of them (13 isomers) which were identified by the characteristic MS/MS fragmentation, have never been reported before. According to the type of reaction, the transformation pathway could be separated into four stages: I) the hydroxyl groups are oxidized to aldehyde or ketone groups; II) aldehyde groups are oxidized to carboxyl groups; III) groups are decarboxylated; IV) groups are deiodinated. Carboxylic acids were found as the major type of iopromide TPs in both roots and leaves. These results contribute to evaluate the fate of such kind of contaminants during phytoremediation treatments. It is important for further studies to give a better understanding of potential mechanisms of iopromide biodegradation.

HC & PS conceived the idea for the manuscript. HC defined the final outline of the manuscript and collected trait information. HC performed all the experiments. HC & AS analyzed the data. HC wrote the first manuscript draft. AS & PS commented on all manuscript versions.

Manuscript 4: Iopromide exposure in *Typha latifolia* L.: Evaluation of uptake, translocation and possible transformation pathway *in planta*.

Hao Cui, Martin Hrabé de Angelis, Peter Schröder

In preparation

This manuscript quantifies uptake, investigates translocation and identifies the transformation pathways of iopromide in *Typha latifolia*. Iopromide is frequently detected in water bodies due to its widespread use as an X- ray contrast agent in medicine. Due to its rapid clearance from the human body and its incomplete removal by standard wastewater treatment, an elevation of its concentration in the environment is observed that might lead to a serious impact on human and environmental health. Alternative removal technologies may be more effective to remove iopromide from the effluents of wastewater treatment facilities, like phytoremediation with aquatic macrophytes. A hydroponic experiment was carried out to assess the fate of iopromide in *Typha latifolia*. The TPs in plant were investigated to predict the possible transformation pathway. The removal process followed first order kinetics with a linear regression R^2 value of 0.983. The iopromide concentration in roots and rhizomes reached a maximum value of 20.70±0.81 and 16.82±1.78 nmol·g^{-1} on the 7th day, respectively, thereafter decreased until the end of experiment. A different result was found in leaves, where iopromide concentration decreased over the whole experimental period. A total of eight TPs were detected in *T. latifolia,* including 23 isomers. The relative content of aldehyde and ketone TPs decreased in roots and rhizomes while the relative content of carboxylic TPs increased. However, the relative content of aldehyde and ketone TPs only showed a slight decrease in leaves while the relative content of carboxylic TPs remained stable during the experimental period. In addition, a significantly increase of decarboxylated TPs was found in leaves, but not in roots and rhizomes. These results indicate that a difference of transformation pathways exists among plant tissues. The findings of this study are important to better understand the transformation pathway of iopromide in plants and to improve phytoremediation technologies for such kind of compounds.

HC & PS conceived the idea for the manuscript. HC defined the final outline of the manuscript and collected trait information. HC performed all the experiments and analyzed the data. The plant X-ray experiment was performed in MH´s institute. HC wrote the first manuscript draft. PS commented on all manuscript versions.

Chapter 4

Discussion

Polar pharmaceuticals are frequently detected in the environment due to their relatively high solubility and mobility in water (Carrara et al., 2008; Chefetz et al., 2008; Matamoros et al., 2012). A major point contributing to their dispersal relates to the fact that in conventional WWTPs, polar pharmaceuticals are not expected to be completely removed by sorption to sludge (Loos et al., 2013; Reemtsma et al., 2006), since they are poorly degradable, and thus may finally be discharged with effluents into water bodies. Although partial degradation implies that these compounds may be further broken down in natural water bodies (Reemtsma et al., 2006), the formation of unknown polar TPs which are eventually more toxic than their parent compounds may increase the environmental risk.

Up to now, the fate of these contaminants has mainly been investigated in the environment, such as water bodies, soil and sediment, where a large variety of these compounds and their TPs have been detected (Drewes et al., 2001; López-Serna et al., 2013; Kosma et al. 2015). However, their fate in organisms, especially in plants is much less investigated. As an important part of the biosphere, plants have significant impact on the transformation of these compounds in the environment. Plants can be used to clean contaminated water by taking up contaminants through their roots, a process which is called "phytoremediation" (Schröder & Collins, 2002). This approach has the superiority in operation simplicity and environmental friendliness above engineering solutions.

This thesis complements the existing knowledge by providing an insight into the fate of polar pharmaceuticals in plants. Both metformin and iopromide can be taken up, translocated and transformed in *T. latifolia*. Short term uptake (1.5h) of metformin into plant roots could be characterized by a linear model and organic cation transporters (OCTs) may be considered as a potential conduit for metformin (Manuscript 1). The hydroponic experiment showed that the bioaccumulation of metformin in roots was much higher than in leaves and rhizomes. methylbiguanide was detected in roots, which was first found as a metformin TP in *T. latifolia* (Manuscript 2). A total of eight iopromide TPs (23 isomers) were detected in *T. latifolia*, which can be separated into four types: 1) aldehyde and ketone TPs; 2) carboxylic TPs; 3) decarboxylated TPs; 4) deiodinated TPs (Manuscript 3). The bioaccumulation of iopromide was lower than the accumulation of metformin in roots and leaves (Manuscript 2; Manuscript 4). The aldehyde and ketone TPs were dominant in roots and rhizomes at the beginning of the experiment, while the carboxylic TPs were dominant at the end. Besides this, the carboxylic TPs were dominant in leaves during the experiment period (Manuscript 4).

4.1 Uptake and translocation of pharmaceuticals in plants

The biological removal process of pollutants by plants includes several mechanisms, i.e. absorption, uptake and degradation. Due to the fact that the concentrations of the polar pharmaceuticals did not show any obvious change in the controls over the experimental period, photodegradation and surface adsorption seem to be negligible for the removal process. In addition, the kind of hydrophilic compounds used (metformin: logP = -2.31; iopromide: logP = -2.95) cannot be expected to strongly adsorb on the walls of plastic vessels and/or the surfaces of roots and rhizomes. Therefore, the main way of removal of polar pharmaceuticals during the experimental period is uptake by plants.

The removal process of both pharmaceuticals (metformin and iopromide) seems to follow first order kinetics at least in the initial phases of the experiments. However, metformin uptake rates on day 28 were far away from the linear trend lines, especially after treatment with low concentrations (Manuscript 2). This was significantly different in iopromide treatment which fitted well to the first order kinetics during the whole experimental period (R^2 = 0.983, Manuscript 4). Metformin is obviously taken up by plants via active transport, while iopromide enters the root via passive diffusion. In the experiments with *T. latifolia*, metformin concentration was obviously high enough so that the removal rate was only dependent on the activity of the transporters. This may explain the deviation of metformin results from the trend line at day 28, because its concentration decreased with incubation time.

Generally, the logP value is used as the important property to predict xenobiotics uptake by plants (Briggs et al., 1982; Trapp et al., 1994). A previous study indicated that organic chemicals with an optimum hydrophobicity (logP = 0.5~3) show great potential of uptake and translocation in plants (Pilon-Smits, 2005). However, and contrary to the latter findings, a recent study showed that even more hydrophilic chemicals also can be well taken up and translocated by plants (Dettenmaier et al., 2009). It is suggested that logP values have only limited predictability for uptake and translocation of xenobiotics by plants (Table 4.1). Since such prediction is based on passive diffusion mechanisms through lipophilic biomembranes, it is appropriate only for neutral compounds.

The uptake and transport process of metformin, a cationic compound, are different from neutral compounds. Interesting differences of uptake and transport processes by *T. latifolia* roots were observed when comparing carbamazepine, metformin and caffeine (Manuscript 1). Dodgen et al. (2015) indicated that for neutral pharmaceutical and personal care products (PPCPs), a positive linear correlation with logD could be observed for bioaccumulation factors (BCFs) of roots. In comparison, no significant correlation was observed between logD and BCFs of roots for cationic compounds.

The result of the pitman chamber experiments showed that metformin can be taken up by roots, but was relatively slow with a rate range of 0.022~0.025 μmol·L·min^{-1}. Thus, this compound is not expected to enter plant roots at a high rate. It also shows that plant uptake of metformin was probably independent of its hydrophilicity. The barrier between roots and leaves for metformin, such as the expression of organic cation transporters, may have more significance than its logP value. Indeed, our results indicate that OCTs may play an important role in uptake and transport of metformin (Manuscript 1). Therefore, the logP value may have limited the predicted translocation at least of compounds like metformin in plants.

The metformin concentration in roots was 1~2 orders of magnitude higher than in rhizomes and leaves. In contrast, iopromide concentration in roots and in rhizomes showed a similar level. This indicated that a barrier could exist between roots and rhizomes for metformin. Plant uptake processes include both, an apoplastic and a symplastic pathway. The apoplastic pathway flow is forced to move into the symplast pathways due to the interruption by the casparian strip. Thus, the active transporters are the only route the ionizable compounds use to enter the vascular system. However, iopromide can enter the rhizomes by passive diffusion since it is a non-ionizable compound so that a similar concentration level of iopromide builds up in roots and rhizomes.

Iopromide concentration in leaves was also much lower than in roots. However, the iopromide concentration in leaves continuously decreased with incubation time while metformin concentration in leaves continuously increased. This may be due to a balance between the compound´s translocation and transformation. Since iopromide has relatively high chemical reactivity and many of its TPs have been found in this study, we can assume that the transformation rate of iopromide was higher than its translocation rate in leaves. On the contrary, metformin was continuously accumulated in leaves probably because its transformation rate was lower than its translocation rate in leaves.

The bioaccumulation factors of metformin (8.37~53.34) were much higher than those of iopromide (0.02~1.04). One reason could be that iopromide is much easier degraded than metformin. This was also supported by our finding that a series of iopromide TPs could be detected in *T. latifolia* (Manuscript 3). A previous study also showed a surprisingly high uptake of metformin in carrot and barley roots indicating that some other processes might be involved in the uptake of this ionizable compound in different species (Eggen et al., 2011).

Table 4.1 The bioaccumulation factor (BAF) of selected pharmaceuticals

Compound	Spiked initial concentration	Incubation time	logP[a]	Plant species	BAF of root	BAF of leaf	Reference
Carbamazepine	1.0 mg·L^{-1}	21 days	2.67	*Scirpus validus*	13.00	0.60	Zhang et al. (2013a)
Naproxen	1.0 mg·L^{-1}	21 days	3.00	*Scirpus validus*	0.30	1.60	Zhang et al. (2013a)
Sulfamethoxazole	232.5 μg·L^{-1}	51 days	0.89	*Brassica rapa var.pekinensis*	0.47	0.07	Herklotz et al. (2010)
Trimethoprim	232.5 μg·L^{-1}	51 days	0.38	*Brassica rapa var. pekinensis*	0.31	0.04	Herklotz et al. (2010)
clofibric acid	1.0 mg·L^{-1}	21 days	2.72	*Scirpus validus*	16.62	32.13	Zhang et al. (2013b)
metformin	10 mg·kg^{-1}	2-3 months	-2.31	*Hordeum vulgare*	approx. 8	approx. 1.5	Eggen et al. (2011)
				Daucus carota ssp. sativus cvs	approx. 2	0.22	Eggen et al. (2011)
	6.5 mg·L^{-1}	28 days		*Typha latifolia*	26.60	1.39	Manuscript 2
Iopromide	15.8 mg·L^{-1}	28 days	-2.95	*Typha latifolia*	0.05	0.02	Manuscript 4
Narasin	10 mg·kg^{-1}	2-3 months	6.59	*Hordeum vulgare*	0.67	0.03	Eggen et al. (2011)
				Daucus carota ssp. sativus cvs	0.05	0.00	Eggen et al. (2011)
Caffeine	1.0 mg·L^{-1}	21 days	-0.13	*Scirpus validus*	0.23	6.42	Zhang et al. (2013c)
Atenolol	0.5 & 5.0 μg·L^{-1}	21 days	0.10	*Cucumis sativus*	approx. 100	approx. 10	Wu et al. (2013)

[a] ACD/Labs.

4.2 Transformation of pharmaceuticals in plants

4.2.1 General transformation of xenobiotics in plants

Plants are known to possess a detoxification system to eliminate toxic pollutants during exposure situations. However, despite some similarities of xenobiotic detoxification in plant and in animal liver, plants do not possess excretory pathways. Therefore, xenobiotics must be transformed to non-toxic TPs and then stored inside plant tissues. The xenobiotic transformation processes have been previously described in three phases: phase I) activation; phase II) conjugation; phase III) compartmentation (Shimabukoro, 1976; Coleman et al., 1997). Sandermann, (1994) compared the metabolic pathways of xenobiotics in plant to that in animals and proposed the significant new concept of the "green liver".

In phase I, the major reaction is oxidation which is mostly catalyzed by the cytochrome P450 system. Reduction and hydrolysis can also be involved in phase I (Coleman et al., 1997). Cytochrome P450s extensively present in microbes, fungi, plants and animals. In humans, they are the principal oxidation enzymes responsible for the metabolism of many pharmaceuticals. Most pharmaceuticals undergo deactivation by cytochrome P450s and excretion from the body. Previous studies showed many xenobiotics could be metabolized by cytochrome P450 system in plants, such as herbicides and insecticides (Schuler, 1996; Robineau et al., 1998). In phase II, the activated xenobiotic compounds are conjugated with an endogenous hydrophilic molecule, such as glucose, glutathione or malonate. The phase II conjugates with lower toxicity and higher water solubility are exported from the cytosol by membrane transporters, which initiate phase III.

4.2.2 Transformation of metformin in plants

The transformation of metformin in *T. latifolia* has been investigated in this study. Only one metformin TP of metformin was found in *T. latifolia*, which could be identified as methylbiguanide. To our knowledge, methylbiguanide has never been reported as a TP of metformin in plants. Metformin shares a similarity in molecular structure (guanidyl group) with some plant nitrogen storage compounds in seeds, such as arginine. However, in contrast to arginine metformin would not be degraded by arginase during germination (Eggen & Lillo, 2012). A previous study showed that metformin can be transformed to guanylurea in plants, but only in reproductive organs and not in vegetative organs (Eggen et al., 2011; Eggen & Lillo, 2012). This is probably because different transformation mechanisms exist between reproductive and vegetative organs. It could also explain the absence of guanylurea in *T. latifolia* in our study. In addition, another reason could be the rapid degradation of guanylurea in *T. latifolia,* which caused that it was not detectable in any of the plant tissues. A recent study showed that guanylurea can be rapidly degraded to guanidine and ammonia by microbes (Perreault et al., 2013).

Since methylbiguanide only contributes to a small part of metformin mass loss, it indicates that methylbiguanide is probably not the dead-end TP. However, the precise mechanism for further transformation of methylbiguanide is not yet clear because intermediates were not observed in our experiments. It is known that the microsomal cytochrome P-450 oxidase system can catalyze guanidine formation from methylguanidine in human liver (Kawata et al., 1983). We suggest that a similar mechanism may also exist in plants. Finally, the formed guanidine can be easily oxidized to urea and then hydrolyzed to ammonia (Clement et al., 1993). This could be an explanation for the total mass loss of metformin during the experiment.

4.2.3 Transformation of iopromide in plants

Transformation of metformin and iopromide were different in *T. latifolia* (Fig 4.1). In total 8 TPs including 23 isomers were identified in *T. latifolia* after iopromide treatment. The transformation of iopromide in *T. latifolia* proceeds via three types of reactions: I) oxidative dehydrogenation; II) decarboxylation; III) deiodination. Demethylation was not found in the transformation pathway of iopromide, though there is a methyl group on the side chain A of the molecule. This may be due to the fact that this methyl group of iopromide is not at the end of the chain, which may decrease its reactivity.

Our study showed that iopromide has a more complex transformation pathway than metformin. Based on the molecular structure of iopromide, it is not difficult to predict that iopromide has a relatively high reactivity. The iopromide TPs may exert some toxicity, especially the aldehydes and ketones. Since iopromide possesses aromatic rings and iodine atoms, it has been hypothezised that it could not be completely degraded in traditional WWTPs (Kormos et al., 2011). The iodine-TPs may have higher toxicity than iopromide, resulting in a greater potential environmental and human health risk. A previous study had indicated that also other iodinated disinfection byproducts such as iodo-trihalomethane and iodo-acid are highly toxic for mammalian cells (Richardson et al., 2008). Iodinated X-ray contrast media (i.e. iopromide) can serve as a source of such iodo-disinfection byproducts (Duirk et al., 2011).

In any case, the present study was able to demonstrate that wetland plant species contribute to the removal of critical pharmaceuticals from water, and accelerate their degradation in the environment. A summary of the reactions described is depicted in the following graph. It will be important to investigate the final fate, i.e. in phase III in plants, where dead-end products might pile up in cell walls or vacuoles. Harvesting such plants, and using the biomass for other purposes could contribute to an efficient removal of the unwanted compounds from the environment.

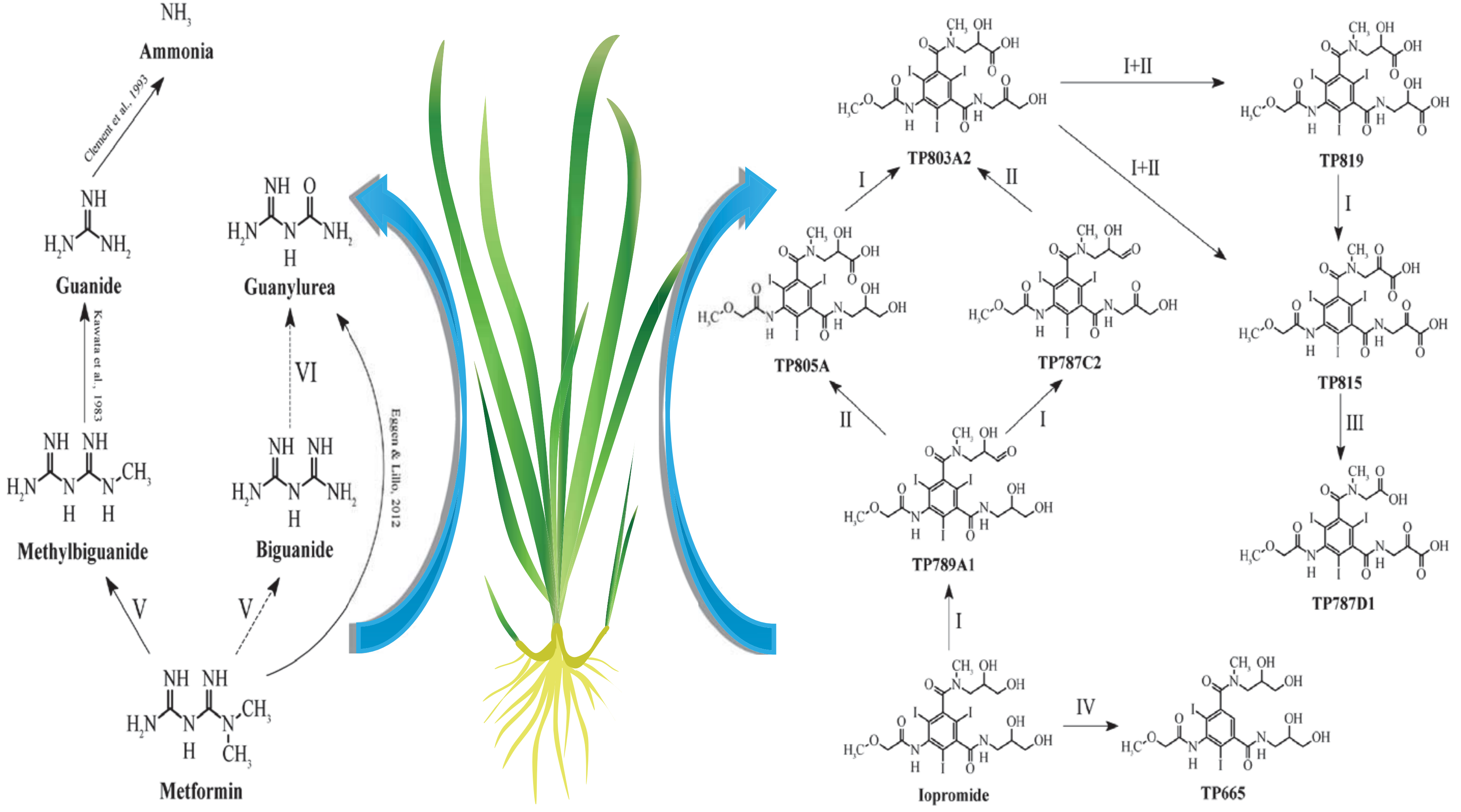

Fig 4.1 Transformation pathway of metformin and iopromide in plants

4.3 Conclusion

The present study confirms that aquatic macrophytes may have potential application in phytoremediation of environmental compartments contaminated by polar pharmaceutical compounds. Metformin and iopromide are both widely detected in water bodies and cannot be completely removed in traditionally operated WWTPs. Both compounds clearly belong to the critical PPCP micropollutants that require special attention, since their incomplete degradation products may cause secondary pollution of our environment. *T. latifolia* has been described as a tolerant species for many pollutants and is therefore widely used in constructed wetlands for phytoremediation. We determined that *T. latifolia* has the formidable ability to remove both pharmaceuticals from water in a series of lab and greenhouse experiments. Now, further studies are needed to evaluate its performance under field conditions which are more related to natural conditions.

Our study provided evidence that *T. latifolia* showed high removal efficiency for both polar pharmaceuticals. High uptake rates into belowground plant parts as well as low transport rates to the aboveground part of plants have been described for the pharmaceuticals. This is represented as a relatively high concentration of the pharmaceuticals in roots while low concentration in leaves. The uptake and transport process of organic cationic compounds such as metformin is different from non-ionic organic compounds. OCTs have been confirmed as a potential conduit for cationic xenobiotics. Their stimulation or overexpression could lead to more efficient uptake.

The TPs of the pharmaceuticals have been identified in different time periods to investigate their transformation pathway in plants. According to our experimental results, both, metformin and iopromide can be metabolized in plants. While metformin can be transformed to the only transformation product methylbiguanide via N-demethylation, iopromide is transformed via four different reactions: 1) hydroxyl groups oxidized to aldehyde and ketone groups; 2) aldehyde and ketone groups oxidized to carboxyl groups; 3) decarboxylation; 4) deiodination.

Pharmaceuticals and their fate have attracted increasing concern in the last decade. An increasing number of publications about their effect on the environment show the importance and urgency of the environmental issues. The present work will help to assess feasibility and practicability of phytoremediation for such polar pharmaceuticals and also to improve our understanding of the transformation pathways of such compounds in plants.

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Appendix A

Manuscript 1-4

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Short term uptake and transport process for metformin in roots of *Phragmites australis* and *Typha latifolia*

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HIGHLIGHTS

- Metformin, carbamazepine and caffeine can be taken up by *P. australis* and *T. latifolia.*
- The uptake process of metformin was not concentration-dependent.
- The characteristic of uptake and transport of metformin is different from carbamazepine and caffeine.
- Quinidine can significantly inhibit the uptake of metformin.
- Organic cation transporters could be considered as a potential channel for metformin in roots.

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ABSTRACT

Metformin (MET) as an emerging contaminant has been detected in surface water and wastewater in numerous countries, due to insufficient retention in classical waste water treatment plants. In order to characterize the uptake of the compound during phytotreatment of waste water, a short term Pitman chamber experiment was carried out to assess the characteristics of MET uptake and transport by roots. Three different concentrations (0.5, 1.0 and 2.0 mmol L^{-1}) were applied to cattail (*Typha latifolia*) and reed (*Phragmites australis*) roots which were used to investigate the uptake mechanism because they are frequently utilized in phytoremediation. In addition, quinidine was used as an inhibitor to assess the role of organic cation transporters (OCTs) in the uptake of MET by *T. latifolia*. The transport process of MET is different from carbamazepine (CBZ) and caffeine (CFN). In both *T. latifolia* and *P. australis*, the uptake processes were independent of initial concentrations. Quinidine, a known inhibitor of organic cation transporters, can significantly affect MET uptake by *T. latifolia* roots with inhibition ratios of 70–74%. Uptake into the root could be characterized by a linear model with R^2 values in the range of 0.881–0.999. Overall, the present study provides evidence that MET is taken up by plant roots and has the potential for subsequent translocation. OCTs could be one of the important pathways for MET uptake into the plant.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) have been recognized as environmental contaminants and attracted increasing concern in the last decade (Daughton and Ternes, 1999). This is due to the fact that these compounds generally are more recalcitrant and cannot be completely removed in conventional wastewater treatment processes so that many of them are detected in the effluents of treatment plants (Ternes, 1998; Kolpin et al., 2002), from where they might enter into our fresh- and groundwater reserves. Hence, these chemicals cause a potential public health problem and an environmental safety risk, such as inhibition of growth of human embryonic cells and decrease of microbial diversity (Zhang et al., 2014). Many studies focus on their influence on aquatic ecosystems, especially on animals. However to date, knowledge about their interactions with plants, such as uptake, transport, and possible metabolism are still limited.

Metformin (MET) is an antidiabetic II drug from the biguanidine class that acts by suppressing glucose production in the liver. It is not metabolized in the human body but excreted unchanged in the urine (Scheen, 1996; Robert et al., 2003). Because of the increasing occurrence of diabetes in the ageing population, it is not surprising that a compound as stable as MET will become detectable in the

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environment. Several studies showed that the concentration of MET in wastewater treatment plant (WWTP) influents and effluents increases steadily and ranges from 20 to 129 $\mu g\ L^{-1}$ and 2.2 to 21 $\mu g\ L^{-1}$, respectively (Scheurer et al., 2009; van Nuijs et al., 2010; Trautwein and Kümmerer, 2011). Some papers reported MET in surface water in the range of 60–3100 $ng\ L^{-1}$ (Vulliet and Cren-Olivé, 2011; Scheurer et al., 2012). These observations are alarming, because the capacities of conventional WWTPs for its removal and degradation may be not sufficient (Oosterhuis et al., 2013).

The uptake of xenobiotics by plants has been reported for a number of organic contaminants including pesticides, veterinary products and especially PPCPs in the last decade (Wu et al., 2010; Herklotz et al., 2010; Gao et al., 2000; Boxall et al., 2006). Predicting the uptake of organic contaminants by plants is critical for evaluating their environmental risk and the potential effectiveness of alternative technologies like phytoremediation. However, we still know little about uptake and transport processes in wetland plants which are used in phytoremediation. Only few research papers show that MET can be taken up by crop plants and bioaccumulates in roots, leaves and seeds (Eggen et al., 2011; Eggen and Lillo, 2012).

In the context of phytoremediation, previous studies have reported the uptake of organic xenobiotics to be mediated by passive diffusion through membranes and within plant tissues (Pilon-Smits, 2005). The octanol:water partition coefficient (log Kow) has been widely used as a descriptor of xenobiotic hydrophobicity which is one of the most important properties influencing xenobiotic uptake by plants (Briggs et al., 1982; Trapp, 1994). However, recent studies indicate that some active processes might be also involved in xenobiotic uptake and translocation. Several substrate specific membrane proteins have been identified in plasma membranes where they seem to be used as transporters for water and organic solutes (Martinoia et al., 2007). Organic cation transporters (OCTs) belong to a family of proteins mediating the transport of charged organic molecules like MET across the cell membrane. They are uniporters and widely distributed in mammalian organs such as liver and kidney (Koepsell and Endou, 2004). Substrates of OCTs have diverse molecular structures, are typically hydrophilic and have low molecular mass. OCT-mediated transport is generally considered electrogenic and independent from sodium. The primary driving force is supplied simply by the electrochemical gradient of the transported compounds or the electronegative membrane potential (Klaassen and Aleksunes, 2010; Zamek-Gliszczynski et al., 2013). However, in recently published studies OCT-mediated transport was also described to occur via cation exchange (Budiman et al., 2000; Pelis et al., 2012). OCTs characterized in plants may be distributed in the vascular tissues of all organs (Küfner and Koch, 2008). It is likely that analogous transmembrane proteins can be considered as potential channels for uptake and transport of MET by plants.

The aim of this study was to (1) investigate uptake and transport of MET with different initial concentrations and plant species using Pitman chamber experiments; and (2) examine the role of OCTs during the transport process by adding quinidine as an inhibitor.

2. Materials and methods

2.1. Chemicals

Metformin HCl (97%), carbamazepine (CBZ, 99%), caffeine (CFN, 99%) and quinidine (98%) were purchased from Sigma–Aldrich (Germany), sucrose (98%) was purchased from Fluka (Germany). Table 1 shows the physicochemical properties of MET, CBZ and CFN. Acetonitrile was HPLC grade and obtained from Roth (Germany), disodium hydrogen phosphate and sodium dodecyl sulfate (SDS) were supplied from Roth (Germany). All chemicals used for nutrient solution were analytical grade.

2.2. Plant material

Typha latifolia and *Phragmites australis* plants were ordered from a local grower (Jörg Petrowsky, Eschede, Germany) and the rhizomes were thoroughly washed in tap water. Plants were grown on perlite in 5 L vessels and then transferred to a greenhouse with 12 h of light/12 h of darkness at 23/18 °C and a humidity of 65%. Nutrients were provided in a modified Hoagland nutrient solution: 2.5 $mmol\ L^{-1}$ K^{+}, 2 $mmol\ L^{-1}$ Mg^{2+}, 2 $mmol\ L^{-1}$ Ca^{2+}, 2 $mmol\ L^{-1}$ SO_4^{2-}, 6 $mmol\ L^{-1}$ NO_3^{-}, 0.5 $mmol\ L^{-1}$ $H_2PO_4^{-}$, 50 $\mu mol\ L^{-1}$ Fe^{2+}, 50 $\mu mol\ L^{-1}$ BO_3^{3-}, 1 $\mu mol\ L^{-1}$ Mn^{2+}, 0.5 $\mu mol\ L^{-1}$ Cu^{2+}, 0.5 $\mu mol\ L^{-1}$ Zn^{2+}, 0.1 $\mu mol\ L^{-1}$ MoO_4^{2-} and the pH was adjusted to 6.0. Plants were acclimatized to greenhouse condition more than two months before they were used in the chamber experiments.

2.3. Pitman chamber experiments

The incubation chambers for present experiments had been designed during previous studies (Pitman, 1971; Schröder et al., 2007). Five roots, approx. 10 cm long, complete with root tips, were carefully cut from selected plants and inserted into the chamber immediately. Each chamber is split into three independent compartments (from left to right named A, B and C) by baffles. Vaseline was applied to all gaps in the chamber to prevent the exchange of solutes between compartments (Fig. 1). For comparison with MET, CBZ and CFN were individually spiked in compartment A at initial concentration of 0.5 mM. Experiments focusing on the transport mechanism of MET were divided into two series: First, both plant species (*T. latifolia* and *P. australis*) were used to determine transport rates; second, only *T. latifolia* was used to investigate the inhibition of OCTs. Compartment A was spiked with MET solution at three different concentrations (0.5, 1.0 and 2.0 $mmol\ L^{-1}$) in the absence of quinidine for the first series and in the presence of quinidine (0.5 $mmol\ L^{-1}$) for the second series. Compartment B and C were filled with 0.03 $mol\ L^{-1}$ sucrose solution in order to provide osmotic pressure in the roots (Tazawa et al., 2001). A chamber receiving an equivalent amount of MET and sucrose solution without roots served as the control. Water samples were collected after 10, 20, 30, 45, 60, 75 and 90 min from both compartments, A and C, and stored at −20 °C for later analyses.

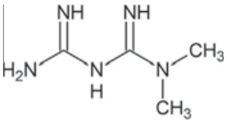
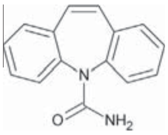
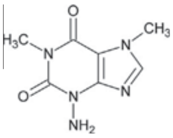
2.4. High-performance liquid chromatography (HPLC) analysis

Methods for pharmaceutical determination in water have been described previously (Kolte et al., 2005). In short, filtrated water samples (0.45 μm) were injected into the HPLC (Varian ProStar 210, Germany) by an auto sampler (Varian ProStar 410, Germany) with an injection volume of 25 μL. An XDB C18 reversed-phase column (5 μm, 4.6 × 50 mm, Agilent, Germany) was used at a column temperature of 40 °C. Detection of MET was at a wavelength of 226 nm using an UV detector (Shimadzu SPD-20, Germany). The mobile phase used was an isocratic mixture of aqueous buffer and acetonitrile (68:32, *v*/*v*) at a flow rate of 1.0 $mL\ min^{-1}$. The buffer consisted of 10 $mmol\ L^{-1}$ disodium hydrogen phosphate and 10 $mmol\ L^{-1}$ sodium dodecyl sulfate (SDS) in distilled water adjusted to pH 7.5 with hydrochloric acid. The mobile phase was mixed, filtered (nylon, 0.45 μm) and degassed.

2.5. Statistical analyses

Data were analyzed for normal distribution. ANOVA was used to determine significant differences between groups by comparing

Table 1
Chemical structure and physicochemical properties of metformin, carbamazepine and caffeine.

Compounds	Metformin	Carbamazepine	Caffeine
Chemical structure			
Molecular weight	129.16	236.27	194.19
pKa	12.33 and 10.27[a]	15.96[a]	−0.92[a]
Log Kow	−4.30 (pH at 7.4)[b]	2.28 (pH at 7.4)[b]	0.28 (pH at 7.4)[b]
Log Dow	−2.31[b]; −2.64[c]; −1.40[d]	2.67[b]; 2.45[c]	−0.13[b]; −0.07[c]

[a] ChemAxon.
[b] ACD/Labs.
[c] EPISuite™.
[d] ChemIDplus advanced.

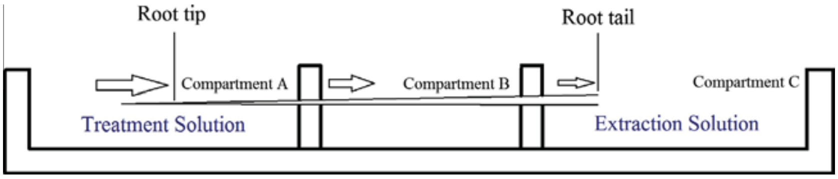


Fig. 1. Cross section of Pitman chamber. Each chamber is split into three independent compartments (from left to right named A, B and C) by baffles. Vaseline was applied to all gaps in the chamber to prevent the exchange of solutes between compartments.

the critical value of variance. These analyses were performed using SPSS v16.0 and comparisons were considered significantly different for $p < 0.05$.

3. Results and discussion

3.1. The versatility of a root chamber to study xenobiotic transport

Studying transport processes in plant tissues with Pitman chambers is a well established method. When *T. latifolia* roots were used to compare transport processes among CBZ, MET and CFN at concentration of 0.5 mM, interesting differences were observed. As is depicted in Fig. 2, CFN was taken up by the roots and transported at high rates, whereas carbamazepine and metformin had low and similar transport rates. In contrast to MET, the intercept of linear fit of CBZ and CFN are very close to zero. This indicates that differences exist between transport processes of cationic compounds like the MET and non-ionic organic chemicals. In fact, the uptake of non-ionic organic chemicals by plant roots could be just a passive, diffusive process (Collins et al., 2006). However, Calderón-Preciado et al. (2011) reported that the uptake of several personal care products, including CFN by crops was well above model predicted concentrations, indicating that other processes might play a role. Since metabolism can be excluded in the Pitman experiment, the differential behavior points to the presence of carriers with a broad substrate spectrum, allowing for the accelerated transport of distinct molecules. This is also stressed by Herklotz and co-authors (2010) who find evidence for the transfer of several pharmaceuticals across intact *Brassica* endodermis tissue which is not possible without the activity of carrier proteins.

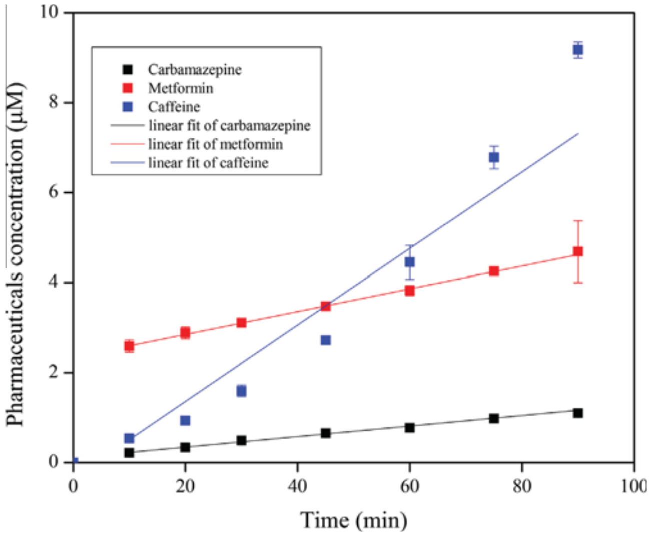


Fig. 2. Concentrations of metformin, carbamazepine and caffeine in the presence of *T. latifolia* at the initial concentration of 0.5 mmol L^{-1} in compartment C. Data points are means of independent experiments; error bar represent SD, $n = 3$.

3.2. Uptake and transport of metformin by roots of different plant species

When MET uptake was scrutinized, further evidence could be collected to confirm the observations made above. MET added to a control Pitman chamber without plant roots, did not cause any decline of MET concentration in compartment A and no diffusion of the compound from compartment A into B or C. On the contrary, in chambers containing plant roots, concentrations of MET steadily increased in compartment C during the experiment (Fig. 3). No contamination was observed in compartment B. After 90 min exposure of *T. latifolia* roots to concentrations of 0.5, 1.0 and 2.0 mmol L^{-1}, MET in compartment C accounted for 0.95%, 0.49% and 0.24% of the initial concentrations, respectively. Similar results were obtained when *P. australis* roots were exposed to the same experimental conditions.

Interestingly, at the end of the experiment, the concentrations of MET transported to compartment C were not significantly ($p < 0.05$) correlated to initial concentrations in compartments A in any treatment. However, the data clearly point to a saturation of the transport process system that might be carrier dependent.

Although the transport of MET by roots was small in comparison to the total amount offered, our studies confirm that MET is taken up by the roots and further transported. To our knowledge, this is the first study using Pitman chambers to investigate the uptake and transport of MET by plant roots. It seems that MET can move into the symplast and is distributed inside the vascular tissue from where it has further potential to reach rhizomes, stems and leaves under the control of transporters.

Some organic xenobiotics have also been suggested to be solely adsorbed on/in plant roots (Schröder and Collins, 2002). It has been observed that root cell walls had a higher sorption capacity for PAH like phenanthrene (Chen et al., 2009). Card et al. (2012) reported the adsorption isotherms of estrogens were linear at mid to high aqueous concentrations and nonlinear at low concentrations. These investigators suspected that a low abundance high affinity sorbent existed in the roots, which could explain the observation of nonlinear adsorption at low concentrations. Other researchers found that the "non-saponifiable and non-hydrolyzable" cuticle fraction could exhibit the most nonlinear behavior (Chen et al., 2005). Although absorption seems to relate to compounds with high log Kow, the relatively high rates of disappearance of MET from the feeding compartment A could also indicate absorption and binding without further transportation.

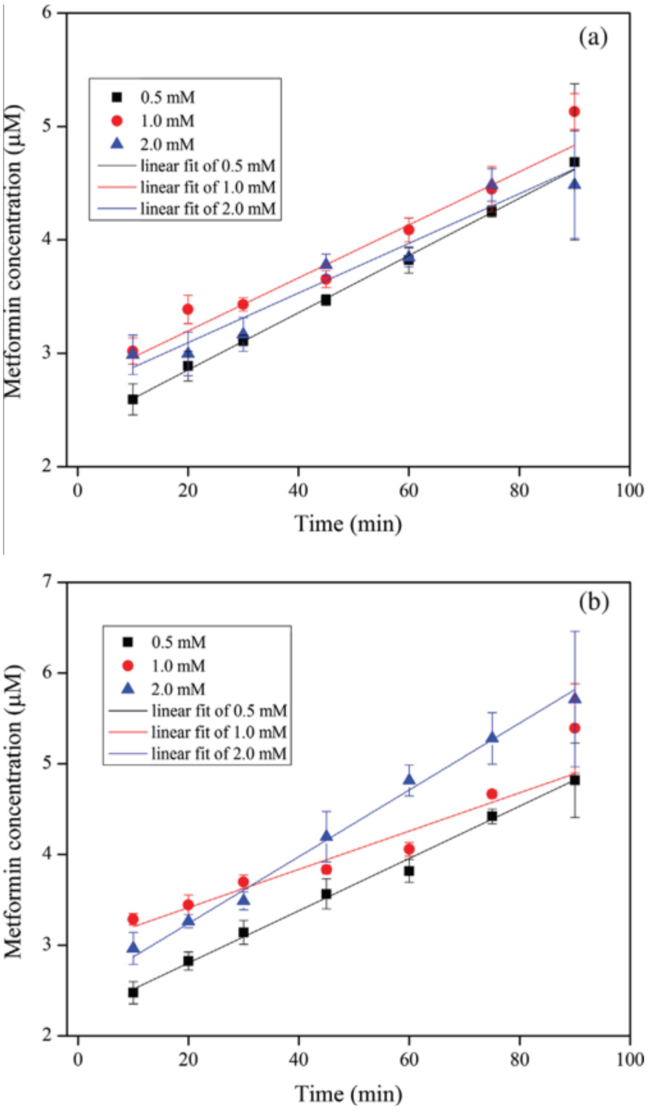


Fig. 3. Transport of metformin into compartment C in the presence of (a) *T. latifolia* and (b) *P. australis*. Data represent mean values of 3 independent biological experiments with 3 replicates ± SD.

The transport rates of MET into *T. latifolia* and *P. australis* in different initial concentrations are summarized in Table 2. For both plant root treatments, the concentrations of MET in compartment C as a function of exposure time was linear, the R^2 values in range of 0.881–0.999 for *T. latifolia* treatments and 0.942–0.993 for *P. australis* treatments (Fig. 2). Previous studies also found other pharmaceuticals such as diclofenac and CFN can be translocated from roots to shoots and the accumulation of pharmaceuticals in the shoots increased as a function of exposure time (Zhang et al., 2012; Zhang et al., 2013b). At the end of our experiments, the concentrations of MET in compartment C were approximately 0.2–1% of the initial concentration in compartment A.

In the present study, the concentrations of MET in compartment A decreased in correspondence to the uptake over time. A maximum of 14.4% MET disappeared from compartment A upon treatment of 2.0 mM for both *T. latifolia* and *P. australis* experiments, and 8.9% (*P. australis*) for the initial concentration of 0.5 mM (Table 2), respectively. A linear relationship between eliminated concentration and exposure time was found in the studies. Statistical analysis showed there are no significant differences ($p < 0.05$) among the treatments with different initial concentration. The R^2 values of different initial concentrations of MET were in the range of 0.503–0.872 for *T. latifolia* treatment and 0.944–0.984 for *P. australis* treatment. Similar results were also reported for other pharmaceuticals (e.g. CBZ, diclofenac and clofibric acid, Zhang et al., 2013a).

We hypothesize that the uptake process is separated in two steps: (1) MET will enter the root tissue by passive diffusion through the apoplastic pathway, where it may also accumulate to a certain percentage; (2) MET is transported across the endodermis into the vascular bundles by active transport via a symplastic pathway. The concentrations in compartment C characterize only the second part of this process while the decrease of concentrations in compartment A characterizes both, including the possible accumulation inside the root tissue and adsorption on the surface of the roots without further movement.

3.3. Uptake and transport of metformin by T. latifolia in the presence of an inhibitor

Quinidine is an inhibitor of MET uptake by OCTs in mammals. We show here that this substance can significantly affect the transport process of MET in *T. latifolia* roots. After 90 min exposure to MET in the presence of the inhibitor, its concentrations in the 0.5, 1.0 and 2.0 mmol L^{-1} treatment increased in compartment C to 1.233, 1.327 and 1.346 μmol L^{-1}, respectively (Fig. 4). The concentration of MET in compartment C was 73.7%, 74.1% and 70.0% lower in the presence of quinidine compared to the absence of quinidine, respectively. This implies that in the presence of quinidine, MET transport is at least in part outcompeted. Still, the residual transport of MET in the roots is concentration dependent. Clearly, the lowest fluxes towards the root are observed at 0.5 mmol L^{-1} MET, where quinidine is present at equimolar concentration. It seems that the quinidine inhibitor, which acts competitively on the transporter protein, is expelled from the binding sites in a typical concentration dependent manner. However, the transport process could not be completely inhibited when higher concentrations of quinidine were added.

Previous studies showed that Arabidopsis OCTs are expressed in vascular tissues of roots (Lelandais-Brière et al., 2007). Subcellular localization also verified OCTs were present in the plasma membrane and tonoplast (Küfner and Koch, 2008). Thus, OCTs can be considered as an important pathway for MET taken up by plant roots. In the present study, we observed a fast transport process

Table 2
The metformin concentrations in compartment C, transport rates and metformin eliminated concentration in compartment A.

	T. latifolia			*P. australis*			*T. latifolia* with quinidine		
	0.5 (mmol L^{-1})	1.0 (mmol L^{-1})	2.0 (mmol L^{-1})	0.5 (mmol L^{-1})	1.0 (mmol L^{-1})	2.0 (mmol L^{-1})	0.5 (mmol L^{-1})	1.0 (mmol L^{-1})	2.0 (mmol L^{-1})
MET concentrations in compartment C (μmol L^{-1})	4.69 ± 0.69	5.13 ± 0.16	4.49 ± 0.47	4.82 ± 0.41	5.39 ± 0.49	5.71 ± 0.75	1.23 ± 0.05	1.33 ± 0.04	1.35 ± 0.05
Transport rates (μmol L^{-1} min^{-1})	0.0250	0.0230	0.0220	0.0290	0.0210	0.0370	0.0023	0.0027	0.0029
MET eliminated concentration in compartment A (μmol L^{-1})	60.3 ± 7.8	50.0 ± 5.0	87.8 ± 48.5	44.5 ± 9.5	36.0 ± 2.2	44.8 ± 4.3	11.8 ± 2.1	14.2 ± 7.6	19.8 ± 4.5

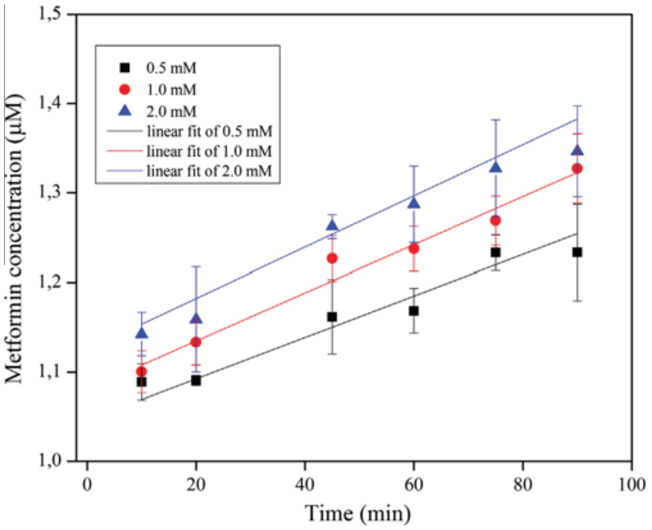


Fig. 4. Transport of metformin into compartment C in the presence of quinidine. Data points depict mean values of independent biological experiments with 3 replicates ± SD.

between 10 min and 90 min, with a positive correlation between initial concentration and transport rate in the presence of quinidine. However, this phenomenon disappeared in the absence of quinidine. This indicated that the uptake and transport of MET by plant roots is a complex process involving parallel activity of several mechanisms. Amino acids can be taken up directly by the roots and many amino acid transporters in plant have been identified (Fischer et al., 1998; Tegeder, 2012). As metformin resembles the molecular structure of arginine, it may be considered a potential substrate for the high affinity arginine transporters. Detaille et al. (2002) reported asymmetrical dimethylarginine, a cationic amino acid transporter inhibitor which can markedly reduce the uptake of MET by *Xenopus* oocytes.

Some authors quote that the movement of PPCPs into plants is a physical process such as passive diffusion (Pilon-Smits, 2005). A passive diffusion driven transport mechanism should be significantly dose-dependent, which was not observed in the present studies. Therefore, the mechanisms for uptake and transport of MET by plants points into a different direction. In addition, it is generally known that many non-selective cation channels (NSCCs) are extensively distributed in the plant cell membrane (Hedrich and Schröder, 1989) that could assist transport processes of xenobiotics to various extents.

In analogy with the absorption process in human liver our research indicates that OCTs may be considered as potential ports for symplastic transport of MET. OCT1, OCT2 and OCT3 are essential for the hepatic uptake of MET (Wang et al., 2002; Higgins et al., 2012; Nies et al., 2011) and other substrates for OCTs were found such as choline and carnitine (Sweet et al., 2001; Lelandais-Brière et al., 2007; Küfner and Koch, 2008). However, OCTs in plant have not been studied in depth as yet (Omote et al., 2006). Therefore, our use of quinidine as OCT inhibitor in the present study delivers first insight in the function of OCTs in plants during an uptake and transport process for cationic xenobiotics. Further studies are required to investigate the transport mechanisms in depth.

4. Conclusions

We demonstrated that MET can be taken up by plant roots and has the potential for subsequent translocation. No significantly difference has been found between *T. latifolia* and *P. australis* for the transport rate. Interestingly, the transport of MET was not correlated to the initial treatment concentrations. Quinidine can significantly inhibit the transport of MET by *T. latifolia* roots, with an inhibition ratios of 70–74%. The transport process of organic cationic compounds like MET is different from non-ionic organic chemicals, and OCTs may be considered as a potential conduit for cationic xenobiotics such as MET. Our studies indicate that plants can take up charged pollutant molecules and even translocate them to their arial parts, which might easily be harvested in a phytoremediation approach, in order to reduce the pollutant load in effluents towards surface waters. Of course, further research is required to explore the role, capacity and inducibility of OCTs and other membrane proteins for pollutant transport, and to screen species used in phytoremediation for such transporters.

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Uptake, translocation and possible biodegradation of the antidiabetic agent metformin by hydroponically grown *Typha latifolia*

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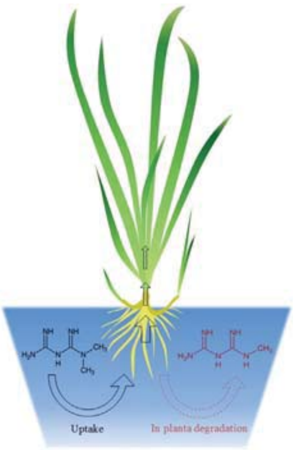
HIGHLIGHTS

- Metformin can be removed from aqueous solution by *Typha latifolia* plants and the removal processes followed first order kinetics.
- Metformin concentration was higher in roots than in leaves and rhizomes.
- First detection of methylbiguanide as a biodegradation product of metformin in plant is reported.
- Guanylurea was degraded at a faster rate than metformin and methylbiguanide by plant enzyme extracts.

GRAPHICAL ABSTRACT



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ABSTRACT

The increasing load of pharmaceutical compounds has raised concerns about their potential residues in aquatic environments and ecotoxicity. Metformin (MET), a widely prescribed antidiabetic II medicine, has been detected in high concentration in sewage and in wastewater treatment effluents. An uptake and translocation study was carried out to assess the ultimate fate of MET in phytoremediation. MET was removed from media by *Typha latifolia*, and the removal processes followed first order kinetics. After 28 days, the removal efficiencies were in a range of 74.0 ± 4.1–81.1 ± 3.3%. In roots, MET concentration was increasing during the first two weeks of the experiment but thereafter decreasing. In contrast, MET concentration was continuously increasing in rhizomes and leaves. Bioaccumulation of MET in roots was much higher than in leaves and rhizomes. As degradation product of metformin in the plant, methylbiguanide (MBG) was detected whereas guanylurea was undetectable. Moreover, MBG concentration in roots was increasing with exposure time. An enzymatic degradation experiment showed the degradation rate followed the order of MET < MBG « guanylurea. This may explain the low concentration of MBG in plant. The findings of this study contribute to understand and evaluate the potential for phytoremediation of such kind of contaminants.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) found in the environment have attracted increasing concern in the last

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decade [1,2]. Many pharmaceuticals are stable chemical entities which are not or only incompletely removed in wastewater treatment plants (WWTPs), and that is why many of them have been detected in WWTP effluents and even in our surface waters [3–5]. The eco-toxicological impact of pharmaceuticals is difficult to predict because of their trace-level concentrations (ranging from ng L^{-1} to μg L^{-1}), potential persistence and biological activity in the aquatic environment [6,7].

Pharmaceuticals inducing environmental risk usually go along with high consumption volumes, but many of them are poorly investigated with regard to their environmental distribution and transport. The antidiabetic II medicine metformin (MET) is one of the most prescribed pharmaceuticals [8–10]. In Germany, MET usage almost tripled in the last 10 years to 1100 tons in 2010, and the trend towards increasing sales numbers grows continuously [11]. MET is metabolized in the human body only to a minor extent and excreted unchanged in the urine [12,13]. Therefore, it is not surprising to find this chemical in WWTPs and in surface water. Previous studies reported MET concentrations in a range of 1.2–118 μg L^{-1} in WWTPs and 0.06–3.1 μg L^{-1} in surface water [5,11,14,15]. Although MET is potentially degraded in activated sludge to a dead-end metabolite, guanylurea, it can still be detected at high concentrations in effluent and surface water because of its high influent load [5].

Phytoremediation is an efficient technology to clean up a variety of organic and inorganic pollutants in soils and waters [16]. Plants and their associated microbes can be used for phytoremediation in constructed wetlands, soils or hydroponic systems [17]. Phytoremediation can be a successful technology for removing PPCPs, which might be implemented as tertiary steps in traditional WWTPs at low operating and maintenance expenses [18,19]. Recent studies have shown that MET can be taken up by crops and vegetables followed by accumulation in leaves [20,21]. However, the final fate of MET in phytoremediation process and its potential removal from effluents is still poorly investigated.

The aims of the current study were: (1) to assess the removal efficiencies of MET from hydroponic solutions by *Typha latifolia* plants; (2) to evaluate the uptake and translocation of MET into roots, rhizomes and leaves; (3) to identify possible biodegradation products in plants.

These results may help to evaluate the environmental fate of MET in wetland plants as well as to promote the further development and application of phytoremediation of similar compounds.

2. Materials and methods

All chemicals and solvents used were of highest quality commercially available as indicated in the Supplementary data (S, Table S1).

2.1. Plant material

T. latifolia L. plants were ordered from a local nursery (Jörg Petrowsky, Eschede, Germany) and the rhizomes were thoroughly washed with tap water. Plants were grown on perlite in 5 L vessels and then transferred to a greenhouse with 12 h of light/12 h of darkness at 23/18 °C and a humidity of 65%. Nutrients were provided to plants by a modified Hoagland nutrient solution (described in the Supplementary data section). Plants were acclimated to greenhouse conditions at least two months before the experiments.

2.2. Experiment setup

The pots were filled with 1.5 L perlite and each pot contained 1 L nutrient solution that was spiked with MET to reach concentrations of 50, 150 and 250 μmol L^{-1} (corresponding to 6.5–32.3 mg L^{-1}), respectively. For each of the three MET concentrations, three assays were set up corresponding to each exposure period studied, i.e., 1, 3, 7, 14 and 28 days. Control plants were grown under the same conditions, only in absence of MET. Furthermore, pots were set as controls (a) without plants but including perlite, and (b) without plants and perlite. In both sets, MET concentrations were not significantly decreasing during the experimental period. Three replicates were treated for each assay and all assays were performed simultaneously. For each exposure time, one assay was harvested. Root, leaf, rhizome and nutrient solutions were collected, frozen and stored, respectively. Additional details for determination of MET and its biodegradation products in plants are described in the Supplementary data.

2.3. Biodegradation in plant tissue enzyme extracts

Crude enzyme extracts of *T. latifolia* roots, rhizomes and leaves were prepared by pestling aliquots of sheared plant tissue under liqu. N_2 and adding 50 mM potassium phosphate buffer (pH 7.0) to the frozen powder. 50 μL stock solution of MET, methylbiguanide (MBG) and guanylurea were added to 950 μL crude enzyme extract to yield a final concentration to 250 μM of each compound, respectively. The reaction solution was mixed and incubated at 25 °C for 60 min, and then quenched by adding 50 μL glacial acetic acid. The resulting solutions were analysed by LC-MS/MS. Additional details are provided in the Supplementary data.

2.4. Analysis for metformin and its biodegradation products by LC-MS/MS

The analysis of water and plant tissue extracts was carried out by solid phase extraction followed by LC-MS/MS. The HPLC system (Varian ProStar 210, Darmstadt, Germany) was coupled to an ion trap mass spectrometer (Varian 500-MS, Darmstadt, Germany) with an electrospray interface operated in the positive ion mode. Separation was achieved using a Synergi Polar-RP 80a column (150 mm × 2 mm, 4 μm, Bischoff, Germany) at a flow rate of 0.3 mL min^{-1}. Further details are available in the Supplementary data.

3. Result and discussion

3.1. Removal of MET from nutrient solution

MET was continuously removed from the nutrient solution by *T. latifolia* during the whole exposure time. By the end of the experiment, the maximum removal efficiency ranged from 74.0 ± 4.1% to 81.1 ± 3.3% for the initial concentrations of 250 μmol L^{-1} and 50 μmol L^{-1}, respectively (Fig. 1a). MET was rapidly removed from the nutrient solution during the initial 7-day period, with removal efficiencies in the range of 39.9–58.9%. The removal processes conformed well to first-order kinetics at rate constants of 0.0969, 0.0655 and 0.0631 day^{-1} for the concentrations of 50, 150 and 250 μmol L^{-1}, respectively (Fig. 1b). In the present study, MET removal efficiencies were significantly related to the exposure time (Pearson correlation coefficient 0.859–0.931, $p < 0.01$). Furthermore, the initial MET concentrations and the MET removal efficiencies clearly fit with a linear relationship at each corresponding exposure time (in all cases, the R^2 value was in a range of 0.950–0.999).

Variations of MET concentration in the nutrient solution were negligible in controls during the experimental period. Abiotic processes such as photodegradation and adsorption to the walls of plastic vessels seem to play only a minor role in MET removal. In

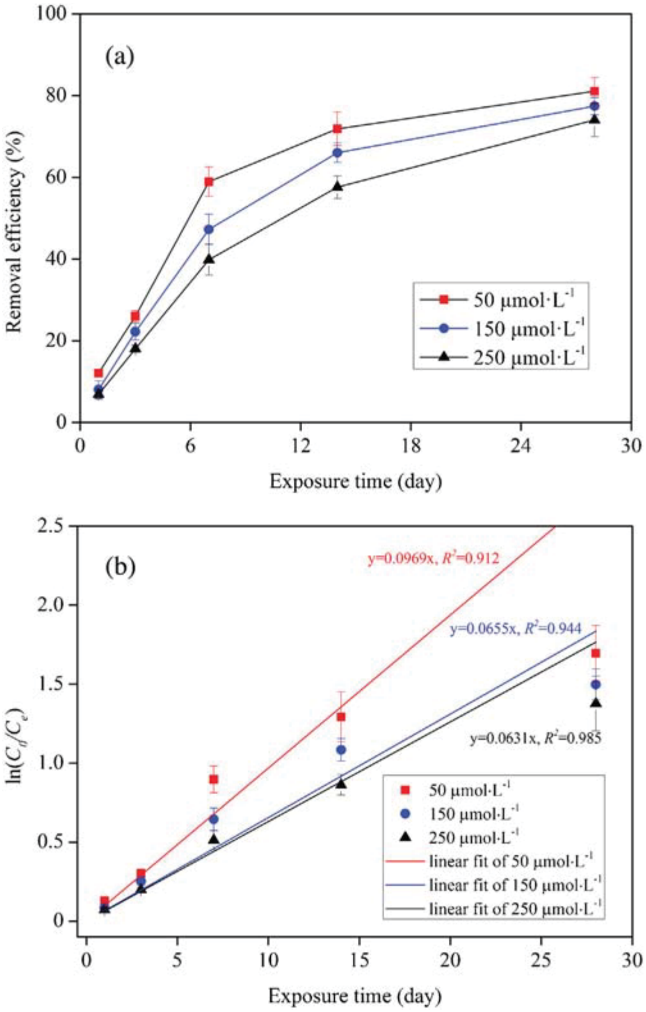

Fig. 1. The removal efficiencies of MET by *T. latifolia* at different exposure time (a); the first-order kinetics fitting of removal process (b). Error bars indicate SD ($n = 3$).

addition, MET as a hydrophilic compound cannot be expected to adsorb on the lipophilic surfaces of roots and rhizomes. Therefore, MET removal from the nutrient solution is expected to depend to a high degree on the uptake by plants.

The removal processes of MET followed first order kinetics. Zhang et al. [22] studied the removal of five different pharmaceuticals by *Scirpus validus* and reported rate constants in a range of 0.023–0.403 day^{-1}. However, the removal process includes different mechanisms such as adsorption, uptake and transformation. Thus, the rate constants should only be considered as a phenomenological value [23]. MET was removed more efficiently from nutrient solutions at lower treatment concentration than at higher treatment concentration. This result is in good agreement with Dordio et al. [24] who observed the same phenomenon in the uptake of carbamazepine by *Typha* spp. Many studies show that the initial concentration of pharmaceuticals and the removal efficiencies clearly fit a linear relationship [24,25].

3.2. Accumulation of MET in roots

In order to quantify the accumulation of MET in plants, the pharmaceutical was scanned in roots, rhizomes and shoots of *T. latifolia*. In fact, MET was detected in all *T. latifolia* tissues of all treated concentration levels. In roots, MET concentrations increased with exposure time up to day 7 in the 50 μmol L^{-1} treatment, but decreased thereafter. For 150 and 250 μmol L^{-1} treatments, MET concentrations in roots started to decrease after day 14 (Fig. 2a). A maximum 11.32 μmol·g^{-1} (FW) of MET was found in plants exposed to 250 μmol L^{-1} treatment after 14 days of exposure. Statistical analysis showed a significant positive correlation between MET concentration in roots and initial concentration in nutrient solution at each time point (Table S2). The bioaccumulation factors (BAFs) are defined as the ratio of MET concentrations in plant tissues to MET concentrations in the nutrient solutions. Table 1 shows the BAFs for roots, ranging from 8.37 to 53.34.

Many uptake processes of xenobiotics are governed by the physico-chemical properties of the contaminant, chemical speciation, and the plant itself. Chemicals of high hydrophilicity (log $P < 0.5$) are not sufficiently adsorbed to roots but may be actively transported through plant membranes [26,27]. Surprisingly, our results demonstrate high MET concentrations and BAFs for roots. This result is also noted by Herklotz and coworkers [28] who find that Salbutamol (log $P = 0.64$) exhibits high bioaccumulation (BAF = 9.048) in cabbage roots. Apparently, MET can be taken up by crops and vegetables from soil while exhibiting a generally high bioaccumulation in roots (BAF = 2–10) [20,21]. By comparison, our results showed higher BAFs than the previous studies in roots. This is probably caused by a higher bioavailability of the compound in the hydroponic systems than in soil systems.

Plant uptake includes both, the apoplastic and the symplastic pathway. The apoplastic pathway is interrupted at the endodermis by the Casparian strip. The casparian strip is a band of cell wall material consisting of suberin and lignin, deposited on the radial and transverse walls of the endodermis to block uncontrolled passive movement of water and chemicals [29]. At this point, the apoplastic flow is forced to move into the symplast pathways, or to cease. Ionizable compounds have low potential for passive diffusion through lipophilic biomembranes. Thus, such compounds are not expected to enter roots at a high rate. However, as previously observed, a surprisingly high uptake and translocation of MET to oilseed rape indicates that some active processes might be involved in the uptake of such a polar and dicationic compound [21]. Our previous study assumed that organic cation transporters may play an important role in the uptake and transport process of MET [30]. Thus, the above mentioned mechanism for the uptake of chemicals by roots may be of general importance for non-ionised compounds [26].

Uptake of MET into the roots was quite rapid and the uptake rates ranged from 0.296 μmol g^{-1} day^{-1} (50 μmol L^{-1} treatment) to 1.461 μmol g^{-1} day^{-1} (250 μmol L^{-1} treatment) up to day 7 (Table S3). This result is in agreement with earlier studies demonstrating that xenobiotics can be taken up by plants within a short time [31,32]. In principle, there should be a balance between uptake and degradation of MET. At the beginning of the experiments, uptake rates were much higher than degradation rates. With time, uptake rates decreased while degradation rates increased, probably because MET concentration decreased in media and MET concentration increased in roots at the same time. MET concentration in roots continuously increased to the maximum critical value when the uptake rates were equal to the degradation rates, and decreased thereafter.

3.3. Translocation of MET from roots to rhizomes and leaves

MET concentrations were in a range from 0.011 to 1.039 μmol g^{-1} (fresh weight) in the rhizomes at day 28 (Fig. 2b), as well as 0.010 to 0.305 μmol g^{-1} (fresh weight) in the leaves (Fig. 2c). MET concentrations in both, rhizomes and leaves increased with exposure time (except 50 μmol L^{-1} treatment for rhizomes). Compared to roots, MET concentrations were relatively low in rhizomes and leaves. After 28 days of exposure, we found the highest BAF values of 4.40 for rhizomes in 250 μmol L^{-1} treatment

Table 1
Bioconcentration factors of MET in roots, rhizomes and leaves of exposed *T. latifolia*.

Spiked initial concentrations (μmol L^{-1})	1 day	3 days	7 days	14 days	28 days
Roots					
50	25.46 ± 1.94	27.44 ± 1.50	38.75 ± 0.96	26.42 ± 3.26	26.60 ± 0.97
150	8.37 ± 0.34	18.04 ± 0.69	44.70 ± 3.30	53.34 ± 2.19	44.13 ± 3.05
250	8.61 ± 1.65	16.65 ± 1.62	43.23 ± 2.93	47.99 ± 6.62	38.06 ± 3.02
Rhizomes					
50	0.20 ± 0.02	0.26 ± 0.01	0.38 ± 0.05	2.00 ± 0.27	1.38 ± 0.15
150	0.31 ± 0.05	0.42 ± 0.01	0.56 ± 0.09	1.46 ± 0.43	3.47 ± 0.20
250	0.33 ± 0.01	0.53 ± 0.04	1.81 ± 0.16	3.94 ± 0.47	4.40 ± 0.34
Leaves					
50	0.18 ± 0.04	0.26 ± 0.03	0.61 ± 0.07	0.66 ± 0.06	1.39 ± 0.32
150	0.12 ± 0.01	0.23 ± 0.03	0.48 ± 0.03	0.59 ± 0.12	1.25 ± 0.24
250	0.09 ± 0.01	0.31 ± 0.05	0.73 ± 0.14	0.90 ± 0.09	1.29 ± 0.09

and 1.39 for leaves in 50 μmol L^{-1} treatment (Table 1). Statistical analysis yielded a significantly positive correlation between MET concentration in rhizomes or leaves and the initial concentration in the nutrient solution at each time point (Table S3).

MET concentrations were relatively low in rhizomes and leaves. Similar results were reported for carrot and barley by Eggen and coworkers [20]. Uptake of other pharmaceuticals has also been previously reported [22,24,28,33]. Our earlier study confirmed that the transport processes of ionic and non-ionic compounds were different. Since ionic compounds can only cross membranes by active transport, the activities of potential transport proteins for these compounds may have more significance than the log *P* value [30]. Another study also indicated that the activity of carrier proteins were necessary for transport of pharmaceuticals by plants [28].

Generally, compounds with an optimum hydrophobicity (log $P = 0.5$–3) are preferred to be taken up and translocated by plants [16,26]. Recent studies indicate that even more hydrophilic chemicals can also have high potentiality of uptake and translocation [34]. However, the present study shows that MET can be taken up by roots at high levels but is poorly translocated to leaves, due to a variety of possible factors including plant species, size, growth conditions, metabolism or volatilization. Furthermore, other studies found plant uptake of xenobiotic organic compounds was independent of hydrophilicity [35,36]. These results suggest that log P may have limited predictive value for uptake and translocation of xenobiotic contaminants by plants.

Plant uptake of neutral compounds has been frequently reported. However, uptake of ionic compounds which included numerous uncertainties has been studied to a much lower extent [37]. Uptake and translocation of ionisable compounds mainly depend on the chemicals pK_a, the solution pH value and the permeability ratio between neutral and ionic molecules. Since MET is present as dicationic molecule in media (pH 6), it was more difficult to predict its environmental fate in these studies [38]. Although the spiked MET concentration was significantly higher than its typical environmental concentrations, it was chosen to allow assessment of the mechanisms involved in plant uptake and biodegradation. However, a hydroponic system may be relatively limited to predict the fate of contaminants in actual engineered conditions or in groundwater. There, reasons for lower bioavailability might include sorption to soil matrix [39]. Therefore, further investigation is still needed, especially in the field.

3.4. Identification of the possible biodegradation products in plant

MBG concentrations increased with the exposure time at all treated concentrations in roots, and a maximum 26.7 nmol g^{-1} (fresh weight) of MBG concentration was found in 250 μmol L^{-1} treatment after 28 days exposure (Fig. 3). We also detected MBG in rhizomes and leaves, but only after 28 days exposure and concentrations were too low to quantitate. Statistical analysis showed a significant positive correlation between MBG concentration in roots and the initial concentration in nutrient solution at each time point (Table S3). In addition, MBG was not detected in water samples, which supports that biodegradation is an in-planta process.

To our knowledge, MBG has never been reported as a biodegradation product of MET in plants. Collin et al. [40] described MBG as one of the primary hydroxyl free radical-induced oxidation products of MET in aqueous solution. *N*-dealkylation is a frequently found metabolic reaction of xenobiotics in plants [41]. Kawata et al. [42] found that the hepatic microsomal cytochrome P-450 oxidase system could also catalyze *N*-demethylation of methylguanidine to guanidine in humans. Trautwein and Kümmerer [43] suggested dealkylation as the mechanism for degradation of MET by microorganisms. However, whether a similar mechanism also exists in plants is still unknown.

Up to now, little knowledge has been collected about in planta biodegradation products of MET. Guanylurea, a stable dead-end metabolite of MET was reported previously in activated sludge [43]. Eggen and coworkers [21] detected guanylurea as a MET related biodegradation product in barley grains, bean pods and potato peel, but not in leaves, rhizomes and roots. However, guanylurea has not been detected, neither in water nor in plant tissues, in our studies. Furthermore, recent studies indicate that guanylurea can still be degraded to guanidine and ammonia rapidly [44]. Under these conditions, guanidine can be oxidized to urea and later hydrolyzed to ammonia [45].

Adsorption and photodegradation did not play important roles in MET removal as only 4.2–6.6% of MET was eliminated from nutrient media in the control. However, a percentage in a range of 33–48% of initial MET concentrations was found in plants at the end of experiment, which was less than the total amount removed from nutrient solutions (58–74%). Since MBG can only contribute a small part of MET mass loss, this indicates that either MBG was not the only dead-end biodegradation product of MET or further degradation of MBG would lead to minor, not detected metabolites.

3.5. Enzymatic degradation experiment

The degradation of selected compounds (MET, MBG and guanylurea) was assessed in crude enzyme extracts from *T. latifolia* roots, rhizomes and leaves, respectively. In comparison to MET and MBG, guanylurea concentrations significantly decreased after 20 min (Fig. 4, S4,5). The highest degradation rates of MET, MBG and guanylurea were 0.061, 0.108 and 0.256 μmol mg protein^{-1} min^{-1}, respectively. The degradation rate is represented by the slope of

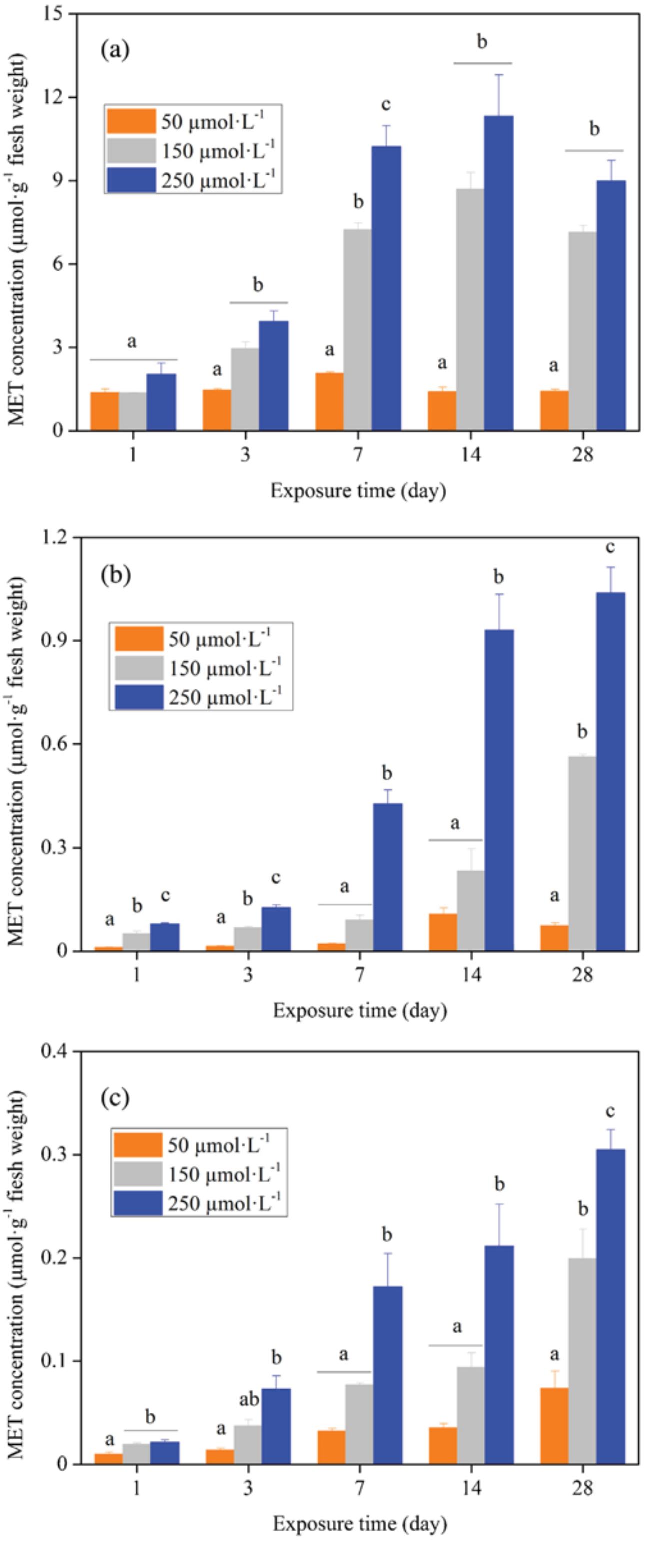

Fig. 2. Concentrations of MET in different tissues of exposed *T. latifolia*. (a) roots; (b) rhizomes; (c) leaves. Error bars indicate SD ($n = 3$).

a linear fit of a degrading process (0–20 min). In all three enzyme extracts, the degradation rates of MET and MBG were much lower than guanylurea (Table S4).

Michaelis–Menten kinetics is a general model to describe enzymatic reactions, but direct comparison of Michaelis–Menten constants (K_m) for enzymes within a crude extract from plant is not reliable [46]. Therefore, we did not fit and calculate the K_m value of the present experimental data. However, previous studies found the K_m value of *N*-demethylation was much higher than N-hydroxylation in hepatic microsomal catalytic degradation of guanidines [42,47]. Since a high K_m indicates low affinity, it had been hypothesized that *N*-demethylation of MET is much more difficult than *N*-hydroxylation, and *N*-demethylation could be the rate-limiting step for the degradation of MET in plant. Therefore, it is possible that the degradation rates could be improved by *N*-demethylation of MET, and after removal of two methyl groups, biguanide could be rapidly degraded even if present in minimal concentration. This could be an explanation for the unanticipated results that we did not detect guanylurea in any of the plant tissues. In addition, previous studies also showed that the *N*-demethylation of pharmaceuticals and herbicides may be mediated by the same microsomal cytochrome P450 fraction in plants [48,49].

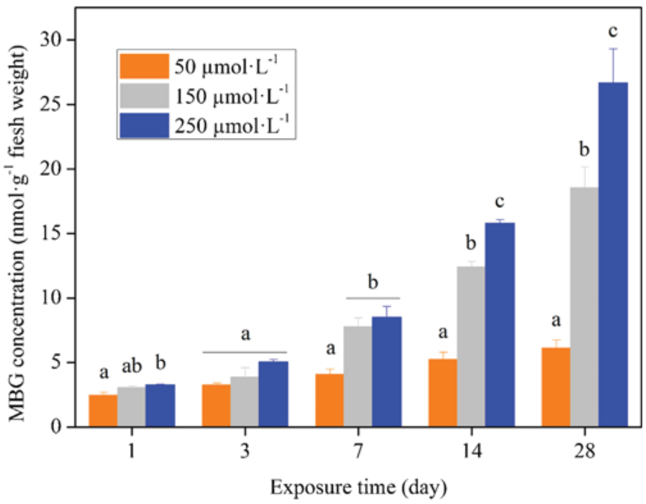

Fig. 3. Concentrations of MBG in roots of exposed *T. latifolia*. Error bars indicate SD ($n = 3$).

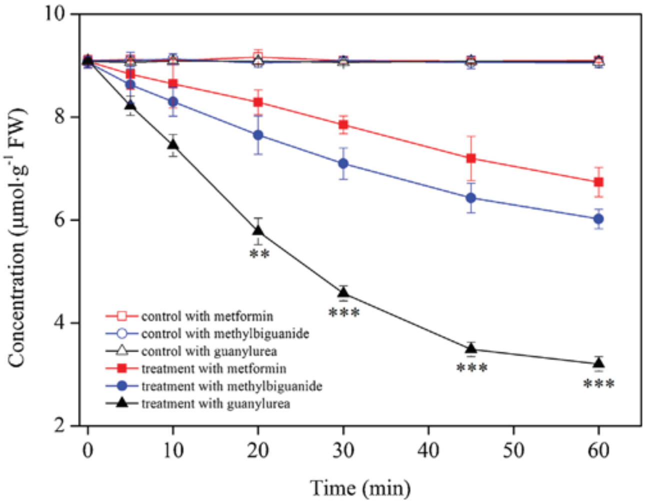

Fig. 4. Concentrations of the selected compounds during exposure to root enzyme extracts. Error bars indicate SD ($n = 3$). 1 mL enzyme extract was from 0.03 g plant material FW. Significant analysis without controls, $^{**}p < 0.01$; $^{***}p < 0.001$.

4. Conclusions

This study investigated the fate of MET in plants used for phytoremediation. The results show that removal processes followed first order kinetics. MET can be taken up from medium, but the translocation via roots to shoots was restricted. Thus, a simply passive diffusion modeling may be not applicable for the ionic compounds due to numerous uncertainties. MBG was first detected as a biodegradation product of MET in planta, and yet its concentration was low. It is important to study the fate of MET and its biodegradation products in plant-based systems. This will help to improve our understanding of phytoremediation for PPCPs. Since plants are the base of the food chain, our finding may also contribute to the fact that root vegetables could impose a higher exposure risk for human than leafy vegetables when from a xenobiotic contaminated environment, e.g., after sewage sludge application. In addition, to identify the possible biodegradation pathways needs further studies.

Acknowledgments

We thank Dr. Christian Huber for his help in the LC-MS/MS analysis and Rudolf Harpaintner for expert technical assistance. Dr. Andrea Kallenberg-Schröder corrected the language and grammar of the manuscript. This research was stimulated by COST Action ES 1202: Conceiving Wastewater Treatment in 2020—Energetic, environmental and economic challenges (Water-2020).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2016.01.054.

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Supplementary data to the Article:

Contents

1. Experimental methods

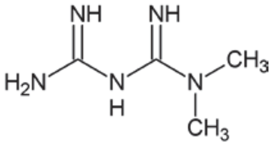
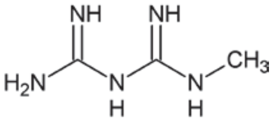
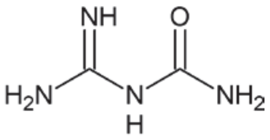
Chemicals. Metformin HCl (MET), methylbiguanide HCl (MBG) and guanylureaare produced by Sigma-Aldrich (Germany). Solvents were LC-MS grade and obtained from Roth (Germany). Ultrapure water was prepared in a Milli-Q water purification system. Inorganic nutrients for modified Hoagland solutions, and all other chemicals were analytical grade.

Nutrients.A modified Hoagland nutrient solution was prepared with the following chemical composition: 2.5 mmol·L^{-1} K^{+}, 2.0 mmol·L^{-1} Mg^{2+}, 2.0 mmol·L^{-1} Ca^{2+}, 2.0 mmol·L^{-1} SO_4^{2-} , 6.0 mmol·L^{-1} NO_3^{-}, 0.5 mmol·L^{-1} $H_2PO_4^{-}$,50 μmol·L^{-1} Fe^{2+}, 50 μmol·L^{-1} BO_3^{3-}, 1μmol·L^{-1} Mn^{2+}, 0.5 μmol·L^{-1} Cu^{2+}, 0.5 μmol·L^{-1} Zn^{2+}, 0.1 μmol·L^{-1} $MoO_4^{2-}$and the pH was adjusted to 6.0.

Experiment Setup. Plants of uniform size were selected for the experiments. Plant roots were rinsed in distilled water and then transferred to 2.5 L pots which contained 1.5 L perlite. Each pot was supplied with 1 L nutrient solution that was spiked with MET at concentrations of 50, 150 and 250 μmol·L^{-1}. The blank plants were grown under the same condition, only in absence of MET. Plants were acclimated to greenhouse conditions at least two months before use. Then all pots, except the controls, received equivalent amounts of MET. For each of the three MET concentrations, three parallel treatments were setup corresponding to each exposure period studied, i.e., 1, 3, 7, 14 and 28 days. Three replicates were treated for each assay and all assays were performed during the same period of time. Additionally, three replicates were set up for the control throughout the experimental period. For each exposure time, one replicate was harvested. Root, leaf and rhizome were collected respectively at the same time, frozen in liquid nitrogen and stored at -80°C. The nutrient solutions remaining in each pot were analysed at each exposure period.

Biodegradation in Plant Tissue Enzyme Extracts. A method for crude enzyme extracts has already been described in the previous study [1].In short, to prepare crude enzyme extracts of *T. latifolia* root, rhizome and leaf tissues, 1 g tissue material were ground in liquid nitrogen, then dissolved and stirred in 50 mM potassium phosphate buffer (pH 7.0) for 30 minutes on ice. The homogenate was centrifuged at 15250 g for 30 minutes at 4°C.5 mM MET, MBG and guanylurea stock solution were also prepared in 50 mM potassium phosphate buffer (pH 7.0).50 μL stock solution of MET, MBG or guanylurea was added to 950 μL crude enzyme extract to yield a final concentration to 250 μM of each compound, respectively. The reaction solution was mixed and incubated at 25 °C in a water bath for 60 min, then reactions were quenched by adding 50 μL glacial acetic acid. The resulting solutions were analysed byLC-MS/MS. Concentration of proteins in the crude enzyme extract (0.018-0.105 mg·mL^{-1}) was measured using the Bradford assay [2].

TableS1. Structure and physicochemical properties of chemicals used in this study

chemicals	structure	molecular weight	pKa	Log D (pH at 7.4)	LogP
Metformin		129.16	12.33 and 10.27[a]	-4.30[b]	-2.31[b]; -2.64[c]
Methylbiguanide		115.14	11.75 and 9.69[a]	-3.14[b]	-1.30[b]; -1.53[c]
Guanylurea		102.10	13.62 and 5.81[a]	-1.82[b]	-1.89[b]; -3.57[c]

[a]ChemAxon, [b]ACD/Labs, [c]EPISuite™

2. Analytical method of LC-MS/MS

Plant Tissue Extracts. The analysis for determination of MET in plants followed a recently published method [3]. Briefly, 0.2 g fresh plant material was extracted with 1.8 mL extraction solution of ammonium acetate/formic acid. The mixture was vortexed, ultrasonicated and centrifuged, the supernatant was filtrated (0.45 μm, nylon) then transferred to a solid phase extraction (SPE) column (Bond Elut LMS, Agilent, Germany), eluted and injected into the LC-MS/MS system. The calibration for extraction processes (including SPE) were performed by use of blank plant tissue spiked with MET and there covery was > 90%.

LC-MS/MS Condition. The HPLC system (Varian ProStar 210, Darmstadt, Germany) was coupled to an ion trap mass spectrometer (Varian 500-MS, Darmstadt, Germany) with an electrospray interface operated in the positive ion mode. Separation was achieved using a Synergi Polar-RP 80a column (150 mm × 2 mm, 4 μm, Bischoff, Germany) with a flow rate of 0.3 $mL·min^{-1}$, and injection volume was 15μL. Eluent A was 0.1% formic acid in water and eluent B was 0.1% formic acid in acetonitrile. The gradient started with 97% of eluent A, after 2.5min it was decreased to 5% within 5 min, held for 2.5 min then increasing the percentage of eluent A back to the initial conditions within 1 min and held for 2min before the end. For MET determination, the precursor ion *m/z* 130.2 (M+H) giving product ions 113.2 and 71.2 was used for quantification and confirmation; for MBG determination, the precursor ion *m/z* 116.2 (M+H) giving product ions 60.2 and 74.2 was used for quantification and confirmation.

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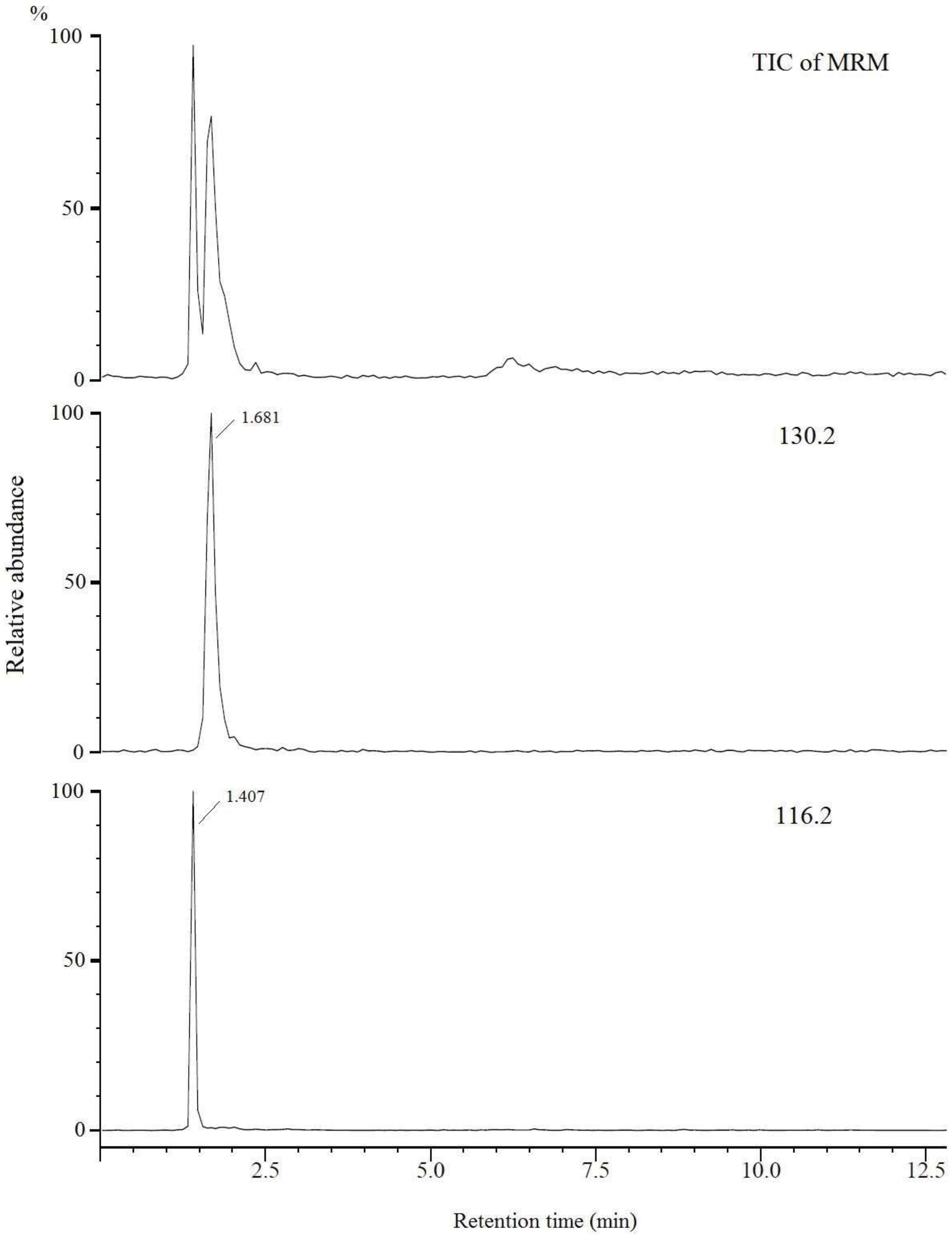

Figure S1. LC-MS/MS chromatograms of 1 μmol·L^{-1}standard sample (a mixture of metformin (m/z 130.2) and methylbiguanide (m/z 116.2)) prepared in extraction solution

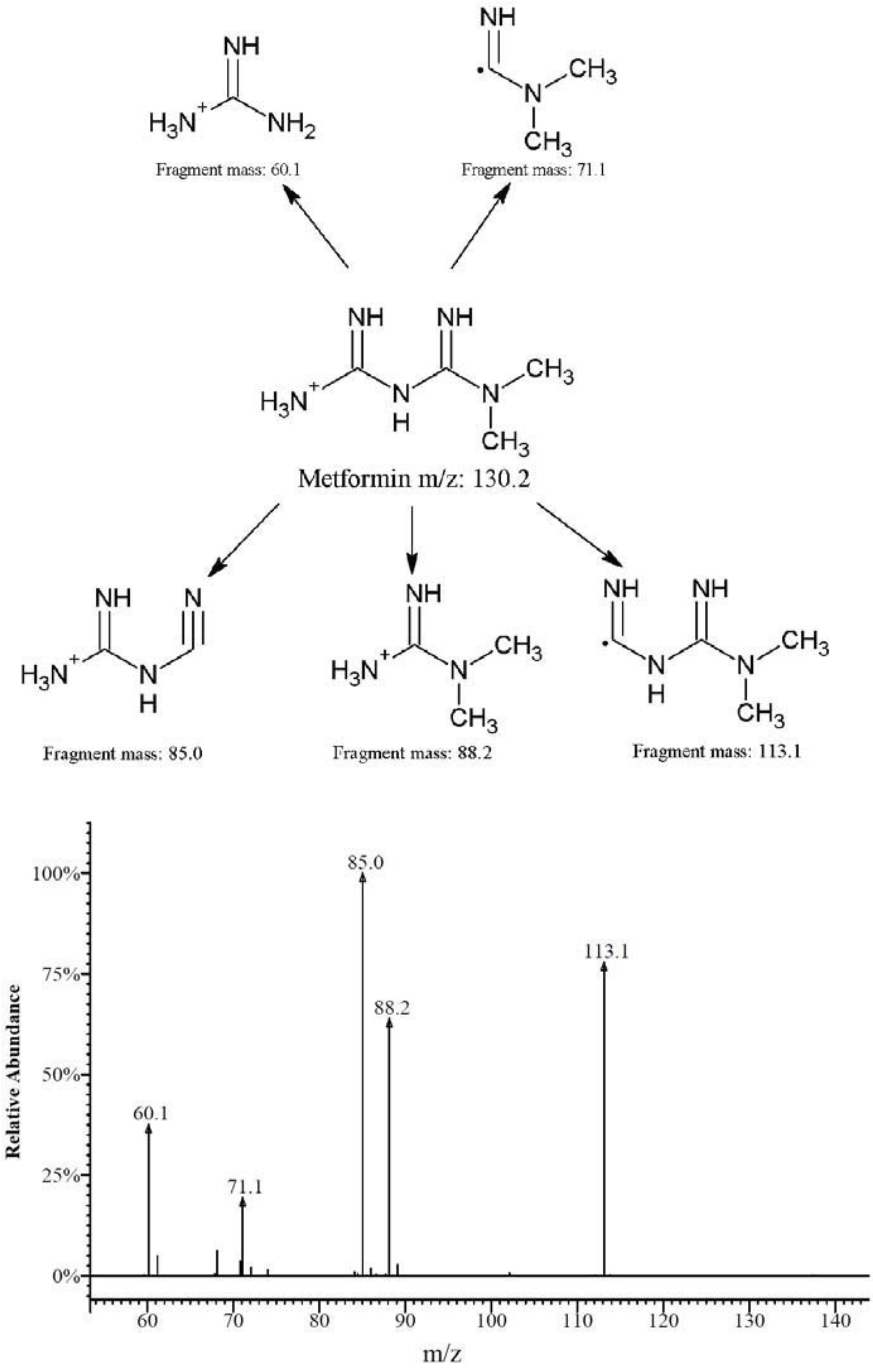

Figure S2. Product ion spectrum of metformin and its proposed fragmentation (MS/MS)

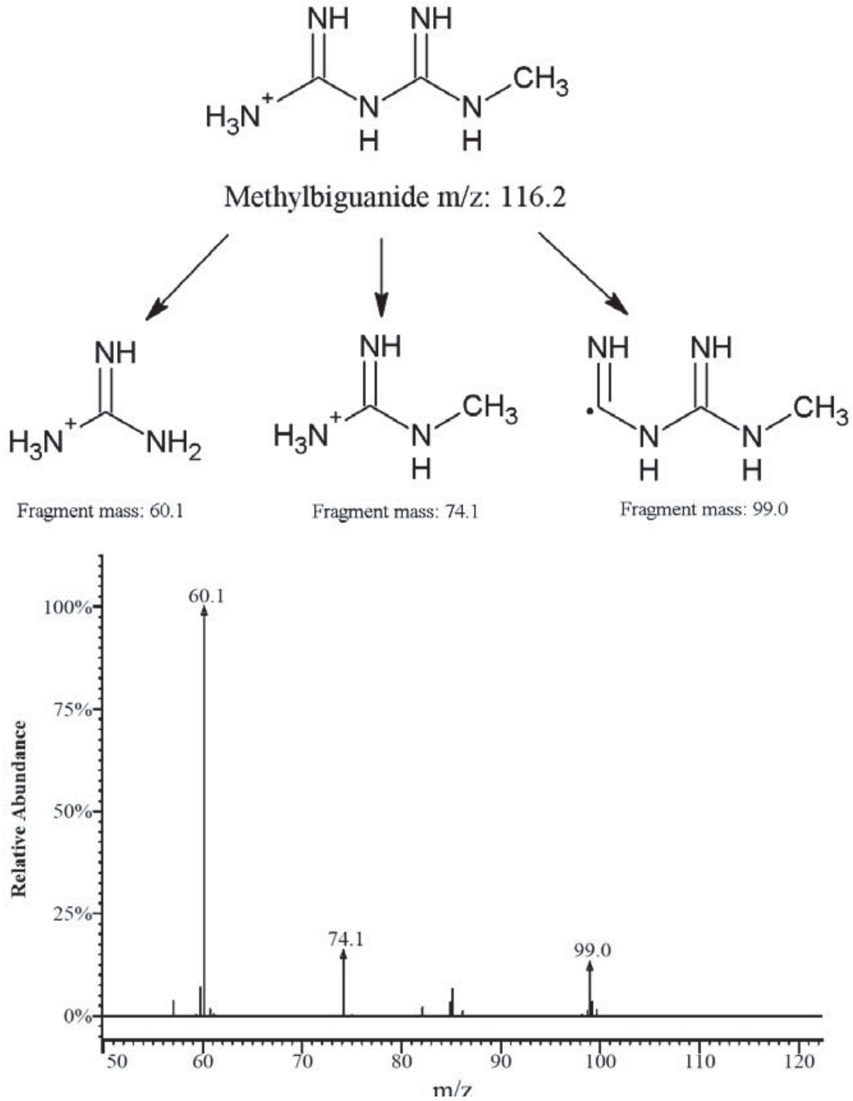

Figure S3. Product ion spectrum of methylbiguanide and its proposed fragmentation (MS/MS)

3. Pearson correlation analysis

Table S2. Metformin and methylbiguanidine concentration in different plant tissues at each time exposure points were analyzed by Pearson correlation (Pearson's coefficient).

Time (days)	MET		MBG	
	roots	rhizomes	leaves	roots
1	0.581*	0.964**	0.893**	0.808**
3	0.930**	0.985**	0.806**	0.752**
7	0.970**	0.919**	0.891**	0.837**
14	0.920**	0.893**	0.885**	0.969**
28	0.941**	0.988**	0.948**	0.949**

*p<0.05; **p<0.01

4. Uptake rates

Table S3.Uptake rates of different plant tissue (μmol·g^{-1}·day^{-1})

Time (day)	Root			Rhizome			Leaf		
	50	150	250	50	150	250	50	150	250
1	1.366±0.137	1.358±0.018	2.038±0.404	0.011±0.001	0.050±0.009	0.079±0.003	0.010±0.002	0.019±0.002	0.022±0.002
3	0.488±0.016	0.983±0.085	1.311±0.129	0.005±0.000	0.023±0.001	0.042±0.003	0.005±0.001	0.012±0.002	0.024±0.004
7	0.296±0.007	1.033±0.035	1.461±0.109	0.003±0.000	0.013±0.002	0.061±0.006	0.005±0.000	0.011±0.000	0.025±0.005
14	0.101±0.011	0.621±0.043	0.809±0.107	0.008±0.001	0.017±0.005	0.066±0.007	0.003±0.000	0.007±0.001	0.015±0.003
28	0.051±0.003	0.255±0.009	0.321±0.026	0.003±0.000	0.020±0.000	0.037±0.003	0.003±0.001	0.007±0.001	0.011±0.001

5. Enzyme extract data

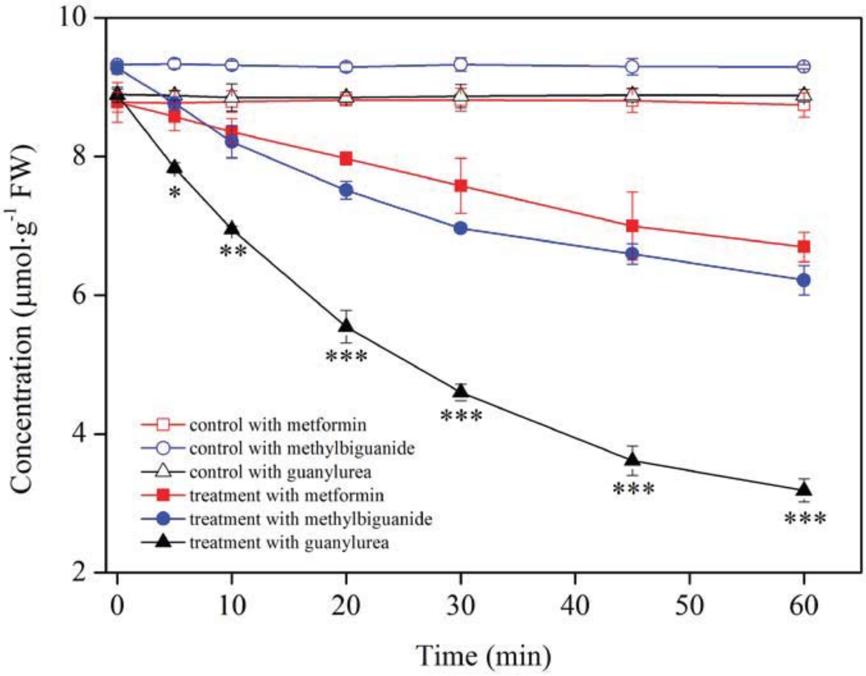

Figure S4. The selected compounds concentrations during of exposure to rhizome enzyme extracts. Error bars indicate SD (n=3).1mL enzyme extract was from 0.03g plant material FW. Significant analysis without controls, $*p<0.05$; $**p<0.01$; $***p<0.001$.

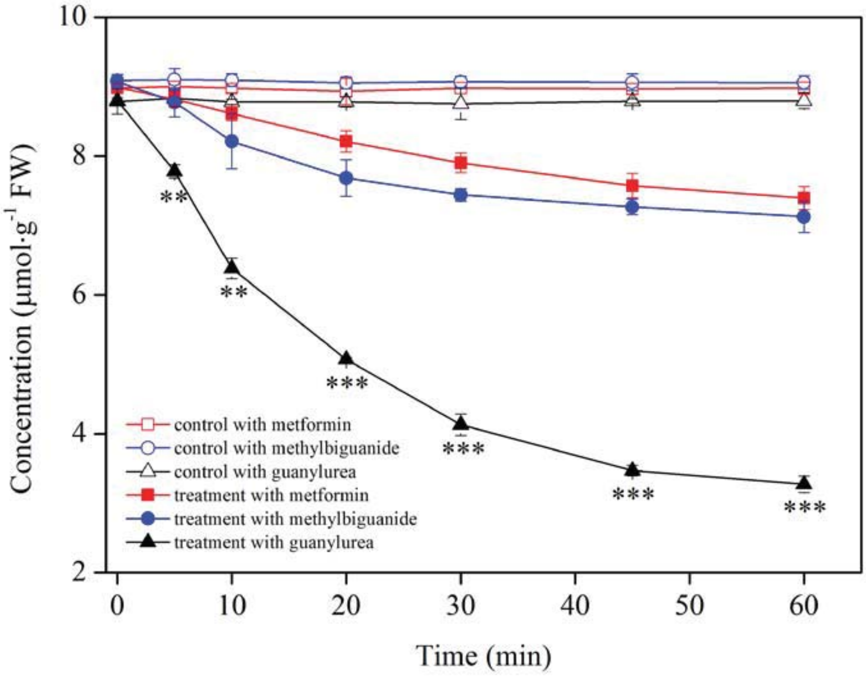

Figure S5.The selected compounds concentrations during of exposure to leaf enzyme extracts. Error bars indicate SD (n=3).1mL enzyme extract was from 0.03g plant material FW. Significant analysis without controls, **$p<0.01$; ***$p<0.001$.

6. Degradation rates

Table S4. The degradation rates (μmol·mg protein^{-1}·min^{-1}) of selected compounds in crude enzyme extracts

	metformin	methylbiguanide	guanylurea
root	0.061±0.025	0.108±0.015	0.256±0.019
rhizome	0.011±0.003	0.024±0.003	0.045±0.003
leaf	0.021±0.005	0.038±0.010	0.101±0.001

Title: Identification of X-ray contrast medium iopromide and its transformation products in *Typha latifolia* L.: Side chain oxidation and deiodination

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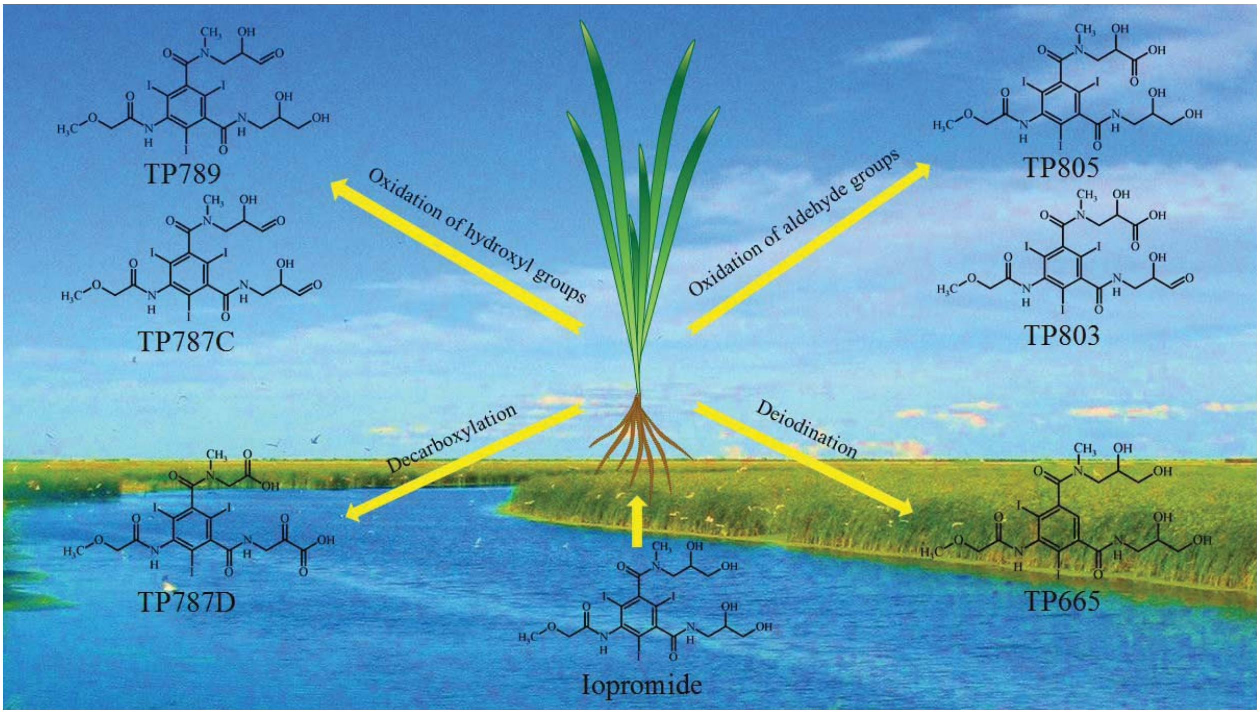
Abstract

Transformation products (TPs) of environmental contaminants have to be clearly identified to predict biodegradation processes and the efficiency of treatment processes. A hydroponic experiment was conducted to assess the fate of iopromide in *Typha latifolia* over a period of 14 days. Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to identify the structure of iopromide TPs. A total of 8 TPs (23 isomers) were detected in *T. latifolia* after the iopromide treatment, especially four of them (13 isomers) which were identified by their characteristic MS/MS fragmentation, have never been reported before. According to the type of reaction, the transformation pathway could be defined as four stages: I) the hydroxyl groups are oxidized to aldehyde or ketone groups; II) aldehyde groups are oxidized to carboxyl groups; III) groups are decarboxylated; IV) groups are deiodinated. Carboxylic acids were found as the major kind of iopromide TPs in both roots and leaves. These results contribute to evaluate the fate of such kind of contaminants during phytoremediation treatments. It is important for the next step to give a better understanding of potential mechanisms of iopromide biodegradation.

TOC/Abstract Art



1. INTRODUCTION

Iodinated X-ray contrast media are widely used for the imaging of human organs and blood vessels during medical diagnosis.[1-4] These compounds are reported to be the most frequently used pharmaceutical classes in hospitals, so that they deserve special scientific attention.[1] High dose contrast media treatments (approximately 100g iodine per person) are applied for diagnostic examinations. After 24 h, they will be completely excreted through the urine in the form of parent compounds.[3] Since these compounds are extremely resistant to biological and chemical degradation, they can be frequently detected in the wastewater effluent and surface water in concentrations ranging from $\mu g \cdot L^{-1}$ to $ng \cdot L^{-1}$.[1,4]

Recent studies have reported that iodinated X-ray contrast media could be effectively removed by several advanced wastewater treatment technologies.[4,5] Nevertheless, one of the main limitations of these technologies may be the formation of transformation products (TPs).[6,7] It has been reported that these iodine compounds might be transformed to even more toxic iodinated disinfection byproducts in the reaction of chlorination.[6,8] Thus, care must be taken to avoid the formation of undesired TPs.

Phytoremediation has shown higher removal efficiencies of emerging contaminants such as pharmaceuticals than conventional technology.[9] Uptake and metabolism of these compounds by plants have been considered as the most important mechanism for removal processes.[10,11] Compared to typical wastewater treatment, phytoremediation can effectively reduce secondary pollution of the environment with TPs because the contaminants are accumulated and/or broken down in the plants, following a metabolism scheme known as the green liver concept.[12] It is proposed that after a phase of activation, where oxidation or hydroxylation are dominant reactions, detoxification proceeds in phase II, before the non-toxic metabolites are excreted or sequestered in vacuole or cell wall during phase III. Therefore, identification of iopromide TPs in plants could improve our understanding of their biodegradation processes and their fate in the environment. Nevertheless, to our knowledge, no information about the transformation products and pathway of iopromide within plant tissues has been published so far.

The aims of the current study were: 1) to evaluate the fate of iopromide in *Typha latifolia* L., which is one of the most widely used plants in constructed wetlands for wastewater treatment; 2) to identify the possible iopromide TPs in plant tissues; 3) to explore the transformation pathway and relative mechanism in plant. These results will contribute to the assessment of environmental fate of such emerging contaminants and further development of phytoremediation.

2. MATERIALS AND METHODS

Chemicals and Plant Materials. Iopromide (98.6%) was purchased from Sigma (Munich, Germany). Solvents and all chemicals were purchased from Roth (Karlsruhe, Germany). Ultrapure water was obtained from Milli-Q water purification system. Young *Typha latifolia* plants, propagated from seeds, were purchased from a local nursery (Jörg Petrowsky, Eschede, Germany) and thoroughly washed with tap water before potting. Plants were grown on perlite and nutrients were provided in irrigation water by a modified Hoagland's nutrient solution.[13] After two months of cultivation, plants uniform in size were selected for exposure experiments. Greenhouse conditions and the composition of nutrient solution are described in the Supporting Information (SI).

Plant Exposure Experiments. Plants were rinsed with distilled water and then transferred to 2.5 L pots. Hoagland's nutrient solution was spiked with iopromide stock solution (2 $mmol \cdot L^{-1}$, dissolved in Hoagland's nutrient solution) to achieve an initial exposure concentration of 20 $\mu mol \cdot L^{-1}$. The inside of the pots was covered with plastic foil to avoid the adsorption to the walls of pots. Each pot was filled with 1 L spiked nutrient solution while 1 L perlite was also added to fix the plant. Plants grown under the same condition but in absence of iopromide were used as blanks and pots without plants were used as controls. Three replicates for treatment, blank and control were exposed simultaneously. After 14 days exposure, plants were harvested and external media were also collected. The plants were carefully washed by tip water, rinsed with distilled water and then dried with blotting paper. The plants were separated as roots and leaves and all the samples were rapidly frozen in liquid nitrogen and stored at -80°C for analysis.

Extraction Procedure and Sample Preparation. Plant material was ground under liquid nitrogen and 0.5 g plant tissue was added to 4.5 ml ultrapure water with 0.1% formic acid. The mixture was homogenized for 30sec, ultrasonicated for 10 min and then centrifuged for 5 min at 10,000 g. The supernatants were filtrated through a 0.45 μm Nylon filter then transferred to solid phase extraction (SPE) cartridges (Oasis HLB, Waters, Germany) which had been preconditioned with 3 mL of methanol followed by 3 mL of ultrapure water. Plant extracts were percolated through the cartridges at a flow rate of 5 mL·min^{-1} and then followed by 3 mL of ultrapure water with 5% methanol for washing the cartridges. After being dried for 5 min, analytes were eluted using 1 mL methanol: acetonitrile solution (50:50) and injected into the LC-MS/MS system.

Identification of Iopromide and its TPs by Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS). Iopromide and its TPs were analyzed using the method described by Schulz et al. and Lutke Eversloh et al.[14,15] Briefly, the HPLC system (Varian ProStar 210, Darmstadt, Germany) was coupled to an ion trap mass spectrometer (Varian 500-MS, Darmstadt, Germany) consisting of an electrospray ionization (ESI) source (operated in positive ionization mode) . Separation was achieved on a Synergi Polar-RP 80a column (150 mm × 2 mm, 4 μm, Bischoff, Germany) and injection volume was 15 μL. Gradient elution was carried out with 0.1% formic acid in water-acetonitrile at a flow rate of 0.3 mL·min^{-1}. For the determination of iopromide, the precursor ion m/z 791.8 $(M+H)^+$ giving fragment ions 773.8 and 572.9 was used for quantification and confirmation. Details on the separation technique and mass parameter settings of the LC-MS/MS are available in SI.

Statistical analysis. Data were analyzed for normal distribution. One-way ANOVA and Tukey's tests ($p<0.05$) were used to determine significant differences between groups. The analysis was performed using SPSS v16.0 (SPSS, Chicago, IL, USA).

3. **RESULTS AND DISCUSSION**

Uptake and Translocation. The iopromide concentration did not decrease significantly in controls during the exposure period, which implies that both photodegradation and adsorption play a minor role in the removal processes (Table SI. 1). Hence, the high removal efficiencies are expected to mainly depend on plant uptake. The concentrations of iopromide were 4.91 ± 0.051 nmol·g^{-1} (FW) in the roots and 0.44 ± 0.07 nmol·g^{-1} (FW) in the leaves, respectively (Table SI. 1), indicating that bioaccumulation might not play an important role during the exposure period. Generally, the hydrophobicity of xenobiotics is used to estimate their uptake potential into plant tissues. Organic compounds with an optimum hydrophobicity (logP = 0.5~3) are preferentially taken up by roots and then translocated in plants.[16] But recently, Bartha et al.[17] indicated that acetaminophen (logP = 0.34) can be taken up by plants and its concentration was higher in roots than in shoots. A similar result was also found in this study for the uptake of iopromide by *T. latifolia*.

Side Chain Oxidative Transformation Products. The iopromide TPs were identified by their exact masses and MS/MS fragment spectra. All the TPs showed a neutral loss of 128 Da in positive ion mode which clearly indicated the loss of HI (Table. 1). Due to the fact that iopromide was the sole source of iodine in the present experiment, it can be confirmed that all the related TPs derived from iopromide only.

A plausible elemental composition of **TP789 (A and B)** is $C_{18}H_{22}O_8N_3I_3$, by a loss of two hydrogens. This is probably achieved by oxidation of the primary hydroxyl group to an aldehyde group or by oxidation of the secondary hydroxyl group to a ketone group, either at side chain A (TP789A) or B (TP789B). The MS/MS fragmentation yields two fragment ions at *m/z* 717.9 and *m/z* 559.1 (Figure SI. 2). The former corresponds to the loss of a propyl group at either side chain A or B, while the latter corresponds to cleavage of the C-N amide bond at side chain A and loss of HI. Although TP789A and B cannot completely be separated on the LC column used because of the great structural similarity, the structural elucidation still can confirm the existence of both TP789 A and B, which is indicated by the characteristic cleavage of C-N-amide bonds at side chain A or B (Figure SI. 2, TP789A→m/z 686.8 and m/z 559.1, TP789B→m/z 701.0 and m/z 572.9), since two signals at different retention times were found in the chromatogram of TP789 (Figure. 1). It is fair to conclude that both aldehyde (TP789A1 and B1) and ketone (TP789A2 and B2) derivatives exist and elute from the column at different retention times.

Following TP789, oxidation of a second hydroxyl group of iopromide led to the formation of **TP787** (Figure SI. 3-4 both at side chain A (**TP787A**) or chain B (**TP787B**) or one at each side chain (**TP787C**)). Similar to TP789, the structure of TP787 can also be confirmed by the characteristic fragments in MS/MS spectra. TP787A is indicated by fragment ions at *m/z* 686.9 (the loss of $C_4H_6O_2N$) and *m/z* 559.2 (the loss of $C_4H_6O_2N$ plus HI) while TP787B is characterized by fragment ions at *m/z* 701.3 (the loss of $C_3H_4O_2N$) and *m/z* 572.9 (the loss of $C_3H_4O_2N$ plus HI). As the characteristic fragment of TP787C at m/z 684.9 has shown three signals in the chromatogram (Figure 1), this can be attributed to the different retention times of the respective aldehyde derivative (TP787C1), ketone derivative (TP787C2) and a combination derivative of aldehyde and ketone group at side chains A and B (TP787C3 and

C4). Therefore, it is proposed that all four reactions took place in our experimental system at the same time.

Similar to TP789A and B, the formation of a carboxyl group at either side chain A or B is proposed for **TP805 (A and B)**, respectively. The existence of both isomers of TP805 is again confirmed by mass spectra. This revealed the cleavage of an N-methylpropylamine group at side chain A or a propylamine group at side chain B (Figure SI. 6, TP805A→m/z 687.1, TP805B→m/z 700.7). Meanwhile, the loss of HI could also be attributed to product ion *m/z* 559.1 and *m/z* 573.1 indicated as TP805A and B, respectively. **TP803 (A and B)** has been found as the further oxidation products which arose from TP805 (A and B). Its chemical formula ($C_{18}H_{20}O_9N_3I_3$) indicates that the primary hydroxyl group of one side chain has been oxidized to a carboxyl group and after that another hydroxyl group has been oxidized to an aldehyde or ketone group at the other side chain. The mass fragments of TP803 (A and B) (Figure SI. 7) follow the typical spectra of dehydration (*m/z* 786.0), cleavage of the amide bond of side chain A (*m/z* 684.9 for TP803A and *m/z* 700.8 for TP803B) and B (*m/z* 715.0 for TP803A and *m/z* 698.9 for TP803B) while elimination of HI (*m/z* 557.3 and 587.0 for TP803A, 573.0 and 571.2 for TP803B) occurred. Similar to TP789, there were also two signals found in the chromatography of TP803 which indicates the possible existence of both aldehyde (TP803A1 and B1) and ketone (TP803A2 and B2) derivatives (Figure 1).

Similar to TP805 (A and B), the structure of **TP819** is proposed by oxidation of both terminal hydroxyl groups of iopromide to carboxyl groups. The MS/MS spectra of TP819 show three fragment ions at *m/z* 773.9, 700.8 and *m/z* 587.2 (Figure SI. 8). By referring to the previous study[14] and mass spectra of iopromide, the loss of a carboxyl group, the methylpropylamine group at side chain A and the propylamine group at side chain B lead to further deiodination corresponding to fragment ions at *m/z* 773.9, 700.8 and 587.2, respectively.

For **TP817 (A and B)**, a chemical formula is proposed as $C_{18}H_{18}O_{10}N_3I_3$, which suggests oxidation of the secondary hydroxyl group at either side chain A (TP817A) or B (TP817B) of TP819. The loss of 116.9 Da ($C_4H_6O_3N$) and 102.9 Da ($C_3H_4O_3N$) is characteristic of TP817A and TP817B while the additional loss of HI can also form the fragment ions at *m/z* 573.2 and 587.4, respectively (Figure SI. 9). **TP815** is formed by oxidation of the second hydroxyl groups to ketone at side chain A or B arose from TP817. The cleavage of the amide bond at side chain A and B form the fragment ions at *m/z* 699.0 and 712.9, respectively.

Another fragment ion with high relative abundance has been found at *m/z* 670.0, corresponding to a loss of HI and OH (Figure SI. 10).

Two decarboxylated TPs have been found as **TP787 (D1 and D2)**. The MS/MS spectra of **TP787D1** indicated fragment ions at *m/z* 712.9, 671.0, 585.0 and 542.8, which were formed by a typical cleavage of C-N-amide bonds at side chain A and B as well as a loss of HI. The characteristic fragment ions were found to be *m/z* 699.0, 684.8, 571.2 and 557.0 for **TP787D2** (Figure SI. 5).

Deiodinated Transformation Products. A deiodinated TP has been found in this study as **TP665**, while the mass suggests an exchange of one iodine atom by hydrogen. It is unknown where in the molecule the dehalogenation occurs. The MS/MS spectra showed fragment ions at *m/z* 648.0, 538.1 and 520.0, corresponding to the loss of H_2O, HI and H_2O+HI, respectively. The cleavage of C-N amide bonds at side chains A or B resulted in fragment ions at *m/z* 561.0 or 575.0 while a loss of HI formed fragment ions at *m/z* 432.9 or 447.3, respectively (Figure SI. 11). A previous study reported several deiodinated transformation products of iopromide in white rot fungus,[18] but not in soil microorganisms.[14] No further derivative hydroxylation of TP665 has been found in this study. It is therefore proposed that TP665 probably formed by reductive dehalogenation, not by substitution reaction. With respect to possible participation of endophytes, although we found TP665 in both roots and leaves, it might still be difficult to distinguish whether this reaction is corresponding to plants only or also to their inhabiting endophytic microorganisms.

Possible Transformation Pathway. A total of 8 TPs (23 isomers) were identified in *T. latifolia* after the incubation with iopromide. According to the type of reaction, the transformation of iopromide in *T. latifolia* could be divided into four reaction types: I) the hydroxyl groups have been oxidized to aldehyde or ketone groups; II) aldehyde groups have been oxidized to carboxyl groups; III) groups have been decarboxylated; IV) groups have been deiodinated (Figure 2). A transformation factor for the TPs was calculated as the ratio between the sum of the TPs peak area and the iopromide peak area under the same analytical conditions. The transformation factors were 1.20 ± 0.10 in roots and 3.96 ± 0.50 in leaves. This indicated that the effect of TPs probably even more important than the parent compound after being exposed to iopromide for two weeks.

The formation of TP789 and TP787 can be explained by reaction type I. This reaction is known to be catalyzed by alcohol dehydrogenases, which are a group of Zn-binding dehydrogenase and commonly existing in higher plants.[19] These dehydrogenases can catalyze alcohols being oxidized into aldehydes or ketones with the reduction of nicotinamide adenine dinucleotide (NAD^+ to NADH). Other studies have shown oxidation of alcohols to aldehydes and ketones by catalysis through peroxidases and monooxygenase in plants, microorganisms and animals.[19,20] Other NADH-dependent enzymes such as methylglyoxal reductase have been reported to catalyze transformation of lactaldehyde into methylglyoxal which would be corresponding to the reaction between TP789 (A and B) and TP787 (A and B).[21]

To our knowledge, aldehydic TPs (TP787A, B and C) were for the first time detected as biotransformation products of iopromide. Kormos et al.[22] indicated that the respective aldehyde TPs of ICM were probably oxidized to the corresponding carboxylates by soil microorganisms. Our results confirm this suggestion (reaction type II) in plants. Aldehyde dehydrogenases are one kind of oxidoreductases responsible for the oxidation of aldehyde to the corresponding carboxylic acid which have been found in different organisms.[23,24] Liu and Schnable[25] showed that homotetrameric aldehyde dehydrogenases can oxidize both aliphatic aldehydes and aromatic aldehydes in maize. Up to now, aldehyde dehydrogenase genes have been identified in many plant species.[26]

In this study, pronounced differences between the TPs composition in roots and leaves have been found. TP789A was the major aldehydic TP in roots, while in leaves, the relative content of both TP789A and TP787C were significantly higher than other aldehydic TPs. Carboxylic TPs were the major kind of iopromide TPs in *T. latifolia* (Figure 3, Figure SI. 12). Among them, TP805A was the major carboxylic TP with significantly high relative content. In leaves, both TP805 (A and B) and TP803A had significantly higher relative content than other carboxylic TPs. Since aldehydes are thought to be dangerous substances with high toxicity for plants, plants obviously possess a self-protection mechanism transforming aldehydic TPs (including TP789 and 787) into carboxylic acid containing TPs (including TP805, 803, 819, 817 and 815, no decarboxylation). This result is also in accordance with the detoxification mechanisms for endogenous aldehydes of plants.[27]

Decarboxylation has been observed in this study following the oxidation of carboxylic TPs. A high relative content of the decarboxylated TPs (TP787D1 and D2) was found in leaves, but not in roots. This indicates that decarboxylation of iopromide might occur probably easier in leaves than in roots (Figure 3). Many different enzymes, such as pyruvate decarboxylases, α-keto acid-dependent dioxygenases, or branched-chain α-keto acid dehydrogenases can catalyze this reaction.[22,28,29] Since no aldehydes were detected as subsequent decarboxylation products of TPs, it is suggested that pyruvate decarboxylases probably do not play a key role in reaction type III. However, whether α-keto acid related enzymes utilize the iopromide TPs for substrates remains unclear and still needs further studies.

During the biodegradation processes of halogenated aromatic contaminants, dehalogenation can occur. Many enzymes have been confirmed to catalyze the dehalogenation of aromatic ring systems, however most reductive dehalogenases have been found in bacteria.[30,31] Surprisingly, a previous study did not observe deiodination of iopromide by bacteria in an active sludge.[14] It remains to be proven whether aerobic or anaerobic conditions or co-metabolism would favour such a microbial reaction. Recently, dehalogenation has also been found in plants. Some studies showed plant nitrate reductase could utilize halogens as electron acceptor to mediate reductive dehalogenation.[32,33] Other studies showed that glutathione S-transferase could also catalyze debromination of polybrominated diphenyl ethers.[32] Currently the research mainly focusses on the dechlorination or debromination of persistent organic pollutants (i.e. polychlorinated biphenyl, polybrominated diphenyl ethers) in plants,[32-34] so far little knowledge about deiodination has been contributed. A recent study found deiodination products during the degradation of iopromide by white rot fungus (*Trametes versicolor*).[18]

Based on the results presented in this and previous studies, it becomes clear that several dehalogenation mechanisms of iopromide may exist in plants. Among them, electron attachment of the iodide could be one mechanism of deiodination.[35] The iodine ions formed by addition of electrons to the atoms. The electron donors could be dissociative electrons and/or some nucleophilic reagents. Nucleophilic substitution may be also one possible mechanism. However, up to now no substitution products have been found. The weak electron-withdrawing property of iodine atoms makes the reaction extremely challenging. Therefore, it is hypothezised that the nucleophilic reagents seem to offer electrons to iodine atoms rather than to attack carbon atoms.

The results of this study clearly suggest that iopromide can be taken up and degraded by *T. latifolia*. Thus, a practical application of wetland plants can be considered as a valid option for treatment of such pharmaceutical compounds, e.g. from hospital waste water. Of course it will be essential to entirely understand the transformation pathways of these pollutants in plants, in order to avoid potential risks of contrast agent derivatives in the environment, and our water reserves. Further research is needed to evaluate performance and optimize design parameters under field conditions.

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Supporting Information contains:

Greenhouse condition, Hoagland’s nutrient solution formula, separation technique and mass parameter settings of the LC-MS/MS. Iopromide and its TPs MS/MS spectra and proposed fragmentation pathway.

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Tables and Figures

Table 1. Identified iopromide and its transformation products in *T. latifolia* with the proposed molecular formula and characteristic MS/MS fragmentation

Figure 1. Representative LC-MS/MS chromatograms of the selected TPs (TP789, TP787C, TP803) in *T. latifolia* exposed to iopromide (20 μmol·L^{-1}) for 14 days.

Figure 2. Proposed transformation pathway of iopromide in *T. latifolia*. I: Oxidation of the hydroxyl groups; II: Oxidation of the aldehyde groups; III: decarboxylation; IV: deiodination.

Figure 3. Relative peak areas of transformation products detected by LC-MS/MS in *T. latifolia* (a) root and (b) leaf exposed to iopromide (20 μmol·L^{-1}) for 14 days. The percent contents are based on the sum peak area of all detected TPs. Error bars indicate SD (n = 3).

Table.1

Compound	Retention time (min)	[M-H]+ (m/z)	Molecular formula	Fragment ions (m/z)	Molecular formula of fragments
Iopromide	5.671	791.8	$C_{18}H_{25}O_8N_3I_3$	773.8	$C_{18}H_{23}O_7N_3I_3$
				572.9	$C_{15}H_{15}O_6N_2I_2$
				558.9	$C_{14}H_{13}O_6N_2I_2$
TP789A1-2	6.165 6.513	789.8	$C_{18}H_{23}O_8N_3I_3$	698.9	$C_{15}H_{14}O_6N_2I_3$
				686.8	$C_{14}H_{14}O_6N_2I_3$
				559.1	$C_{14}H_{13}O_6N_2I_2$
TP789B1-2	6.165 6.513	789.8	$C_{18}H_{23}O_8N_3I_3$	701.0	$C_{15}H_{16}O_6N_2I_3$
				699.9	$C_{15}H_{17}O_5N_3I_3$
				572.9	$C_{15}H_{15}O_6N_2I_2$
TP787A	6.498	787.8	$C_{18}H_{21}O_8N_3I_3$	718.1	$C_{15}H_{19}O_6N_3I_3$
				686.9	$C_{14}H_{14}O_6N_2I_3$
				559.2	$C_{14}H_{13}O_6N_2I_2$
TP787B	6.498	787.8	$C_{18}H_{21}O_8N_3I_3$	701.3	$C_{15}H_{16}O_6N_2I_3$
				700.0	$C_{15}H_{17}O_5N_3I_3$
				572.9	$C_{15}H_{15}O_6N_2I_2$
TP787C1-4	6.213	787.8	$C_{18}H_{21}O_8N_3I_3$	716.0	$C_{15}H_{17}O_6N_3I_3$
	6.498			698.9	$C_{15}H_{14}O_6N_2I_3$
	6.667			571.2	$C_{15}H_{13}O_6N_2I_2$
TP805A	6.307	805.8	$C_{18}H_{23}O_9N_3I_3$	714.9	$C_{15}H_{14}O_7N_2I_3$
				587.2	$C_{15}H_{13}O_7N_2I_2$
				559.1	$C_{14}H_{13}O_6N_2I_2$
TP805B	6.307	805.8	$C_{18}H_{23}O_9N_3I_3$	760.2	$C_{17}H_{21}O_7N_3I_3$
				700.7	$C_{15}H_{16}O_6N_2I_3$
				573.1	$C_{15}H_{15}O_6N_2I_2$
TP803A1-2	6.263 6.611	803.8	$C_{18}H_{21}O_9N_3I_3$	715.0	$C_{15}H_{14}O_7N_2I_3$
				684.9	$C_{14}H_{12}O_6N_2I_3$
				557.3	$C_{14}H_{11}O_2N_2I_2$
TP803B1-2	6.263	803.8	$C_{18}H_{23}O_9N_3I_3$	700.8	$C_{14}H_{12}O_7N_2I_3$
	6.611			698.9	$C_{15}H_{14}O_6N_2I_3$

				571.2	$C_{15}H_{13}O_6N_2I_2$
TP819	6.458	819.8	$C_{18}H_{21}O_{10}N_3I_3$	715.1	$C_{15}H_{14}O_7N_2I_3$
				700.8	$C_{14}H_{12}O_7N_2I_3$
				587.2	$C_{15}H_{13}O_7N_2I_2$
TP817A	6.067	817.8	$C_{18}H_{21}O_{10}N_3I_3$	713.0	$C_{15}H_{12}O_7N_2I_3$
				700.9	$C_{14}H_{12}O_7N_2I_3$
				573.1	$C_{14}H_{11}O_7N_2I_2$
TP817B	6.067	817.8	$C_{18}H_{21}O_{10}N_3I_3$	714.9	$C_{15}H_{14}O_7N_2I_3$
				699.0	$C_{14}H_{10}O_7N_2I_3$
				587.1	$C_{15}H_{13}O_7N_2I_2$
TP815	5.554	815.8	$C_{18}H_{17}O_{10}N_3I_3$	712.9	$C_{15}H_{12}O_7N_2I_3$
				670.0	$C_{18}H_{14}O_9N_3I_2$
				585.0	$C_{15}H_{11}O_7N_2I_2$
TP787D1	6.129	787.8	$C_{17}H_{17}O_9N_3I_3$	712.9	$C_{15}H_{12}O_7N_2I_3$
				671.0	$C_{13}H_{10}O_6N_2I_3$
				542.8	$C_{13}H_9O_6N_2I_2$
TP787D2	6.129	787.8	$C_{17}H_{17}O_9N_3I_3$	699.0	$C_{14}H_{10}O_7N_2I_3$
				684.8	$C_{14}H_{12}O_6N_2I_3$
				557.0	$C_{14}H_{11}O_6N_2I_2$
TP665	5.671	665.8	$C_{18}H_{26}O_8N_3I_2$	575.0	$C_{15}H_{17}O_6N_2I_2$
				561.0	$C_{14}H_{15}O_6N_2I_2$
				520.0	$C_{18}H_{23}O_7N_3I$

Fig.1

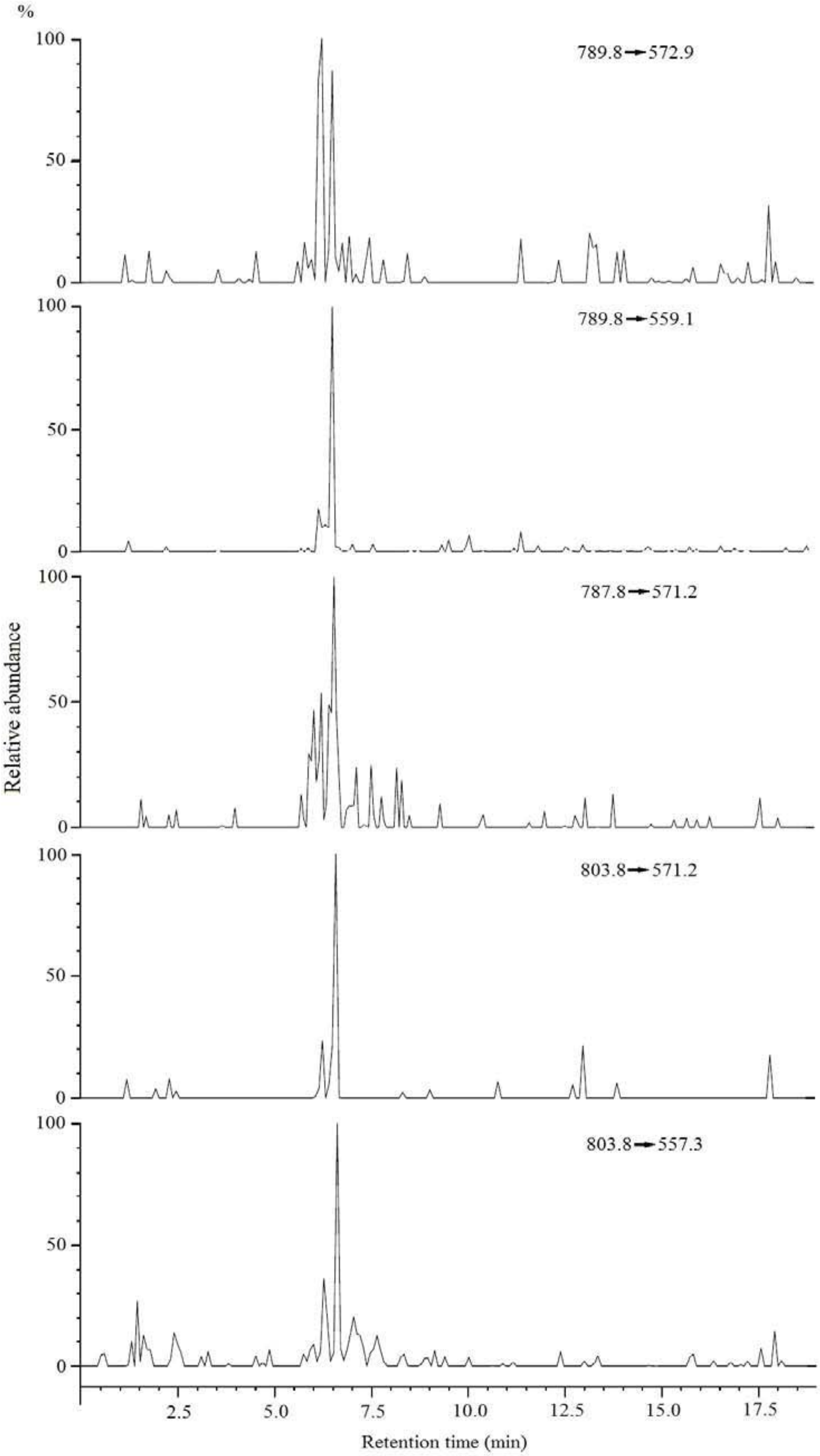

%
100
50
0
789.8→572.9
789.8→559.1
787.8→571.2
803.8→571.2
803.8→557.3
Relative abundance
2.5
5.0
7.5
10.0
12.5
15.0
17.5
Retention time (min)

Fig.2

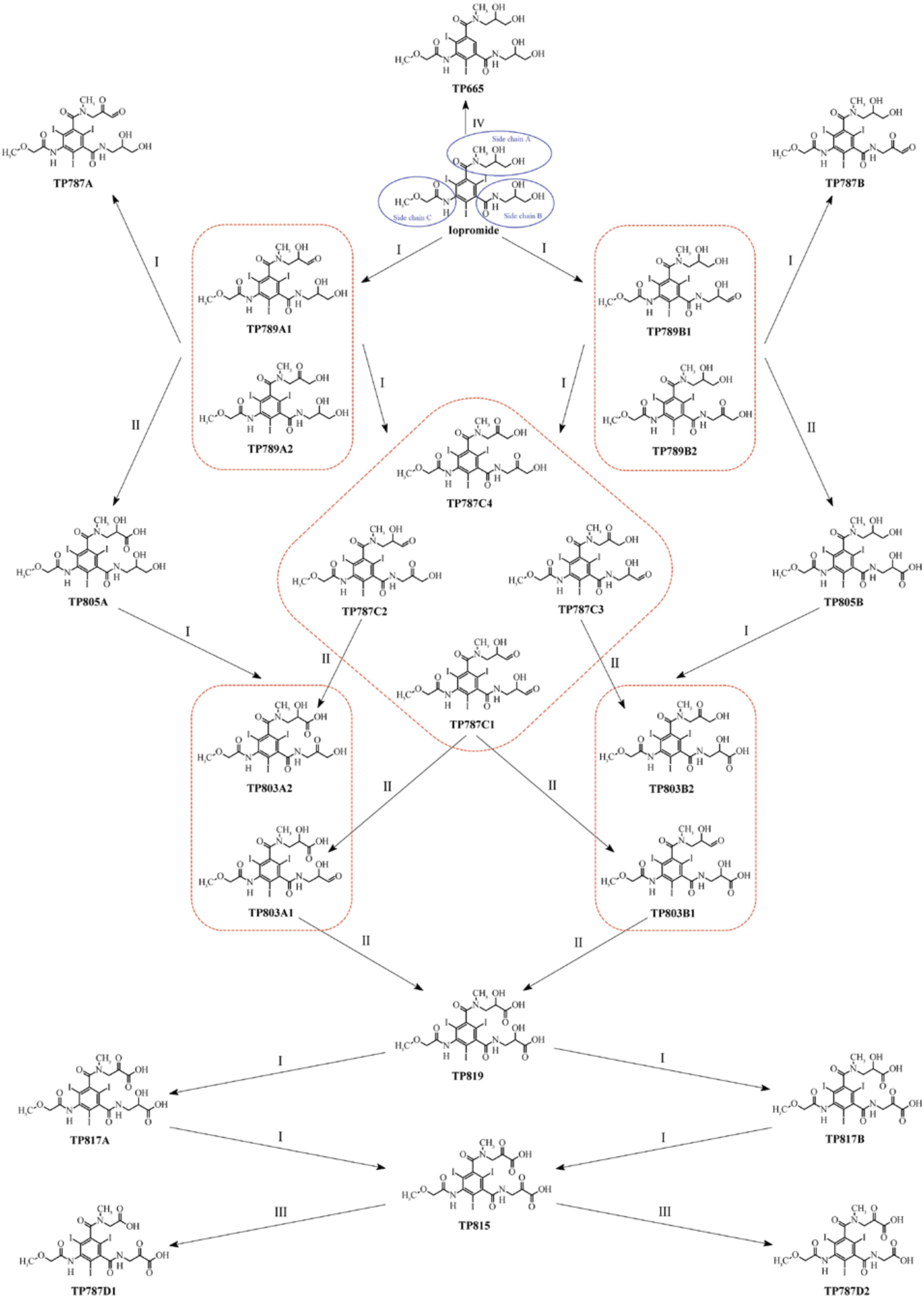

TP665
IV
Side chain A
Side chain B
Side chain C
Iopromide
TP787A
TP787B
I
TP789A1
TP789A2
TP789B1
TP789B2
II
TP805A
TP805B
TP787C4
TP787C2
TP787C3
TP787C1
TP803A2
TP803A1
TP803B2
TP803B1
TP819
TP817A
TP817B
TP815
III
TP787D1
TP787D2

Fig.3a

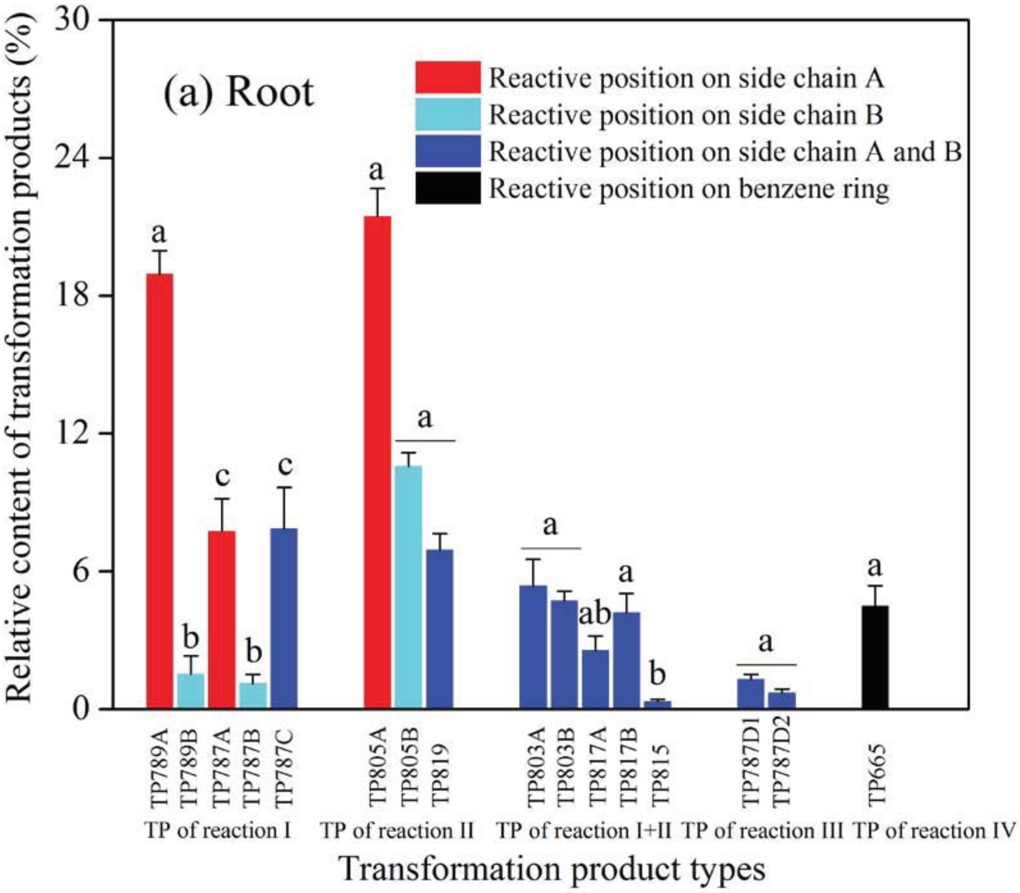
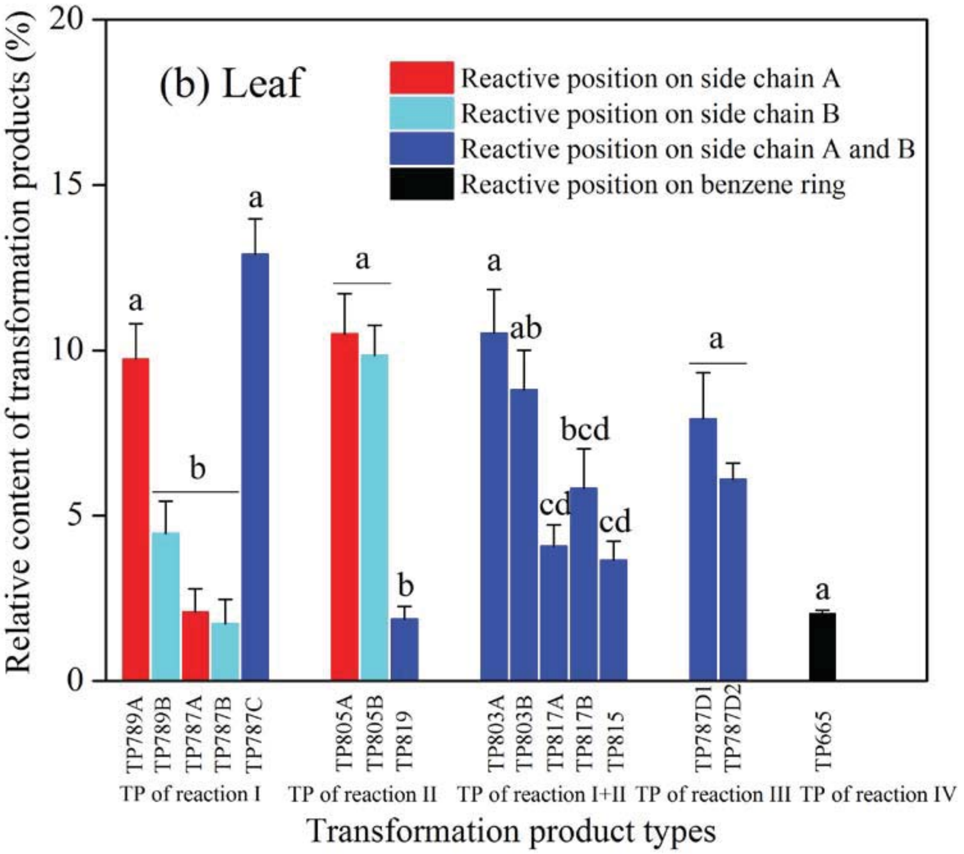

(a) Root
Reactive position on side chain A
Reactive position on side chain B
Reactive position on side chain A and B
Reactive position on benzene ring
Relative content of transformation products (%)
0
6
12
18
24
30
a
b
c
b
c
a
a
a
ab
a
b
a
a
TP789A
TP789B
TP787A
TP787B
TP787C
TP805A
TP805B
TP819
TP803A
TP803B
TP817A
TP817B
TP815
TP787D1
TP787D2
TP665
TP of reaction I
TP of reaction II
TP of reaction I+II
TP of reaction III
TP of reaction IV
Transformation product types

Fig 3b


(b) Leaf
Reactive position on side chain A
Reactive position on side chain B
Reactive position on side chain A and B
Reactive position on benzene ring
Relative content of transformation products (%)
0
5
10
15
20
a
b
a
a
b
a
ab
cd
bcd
cd
a
a
TP789A
TP789B
TP787A
TP787B
TP787C
TP805A
TP805B
TP819
TP803A
TP803B
TP817A
TP817B
TP815
TP787D1
TP787D2
TP665
TP of reaction I
TP of reaction II
TP of reaction I+II
TP of reaction III
TP of reaction IV
Transformation product types

Supporting Information to the Article:

Contents

1. Greenhouse conditions and Hoagland's nutrient solution formula

Greenhouse conditions.

illumination	12 h of light / 12 h of darkness
temperature	23°C at day / 18°C at night
humidity	65%

Hoagland's nutrient solution formula.

K^+	2.5 mmol·L^{-1}
Mg^{2+}	2.0 mmol·L^{-1}
Ca^{2+}	2.0 mmol·L^{-1}
SO_4^{2-}	2.0 mmol·L^{-1}
NO_3^-	6.0 mmol·L
$H_2PO_4^-$	0.5 mmol·L
Fe^{2+}	50 µmol·L^{-1}
BO_3^{3-}	50 µmol·L^{-1}
Mn^{2+}	1 µmol·L^{-1}
Cu^{2+}	0.5 µmol·L^{-1}
Zn^{2+}	0.5 µmol·L^{-1}
MoO_4^{2-}	0.1 µmol·L^{-1}

pH was adjusted to 6.0.

2. LC-MS/MS Conditions

LC parameters:

Column Temperature: 30°C

Injection Volume: 15 μL

Solvent A: Water with 0.1% formic acid

Solvent B: Acetonitrile with 0.1% formic acid

Gradient program:

Time / min	Solvent A / %	Solvent B / %
0	90	10
2.5	90	10
12.5	5	95
16	5	95
17.5	90	10
19	90	10

MS parameters:

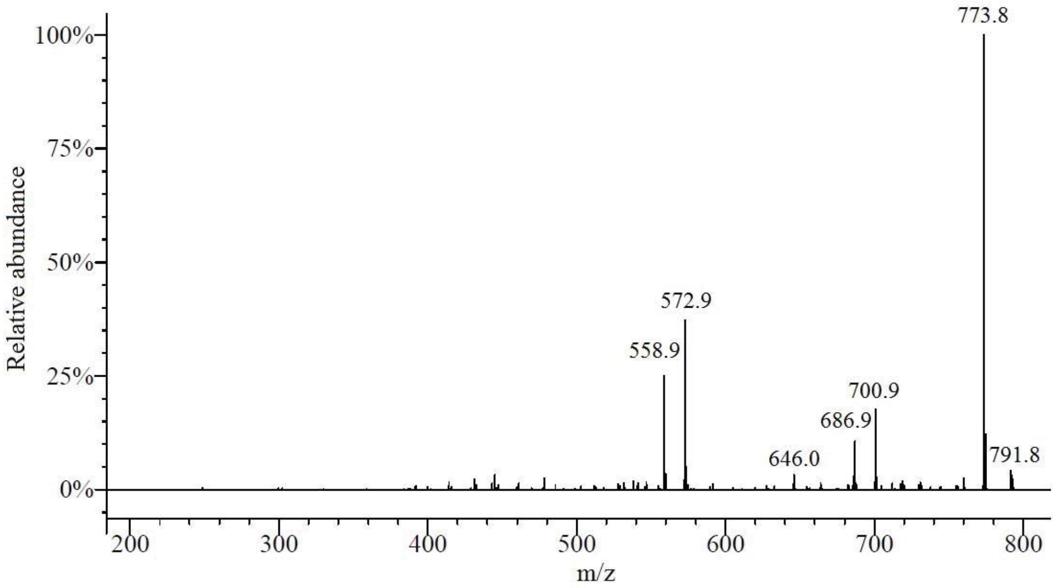
Ion source: ESI+

Scan Type: MS, MS^2

Mass range: 100-1500 m/z

Fragmentation: CID

3. Iopromide and its TPs MS/MS spectra and proposed fragmentation pathway



Iopromide

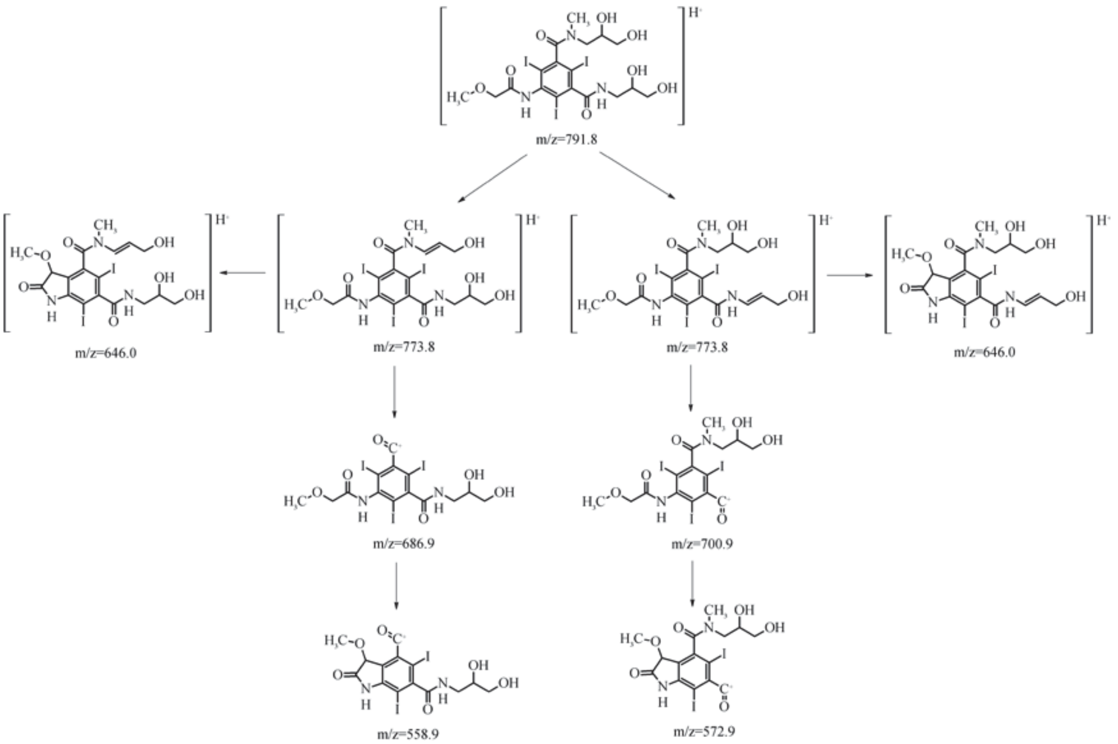
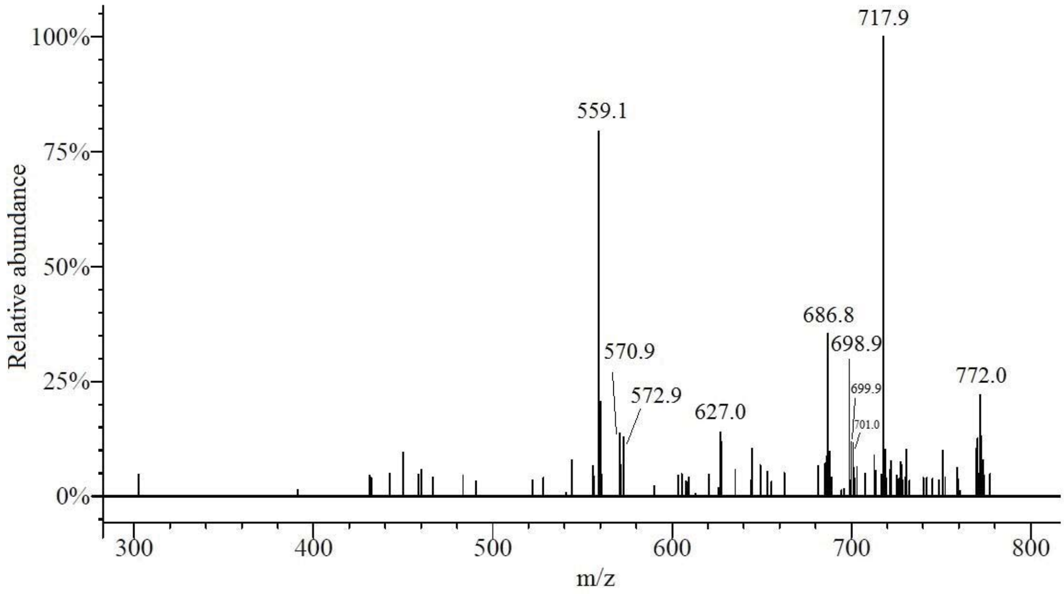
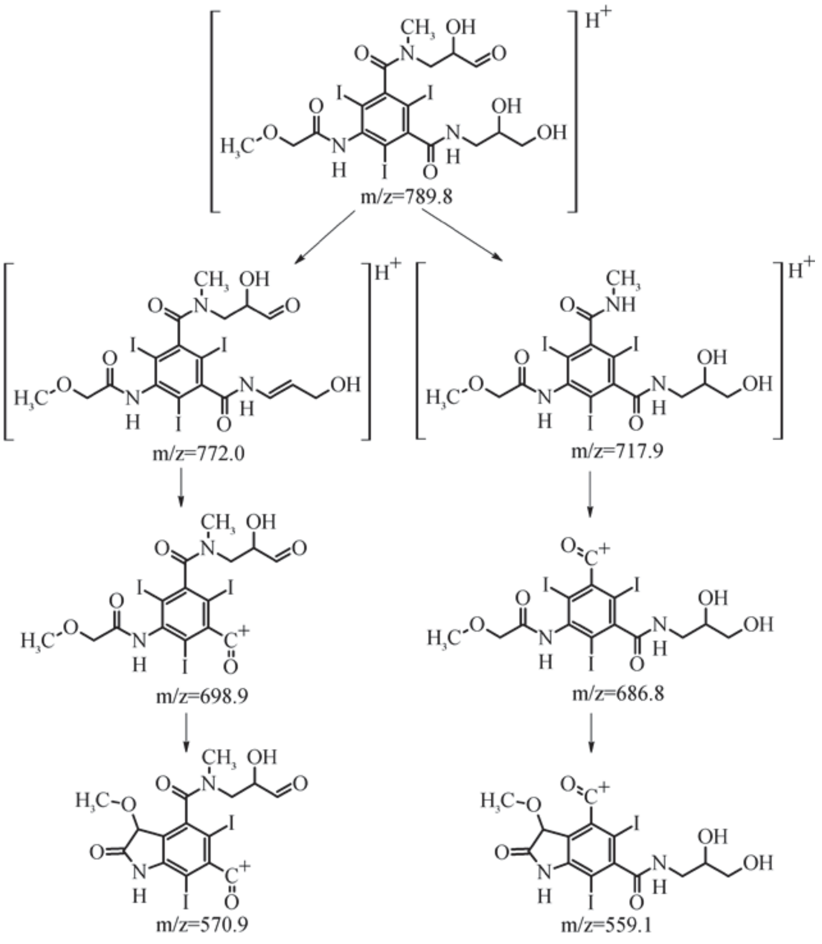


Fig SI.1 Iopromide MS/MS spectra and proposed fragmentation pathway



Relative abundance
100%
75%
50%
25%
0%
m/z
300
400
500
600
700
800
717.9
559.1
570.9
572.9
627.0
686.8
698.9
699.9
701.0
772.0

TP789A



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m/z=772.0
m/z=717.9
m/z=698.9
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m/z=570.9
m/z=559.1

TP789B

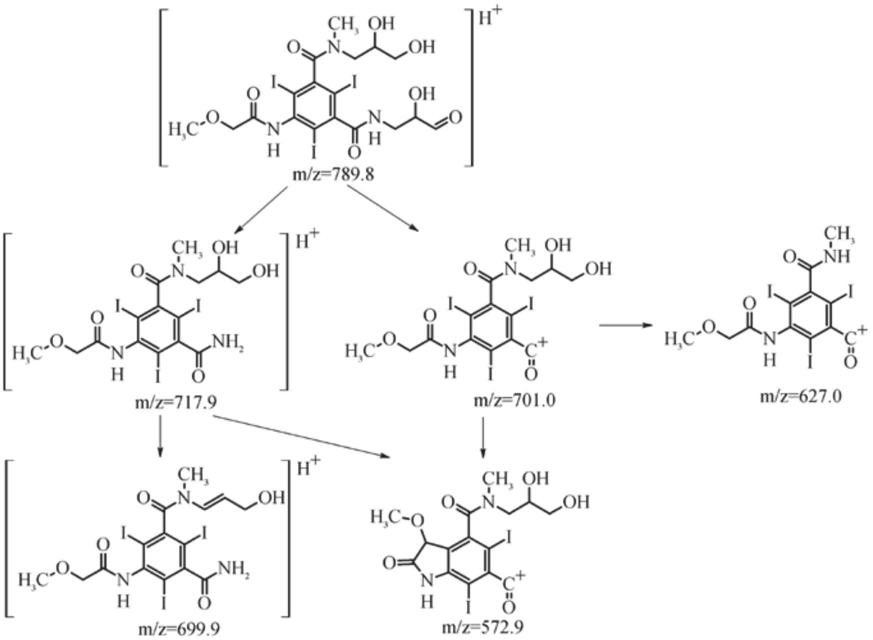
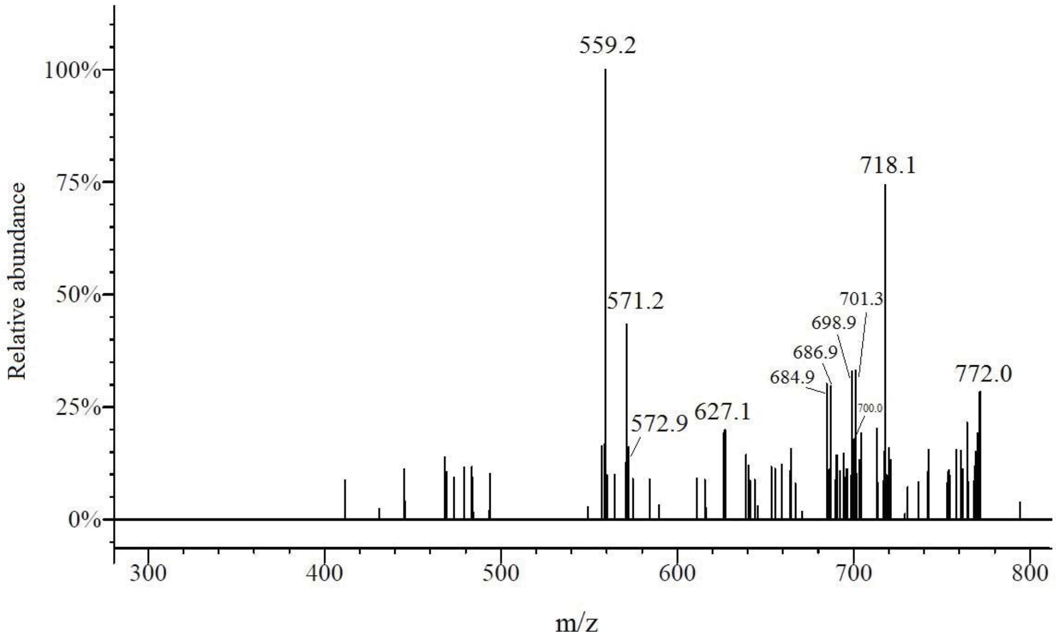


Fig SI.2 TP789 MS/MS spectra and proposed fragmentation pathway


Relative abundance
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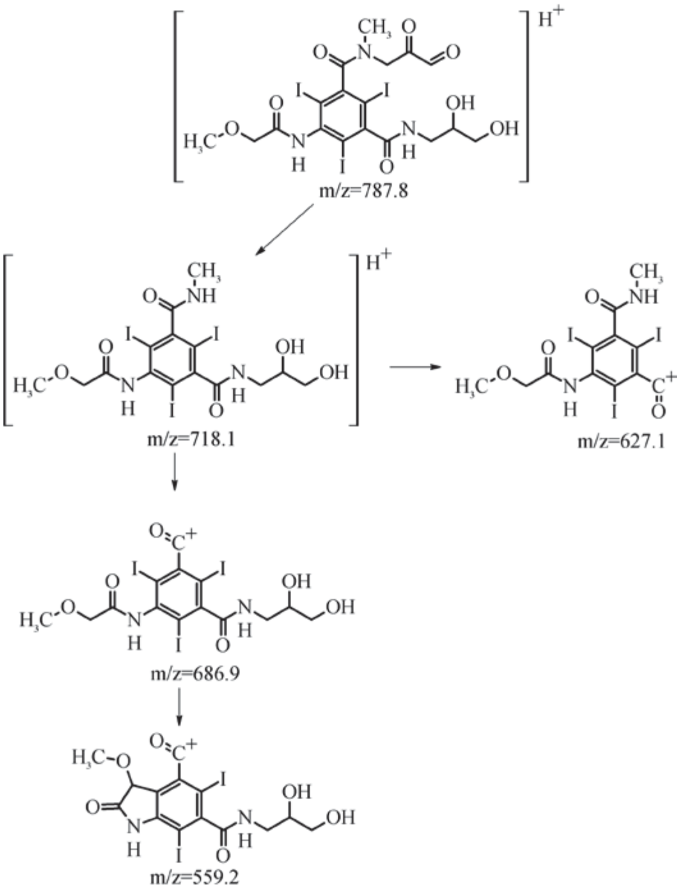
75%
50%
25%
0%
300
400
500
600
700
800
m/z
559.2
571.2
572.9
627.1
684.9
686.9
698.9
701.3
700.0
718.1
772.0

TP787A


H+
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H+
m/z=718.1
m/z=627.1
m/z=686.9
m/z=559.2

TP787B

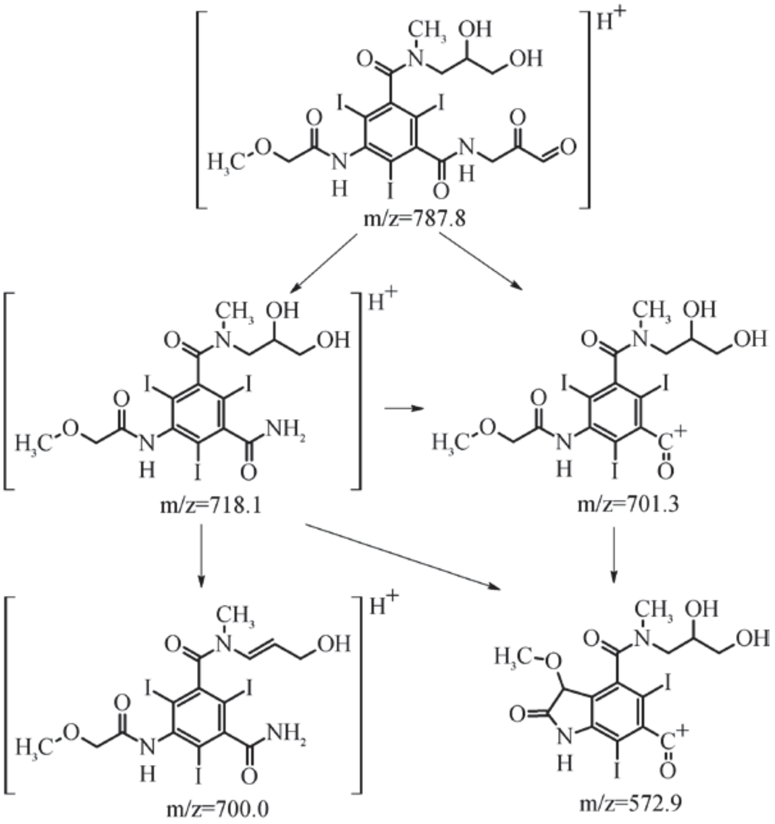
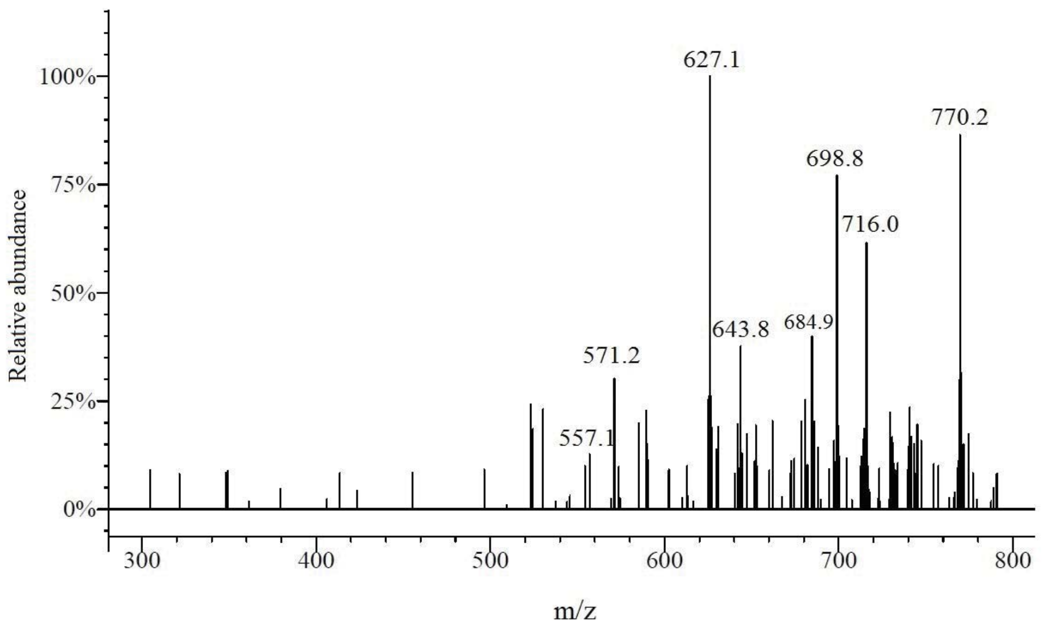


Fig SI.3 TP787A and B MS/MS spectra and proposed fragmentation pathway



TP787C

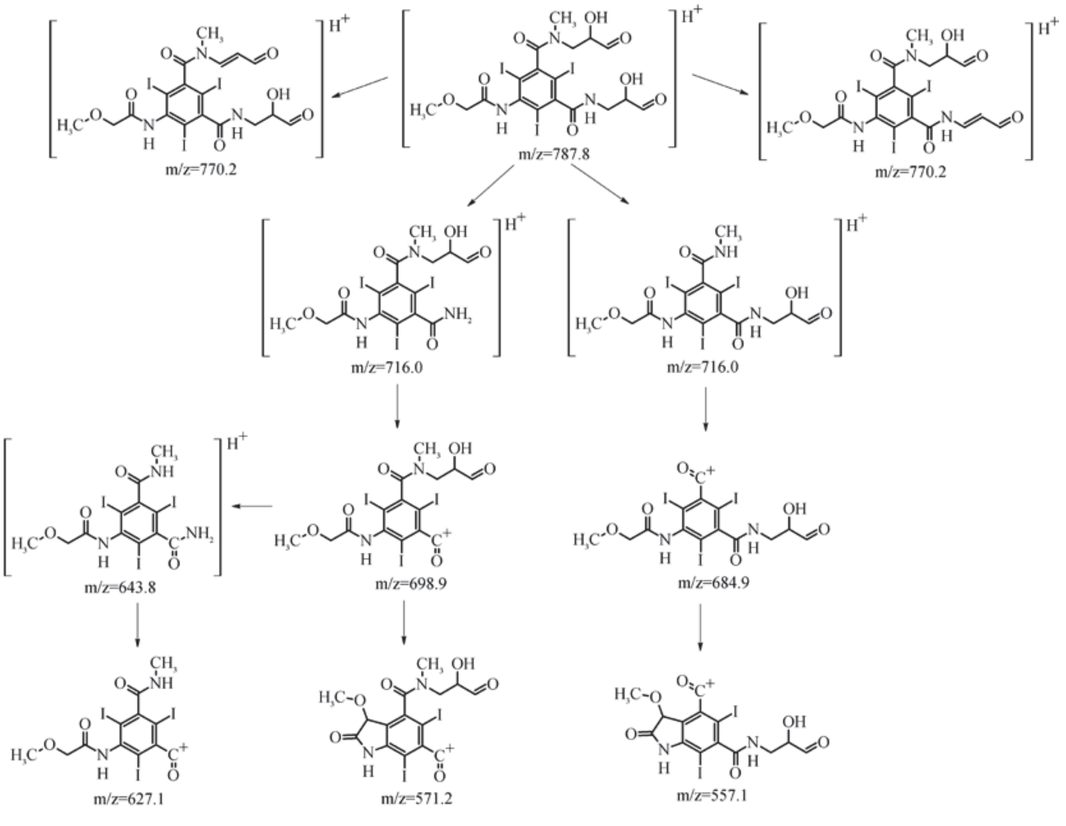
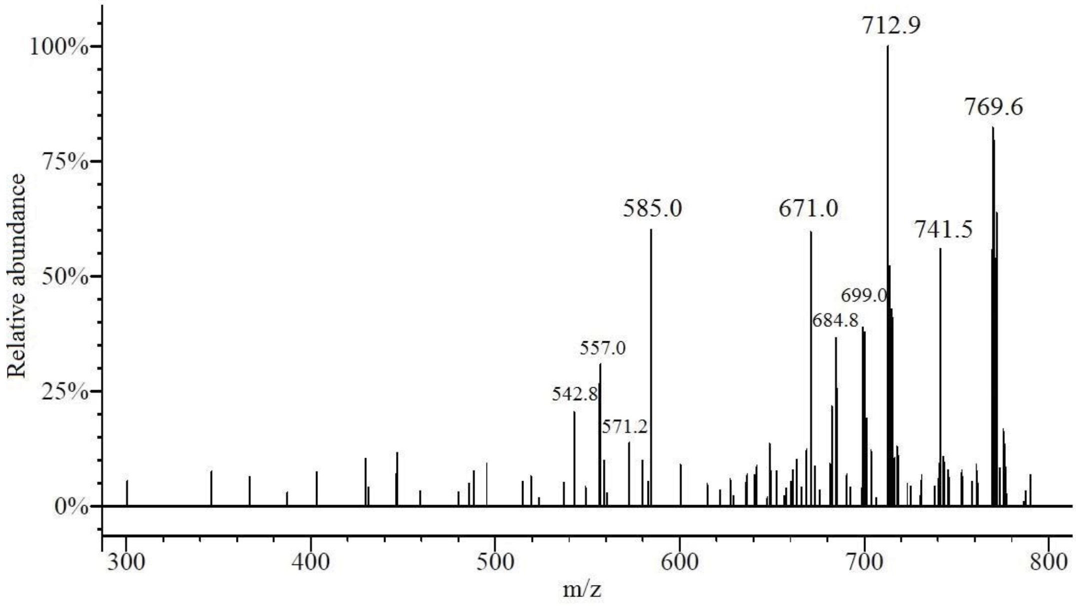


Fig SI.4 TP787C MS/MS spectra and proposed fragmentation pathway


Relative abundance
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75%
50%
25%
0%
m/z
300
400
500
600
700
800
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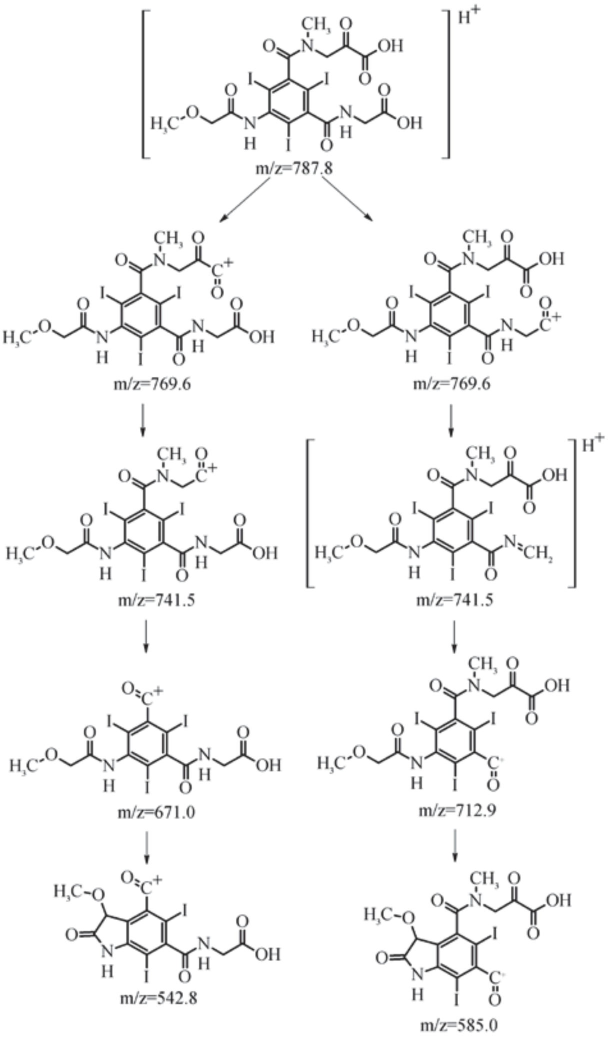
585.0
671.0
741.5
699.0
684.8
557.0
542.8
571.2

TP787D1


H+
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m/z=769.6
m/z=769.6
m/z=741.5
H+
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m/z=671.0
m/z=712.9
m/z=542.8
m/z=585.0

TP787D2

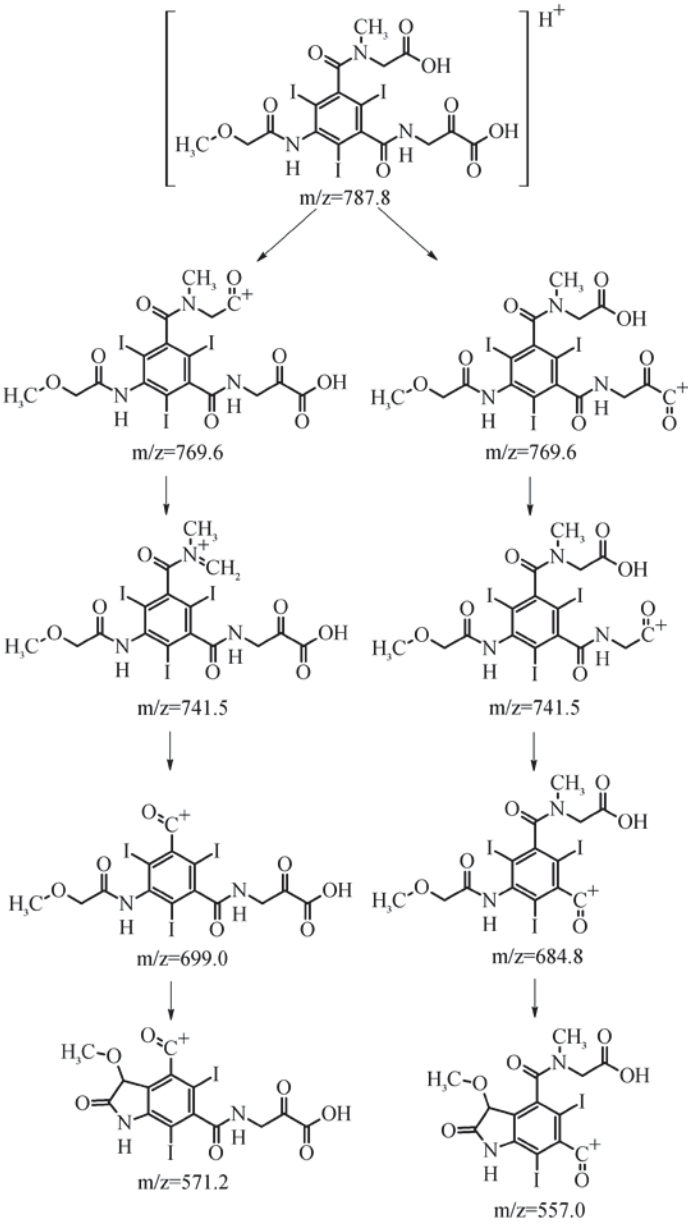
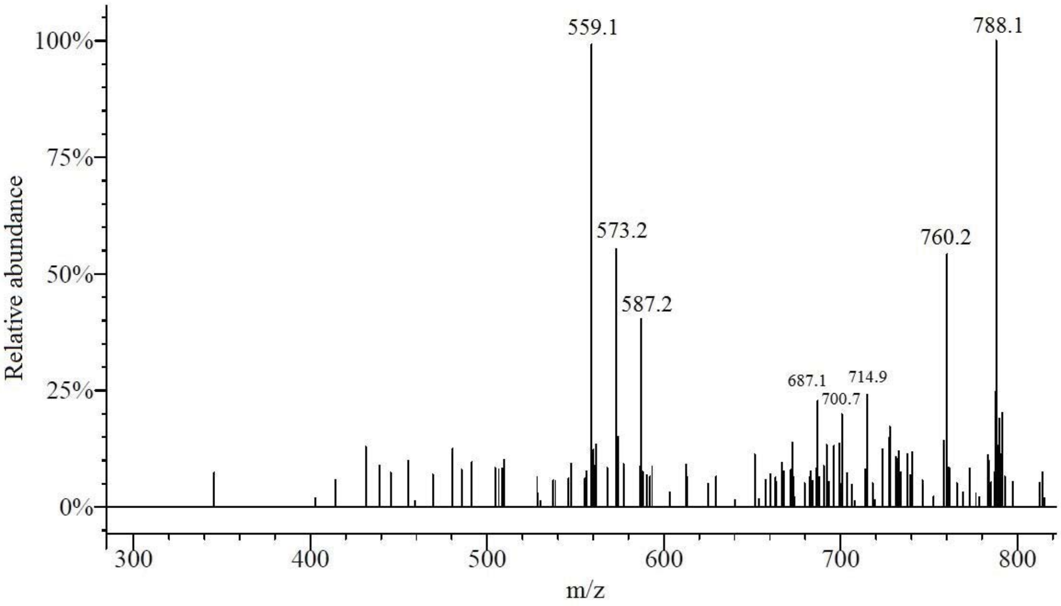
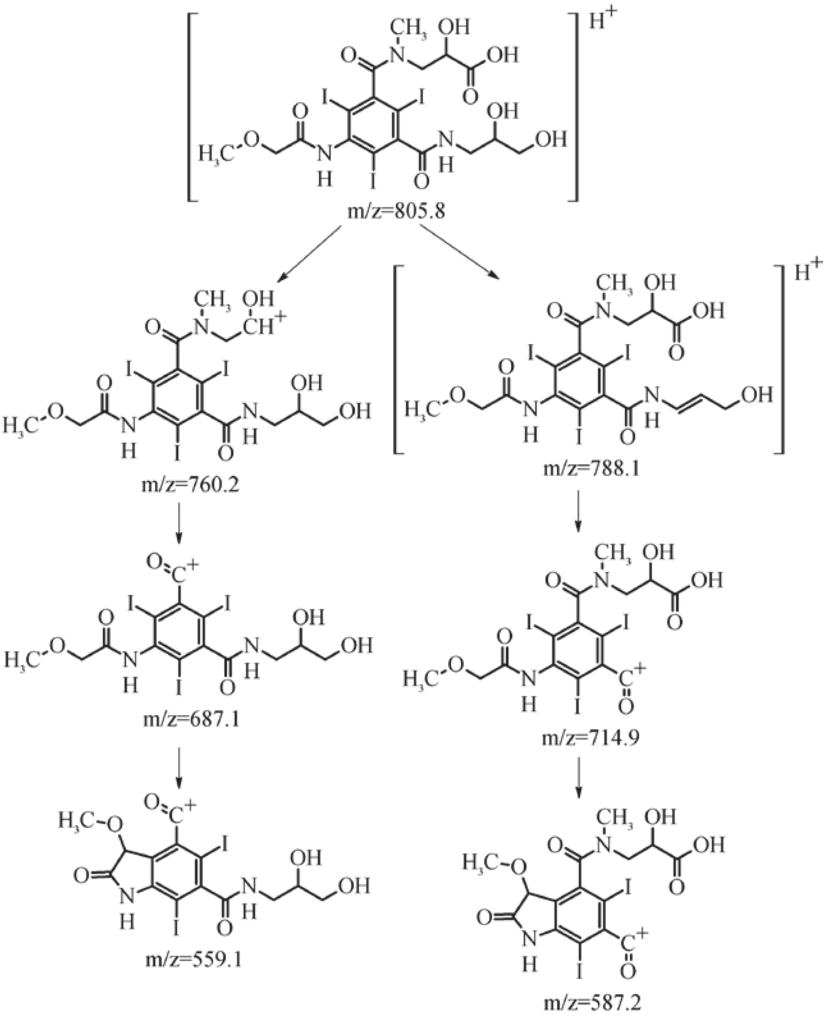


Fig SI.5 TP787D MS/MS spectra and proposed fragmentation pathway


559.1
788.1
573.2
760.2
587.2
687.1
714.9
700.7
100%
75%
50%
25%
0%
Relative abundance
300
400
500
600
700
800
m/z

TP805A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m/z=687.1
m/z=714.9
m/z=559.1
m/z=587.2

TP805B

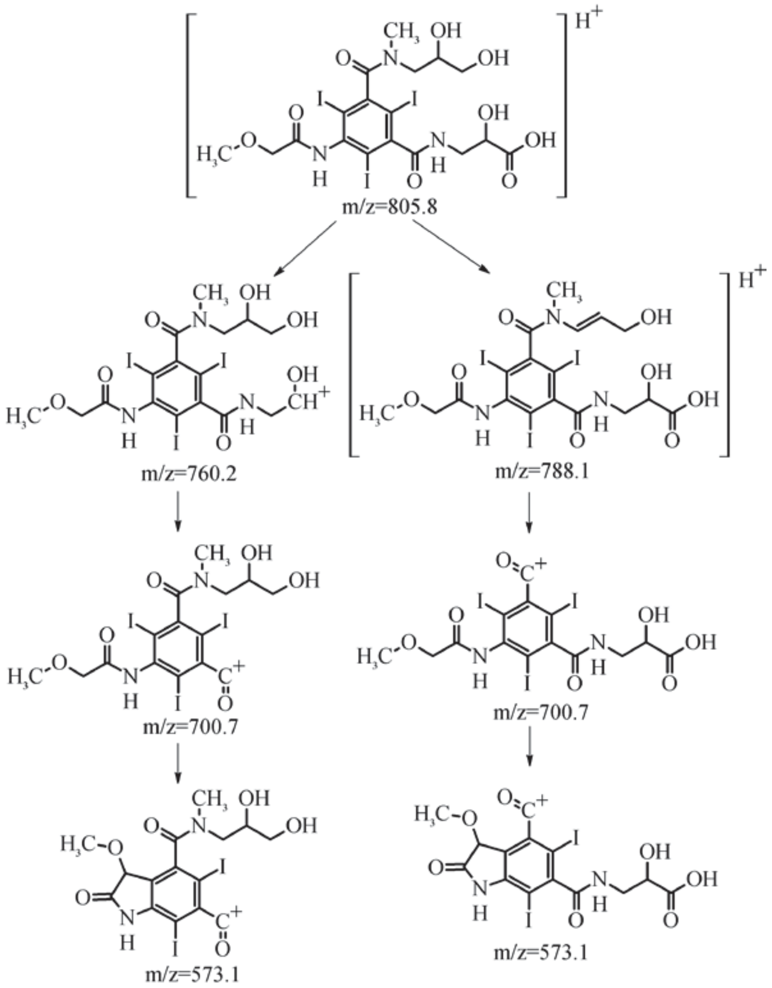
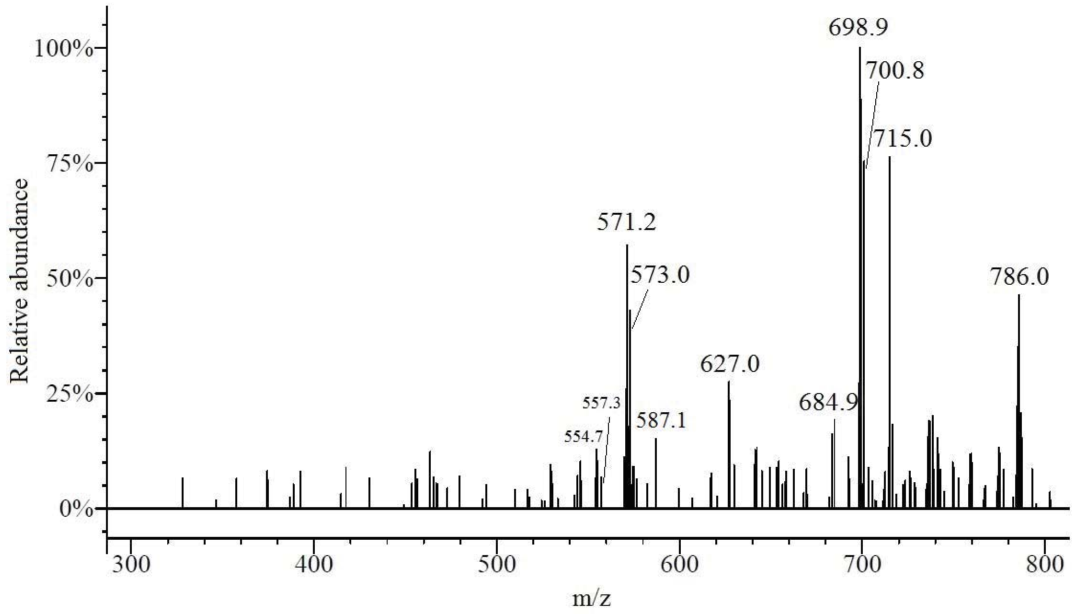


Fig SI.6 TP805A and B MS/MS spectra and proposed fragmentation pathway


Relative abundance
100%
75%
50%
25%
0%
m/z
300
400
500
600
700
800
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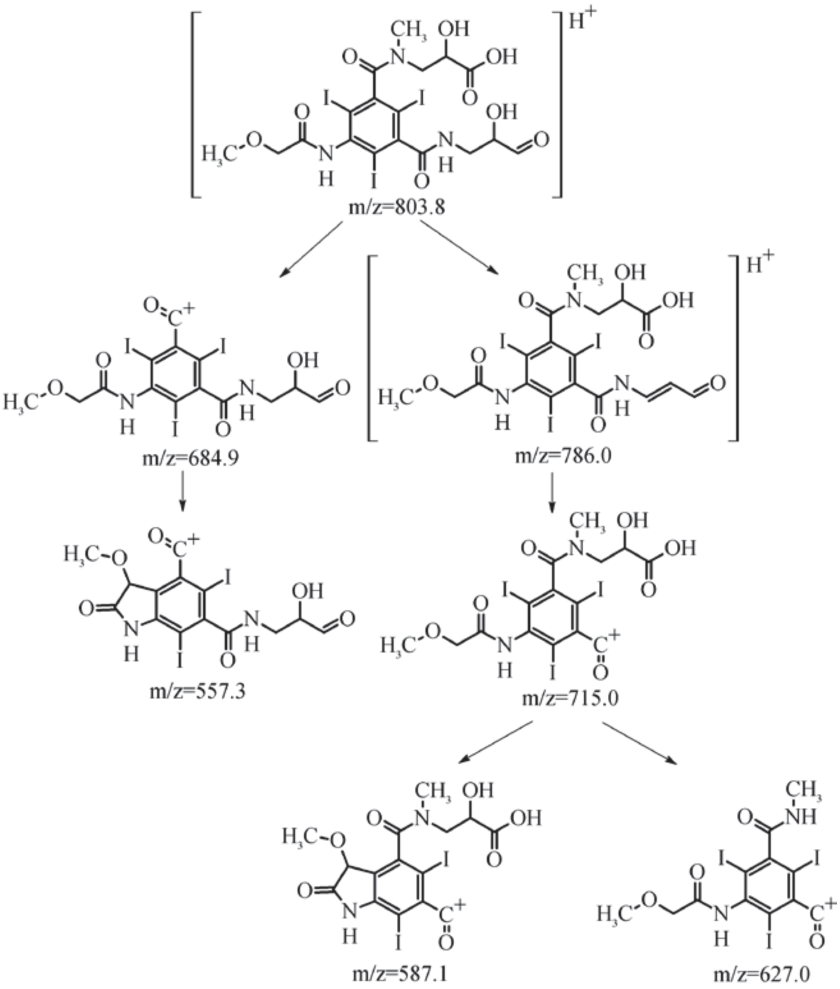
571.2
573.0
786.0
627.0
684.9
557.3
587.1
554.7

TP803A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TP803B

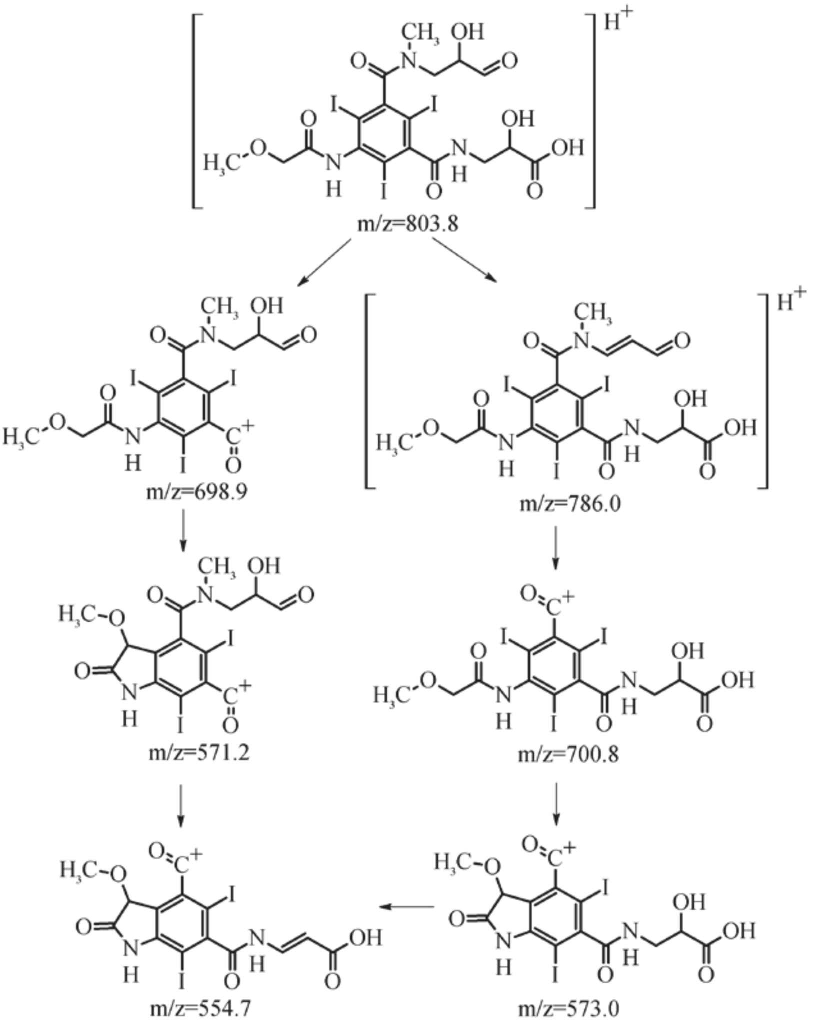
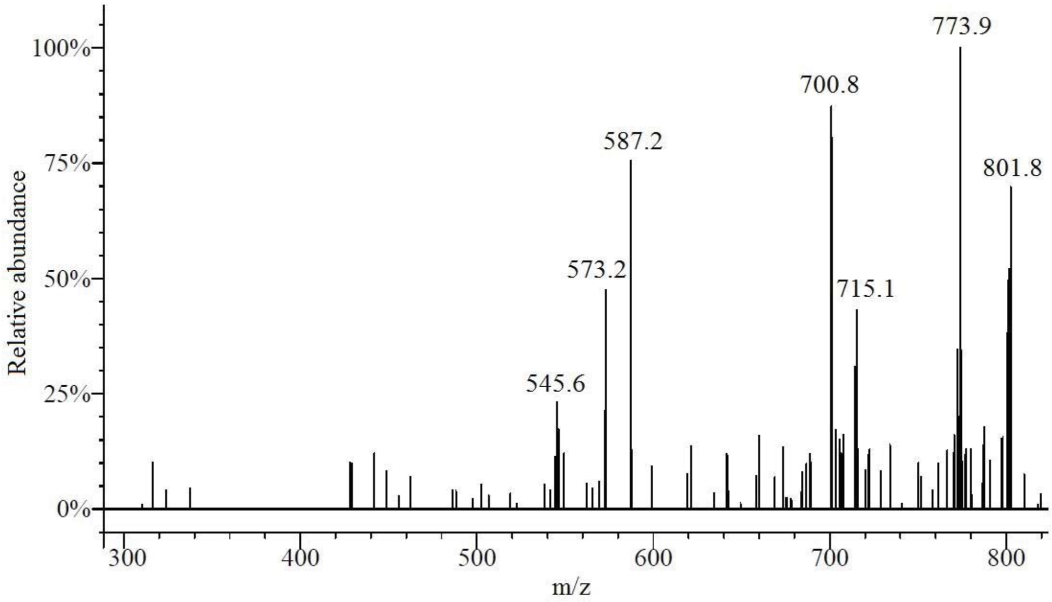


Fig SI.7 TP803A and B MS/MS spectra and proposed fragmentation pathway



TP819

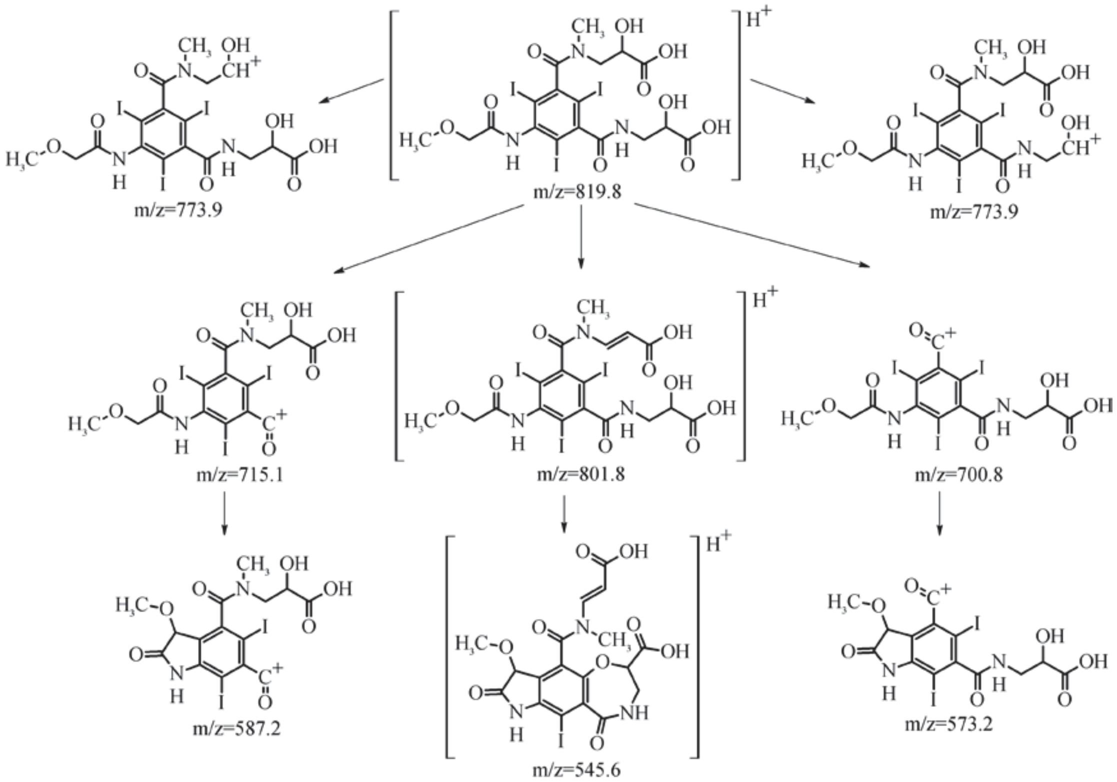
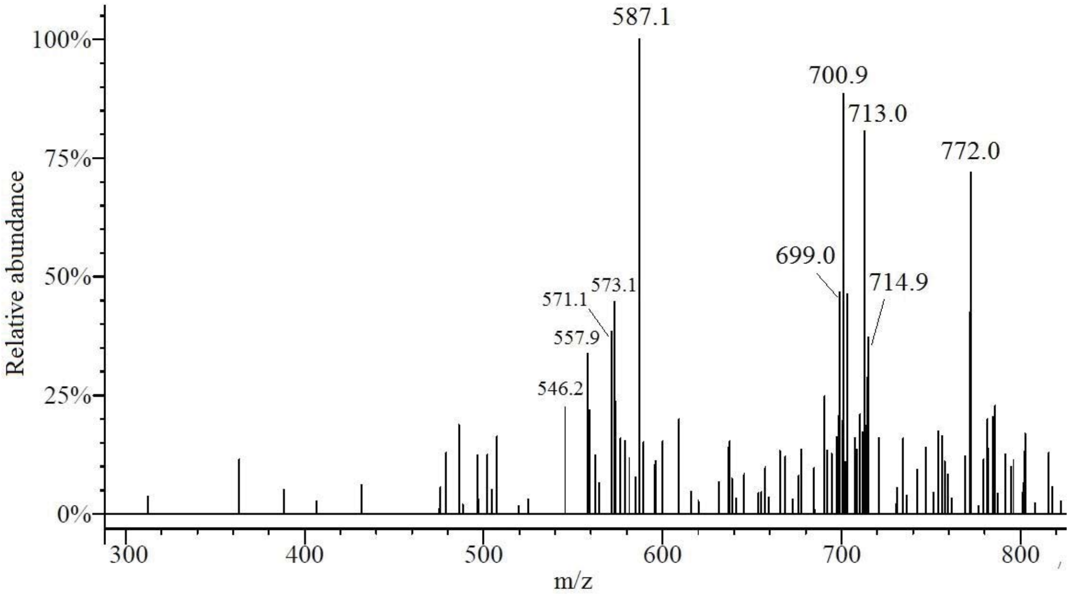
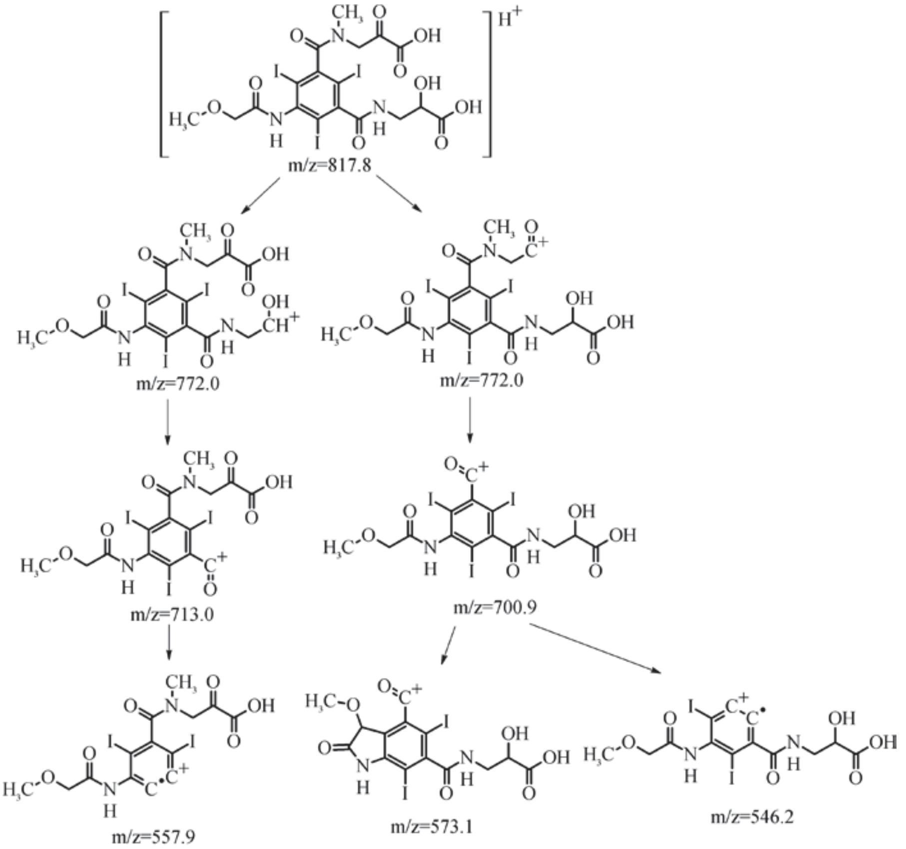


Fig SI.8 TP819 MS/MS spectra and proposed fragmentation pathway


587.1
700.9
713.0
772.0
699.0
714.9
573.1
571.1
557.9
546.2
100%
75%
50%
25%
0%
Relative abundance
300
400
500
600
700
800
m/z

TP817A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TP817B

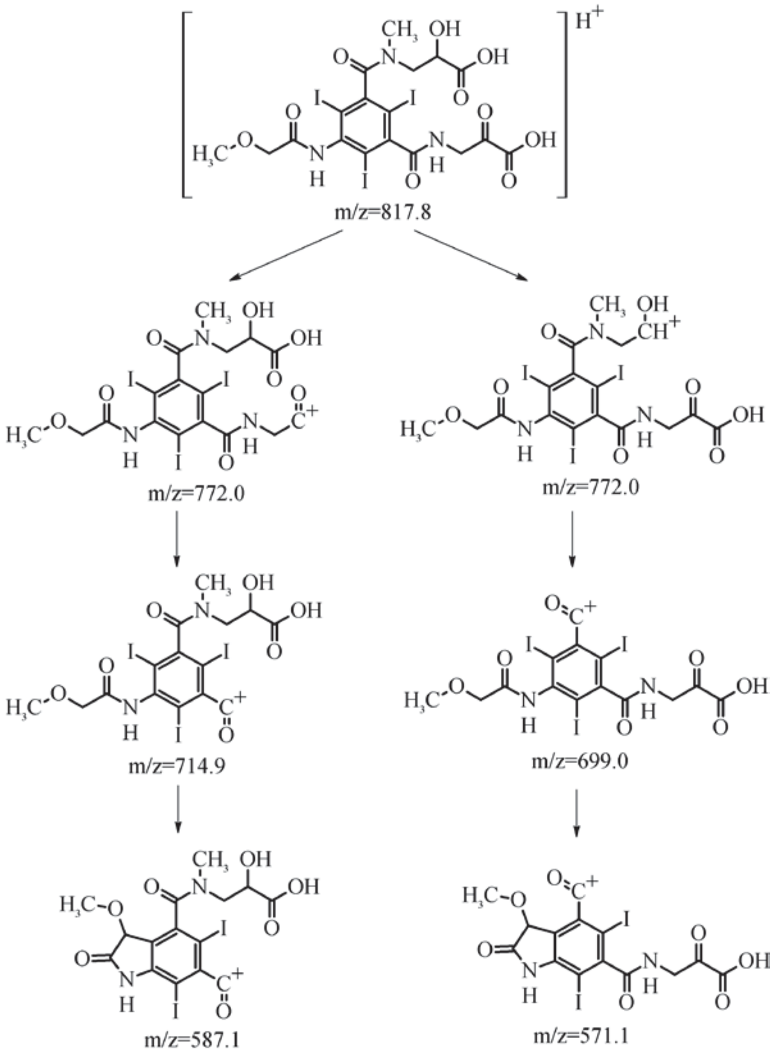
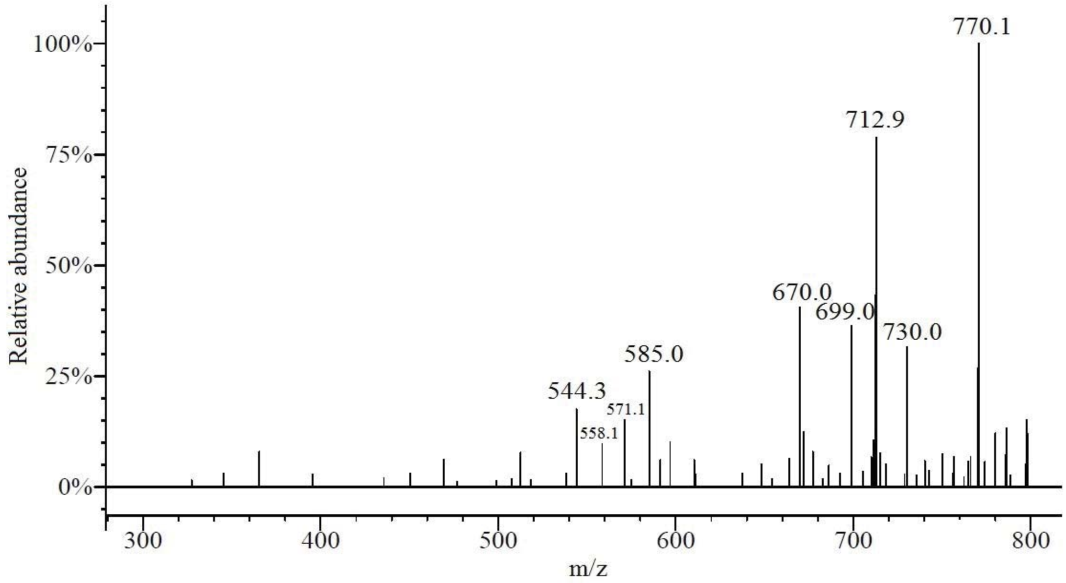


Fig SI.9 TP817A and B MS/MS spectra and proposed fragmentation pathway



TP815

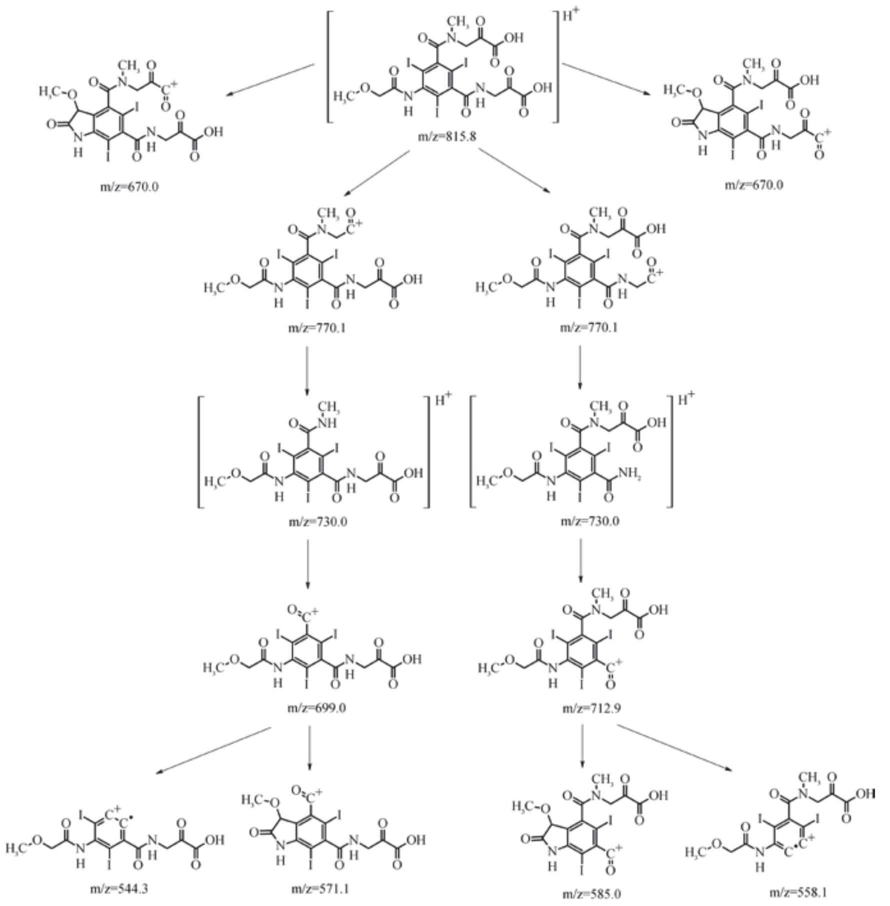
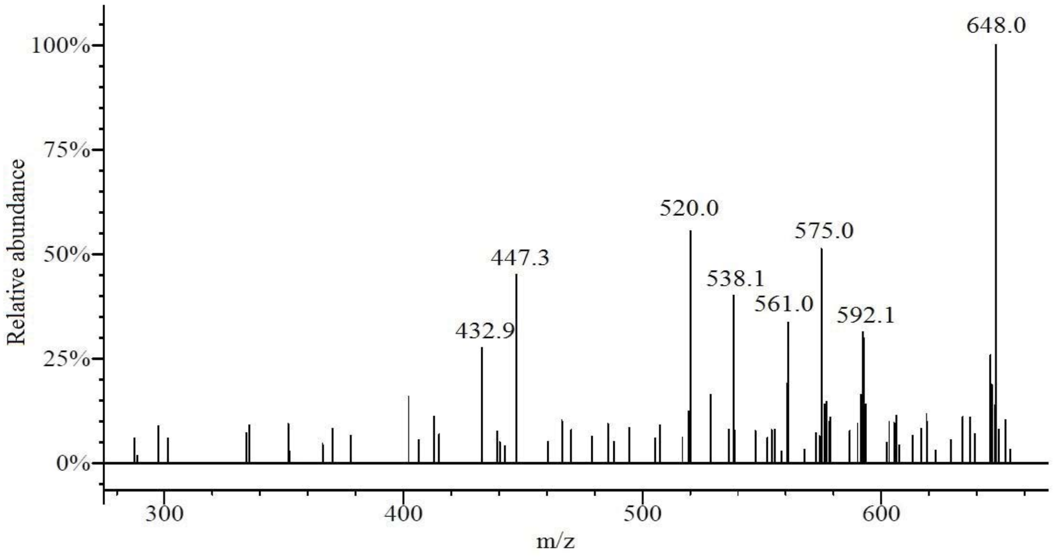


Fig SI.10 TP815 MS/MS spectra and proposed fragmentation pathway



TP665

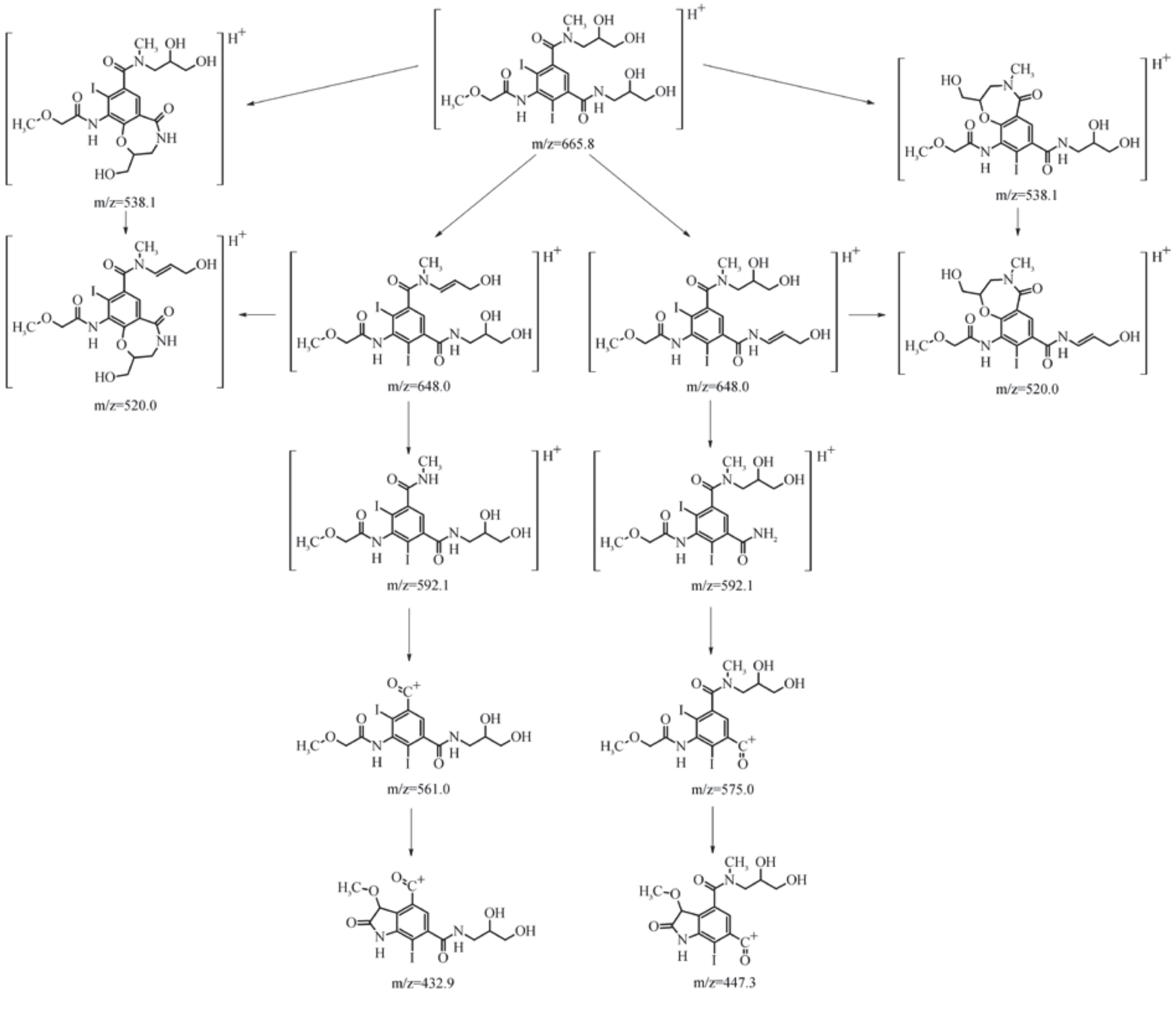


Fig SI.11 TP665 MS/MS spectra and proposed fragmentation pathway

4. Relative content of TPs in *T. latifolia* tissues

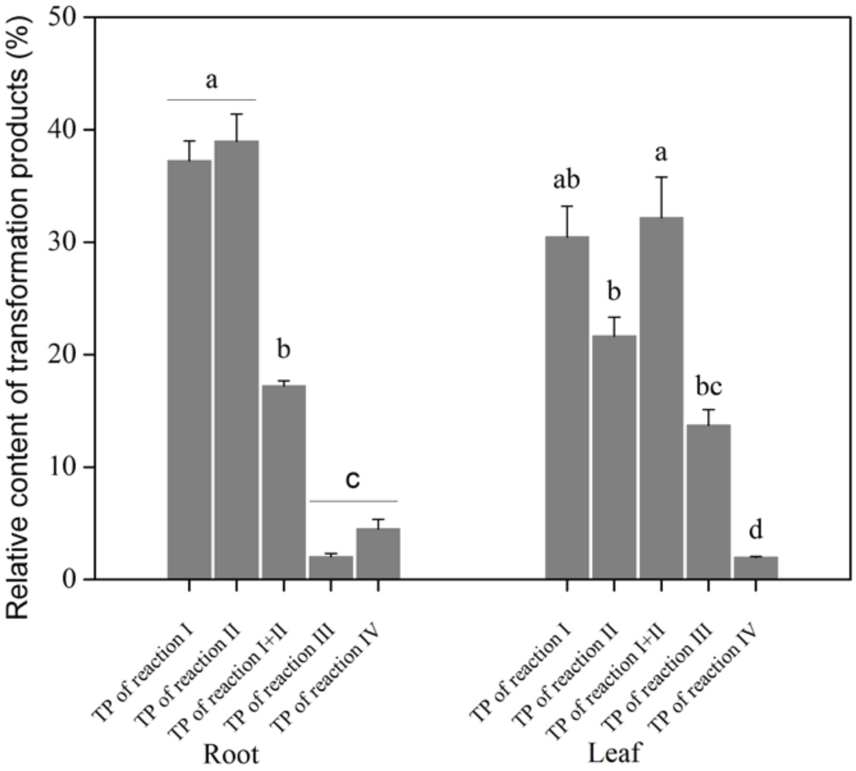


Fig SI. 12 Relative peak areas of different kinds TPs detected by LC-MS/MS in *T. latifolia* root and leaf incubated with iopromide after 14 days.

5. The concentrations of iopromide in the medium and plant tissues.

Table SI. 1 The concentrations of iopromide in the medium and in exposed *T. latifolia* tissues. Controls and treatments were found to be statistically different at the $p < 0.05$ level.

A	Iopromide concentration in media [$\mu mol \cdot L^{-1}$]	
	t=0 d	t=14 d
control pots	20.40 ± 0.15	19.76 ± 0.14
planted pots	19.88 ± 0.21	6.42 ± 0.73

B	Iopromide concentration *in planta* [$nmol \cdot g^{-1}$ (FW)]	
	roots	leaves
control plants	n.d.	n.d.
treated plants	4.91 ± 0.51	0.44 ± 0.07

Title: Iopromide exposure in *Typha latifolia* L.: Evaluation of uptake, translocation and possible transformation pathway *in planta*.

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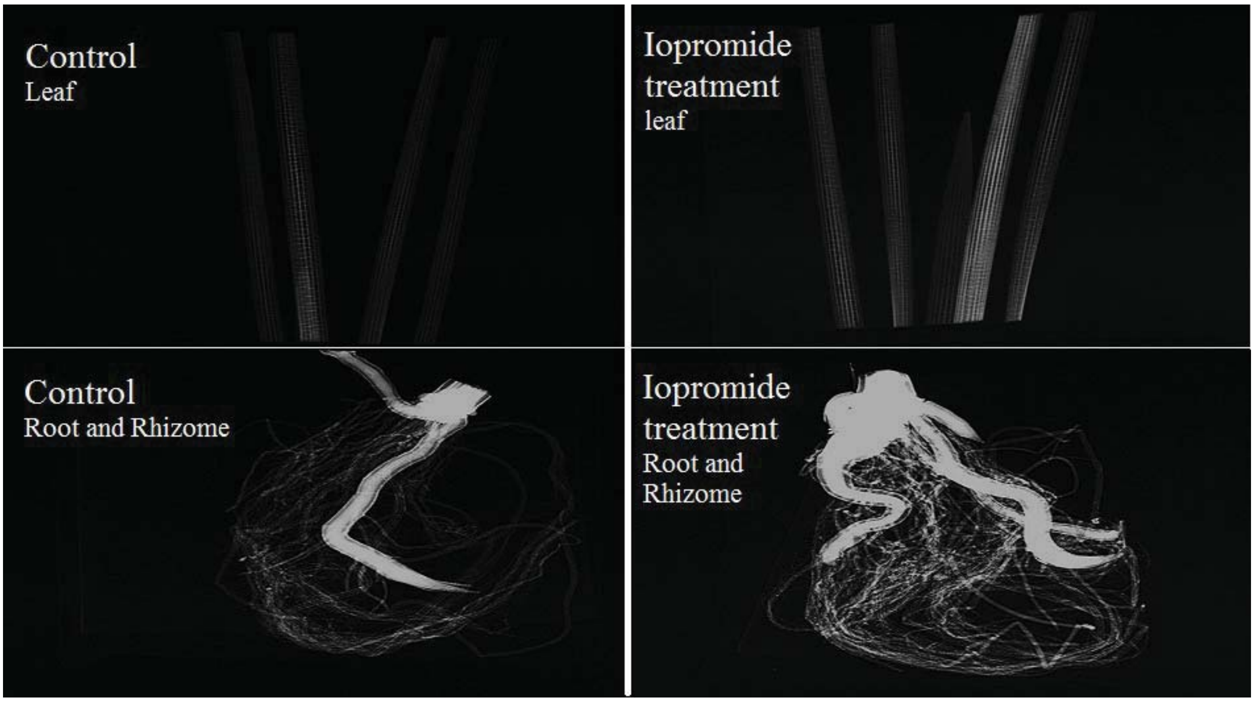
Abstract

Iopromide is frequently detected in water bodies due to its widespread use as an X- ray contrast agent in medicine. Due to its rapid clearance from the human body and its incomplete removal by wastewater treatment, an elevation of its concentration in the environment is observed that might lead to a serious impact on human and environmental health. Alternative removal technologies may be more effective to remove iopromide from the effluents of wastewater treatment facilities, like phytoremediation with aquatic macrophytes. A hydroponic experiment was carried out to assess the fate of iopromide in *Typha latifolia*. The transformation products (TPs) in plant were investigated to predict the possible transformation pathway. The removal process followed first order kinetics with a linear regression R^2 value of 0.983. The iopromide concentration in roots and rhizomes reached a maximum value of 20.70±0.81 and 16.82±1.78 $\text{nmol}\cdot\text{g}^{-1}$ on the 7th day, respectively, thereafter decreased until the end of experiment. A different result was found in leaves, where iopromide concentration decreased over the whole experimental period. A total of eight transformation products were detected in *T. latifolia*, including 23 isomers. The relative content of aldehyde and ketone TPs decreased in roots and rhizomes while the relative content of carboxylic TPs increased. However, the relative content of aldehyde and ketone TPs only showed a slight decrease in leaves while the relative content of carboxylic TPs remained stable during the experimental period. In addition, a significantly increase of decarboxylated TPs was found in leaves, but not in roots and rhizomes. These results indicate that a difference of transformation pathways exists among plant tissues. The findings of this study are important to better understand the transformation pathway of iopromide in plants and to improve phytoremediation technologies for such kind of compounds.

TOC/Abstract Art



1. INTRODUCTION

Iodine containing X-ray contrast media are used to enhance the contrast of structures within the body in medical imaging. Especially the non-ionic iodine contrast media are widely used today as they are quickly spread and stable in the human body. Since increasingly higher amounts of such compounds are in use throughout Europe, it is not surprising that such compounds are frequently detected in wastewater effluents and surface waters.[1,2] Iodine contrast media can form many transformation products (TPs) during wastewater and drinking water treatment processes, which might possess higher toxicity than the parent compounds.[3] Therefore, chronic exposure to these compounds may represent a non-negligible risk for aquatic organisms and human health.

Iopromide is one of the most frequently used X-ray contrast media. With high doses of usage (15g) and rapid excretion, it leaves the body almost unchanged (>95%).[4-6] Hence it is not surprising that this contrast agent has been detected in wastewater and in surface water at concentrations ranging from $ng \cdot L^{-1}$ to $\mu g \cdot L^{-1}$.[7,8] Despite its beneficial properties, iopromide has been shown to induce oxidative stress and apoptosis in human neutrophils.[9] And, importantly, the scientific concern is more on the formation of iodinated disinfection byproducts (DBPs). Previous studies have detected many toxic DBPs formed by chlorination of X-ray contrast media.[3,10] Richardson et al.[11] reported that iodinated DBPs have enhanced mammalian cell toxicity as compared to their brominated and chlorinated analogues. However, up to now, the generated DBPs cannot be effectively removed during sewage water treatment processes.

Phytoremediation is a plant-based approach to water remediation that takes advantage of low cost, simple operation and environmental friendliness.[12] An increasing number of studies found that aquatic plant-based systems have been highly efficient in the removal of emerging contaminants.[13-16] In contrast to the traditional wastewater treatment process, phytoremediation can realize the removal of contaminants from the aqueous phase by plant uptake, and then these contaminants can be accumulated or degraded in the plants. Hence, this technology could effectively avoid the secondary pollution by TPs and reduce environmental and human health risks. But so far, specific investigations of plant uptake and biological transformation are scarce.[17]

In this study, the uptake and translocation of iopromide were studied using cattail (*Typha latifolia*) cultivated in hydroponic exposure. To highlight the transformation of iopromide in plants, the tissue distribution of iopromide and its TPs during different incubation times were assessed. Based on the results obtained in this study, the mechanism of plant-based transformation of iopromide has been further explored.

2. MATERIALS AND METHODS

Chemicals. Iopromide (98.6%) was purchased from Sigma (Germany). Solvents were LC-MS grade and obtained from Roth (Germany). All other chemicals were analytical grade. Ultrapure water was obtained from a Milli-Q water purification system.

Plant Materials. *Typha latifolia* plants were ordered from a local plant nursery (Jörg Petrowsky, Eschede, Germany) and carefully washed with tap water to remove adherent soil and debris. Plants were grown in perlite in 5L pots under greenhouse conditions with 12h of light/12h of darkness at 23/18°C and a humidity of 65%. Nutrients were provided in water by a modified Hoagland's nutrient solution. Plants uniform in size were selected for hydroponic experiment after acclimated to greenhouse conditions for two months. The composition of the nutrient solution was: 2.5 $mmol \cdot L^{-1}$ K^+, 2.0 $mmol \cdot L^{-1}$ Mg^{2+}, 2.0 $mmol \cdot L^{-1}$ Ca^{2+}, 2.0 $mmol \cdot L^{-1}$ SO_4^{2-} , 6.0 $mmol \cdot L^{-1}$ NO_3^-, 0.5 $mmol \cdot L^{-1}$ $H_2PO_4^-$,50 $\mu mol \cdot L^{-1}$ Fe^{2+}, 50 $\mu mol \cdot L^{-1}$ BO_3^{3-}, 1 $\mu mol \cdot L^{-1}$ Mn^{2+}, 0.5 $\mu mol \cdot L^{-1}$ Cu^{2+}, 0.5 $\mu mol \cdot L^{-1}$ Zn^{2+}, 0.1 $\mu mol \cdot L^{-1}$ $MoO_4^{2-}$and the pH was adjusted to 6.0.

Hydroponic Experiment Setup. Plant rhizomes were rinsed with distilled water and then transferred to 2.5 L plastic pots. The pots were covered with plastic package inside to prevent the potential adsorption of iopromide on the pot surface. Each pot contained 1 L nutrient solution and 1L perlite to fix the plant. The nutrient solution was spiked with iopromide to reach a final initial concentration of 20 $\mu mol \cdot L^{-1}$. Pots with equal concentration of iopromide but without plants were used as controls. Three replicates of controls and each exposure period studied were set up, i.e., 1, 3, 7, 14 and 28 days. For each exposure period, root, leaf, rhizome and nutrient solutions were collected, frozen and stored at -80°C.

Analysis for Iopromide and its TPs by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). The determination of iopromide in water and plant followed our previous work. Briefly, 0.5 g fresh plant material was ground under liquid nitrogen and then extracted with 4.5 mL extraction solution (water with 0.1% formic acid). The mixture was homogenized, ultrasonicated, centrifuged and then the supernatant was filtrated through a 0.45 µm Nylon filter. The filtrates were transferred to a solid phase extraction (SPE) column (Oasis HLB, Waters, Germany), percolated, washed and eluted. The analytes were injected into the LC-MS/MS system.

The HPLC system (Varian ProStar 210, Darmstadt, Germany) was coupled to an ion trap mass spectrometer (Varian 500-MS, Darmstadt, Germany) consisting of an electrospray ionization source. Separation was achieved on a Synergi Polar-RP 80a column (150 mm × 2 mm, 4µm, Bischoff, Germany) at a flow rate of 0.3 $mL \cdot min^{-1}$. For the determination of iopromide, the precursor ion *m/z* 791.8 $(M+H)^+$ giving fragment ions *m/z* 773.8 and *m/z* 572.9 was used for quantification and confirmation.

3. **RESULTS AND DISCUSSION**

Removal of Iopromide from the Medium. Iopromide was removed from the nutrient solution by *T. latifolia*, and the removal efficiency increased with the exposure time. The maximum removal efficiency was 86.6±2.3% after 28 days (Figure 1). The removal process satisfied the first-order kinetic equation as well as the decay rate constant of 0.0783 day^{-1} with a linear regression R^2 value of 0.983 (Figure 2). No significant variation of iopromide concentration in the medium was found in controls during the whole experimental period.

Previous studies showed that pharmaceuticals can be removed by plants. Dordio et al.[18] reported carbamazepine removal by *Typha spp.* with removal efficiencies ranging between 56%~82% for different initial concentrations. Reinhold et al.[19] indicated that in an active duckweed reactor which consisted predominantly of *L. minor* or *L. punctata*, ibuprofen concentrations decreased linearly to 47.5% depletion in 9 d. Zhang et al.[15] found the removal processes of five different pharmaceuticals by *Scirpus validus* were also following first-order, and the decay rate constants in a range of 0.023~0.403 day^{-1}. Adsorption and photodegradation of iopromide seem to play only a minor role during the experimental period as no significant variation has been found in controls. Therefore, the elimination of iopromide is expected to be highly dependent on the uptake by plants.

Uptake and Translocation of Iopromide by *T. latifolia*. Iopromide was detected in all plant tissues, including roots, rhizomes and leaves during the whole exposure period. When plant parts were x-rayed in a standard x-ray apparatus, lignified or suberized tissues appeared greyish/white, with a higher intensity in freshly developed rhizomes, and cell walls. Plants treated with iopromide, however, showed distinctly higher whitening in roots, rhizomes, and stronger display of the veins of the leaves (Figure S1). In roots and rhizomes, iopromide concentrations increased with exposure time up to day 3, remained stable until day 7 and decreased thereafter (Figure 3). The maximum iopromide concentration in roots was found to be 20.70±0.81 nmol·g^{-1} (fresh weight) while in rhizomes 16.82±1.78 nmol·g^{-1} (fresh weight) accumulated after 7 days of exposure. Iopromide concentration in leaves decreased constantly with exposure time (Figure 3). The iopromide concentration in leaves was 2.00±0.22 nmol·g^{-1} (fresh weight) at day 1, and gradually decreased to 0.34±0.05 nmol·g^{-1} (fresh weight) at day 28. Compared to roots and rhizomes, iopromide concentration was relatively low in leaves. Table S1 showed the bioaccumulation factors for all plant tissues, ranging from 0.02 to 1.04.

Plant uptake includes both, the apoplastic and symplastic pathway. The apoplastic flow is finally forced to move into the symplast pathways as the root endodermis contains the casparian strip, blocking the uptake of undesirable compounds.[20] Thus, the transmembrane transport of contaminants plays a decisive role in the uptake processes. Organic contaminants tend to move into plant roots driven by passive diffusion.[21] Therefore, uptake of xenobiotics is depended on their physico-chemical properties, especially their hydrophobicity. Organic compounds of low hydrophobicity ($\log P < 1$) are not sufficiently taken up by plants.[22] Not surprisingly, our results demonstrate higher iopromide concentration in roots than in leaves. Our result is consistent with that of Herklotz and coworkers[23] who find that Salbutamol ($\log P = 0.64$), a high hydrophilic compound, also exhibits strong absorption in cabbage roots.

Uptake of iopromide into the roots was relatively fast up to day 3 (Figure 3). This result is in line with previous studies showing uptake of xenobiotics by plants within a short time.[24,25] Similar results were also found for other pharmaceuticals in previous studies.[13,23,25] Generally, organic compounds with an optimum hydrophobicity ($\log P = 0.5 \sim 3$) seem to be taken up by plant roots and can then be translocated in plant tissues.[21,22] However, recent studies indicate that hydrophilic chemicals can also have great potentiality to be taken up and translocated by plants.[26]

The marked decrease of iopromide concentration in leaves suggests that iopromide is successively transformed in plant tissues. In theory, there should be an equilibrium between iopromide intake and iopromide dissipation. In roots and rhizomes, the iopromide intake mainly represents uptake while the iopromide dissipation is composed of translocation to leaves and transformation. In leaves, the iopromide intake mainly consists of translocation from roots and the iopromide dissipation includes transformation. At the onset of the experiments, the intake rate was higher than the disappearance rate in roots and rhizomes. Since the iopromide concentration decreased in medium with time, the intake rate decreased while the disappearance increased. As a result, iopromide concentrations in roots and rhizomes increased to a high level remained constant for some time and decreased thereafter.

Proposed Transformation pathway of Iopromide in *T. latifolia*. A total of 8 TPs including 23 isomers were detected when iopromide was metabolized by *T. latifolia*. The structures of these TPs were identified by LC-MS/MS, and to unravel the transformation pathways, these TPs were analyzed at five different time points during incubation. Iopromide transformation by *T. latifolia* could be divided into four reactions: I) the hydroxyl groups being oxidized to aldehyde or ketone groups; II) aldehyde groups being oxidized to carboxyl groups; III) decarboxylation; IV) deiodination.

The temporal progression of TPs formation in roots is shown in Figure 4. There is a trend of decrease in the content of TP789A and B with the extension of inoculation time. The sum of the relative content of TP789A and B were 48.3% at day 1, then continually decreased to 7.0% at day 28. This indicates that TP789A and B are probably precursor compounds for most of the other TPs. Thus, the hydroxyl group oxidized into aldehyde or ketone groups seems to be the first step of iopromide transformation in *T. latifolia* roots. TP787 A, B and C were formed from TP789 by further oxidation of the hydroxyl group. The relative content of TP787A increased until day 14 then remained stable similar to TP787B for which also no significant variation was observed. The relative content of TP787C increased in the first 14 days and thereafter decreased.

Reaction I is known and approved to be catalyzed by alcohol dehydrogenases, which commonly exist in bacteria and higher plants.[27] Nicotinamide adenine dinucleotide (NAD^+) is the acceptor during the enzymatic oxidative dehydrogenation process. Some studies indicate that peroxidase and monooxygenase can also be responsible for this type of reaction in plants.[27,28] Our results show an increasing relative content of TP787A than TP787B over the time. This is probably due to the difference between the structure of side chain A and side chain B. Many NADH-dependent enzymes (i.e. methylglyoxal reductase) may correspond to the transformation from TP789 (A and B) and TP787 (A and B).

The relative content of TP805A in roots and rhizomes increased in the first two weeks followed by a plateau. In leaves, the relative content of TP805A decreased with time. For TP805B in roots and rhizomes, the relative content decreased until day 7, then increased with time, while no significant trend was found in leaves (Figure 5).

Generally, carboxylic TPs were formed from aldehyde TPs. Aldehyde dehydrogenases, which are probably responsible for this chemical reaction, have been found in different organisms.[29,30] Several aldehyde dehydrogenase genes have been identified in plant species.[31,32] It has been shown that aldehyde dehydrogenases can oxidize both aliphatic aldehydes and aromatic aldehydes in maize.

The relative content of TP803A in roots was stable in the first two weeks. After that, an increase followed, while in rhizomes it showed a continuous increasing trend. For TP803B, a stable period in the first week followed by an increase was observed in both roots and rhizomes. In leaves, the relative content of both TP803A and B remained stable in the first week, and thereafter increased until the end of incubation. The total relative content of both TP803A and B presents a steadily increasing trend in all plant tissues, except on day 7 when it decreased slightly in leaves (Figure 5).

The relative content of TP819 showed an increasing trend in roots, while on the contrary, a decreasing trend in leaves was observed. The relative content increased in the first two weeks followed a slight decrease in rhizomes. The relative content of both TP817 A and B remained stable in the first week in all plant tissues then started increasing in roots and rhizomes but decreasing in leaves. The relative content of TP815 reached a maximum of 0.69% at day 7 in roots, while 1.70% at day 3 in rhizomes. An increasing trend of the relative content of TP815 with time was found in leaves (Figure 5).

The precise mechanism of the formation of carboxylic TPs is not yet known. Mechanisms involving the oxidation of lactic acid are possible, and such a reaction may be catalyzed by lactate dehydrogenase in plant. Glycolate oxidase catalyzes the oxidation of glycolic acid to glyoxylate in the peroxisomes during photorespiration.[33] The glycolate oxidase is mainly expressed in green leaves, and may be responsible for the formation of TP815.[34] This could be an explanation for the increasing trend of the TP815 relative content in leaves with time. Because TP819 and TP817 could be further oxidized, the product accumulating in leaves might be TP815 which is probably also the end product of the carboxylic TPs type. Carboxylic TPs were the major kind of iopromide TPs in *T. latifolia* (Figure 5). Aldehydes are known to be toxic to the plants. Therefore, an oxidation of aldehydes TPs to carboxylic TPs in plant tissues would be expected. This transformation is in accordance with the aldehyde detoxification mechanisms in plants.

The relative content of TP787D decreased in the first two weeks in roots and rhizomes, thereafter it increased until the end of incubation time. On the contrary, the relative content of TP787D increased in the first two weeks in leaves before its concentration reached a plateau (Figure 6). Based on molecular structure of TP787D, we suggest TP787D is probably formed from decarboxylation of TP815. Many different enzymes can catalyze this reaction, such as α-keto acid-dependent dioxygenases and branched-chain α-keto acid dehydrogenases.[35,36]

The relative content of TP665 reached a maximum of 4.50% at day 14 in roots, thereafter decreased to 1.33% at the end of incubation. In leaves, the relative content of TP665 showed a slight increasing trend until day 14 and a stronger accumulation until the end of incubation, while a broadly stable trend has been found in rhizomes (Figure 6).

Dehalogenation has been found in plants during the degradation of halogenated aromatic contaminants. Wang et al.[37] reported that debromination was catalyzed in maize after the exposure to polybrominated diphenyl ethers. Recently, Sun et al.[38] also found debromination from brominated diphenyl ether-47 to brominated diphenyl ether-28 in young pumpkin plants. However, further debromination products were not detected in those studies. This is similar to our results which only found deiodination from iopromide to TP665, but no further deiodinated TPs.

Many reductive dehalogenases have been found in bacteria which are responsible for catalyzing dehalogenation of aromatic ring systems.[39,40] However, little information has been found in plants. Glutathione S-transferase can catalyze the reductive degradation of xenobiotics in plants. However, the related intermediates (i.e. glutathione conjugates) were not detected in this study. We suggest this is probably due to steric effects of the side chains which prompt the glutathione leaves from the aromatic ring.

The results of this study clearly show that iopromide can be taken up by *T. latifolia* and the removal efficiency increases with exposure time. Therefore, phytoremediation can be an effective way to eliminate such polar pharmaceuticals from water. The maximum iopromide concentration in roots and rhizomes was found at 3rd day which indicated iopromide can be rapidly degraded in plants. Thus it is essential to clearly understand the transformation pathway of such pollutants in plants. Further research is needed to evaluate the toxicity of many iopromide TPs.

Acknowledgments

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Supporting Information contains:

X-ray analysis condition, bioaccumulation factors of iopromide in different plant tissues.

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Tables and Figures

Figure 1. The removal efficiencies of iopromide by *T. latifolia* at different exposure time. Plotted values are means ± SD of three replicates.

Figure 2. The first-order kinetics fitting of the removal process. Plotted values are means ± SD of three replicates.

Figure 3. Concentrations of iopromide in different tissues of exposed *T. latifolia*. Plotted values are means ± SD of three replicates.

Figure 4. Relative peak area trends of aldehyde and ketone transformation products identified in *T. latifolia* during the experimental periods. Plotted values are means ± SD of three replicates.

Figure 5. Relative peak area trends of carboxylic transformation products identified in *T. latifolia* during the experimental periods. Plotted values are means ± SD of three replicates.

Figure 6. Relative peak area trends of decarboxylated and deiodinated transformation products identified in *T. latifolia* during the experimental periods. Plotted values are means ± SD of three replicates.

Figure 1

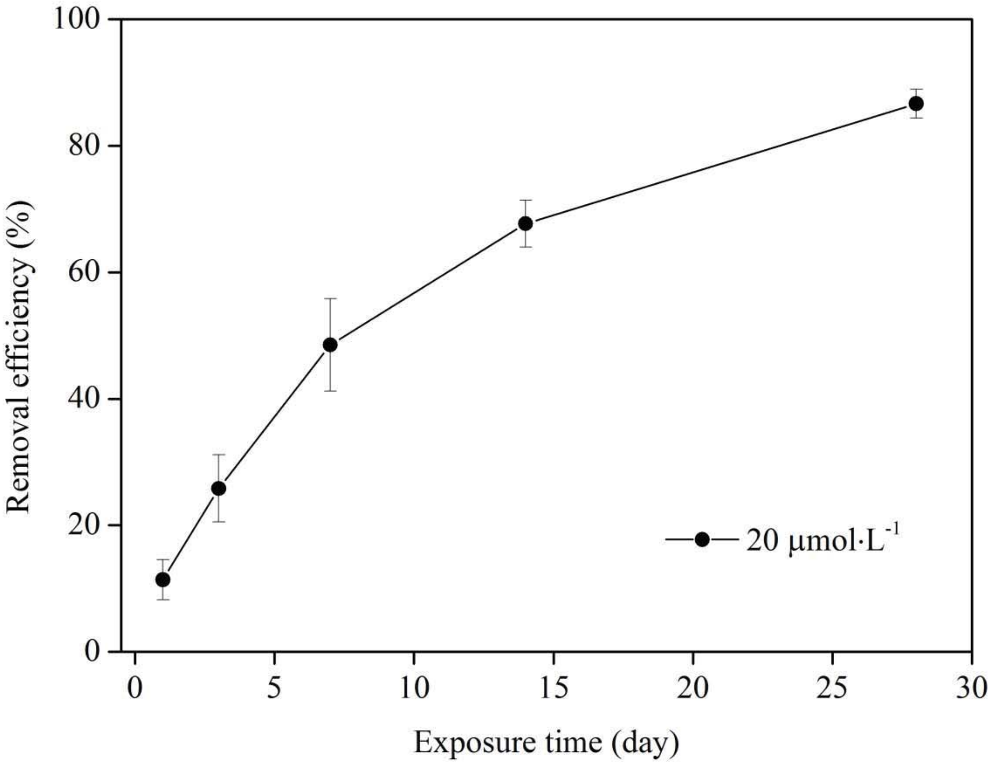
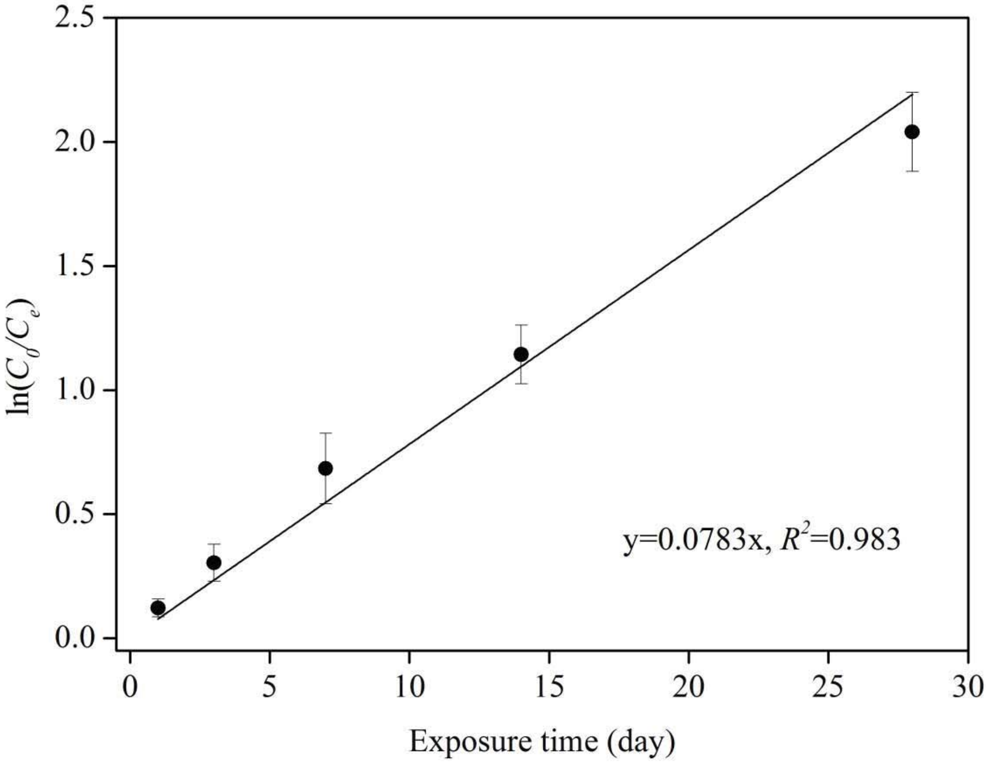

100
80
60
40
20
0
Removal efficiency (%)
0
5
10
15
20
25
30
Exposure time (day)
20 μmol·L-1

Figure 2



$\ln(C_0/C_e)$
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
Exposure time (day)
y=0.0783x, R^2=0.983

Figure 3

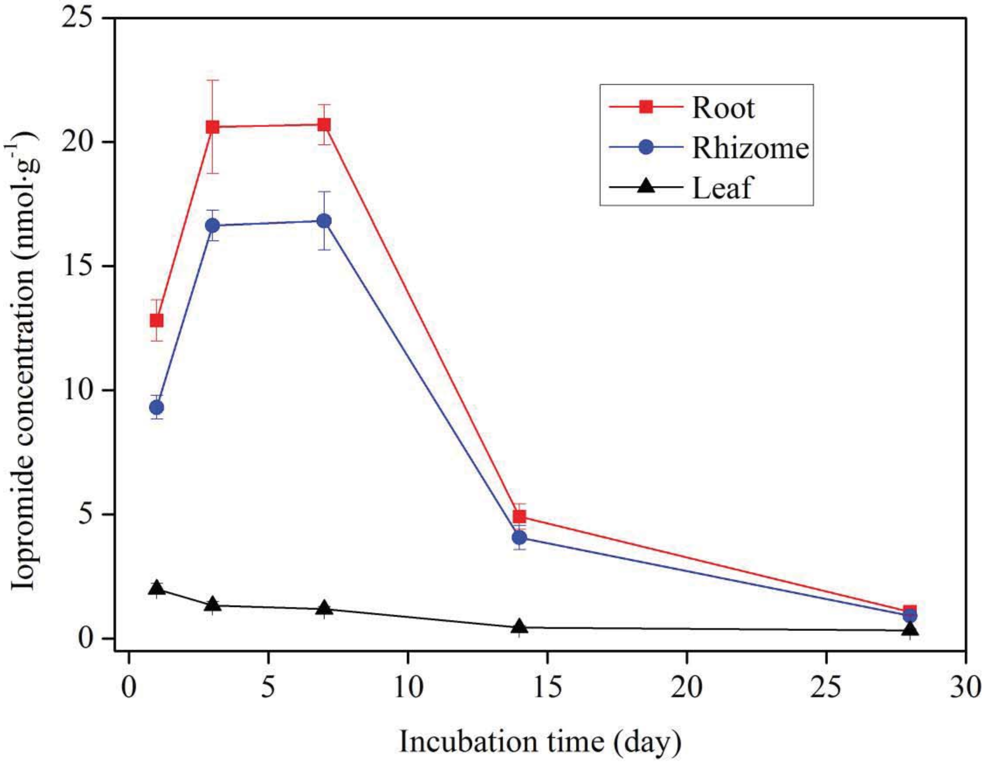
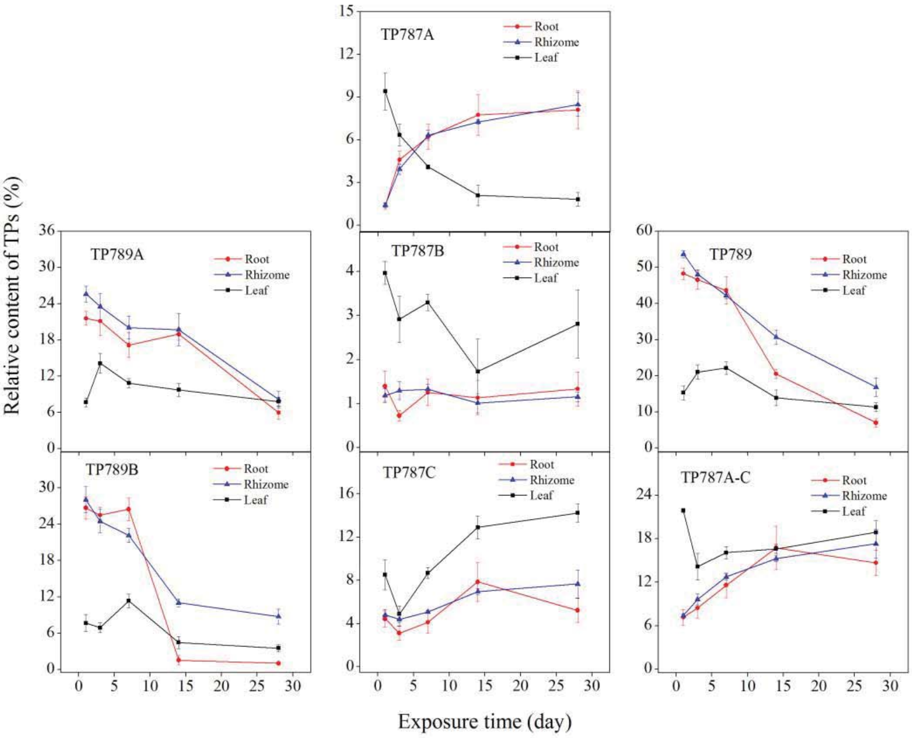

25
20
15
10
5
0
Iopromide concentration (nmol·g⁻¹)
0
5
10
15
20
25
30
Incubation time (day)
Root
Rhizome
Leaf

Figure 4



Relative content of TPs (%)
Exposure time (day)
TP787A
TP789A
TP787B
TP789
TP789B
TP787C
TP787A-C
Root
Rhizome
Leaf
0
3
6
9
12
15
0
6
12
18
24
30
36
0
1
2
3
4
0
10
20
30
40
50
60
0
6
12
18
24
30
0
4
8
12
16
0
6
12
18
24
0
5
10
15
20
25
30

Figure 5

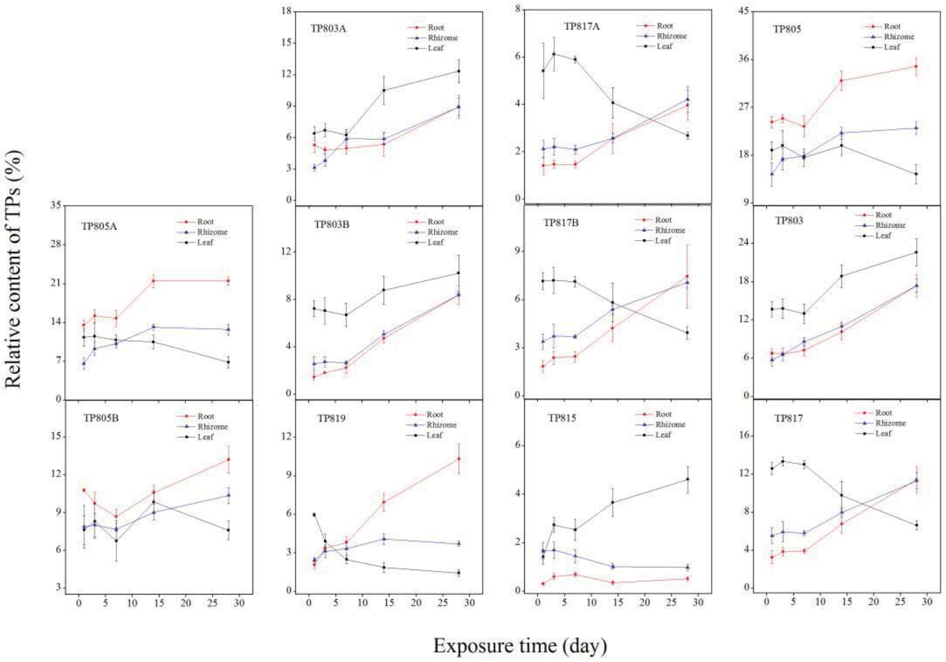
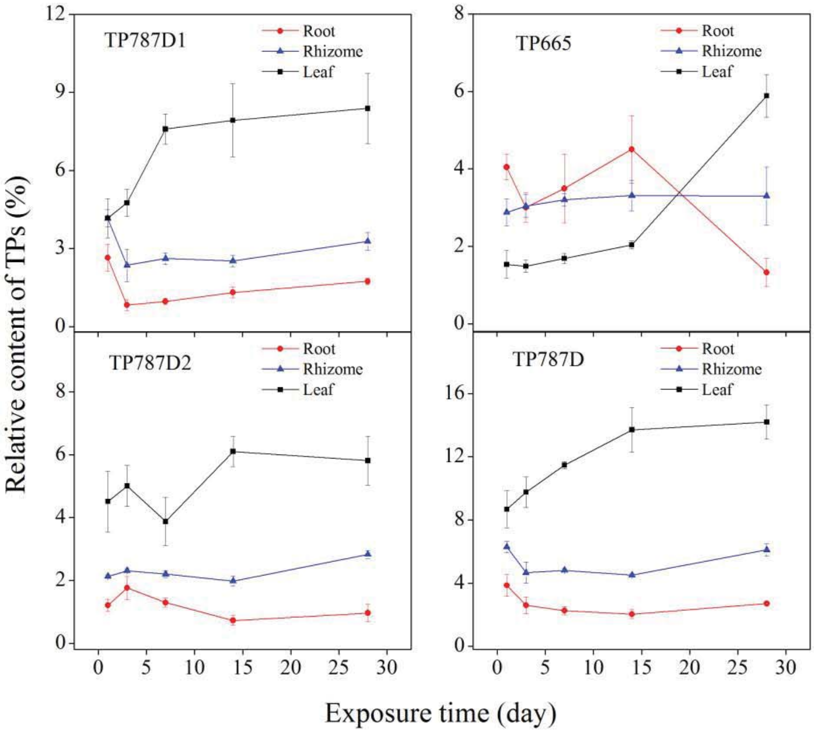

Relative content of TPs (%)
TP803A
TP817A
TP805
TP805A
TP803B
TP817B
TP803
TP805B
TP819
TP815
TP817
Root
Rhizome
Leaf
0 5 10 15 20 25 30
Exposure time (day)

Figure 6


TP787D1
TP665
TP787D2
TP787D
Root
Rhizome
Leaf
Relative content of TPs (%)
Exposure time (day)
0
3
6
9
12
2
4
8
16
0
5
10
15
20
25
30

Supporting Information to the Article:

Contents

1. X-ray analysis

Plants were treated in hydroponic systems with iopromide for 3 days. They were thoroughly washed with tap water to remove any adherent dead material or adsorbed iopromide, and dried with tissue paper before controls and treated plants were cut into pieces of similar size and length. Plant pats were sealed in plastic bags and submitted immediately to X-ray-analysis at identical exposure time. For image analysis and better comparability, contrast and brightness of both, control and exposure photograph were set to equal values. The X-ray analysis was performed using a Faxitron MX-20 DC-12 system (Faxitron Bioptics, LLC, 3440 E Britannia Dr, Suite 150, Tucson, Arizona 85706 USA) under automated setting. Images were taken by the implemented camera system and stored as digital files. Details about the system condition can be found in a previous study.[1]

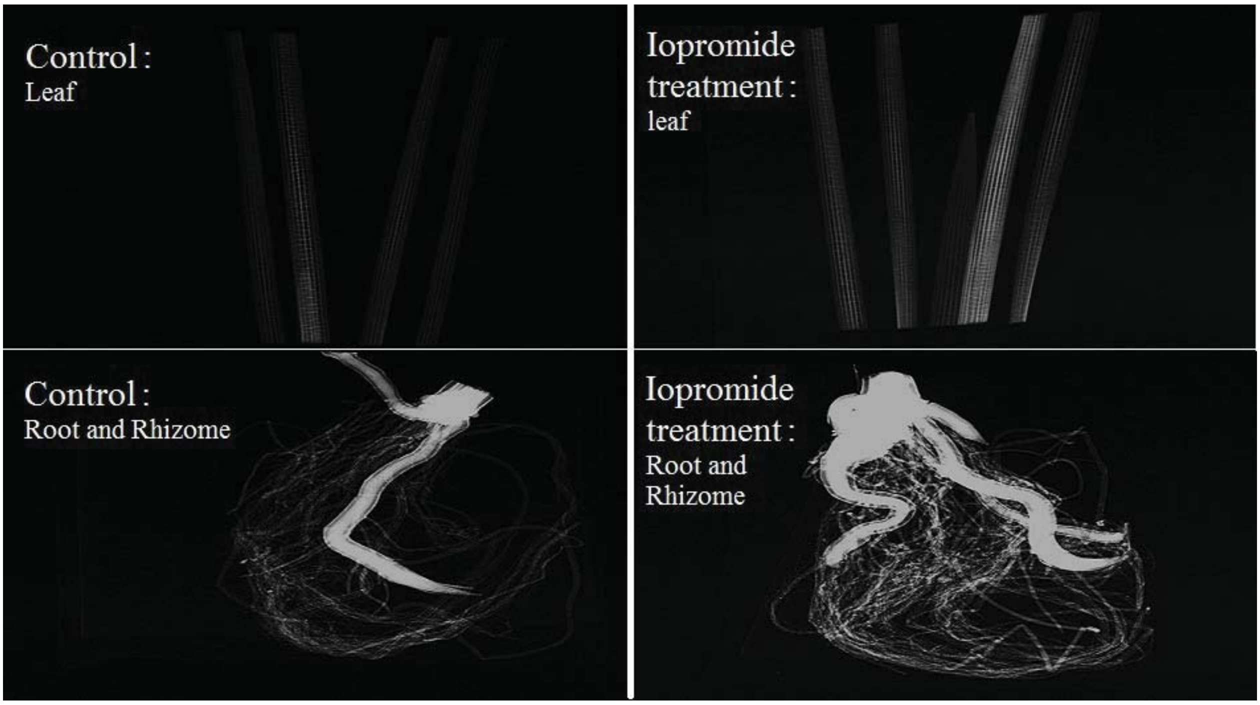


Fig S1. X-ray analysis of control and iopromide treated plants.

Reference

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2. Bioaccumulation factors of iopromide in different plant tissues

Table S1. Bioconcentration factors of iopromide in roots, rhizomes and leaves of exposed *T. latifolia*.

	1 day	3 days	7 days	14 days	28 days
Roots	0.644±0.040	1.039±0.105	1.041±0.033	0.248±0.028	0.054±0.006
Rhizomes	0.468±0.021	0.837±0.023	0.846±0.056	0.205±0.026	0.046±0.006
Leaves	0.101±0.012	0.067±0.008	0.060±0.005	0.022±0.004	0.017±0.002

Appendix B

Curriculum Vitae

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Education Background

1. Sep 2012-Aug 2016, PhD student at Helmholtz Zentrum München
2. Sep 2009-Jul 2012, Master of Agronomy at Graduate University of Chinese Academy of Sciences, Beijing, China.
3. Sep. 2005 - Jul. 2009, Bachelor of Environment Science at China University of Mining and Technology, Jiangsu, China.

Publications

1. Cui, H & Wang, S.P., 2012. Eco-toxicity and molecular biomarkers of antibiotics in soil. Asian Journal of Ecotoxicology 7(2): 113-122.

2. Cui, H & Wang, S.P., 2012. Adsorption characteristics of ciprofloxacin in ustic cambosols. Chinese Journal of Environmental Science 33(8): 333-338.

3. Cui, H., Wang, S.P., Jia, S.G., Zhang, N., Zhou, Z.Q., 2013. Influence of ciprofloxacin on the microbial catabolic diversity in soil. Journal of Environmental Science and Health, Part B, Pesticides, Food Contaminants, and Agricultural Wastes 48(10): 869-877.

4. Cui, H., Wang, S.P., Fu, J., Zhou, Z.Q., Zhang, N., Guo, L., 2014. Influence of ciprofloxacin on microbial community structure and function in soils. Biology and Fertility of Soils 50(6): 939-947.

5. Cui, H., Hense, B.A., Müller, J., Schröder, P., 2015. Short term uptake and transport process for metformin in roots of *Phragmites australis* and *Typha latifolia*. Chemosphere 134: 307-312.

6. Cui, H & Schröder, P., 2016. Uptake, translocation and possible biodegradation of the antidiabetic agent metformin by hydroponically grown *Typha latifolia*. Journal of Hazardous Materials 308: 355-361.

Conference Contributions

1. Poster presentation in international conference on groundwater contamination and water system security, Dec 1-2, 2011, Beijing, China.
2. Poster presentation in 15th EuCheMS international conference on chemistry and the environment, Sep 20-24, 2015, Leipzig, Germany.