Organic Chemistry I/2, NW II, Faculty of Biology, Chemistry and Geology, University of Bayreuth, Germany

Metabolism of metamizol in early stages of the incubated hen's egg

L. KIEP, S. MADERNER and K. SEIFERT

In a previous paper we introduced a model for studying the metabolism of xenobiotics in the incubated hen's egg. This model is characterized by application of the xenobiotic into the yolk sac and identification of metabolites in the allantoic fluid (AF). Depending on the stage of development, the incubated hen's egg can be used both conservatively – in the sense of an animal experiment – and alternatively as a complementary method (appr. first half of incubation period). In the present investigation we used the sodium salt of [(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N*-methyl-amino]methanesulfonic acid (**1**; metamizol, dipyrone®) as model substance. Concentrations of **1** up to 32 mg/ egg did not affect the viability of the incubated eggs. After inoculation of **1** (10 mg/egg) on the sixth day of incubation (DI) eight compounds were identified in the AF on DI 11 (TLC, GC, GC-MS, synthesis, MS, ^{13}C NMR): 4-methylamino-(**2**), 4-amino-(**3**), 4-hydroxy-(**4**), 4-acetylamino-(**5**), 4-*N*-acetyl-N-methylamino-(**6**), 4-*N*-hydroxyacetyl-N-methylamino-(**7**), 4-*N*-hydroxyoxalyl-N-methylamino-(**8**) and 4-*N*-formyl-N-methylamino-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one (-antipyrine; **9**), respectively. Main metabolites are **2** and **5**; **4** and **7** occur as sulfate or glucuronide conjugates. Compound **7** is also eliminated in it's free form. To our knowledge **7** has been detected as a new metabolite of **1** for the first time. The metabolic route of **7** was elucidated after application of **6**. Compounds **8** and **9** are artefacts of **7**. The data presented here demonstrate the usefulness of the developed model also in early stages of the incubated hen's egg. Consequently, the model is suitable as a complementary method for xenobiotic metabolism.

1. Introduction

For metabolism studies of xenobiotics numerous *in vitro* models have been established, whose statement for mechanistic questions is generally accepted [1]. A predictive value of these models for the *in vivo* situation is only existing, when they are included in a test battery. Hereby animal experiments – which are necessary nevertheless – can be reduced to the essential extent.

Starting from the biochemical assumptions of the bird's embryo for functionalization and conjugation of selected model substances we introduced an *ex vivo* model for studying the metabolism of xenobiotics [2]. This simple, inexpensive and robust model which is positioned between *in vitro* and *in vivo* assays has firstly been described by Kiep [3]. The model is characterized by application of the xenobiotic into a feeding compartment (yolk sac) of the incubated hen's egg on DI 6 and by identification of metabolites in the excretion medium (AF) of the embryonic kidneys of the following DI's.

The fundamental suitability of this model in the meantime could be confirmed by investigations of Neugebauer [4] with the substrate (+)-methamphetamine as well as by Clement and Mladek using the model substances 7-ethoxy-coumarin [5] and p-nitrophenol [6]. Depending on the stage of development the incubated hen's egg can be used both conservatively – in the sense of an animal experiment – and alternatively as a complementary method [7]. Up to the end of the first half (DI 11) of the incubation period a missing or limited sensitivity of the embryo has to be considered [8]. This refinement in terms of the three Rs concept of alternatives pioneered by Russel and Burch [9] is also in accordance with the British law of animal rights [10]. In the course of further harmonization of the rules of law in the European Union such a regulation is to be expected in the German animal rights as well.

The following investigations report on the metabolism of **1** in early stages of the incubated hen's egg. The biotransformation of **1** in animal and human [11–14] is similar to that of aminophenazone (4-dimethylaminoantipyrine). After **1**-application compound **2** is formed by spontaneous, nonenzymatic hydrolysis [15]. In case of aminophenazone, **2** is the product of oxidative demethylation [16]. The further metabolic way with both substrates leads to identical main metabolites.

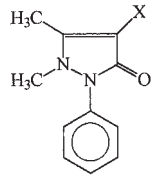
In vitro the *N*-demethylation of aminophenazone to **2** and **3** with hepatic cell cultures [17–20] and liver microsomes [21–27] of chick embryos is well established. The *N*-demethylation of **2** to **3** with liver microsomes from chick embryos of DI 16 was firstly described by Brodie and Maickel [28] forty years ago.

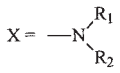
2. Investigations and results

On DI 6 a sterile, aqueos solution (0.1 ml) of the substrate was inoculated into the incubated egg. The biological assay was stopped by freezing the eggs on DI 11. The limitation to this total time of incubation represents the only alteration of the method which has already been published in detail [2]. The AF (6.5–8 ml/egg) was aspirated after thawing the eggs at room temperature. For evaluation of the substrate's dose for the investigations of biotransformation, doses of **1** were applicated following a geometric progression by the factor of two on DI 6. The test was evaluated and terminated on DI 11. Concentrations of **1** up to 32 mg/egg did not affect the viability of the incubated eggs. For metabolism studies of **1** a dose of 10 mg/egg was chosen.

For detection of free metabolites, the AF was adjusted to pH 8–9 and extracted with dichloromethane repeatedly. The aqueos layer was neutralized and for detection of conjugated metabolites hydrolyzed with hydrochloric acid and β-glucuronidase/arylsulfatase, respectively. After hydrolysis the fluid was adjusted to pH 3–4 or pH 5–6, injected onto an Extrelut-column and then eluted again with dichloromethane. The dichloromethane extracts were dried with anh. sodium sulfate and evaporated to dryness *in vacuo*. The residues were taken up in an appropriate solvent. First the present 4 metabolites (**2**, **4**, **5** and **6**) could definitely be detected by TLC analysis with different solvent systems (solv. I–III) in comparison to identically treated control AF from uninjected eggs (Table 1). Compound **4** was found after hydrolysis only.

Table 1: Structure of the compounds identified in the AF after inoculation of 1





X = —OR$_3$

Compd.	R_1	R_2	R_3
1	$-CH_2SO_3Na$	$-CH_3$	
2	−H	$-CH_3$	
3	−H	−H	
4			−H
5	$-COCH_3$	−H	
6	$-COCH_3$	$-CH_3$	
7	$-COCH_2OH$	$-CH_3$	
8	−COCOOH	$-CH_3$	
9	−CHO	$-CH_3$	

The results of TLC were confirmed by the following GC investigations (Table 2) in comparison with authentic reference substances and additionally by GC-MS analysis (Table 3). Regarding the results of enzymatic hydrolysis **4** is present in the AF either as sulfate or as glucuronide. In the AF a further substance could be detected by GC-MS analysis. According to it's MS, the molecular ion peak appeared at m/z 289 and the main fragment peaks were observed at m/z 217, 98, 83 and 56, respectively. These fragment ions are common for derivatives of 4-*N*-methylaminoantipyrine [29, 30] and were also observed for **2**, **6** and **9** (Table 3). The splitting of carbon dioxide from the molecular ion under formation of **9** led to an unambiguous identification of **8**. As **8** is a carboxylic acid which was detected in the alkaline AF extract, the assumption was obvious that this was an artefact due to previous treatment. The native metabolite **7** could be detected (TLC, GC, GC-MS, synthesis, MS, ^{13}C NMR) to our knowledge for the first time. This new metabolite of **1** is eliminated from chick embryo in both the free and the conjugated form (sulfate or glucuronide). Compound **9** is an artefact and arises from **8** by decarboxylation.

As **3** in it's free form could not be found after inoculation of **1** we applicated this substance on DI 6 (5 mg/egg) and analyzed the AF of DI 11 as described above. In it's free form only **5** could be identified. After enzymatic hydrolysis **4** was also found. Consequently, **3** is a 'transit' metabolite which is converted to **5** by acetylation and to **4** by deamination followed by sulfatation or glucuronidation. For elucidation of biogenesis of **7** on DI 6 we applicated compound **6** (2.5 mg/egg), which has been detected as a metabolite after inoculation of **1** and analysis of the AF of DI 11 as described before. In their free form **5** and **7** could be identified. After enzymatic hydrolysis **7** was additionally characterized as sulfate or glucuronide, respectively. Consequently, **6** is biotransformed to **5** by dealkylation of the 4-N-methyl group and by hydroxylation of the 4-N-acetyl group to **7**.

Table 2: Chromatographic data of substances 2–9

Compd.	TLC* R_F value in the solvent system			GC** retention index
	I	II	III	
2	0,70	0,55	0,38	1995
3	0,58	0,48	0,33	1974
4	0,31	0,27	0,51	1858
5	0,48	0,29	0,21	2277
6	0,58	0,36	0,33	2340
7	0,31	0,17	0,26	2529
8	–	–	–	2317
9	0,58	0,40	0,36	2331

* s. 4.6.2.; ** s. 4.6.3.; –: not found by TLC analysis

Table 3: MS main fragmentation pattern of compounds 2–9

Compd.	Structure determining ions in the EI-MS (m/z)
2	217 ($M^{+\cdot}$), 98 ($M^{+\cdot} - C_6H_5NCO$ = a), 83 (a $- \cdot CH_3$ = b), 56 (b − HCN)
3	203 ($M^{+\cdot}$), 84 ($M^{+\cdot} - C_6H_5NCO$ = c), 56 (c $- H_2CN$)
4	204 ($M^{+\cdot}$), 85 ($M^{+\cdot} - C_6H_5NCO$ = d), 56 (d $- \cdot CHO$)
5	245 ($M^{+\cdot}$), 203 ($M^{+\cdot} - COCH_2$ = e), 84 (c), 56
6	259 ($M^{+\cdot}$), 217 ($M^{+\cdot} - COCH_2$ = f), 98 (a), 83 (b), 56
7	275 ($M^{+\cdot}$), 245 ($M^{+\cdot} - CH_2O$ = g), 217 (g − CO = f), 98 (a), 83 (b), 56
8	289 ($M^{+\cdot}$), 245 ($M^{+\cdot} - CO_2$ = g), 217 (f), 98 (a), 83 (b), 56
9	245 ($M^{+\cdot}$), 217 ($M^{+\cdot} - CO$ = f), 98 (a), 83 (b), 56

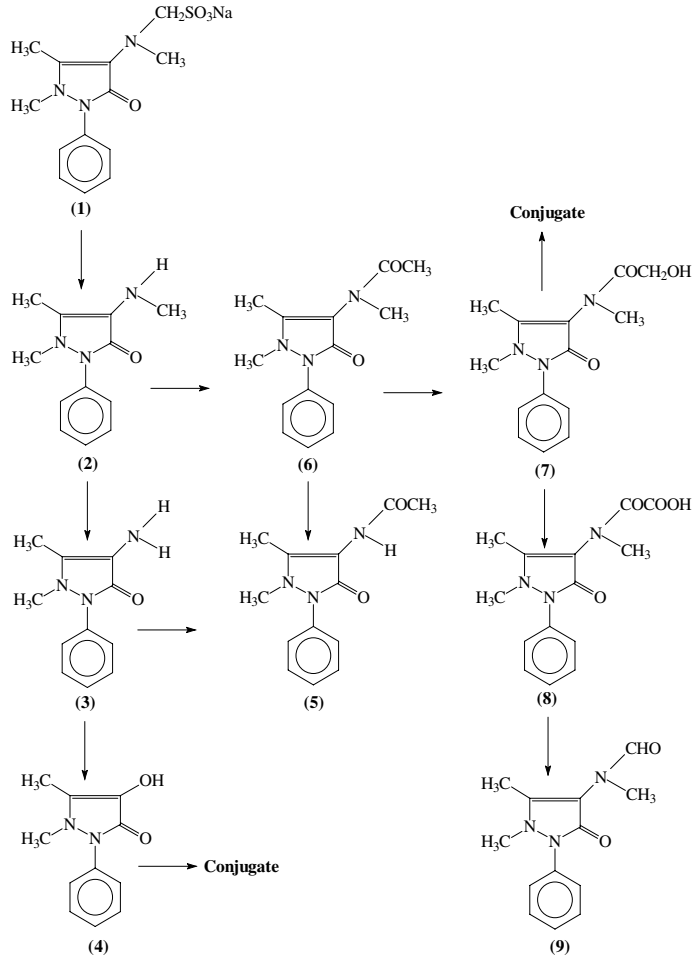
3. Discussion

As in the case of animals and humans [15], compound **1** is not detectable after application into the incubated hen's egg since absorption is preceded by nonenzymatic hydrolysis of **1** to **2**. Also the main metabolic pathway of **1** in the embryonated egg is corresponding to that in animals and humans [11–14] (Scheme). After N-demethylation of **2** to **3** N-acetylation to **5** follows. In contrast to the aforementioned species, however, in the hen's egg no compound **3** in it's free form could be found, which is also the case after inoculation of this substrate. As the ratio of **5** to **3** determines the typ of acetylation [31], the chick embryo has to be categorized as an 'ultra rapid acetylator'. Besides acetylation of **3** to **5** as a sideway of metabolism in the incubated egg additionally an acetylation of **2** to **6** has been proved. Substrates of the arylamine N-acetyltransferases in animal and human usually are primary amines [32]. Acetylation of a secondary amine only rarely occurs. This for instance is the case in aminophenazone metabolism in humans [16, 33] in which **2** is biotransformed into **6**. As a metabolite of **1** up to now **6** has only once been described in rat and humans [14].

Furthermore, in the embryonated egg **6** is partly metabolized to **7**. This transformation, characterized by hydroxylation of an acetamido group, is a rare way of biotransformation and was firstly described by Kiese and Lenk [34] for different derivatives of acetanilide in rabbits. Using the substrates 2-acetamidothiazole and 2-acetamido-4-phenylthiazole this metabolic way could also be confirmed in rat [35]. Interestingly, Volz et al. [14] after application of **1** in human detected compounds **8** and **9** which in the chick embryo were identified as artefacts of **7**. Definitely, **9** is an artefact. Should **8** be a **1**-metabolite in humans [14] it's formation is only possible when **7** is present and therefore the hydroxylation of the 4-N-acetyl group of **6** is a prerequisite.

After application of aminophenazone to rats Goromaru et al. [36] detected 5-hydroxymethyl-4-*N*-acetyl-*N*-methyl-

Scheme



aminoantipyrine, which has a structure that is isomeric to **7**. No indications for hydroxylation of the 5-methyl group could be obtained after inoculation of **6** into the incubated egg. Besides hydroxylation of **6** to **7** as a sideway in the hen's egg additionally an 4-N-demethylation of **6** to **5** has been detected. Compared to the transformation of **2** to **3** the extent of this N-demethylation is only small. For various *N*-formyl-*N*-methylphenylalkylamines within the 9000 g-supernatant of rat liver homogenates Borchert et al. [37] could prove a deminished rate of turnover in the N-demethylation in comparison to the non-N-formylated compounds and explained this by a sterical inhibition, which is to be discussed for transformation of **6** to **5** in the incubated hen's egg as well.

4-Formylaminoantipyrine [12–14] could not definitely be detected in the AF of the embryonated egg. Compound **4**, which is a reliably identified metabolite of **1** [11–14] was also found in the hen's egg in it's conjugated form (sulfate/glucuronide). With liver microsomes of 18 days old chick embryos Rifkind et al. [25] could firstly identify **4** in this species, using phenazone (antipyrine®) as a substrate. Neugebauer [38] found the **4**-sulfate in the AF after application of phenazone into the egg albumen of the incubated hen's egg.

In spite of intensive investigations during the last five decades the **1**-metabolism in animals and humans, however, could not be completely elucidated. In a recent paper [39] oxalylhydrazides are presumed to be potential metabolites of **1**, which arise *in vitro* by opening of the pyrazolinone ring of **2** and **3** under physiological conditions. *In vivo* these compounds could not be found up to now.

Concerning the enzymes of metabolism of the 4-methylamino substituted pyrazolinones there is no complete clarity. Niwa et al. [40, 41] could detect a high rate of turnover in 4-N-demethylation of aminophenazone by the cytochrome P-450 (CYP)-isozymes CYP2C19 and CYP2B6 of human liver, exprimed in *Saccharomyces cerevisiae*. Agúndez et al. [42, 43] on the other hand found significant correlation between 4-N-demethylation of this substrate and the activity of CYP1A2. In investigations with human liver microsomes a 4-N-demethylation of **2** was found, inhibitionable by ketoconazole, which indicates activity of CYP3A4 [44]. Lautenschlager et al. [45] supposed CYP2B6 to be the isozyme responsible for catalysis of **2** to **3**. Participation of the polymorphic arylamine N-acetyltransferase 2 (NAT2) in acetylation of **3** to **5** has been validated [46].

The CYP subfamilies of the CYP isozymes involved in Phase I metabolism of aminophenazone and **1** in animal and human are also present in chick embryo [47–51]. Xenobiotic-metabolizing enzyme activities already appear in early stages of embryogenesis [52–54]. Using substrate **1** during the first half of the incubation period this could be confirmed *in ovo*. Moreover, the ability of the chick embryo for conjugation of foreign substances, as N-acetylation [55], is also prematurely evident. Up to DI 11 besides acetylation of the primary 4-amino group of **3** additional acetylation of the secondary 4-methylamino group of **2**

was found. In this way this rare reaction of biotransformation using **1** as substrate could also be proved for this species.

The results presented here using **1** as a substrate demonstrate the usefulness of the developed model – also in early stages of the incubated hen's egg – for studying the xenobiotic metabolism. The crucial advantage of the model in contrast to the *in vitro* methods on one hand consists of the 'natural' standardization and on the other hand of taking pharmaco- and toxicokinetical aspects into account. After inoculation of the xenobiotic into a feeding compartment of the incubated hen's egg absorption, distribution, metabolism and excretion occur as in a complete organism. The model includes both Phase I and Phase II reactions of metabolism and only the bioavailable amount of the foreign compound is metabolized.

As in any other model not only advantages but also disadvantages have to be discussed. Depending on the phylogenetic distance to other species these disadvantages are especially the differences in the sequence of amino acids of the CYP isozymes, which does not limit the predictive value for the *in vivo* situation by including in a test battery. Neugebauer [38] investigated the metabolism of six drugs in the incubated hen's egg and could detect biotransformation reactions occurring in animals and humans.

The developed model for the investigation of xenobiotic metabolism fits into a line of further models for pharmaco- and ecotoxicological questioning: Chick Embryotoxicity Screening Test (CHEST) [56], Hen's Egg Test/Chorioallantoic Membrane (HET-CAM) [57], Hen's Egg Test for Micronucleus Induction (HET-MN) [58], Frog Embryo Teratogenesis Assay/*Xenopus* (FETAX) [59] and the test on acute toxicity in fertilized eggs of *Danio rerio* (fishegg test) [60]. Common characteristic of these models is the use of early developmental stages of a lower vertebrate with missing or limited sensitivity. These models are highly organized testing systems with complex mechanisms of regulation and coordination.

4. Experimental

4.1. Instruments and materials

MS analyses were measured on a Finnigan MAT 8500 mass spectrometer (Bremen, Germany). Electron impact was used as ionization method (ionization energy 70 eV, ion source temperature 250 °C). ^{13}C NMR spectra were recorded on a Bruker AC 250 (62.5 MHz). The chemical shifts refer to $CDCl_3$ ($\delta = 77.0$ ppm). Melting points are uncorrected and were determined in open capillary tubes on a SDV 500 Plus apparatus (HWS Labortechnik, Mainz, Germany). TLC, GC and GC-MS are described in detail below. Metamizol (**1**) was obtained as a commercial preparation for injection (Berlosin®-inject) from Berlin-Chemie AG, Germany. Berlosin-inject was used as a stock solution (metamizol-sodium monohydrate 500 mg/ml) for metabolism studies. The test solutions were prepared by diluting the stock solution with sterilized water under aseptic conditions. The reference substance 4-methylaminoantipyrine (**2**) was generously donated by Berlin-Chemie AG. 4-Aminoantipyrine (**3**), β-glucuronidase/arylsulfatase and Extrelut® NT3-prepacked glass columns were purchased from Merck, Darmstadt, Germany. 4-Hydroxyantipyrine (**4**) was obtained from Aldrich, Steinheim, Germany. All other materials were obtained from commercial suppliers and used without further purification.

4.2. Synthesis of the reference substances

4.2.1. 4-Acetylaminoantipyrine **(5)**

Synthesis according to [61]; M.r.: 197–198 °C; MS (EI): m/z (%) = 245 $[M]^{+\cdot}$ (38), 229 (9), 203 (31), 137 (18), 119 (3), 91 (3), 84 (45), 77 (9), 56 (100).

4.2.2. 4-N-Acetyl-N-methylaminoantipyrine **(6)**

Synthesis according to [61]; M.r.: 153–154 °C; MS (EI): m/z (%) = 259 $[M]^{+\cdot}$ (38), 217 (37), 123 (14), 119 (2), 98 (11), 91 (2), 83 (18), 77 (5), 56 (100).

4.2.3. 4-N-Formyl-N-methylaminoantipyrine **(9)**

Synthesis according to [62]; M.r.: 106–107 °C; MS (EI): m/z (%) = 245 $[M]^{+\cdot}$ (20), 217 (74), 123 (11), 119 (3), 98 (30), 91 (2), 83 (50), 77 (11), 56 (100).

4.2.4. 4-N-Hydroxyacetyl-N-methylaminoantipyrine **(7)**

To a solution of 217 mg (1 mmol) of **2** in CH_2Cl_2 (10 ml) a solution of 226 mg (2 mmol) of chloroacetyl chloride in CH_2Cl_2 (10 ml) was added dropwise. The reaction mixture was stirred at RT for 4 h. The solvent was distilled off, the residue dissolved in H_2O (5 ml) and the resulting solution was neutralized with $NaHCO_3$. To the solution solid NaCl (1 g) was added and then extracted with CH_2Cl_2 repeatedly. The organic layer was washed with H_2O, dried with Na_2SO_4 and the solvent was removed. The resulting residue (245 mg = 0,84 mmol of 4-*N*-chloroacetyl-*N*-methylaminoantipyrine) was dissolved in MeOH (15 ml) and 821 mg (8.4 mmol) anh. KOAc was added to the solution. The mixture was stirred and refluxed for 24 h, the solvent distilled off and the residue was dissolved in H_2O (10 ml). Solid NaCl (2 g) was added to the solution and then extracted with CH_2Cl_2 repeatedly. The organic layer was washed with H_2O, dried with Na_2SO_4 and after that the solvent was distilled off. The residue was recrystallized from EtOAc. Yield: 190 mg (83%); M.r.: 80–82 °C; MS (EI): m/z (%) = 275 $[M]^{+\cdot}$ (14), 245 (8), 218 (32), 217 (30), 153 (9), 123 (22), 119 (12), 98 (8), 91 (11), 83 (14), 77 (12), 56 (100); $C_{14}H_{17}N_3O_3$: calcd. 275.1269, found 275.1269; ^{13}C NMR ($CDCl_3$): δ (ppm) = 9.5 (5-CH_3), 34.6* (1-NCH_3), 35.1* (4-NCH_3) [*exchangeable], 59.2 (4-NCO$\underline{C}H_2$OH), 110.3 (C-4), 123.6 (C-2′, C-6′), 126.6 (C-4′), 128.4 (C-3′, C-5′), 133.1 (C-1′), 150.6 (C-5), 160.2 (C-3), 172.5 (4-N$\underline{C}$OCH_2OH).

4.3. Hen's eggs for testing

Fertilized 'White Leghorn' eggs of free-range hen's were obtained from a local supplier (Mewes, Brunow, Germany). The weight of the eggs ranged from 55 g to 65 g. The eggs were first candled to discard those were defective. Prior to incubation the eggs were stored at 10 °C and 90% relative humidity for 24 h with the large ends up.

4.4. Biological assay

The eggs were incubated horizontally in a commercial incubator at 37.5 °C (±0.5 °C) and 60% (±5%) relative humidity and turned every 6 h. At DI 6 the eggs were candled and infertile eggs and those containing dead embryos were discarded. By use of a dentist's drill a 1–2 mm diameter hole was made in the shell directly over the air cell, without damaging the subjacent shell membrane. Two hours before injection, the eggs in the incubator were stored in an upright position with the large ends up. Then, the blunt end of each egg was cleaned with ethanol (70%) and 0.1 ml of the test solution was injected vertically into the yolk sac; controls were treated with 0.1 ml of the vehicle (sterilized water) only. After sealing the hole with melted paraffin, the eggs were further incubated in a horizontal position and candled daily for viability. On DI 11 the biological assay was terminated by placing the eggs into a freezer at −18 °C for 30 min. After thawing to RT, the egg shell over the air chamber was prepared off and the inner egg membran was removed. Then, with a syringe the chorioallantoic membrane was penetrated and the AF was aspirated.

4.5. Evaluation of the substrate's dose

Embryonated eggs (DI 6) were divided into five experimental groups and one control group, with five eggs in each. The eggs in the experimental groups were injected with 0.1 ml of the **1**-solutions containing 2 mg, 4 mg, 8 mg, 16 mg and 32 mg, respectively. The control group was inoculated with the vehicle (sterilized water) only. After examination (DI 11) the toxicological test was stopped by freezing the eggs.

4.6. Analysis of the allantoic fluid (AF)

4.6.1. Sample preparation

For extraction of metabolites, the AF of 5 eggs was combined. Five milliliters of the pooled AF were adjusted to pH 8–9 with 1 M $NaHCO_3$ solution and extracted with CH_2Cl_2 repeatedly. The combined organic layers were dried with anh. Na_2SO_4 and evaporated to dryness *in vacuo*. For TLC analysis (4.6.2.) the residue was dissolved in MeOH (100 µl) and for GC (4.6.3.) and GC-MS analysis (4.6.4.) in an appropriate solvent (CH_2Cl_2, Me_2CO, EtOAc), respectively. The aqueos layer was neutralized with 1 M HCl and for the detection of conjugated metabolites, the hydrolysis was performed under the following conditions: (i) 5 ml of the solution containing 0.5 ml HCl (37%) was stirred and heated for 1 h on a boiling water bath. After cooling the solution was adjusted to pH 3–4. (ii) 5 ml of the solution was incubated with β-glucuronidase/arylsulfatase (0.1 ml) at 37.5–38.5 °C for 24 h in acetate buffer (pH 5.5). The hydrolyzed fluids were injected onto Extrelut NT3-columns and eluted with CH_2Cl_2 according to the manufacture's instructions. Then, the eluates were worked up and analyzed as described before. Samples of control AF (5 ml) from uninjected eggs were treated identically.

4.6.2. Thin layer chromatography

TLC analyses were carried out on silica gel 60 F_{254} aluminium sheets (Merck), with the following solvent systems: I: $CHCl_3$/MeOH/Et_2O/NH_3 (25%) 50 : 10 : 12 : 2 [63]; II: BuOAc/Me_2CO/n-BuOH/NH_3 (10%) 25 : 20 : 15 : 5 [64]; III: CH_2Cl_2/Me_2CO/MeOH 35 : 10 : 5. From the sample (4.6.1.) 1–5 µl were applied and the chromatogram developed over a path of 15 cm in a paperlined chamber, previously left to equilibrate for at least 30 min at RT. The spots were visualized by UV absorption at 254 nm.

4.6.3. Gas chromatography

GC was performed using a Thermo Quest CE gas chromatograph (Rodano, Italy) with a DB-5 fused silica capillary column (J & W Scientific), 30 m × 0.32 mm internal diameter and 0.1 µm film thickness. Two microliters of the sample (4.6.1.) and 0.2 µl of a solution of hydrocarbons (C_9H_{20} to $C_{26}H_{54}$ in hexane) were injected in the splitless mode. The temperature of the injector and the detector (FID) were 270 °C and 290 °C, respectively. The carrier gas was H_2 with a flow rate of 2 ml/min; split ratio 1 : 10. Temperature program: 80 °C for 3 min, 3 °C/min to 280 °C, 280 °C for 15 min. Retention indices were calculated according to [65].

4.6.4. Gas chromatography – mass spectrometry

A gas chromatograph Hewlett Packard model 5890 series II (Böblingen, Germany) combined with a Finnigan MAT 95 doublefocussing sector field mass spectrometer (Bremen, Germany) was used. GC separation was performed on a DB-5 fused silica capillary column (J & W Scientific), 30 m × 0.32 mm internal diameter and 0.25 µm film thickness. Two microliters of the sample (4.6.1.) and 0.2 µl of a solution of hydrocarbons (C_9H_{20} to $C_{26}H_{54}$ in hexane) were injected in the splitless mode at an injector temperature of 280 °C. Carrier gas was H_2 with a flow rate of 2 ml/min; split ratio 1 : 10. The interface was temperated at 290 °C. MS were recorded in the electron impact ionization mode at 70 eV and an ion source temperature of 250 °C. Temperature program (compounds **2**–**7**, **9**): 50 °C to 300 °C, heat rate 3 °C/min, 300 °C for 10 min. Temperature program (compound **8**): 50 °C to 80 °C, heat rate 3 °C/min, 80 °C to 300 °C, heat rate 10 °C/min, 300 °C for 4 min.

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Dr. L. Kiep
Ringstr. 4
D-19372 Brunow/Mecklenburg