A Study of the Metabolic Degradation of an Insect Juvenile Hormone Analog Using Different Radiolabeling

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The fate of an insect juvenile hormone analog applied to the insect body of the flesh fly (*Sarcophaga bullata*) or the tsetse fly (*Glossina palpalis*), respectively, was studied using three different radiolabeled positions in the parent biologically active compound **1**. Several metabolites were found and analyzed. A mechanism of degradation of the applied molecule was designed using a combination of several radioanalytical methods. A unique monitoring of the applied compound and its metabolites was provided, based on the different radiolabeling of the structure (*cf.* **1a**–**1c**), and established their fate in an insect body during a 10-day experiment. A ^{14}C and ^{3}H radiolabeling, respectively, was employed to synthesize three different radiolabeled forms **1a**–**1c** derived from the parent non-labeled **1**. A combination of three different ways of radiolabeling resulted in an advantage in tracing the metabolic pathway of degradation of the employed compound **1** in its radiolabeled forms **1a**–**1c**.

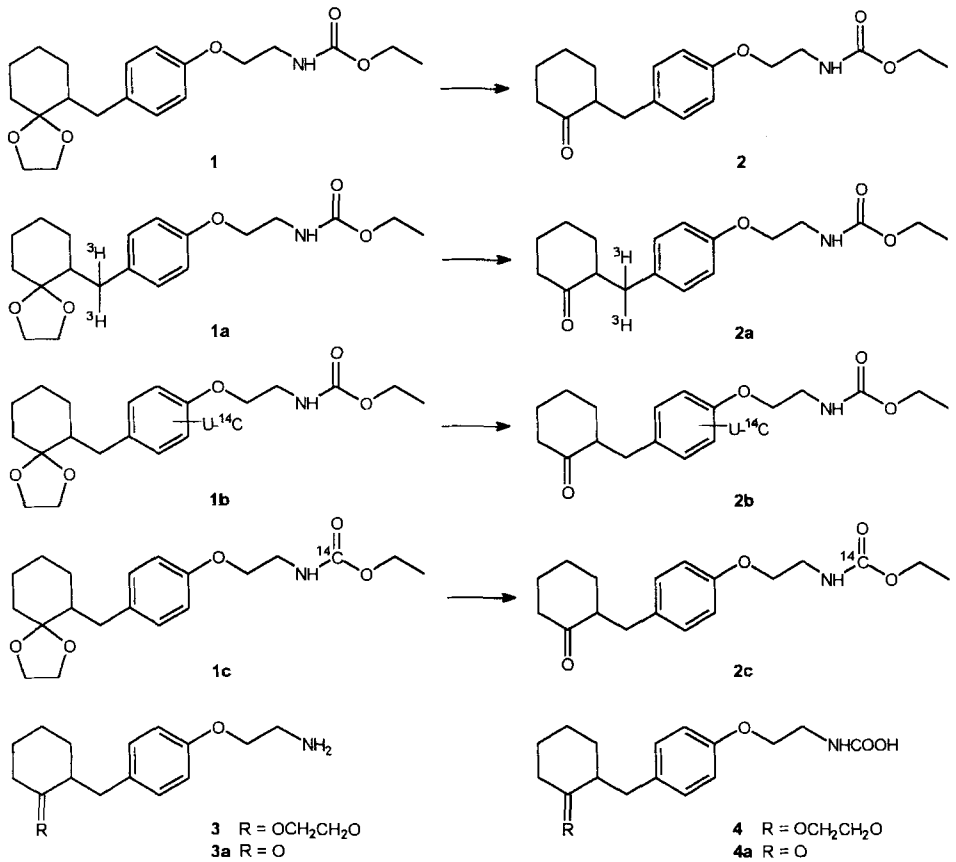
Introduction. – An intensive investigation of insect juvenile hormone analogs has started since mid-1960s. Since mid-1970s, a commercial development of insect juvenile hormone analogs has taken place, and has contributed by its approach to designing many structures displaying remarkable biological-activity values on different insect pests [1]. A series of insect juvenile hormone analogs [2], structurally derived from 2-(4-hydroxybenzyl)cycloalkanones, has been developed since 1975. A long-term investigation of these compounds has resulted in an augmentation of our knowledge on the structure-activity relationships between the structures of the compounds and their effects on several non-related insect species. Several observations in the area of the structure-activity relationships have been applied in the structure design [2]. Nevertheless, a basis of the mode of action of the insect juvenile hormone analogs has not yet been fully understood.

To understand the mode of action of insect hormones, a photoaffinity labeling of juvenile-hormone-binding proteins was developed by *Prestwich et al.* [3], who designed this effective approach to study biochemical processes in insects. In this approach, a ^{3}H labeling of a structure of either insect juvenile hormone or its analog was accompanied by the presence of a group which can be photoactivated in the same molecule. An irreversible binding of this radioisotopically labeled structure to a juvenile-hormone-binding protein was enabled through an irreversible transformation of this group in the presence of selected functionalities present in the active site of the juvenile-hormone-

binding protein. A complex occurring after photoaffinity attachment to juvenile-hormone-binding protein could be easily traced and isolated due to the presence of the ^3H labeling.

Radiolabeling of biologically active compounds for a study of their fate in an insect body has been generally considered as a highly sensitive and effective technique. *Tykva* and *Bennettová* [4] developed a quantitative analytical method consisting in a determination of the radioactivity microdistribution within an insect body sectioned previously at $-20°$ in a cryostat. A computer-aided arrangement with a special semiconductor detector was used enabling a quantitative analysis of the penetration, distribution, and excretion of an applied compound and/or its metabolites.

Scheme 1. *Metabolic Pathway of Biologically Active Insect Juvenile Hormones*



The target insect-juvenile-hormone analog was selected on the basis of a detailed screening, of more than 20 non-labeled compounds and a wide variety of insect species. Finally, two fly species were selected for this investigation, the flesh fly (*Sarcophaga bullata*) and the tsetse fly (*Glossina palpalis*). The selected juvenile-hormone analog was labeled by either ^{14}C or ^3H in three different positions (*cf.* **1a**–**1c**; *Scheme 1*) to detect as many as possible radiolabeled metabolites.

The aim of this research was divided into the following partial goals:

i) Monitoring of a radioactivity distribution after topical application to the insects was studied using a microscale quantitative analysis of radioactivity within the whole insect body as well as in selected body parts. This arrangement enabled a fully automatic quantitative evaluation of a radioactivity microdistribution aided by using a software developed for this purpose. A total radioactivity of selected body parts was measured with a liquid scintillation spectrometer using isolated insect organs.

ii) A quantitative analysis of the extracts of selected parts of the insect body, and those of the insect excreta was accomplished using a reverse-phase radio-HPLC (*i.e.*, a HPLC system equipped with a combination of a UV/VIS detector and a radioactivity detector) combined with two simple chemical reactions used for the chemical correlation of the structures.

iii) A GC/MS analysis of selected extracts or that of their separated fractions contributed to the identification of as many as possible detected metabolites, which could not be identified using direct or indirect approach during the HPLC analysis.

Results and Discussion. – Application of a radiolabeled insect juvenile hormone analog topically to the insect body, and subsequent monitoring of the distribution of the original radiolabeled compound and its radiolabeled metabolites, contributed to a study of the radioactivity pathway within the insect organism. Three different radiolabeled forms [5–7] **1a**–**1c** (*Scheme 1*) of the biologically active compound **1** have been designed to represent a complex approach for studying the degradation of the molecule of the selected insect juvenile hormone analog **1**. Radiolabeling of **1** both by ^{3}H in the CH_2 junction unit between the two rings (→ **1a**) and by ^{14}C at the carbamate function (→ **1c**) assisted in tracing the early stages of the degradative procedure. Radiolabeling by ^{14}C at the central aromatic ring of the molecule (→ **1b**) was designed as a long-term persistency center of radioactivity in the insect body, which assisted in monitoring the way of a radiolabeled compound from the surface of the insect cuticule throughout the insect body, to be finally detected in the insect excreta. Evaluation of the experimental data showed the fate of the molecule in the insect body, and located the distribution of radiolabeled compounds within the insect body during certain period of the life of the tested fly.

To eliminate the possibility of an abiotic degradation of the applied juvenile hormone analog within the insect body, the pH values of its isolated parts were measured. No value was found to decrease below pH 6.93, and, therefore, the compounds identified by a radio-HPLC analysis represent the metabolites of the parent structure.

Figs. 1 and *2* show component ratio or a fraction ratio of the radioisotopically labeled components of the body extracts or those of the extracts of the excreta of *Glossina palpalis* and *Sarcophaga bullata*, respectively. It should be pointed out that *Fig. 1*, summarizing an analysis of insect body extracts, shows a ratio of the appropriate radioisotopically labeled components found in the insect organism at the moment of the extraction procedure. On the contrary, an analysis of insect excreta is displayed in *Fig. 2*, representing the average ratio of the radioisotopically labeled components, which were excreted by the insect species studied from the beginning of the experiment up to its end, including the *in vitro* formed metabolites, *i.e.*, until the moment of the extraction of the above identified excreta. This note is important for evaluation of the obtained results.

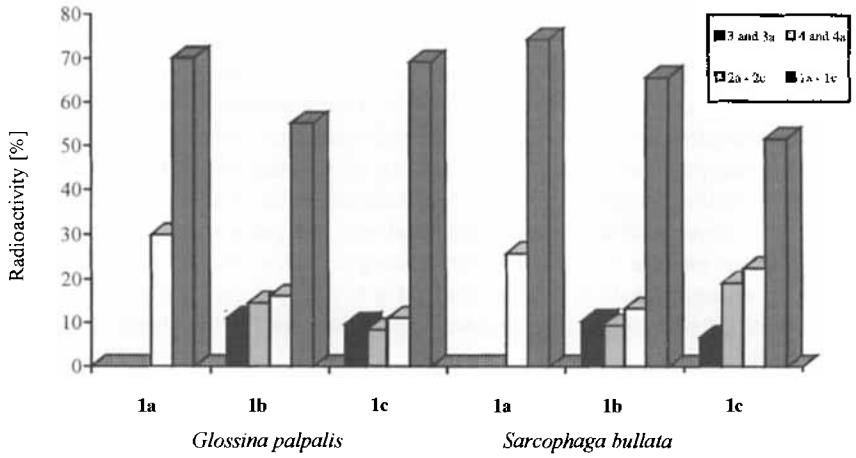


Fig. 1. *Radioactivity distribution in the insect-body extracts.* The values given in percents are based on a radio-HPLC analysis, and they determine relative ratios of radiolabeled compounds **1**–**4** found in the analyzed insect body extracts.

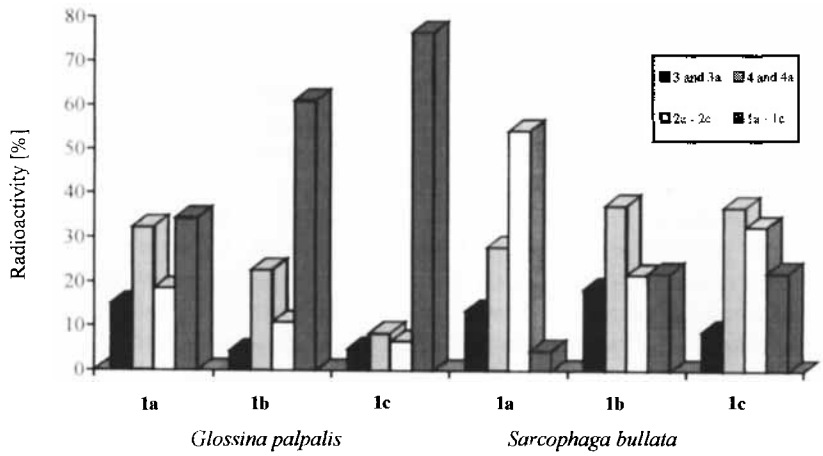


Fig. 2. *Radioactivity distribution in the insect excreta.* The values given in percents are based on a radio-HPLC analysis, and they determine relative ratios of radiolabeled compounds **1**–**4** found in the analyzed extracts of insect excreta.

1. *Radioanalysis.* The total radioactivity of an isolated part of an insect body was measured after its dissolving in a scintillation cocktail using a liquid scintillation spectrometer. The measured samples were lost during application of this relatively tedious technique, which, moreover, disabled to determine the radioactivity distribution within the analyzed sample. For these reasons, whole-body sections or preparations of selected organs were analyzed as well, using a PC-aided scanning under special semiconductor detectors [8]. This arrangement enabled a fully automatic, quantitative evaluation of the radioactivity distribution, including a sample outline by the applied PC software [9].

2. *A Comparison of the Insect Metabolic Systems.* Comparing both, the analysis of the extract of insect bodies and that of the insect excreta, a considerable difference was observed between the respective ratios of the radiolabeled metabolites produced by *Glossina* species and those by *Sarcophaga* species. The metabolic system of the *Sarcophaga* species works more quickly and more destructively of the two tested ones. It is not a topic of this paper to study a biochemical reasoning for this finding. Attention will be given to this phenomenon in the future.

3. *Analysis of the Extracts of the Insect Bodies.* Metabolism of the structure used, **1a**–**1c**, for treatment of insect individuals tested follows the same way with both tested insect species, *i.e.*, *Glossina palpalis* and *Sarcophaga bullata*. It is important to define which potential metabolites of the respective structures used, **1a**–**1c**, will keep a radioisotopic labeling (because only those may be traced during the radioanalysis), and which of them will lose it. None of the metabolic steps discussed below was found to proceed in the quantitative way.

Liberating the metabolites **2a**–**2c** (*Scheme 1*) from the corresponding structures applied, **1a**–**1c**, was the first step in the metabolic pathway (*Scheme 1*). Regardless of the location of the radioisotopic labeling, the compounds **2a**–**2c** will keep the radioisotopic labeling after passing the above described degradative step. Compounds **2a**–**2c** were found during a radio-HPLC analysis of the extracts of the insect bodies with both insect species studied. Their retention times were compared with that of **2**, which was used as a reference compound.

An enzyme-mediated transformation of the methylene junction unit located between the two rings was the second metabolic step in the pathway studied. The potential metabolites of the ^{3}H-labeled compounds, **1a** and **2a**, will lose radioisotopic labeling completely. This means that the metabolites capable of proving this degradative step cannot be traced more on the basis of the ^{3}H-labeling. Evaluation of the experimental observation proved that no radioisotopically labeled fractions were found in both polar and medium-polar areas of the reverse phase radio-HPLC chromatograms, *i.e.*, none of the ^{3}H-labeled compounds **3**, **3a**, **4**, or **4a** (*Scheme 1*) were found. This finding indicates that the methylene junction unit had been attacked before the carbamate function was treated.

The third step in the metabolic pathway was an enzyme-mediated treatment at the carbamate function. This process resulted in two series of products: either producing a terminal primary amine function (loss of the EtOCO group), resulting in **3** and/or **3a**, or leading to a terminal COOH function (an ester-bond fission), resulting in **4** and/or **4a**. When **1c** was used in the experiments, a part of the metabolites kept a radioisotopic labeling (**4** and **4a**), while another part (the NH_2-terminated ones) lost it due to the loss of the radioisotopically labeled ^{14}C of the carbonyl subunit (**3** and **3a**). However, when **1b** was used in the experiments, all the metabolites resulting from this step kept radioisotopic labeling. The metabolites **4** and **4a** occurred within the medium-polar area of the reverse-phase radio-HPLC chromatograms, while **3** and **3a** occurred within the polar area. The amine **3** was synthesized recently in its non-labeled form [10][11], and was proved to be an extremely polar compound. A higher content of all radioisotopically labeled components **3**, **3a**, **4**, and **4a** was observed in the experiments with **1b**, when compared with the results of the analysis obtained with **1c**. Metabolites of **1a** were no more radiolabeled in this step.

4. *Analysis of the Excreta.* All compounds applied or occurring during the metabolic process, both in their radioisotopically labeled or non-labeled forms, were stepwise excreted by insect, respecting the rules of excretion. Detecting radioisotopically labeled components both in the polar and the mid-polar areas of the reverse phase radio-HPLC chromatograms (**3**, **3a**, **4**, and **4a**) during the analysis of the excreta in such experiments, after application of **1a**, led to the assumption that the metabolic pathway continues in the excreta *in vitro*.

During the *in vivo* metabolic process the structures studied, **1a**–**1c**, were metabolized by a stepwise degradation discussed above. The *in vitro* metabolic mechanism of degradation of either structure studied proceeded in a different way. At this point, it is necessary to remember that all compounds and metabolites are stepwise excreted by the insect individuals.

Starting with the compounds applied to the insect species, **1a**–**1c**, and with the *in vivo* occurring metabolites **2a**–**2c**, **3**, **3a**, **4**, and **4a**, all compounds were subjects of a further metabolic degradation *in vitro*. Evaluation of the *in vitro* metabolism of **1a**–**1c** and **2a**–**2c** resulted in a finding that the initial step of the *in vitro* metabolic process consists in a transformation of the carbamate moiety. The metabolic products displayed a medium or a high polarity, and kept their radioisotopic labeling in most cases, regardless of the labeling position. Further degradation of the molecules **1a**–**1c**, following the mechanism of degradation at the CH_2 junction unit between the two rings, resulted in the non-labeled metabolites, when **1a** was applied on both insect species studied. A certain number of metabolites occurred in either the polar or the medium-polar areas (**3**, **3a**, **4**, and **4a**) of the reverse-phase HPLC chromatograms in their non-labeled forms (UV detection) after application of **1c** due to a loss of the ^{14}C-labeled ethoxycarbonyl subunit. Such metabolites (non-labeled **3**, **3a**, **4**, and **4a**) are no more traceable by a radioactivity detection, and, therefore, a relatively lower quantity of the radioisotopically labeled compounds **3**, **3a**, **4**, and **4a**, resulting from **1c**, is explained in a relation to the results obtained with **1b**. Because the metabolic process is continuous, a continuous metabolic degradation of the components of the polar fractions could result in other metabolites, including non-labeled compounds. These metabolites were studied using a GC/MS analysis (see *Sect. 6*).

5. *Auxiliary Experiments.* To establish the structure of **3**, **3a**, **4**, and **4a** (a comparison with the synthetic reference compound is only possible with **3**), the approach already used with non-labeled compounds [12], was applied. If **3**, **3a**, and/or **4**, **4a** are present in the extracts, their reaction with the selected reagents results in the known compounds. The experiments were studied by a reverse-phase radio-HPLC analysis using a [U-^{14}C] labeled (**1b**) sample extract:

i) The amines **3** and **3a** react with ClCOOEt yielding the appropriate carbamate compounds **1b** and **2b** (*Scheme 2*).

ii) The amines **3** and **3a** may also react with CH_2N_2 affording corresponding secondary and/or tertiary amines (*Scheme 2*).

iii) The carbamic acids **4** and **4a**, which only occur in the crude extracts but cannot be isolated therefrom, give a reaction with CH_2N_2 yielding methyl esters **1d** or **2d**, the analogs of the appropriate carbamate compounds **1a**–**1c** and **2a**–**2c** (*Scheme 2*).

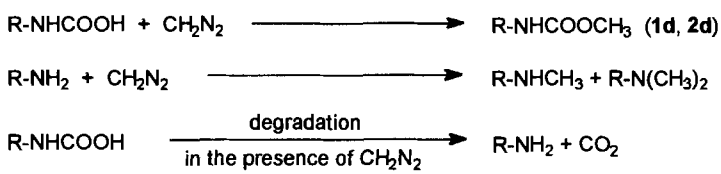
iv) A side process was observed, involving CH_2N_2 treatment of the NHCOOH moiety of **4** and **4a** yielding the amines **3** and **3a**. An increase in concentration of **3** and

3a was observed after the treatment of a sample extract in the presence of an excess of CH_2N_2. It might be caused by expected instability of **4** and **4a** (a free carbamic acid does not exist, even if its esters are stable compounds). Based on the observations described above, the occurrence of **4** and **4a** was established in the extracts by a subsequent reaction with CH_2N_2 affording **1d** and/or **2d** (*Scheme 2*). The result is in accordance with the earlier investigation [12].

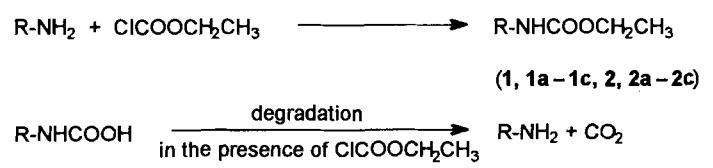
v) Based on the structure and instability of **4** and **4a**, it is assumed that alkylation/esterification of **4** and **4a** in the presence of ClCOOEt (*Scheme 2*) affords **1b** and/or **2b**. The instable acids **4** and **4a** are subjects of a degradation resulting in **3** and **3a**, which react immediately with ClCOOEt.

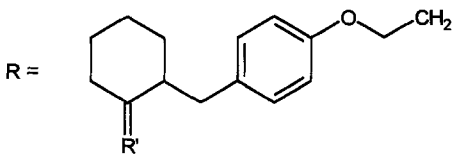
Scheme 2. *Chemical Treatment of Radiolabeled Samples, and the Structures of the Primary Amine, the Alkylcarbamic Acid, and the Compounds* **1d** *and* **2d**

A Radiolabeled Sample of an Extract + CH_2N_2:



A Radiolabeled Sample of an Extract + $ClCOOCH_2CH_3$:





1 and **1a–1d**, R' = OCH_2CH_2O
2 and **2a–2d**, R' = O

The above described results lead to the following conclusions:

1) *CH_2N_2 as the Derivatizing Reagent.* First, it should be noticed that the chromatographic behavior of both **1d** and **2d** does not differ from that of **1a–1c** and **2a–2c**, respectively, under the reverse-phase HPLC conditions used (*cf. Experimental*). An increase of radioactivity was detected by a radio-HPLC analysis, when the metabolites

1b and **2b** were used. An increase of the content of a more polar compound was also observed, indicating the presence of the amines **3** and **3a**. A chemical degradation of the instable acids **4** and **4a** was expected. Calculation of the radioactivity difference proved that the increase of radioactivity in one part of the chromatogram is equivalent to a decrease of radioactivity of other peaks (*cf. Table*).

2) *ClCOOEt as the Derivatizing Reagent.* Using a radio-HPLC detection, no more radiolabeled compounds were found in the polar and in the medium-polar fraction. The primary amines **3** and **3a** were quantitatively transformed into the structures **1b** and **2b**. A degradation of **4** and **4a** was again expected, producing **3** and **3a**, which were immediately transformed into **1b** and **2b** (*cf. Table*).

Table. *Radioactivity Distribution and Quantitative Evaluation of the Experiments Using a Radio-HPLC Analysis*[a])

Sample	**4** and **4a**	**3** and **3a**	**2b** or **2d**	**1b** or **1d**	Total	Total [%]
Original	30,787	9,341	32,536	37,190	109,854	100
Sample + CH_2N_2[b])	0	38,102	36,057	38,222	112,381	102
Sample + ethyl chloroformate[b])	0	0	54,948	58,478	113,426	103

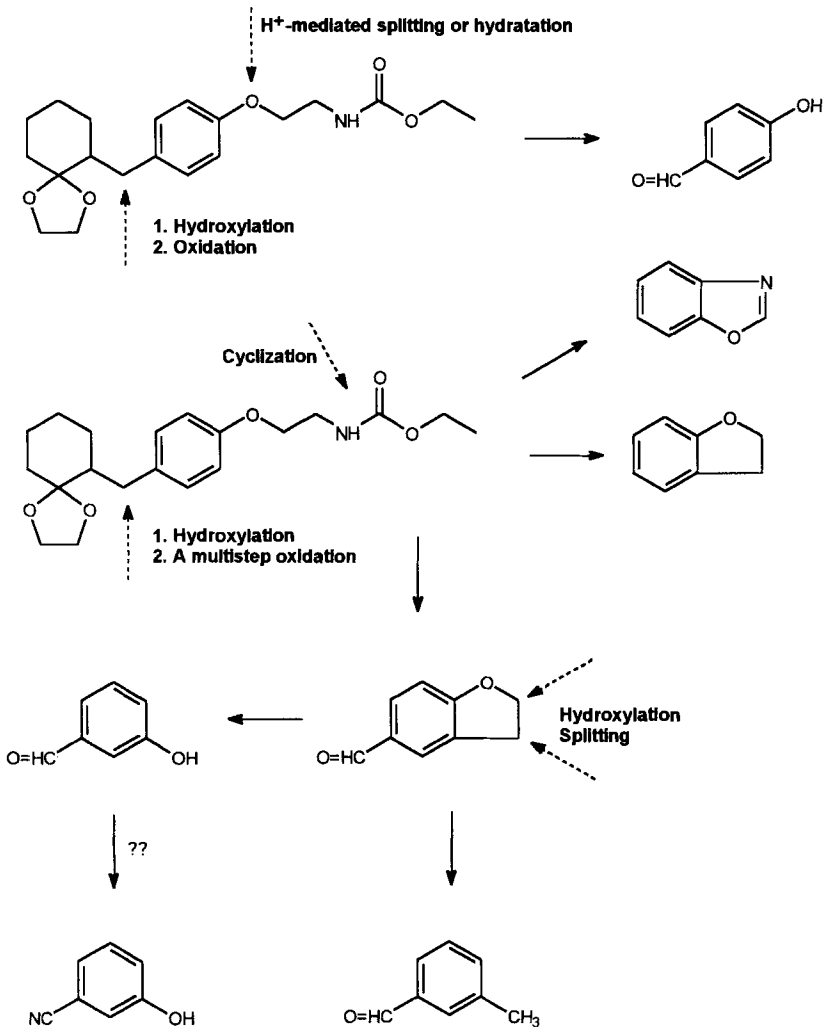
[a]) The values correspond to the area of the appropriate peaks taken from the radio-HPLC analysis. [b]) Reaction of a sample with the indicated reagent was run before injecting into the HPLC system. The compounds **1d** or **2d** occur in a reaction of **4** or **4a** with CH_2N_2; however, their behavior under the reverse-phase HPLC analysis is identical with that of **1b** or **2b**.

The values given for the individual compounds (**1b**, **1d**, **2b**, **2d**, **3**, **3a**, **4**, and **4a**) in the *Table* represent the appropriate radiochromatographic peak areas calculated by a PC software. The 'Total' value means a sum of the area values corresponding to the sample. The total peak area of the original sample was taken to be equal to 100%. The total values given in the *Table* are in no connection to the total radioactivity of 370 kBq for **1a** or 185 kBq for **1b** or **1c**, which were applied in other experiments described (*i.e.*, to the flies). The auxiliary experiments were carried out with a randomly selected extract resulting from an experiment, in which radioactive material was previously applied to the flies as described. In the auxiliary experiments, an extract sample was diluted to a 1-ml scale volume, and separated into three identical partial volumes. A chromatographic sample (usually 5 µl) was analyzed by the radio-HPLC, and the results are summarized in the first row of the *Table*. A reaction of the second and the third partial volume (*cf.* above) of the original sample with CH_2N_2 or ClCOOEt, respectively, was realized, and the analytical samples (adequate volumes, reflecting the increase of the volume of the respective reaction mixture) were subjected to a radio-HPLC analysis. The results are summarized in the second and the third rows of the *Table*. Comparing the total radioactivity measured in these three samples, a very small variation in the total radioactivity detected was measured; a 3% range (*i.e.*, 100–103% of total; *cf. Table*) seems to be within the experimental error. This finding indicates that no radioactivity was lost during the auxiliary experiments, establishing the occurrence of the structures **1b**, **1d**, **2b**, **2d**, **3**, **3a**, **4**, and **4a** (*i.e.*, the compounds mentioned in the *Table*).

6. *Further Metabolites.* After establishing the structures of the metabolites **2a**–**2c**, **3**, **3a**, **4**, and **4a** by comparing them either with synthetic reference compounds (**2** and **3**) or

by applying simple chemical correlation reactions (**3**, **3a**, **4**, and **4a**), attention was focused at further metabolites, which were expected to be small molecules with retention times within the introductory 5 min of the reverse-phase radio-HPLC analysis. Two most intensive peaks in this part of chromatogram were separated and subjected to a GC/MS analysis, which showed that each of these two fractions is a mixture of several compounds. The mass spectra recorded were compared with the reference-data library. Only the structures were considered by which 'Purity' and 'Fit' values, defined by the processing method of the instrument, were higher than 80%. In *Scheme 3*, suggested ways of splitting of the molecule given by the formula **1** are shown.

Scheme 3. *The Structure of Potential Metabolites Suggested on the Basis of a GC/MS Analysis*



7. *Radiolysis.* To test the stability of **1a**–**1c** during the experimental work, we found that the methanolic solutions of the ^{14}C-labeled samples of **1a**–**1c**, if stored at room temperature for several months, are subject to partial radiolysis. The radiolytic products, *i.e.*, the compounds **2a**–**2c**, occurred in quantities not exceeding 1.2% of the mixtures **1a**/**2a**–**1c**/**2c**. If methanolic solutions of **1a**–**1c** were stored at −18°, the purity of the radiolabeled compounds **1a**–**1c** did not change for more than 1 year. In turn, a non-labeled sample of the compound **1** was exposed to an intensive γ-irradiation for 3 weeks. After that time, two similar peaks (*i.e.*, **1** and **2**) were found together with several minor degradation products (some of those shown in *Scheme 3*) at the same retention-time intervals in the reverse-phase HPLC analysis.

8. *Radioactivity Distribution within an Insect Body.* A detection of a radioactivity microdistribution after application of the radiolabeled forms **1a**–**1c** of the target compound **1** in both fly species tested resulted in relatively equal organ values by both detection methods. The former method consisted in isolation of the respective organ, in which the total radioactivity was measured subsequently. The latter method consisted in preparation of a whole-body section, which included the studied organ, and the radioactivity microdistribution was measured using this body section. The radioactivity microdistribution in the organs was evaluated by a PC system. The radioactivities of the metabolites (**1a**–**1c**, **2a**–**2c**, **3**, **3a**, **4**, and **4a**) in selected insect body parts (*i.e.*, in ovaries, head, or gut) were different in time after application of the labeled compound **1**. This indicates that the metabolic steps described in *Sect. 3* proceeded in various parts of the insect body differently. A detailed study is in progress, and will be published later.

A combination of the radioactivity data obtained with the degradation scheme proposed in this paper (*Schemes 1–3*) could be a decisive contribution to the investigation of a mode of action of the target juvenile hormone analog. The described methodology can be applied to other compounds as well. Moreover, the degradation studies could be useful for assessment of environmental safety [13].

Experimental. – *Synthesis of Radiolabeled Compounds.* The synthesis of **1a** has already been published in [5] using a CESG (Catalyzed Exchange in Solution with Gas) method. The total synthesis of **1b** was also published in [6] starting from 4-hydroxy[ring-U-^{14}C]benzoic acid. The synthesis of **1c** has not been published yet [7].

Entomological Testing. The females of *Sarcophaga bullata* and *Glossina palpalis*, less than 24 h old, were used for testing. An acetone soln. (5 µl) of a radiolabeled compound (**1a**–**1c**) was applied on the upper part of thorax, representing either 370 kBq of **1a**, or 185 kBq of **1b** or **1c**.

Radioactivity Distribution in the Insect Organism. A *Beckman LS 6500* liquid scintillation spectrometer was used to monitor a radioactivity distribution in the insect body. A *BTS 450* scintillation cocktail was used to dissolve isolated parts of an insect body. To measure radioactivity within a whole-body section by microscanning, a PC-controlled semiconductor topographic equipment [9] was applied.

A Reverse-Phase Radio-HPLC of the Extracts. The radio-chromatographic system consisted of a *Waters 600* HPLC instrument equipped with a *Waters* solvent delivery system, a *Waters 490E* UV detector, a *Beckman 171* radioactivity detector, and a PC for data management. Analyses were performed using a *Merck* stainless steel anal. column (i.d. 150 × 4 mm) packed with a 5-µm *LiChrosphere 100 RP-18*. Chromatograms were run using a gradient program (*A*, MeOH; *B*, H_2O) at a flow rate 0.8 ml · min^{-1}. The following gradient profile was set: 0–10 min, *A*/*B* 30:70, an isocratic profile; 10–20 min, *A*/*B* 30:70 to 80:20, a linear gradient; 20–40 min, *A*/*B* 80:20, an isocratic profile [14]. For the radio-HPLC, MeOH/$CHCl_3$ 1:1 was used to extract whole bodies of the tested insect species (5 ml per individual) after repeated washing on the day 8 after application of the radiolabeled juvenile hormone analogs **1a**–**1c**.

GC/MS Analysis. A splitless technique (sensitivity of *ca.* 1 ng) was used during a cap. GC/MS measurements, employing a system consisting of a *Varian 3400* GC instrument coupled with a *Finnigan ITD 800* MS detector, and a *DB-5* column (i.d. 30 mm × 25 mm). Injection chamber temp. was set to 200°, He stream to 30 ml · s^{-1}. Temp.

program after injection: 200° were kept for 2 min, then a gradient up to 250° was applied with a speed 5°/min, and then 250° were kept for 10 min. A *NIST/EPA/NIH* mass spectral database (over 38,000 entries) was used as reference database.

Conclusions. – *1*) The mechanism of metabolic degradation of the applied compounds, **1a**–**1c**, has been studied using a reverse-phase radio-HPLC and a GC/MS analyses.

2) The metabolites **2a**–**4a** and the starting compounds **1a**–**1c**, *ca.* 70% of radioactive material detected by the radio-HPLC analysis, were traced by two simple reactions for correlation analysis. Reference compounds were also used, especially to identify **1**–**3** (*Scheme 2*). A calculation of the radioactivity distribution after the chemical reactions confirmed the expected changes in a radioactivity distribution. The sum of the radioactivity resulting from counting the traced compounds **1a**–**4a** changed only slightly (*cf.* column 'Total' [%] in the *Table*). The values are given in the *Table* as the areas of the appropriate peaks in the radio-HPLC, and expressed in percent scale in the last column (100% radioactivity is a sum of the radioactivity of the sample selected for the correlation analyses). The area values are correlated for identical sample volumes.

3) Several further potential metabolites (the molecules with mol. wt. ≤ 130) were found using a combination of separation of two fractions by a reverse-phase HPLC analysis with subsequent GC/MS analysis. Those compounds, including suggested metabolic ways of their production in insect body, are summarized in *Scheme 3*.

4) A long-term exposure (several months) of a methanolic solution of **1b** or **1c** to a room temperature results in a self-radiolytic degradation of the structures **1b** and **1c** to mixtures **1b**/**2b** or **2c**/**2c**, respectively, together with several other metabolites (some of those shown in *Scheme 3*). The same result was obtained by a 3-week γ-irradiation of non-labeled **1**.

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