In vitro production of adaline and coccinelline, two defensive alkaloids from ladybird beetles (Coleoptera: Coccinellidae)

Pascal Laurent [a], Jean-Claude Braekman [a,*], Désiré Daloze [a,1], Jacques M. Pasteels [b]

[a] *Laboratory of Bio-Organic Chemistry, Department of Organic Chemistry, Free University of Brussels, CP 160/07, Av. F. D. Roosevelt, 50–1050 Brussels, Belgium*
[b] *Laboratory of Cellular and Animal Biology, Free University of Brussels, CP 160/12, Av. F. D. Roosevelt, 50–1050 Brussels, Belgium*

Abstract

In vitro experiments using [1–^{14}C] and [2–^{14}C]acetate were devised to study the biosynthesis of the defensive coccinellid alkaloids adaline and coccinelline in *Adalia 2-punctata* and *Coccinella 7-punctata*, respectively. The labelled alkaloids obtained in these experiments had a specific activity about ten times higher than that of the samples obtained in feeding experiments. This in vitro assay has enabled us to demonstrate that these two alkaloids are most likely biosynthesised through a fatty acid rather than a polyketide pathway, that glutamine is the preferred source of the nitrogen atom and that alkaloid biosynthesis takes place in the insect fat body. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

When attacked, coccinellid beetles emit droplets of hemolymph from the tibio–femoral joints of their legs (reflex bleeding). It has been demonstrated that the deterrency exhibited by many species of coccinellids towards potential predators results from the presence of alkaloids in that fluid. Over 50 alkaloids have been characterised from ladybirds up to now, including acyclic amines, piperidines, pyrrolidines, perhydroazaphenalenes, "dimeric" alkaloids, azamacrolides and homotropanes (Daloze et al., 1995; King and Meinwald, 1996). See Fig. 1.

Although many biological and synthetic studies of these alkaloids have been performed, the biosynthetic pathways through which they are formed have been the subject of only a handful of investigations. In *Coccinella 7-punctata*, the perhydroazaphenalene alkaloid coccinelline (**1**) was shown to be labelled after the beetles had been fed with [1−^{14}C] and [2−^{14}C]acetate. Results of

degradation experiments on the labelled coccinelline were in agreement with a polyacetate origin for that compound (Tursch et al., 1975). After feeding the same labelled precursors to *Adalia 2-punctata*, radioactive adaline (**2**) was obtained. Chemical degradation of this sample led also to the conclusion that adaline must derive from the condensation of seven acetate units (Laurent et al., 2001). However, it is not known yet if this alkaloid is biosynthesised through a fatty acid (Route A, Fig. 2) or a polyketide (Route B, Fig. 2) pathway.

On the other hand, incorporation experiments with ^{2}H-labelled oleic acid and ^{2}H- and ^{13}C,^{15}N-labelled L-serine have shown that epilachnene (**3**), a member of the azamacrolide group of alkaloids produced by the pupa of the Mexican bean beetle *Epilachna varivestis*, is biosynthesised from oleic acid and L-serine (Attygalle et al., 1994, 1999).

The feeding technique used in previous works is time-consuming and leads to very low specific incorporation rates (SIR) when little advanced precursors such as [1−^{14}C] and [2−^{14}C]acetate are employed (about 0.01% at best). Thus, to gain a better understanding of the biosynthetic pathways along which defensive ladybird alkaloids are formed, we needed a more efficient and
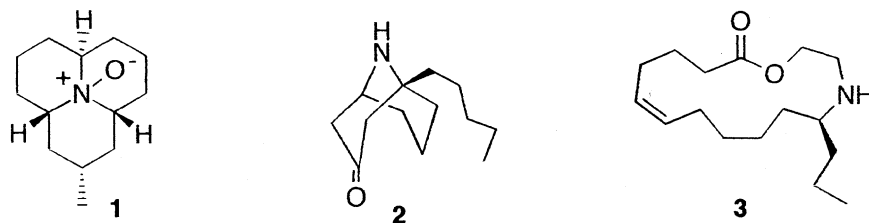
Fig. 1. Structures of coccinelline (**1**), adaline (**2**), and epilachnene (**3**).
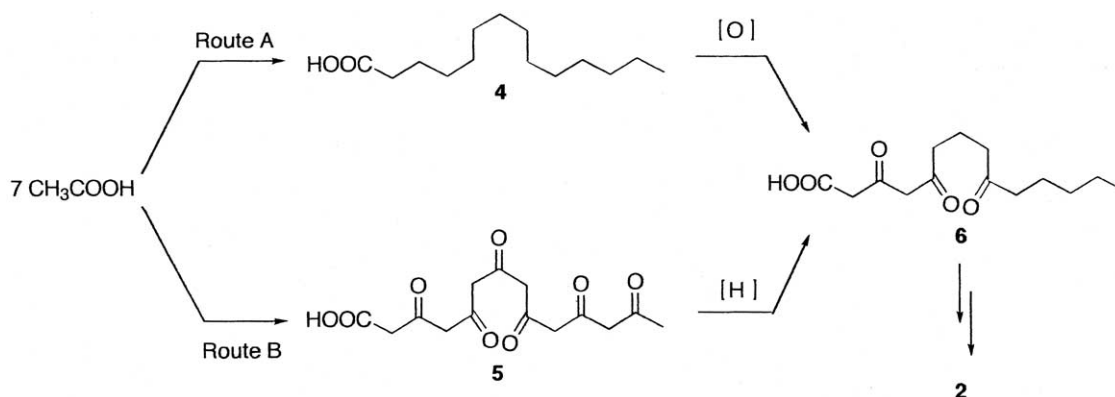

Fig. 2. Hypothetical biosynthetic pathways for the formation of adaline (**2**).

practical incorporation technique. We report here that this has now been achieved by an in vitro incubation assay using ladybird tissues. With this technique in hand, we have been able to answer several important questions pertaining to the mechanism of the biosynthesis of coccinelline (**1**) and adaline (**2**). In particular, we have conducted experiments: (i) to distinguish between a fatty acid and a polyketide origin for these two alkaloids; (ii) to determine the source of their nitrogen atom; and (iii) to find the anatomical localisation of alkaloid biosynthesis in these beetles.

2. Materials and methods

2.1. General chemical techniques

The ^{13}C NMR spectra were recorded at 75.4 MHz with a Bruker Avance TM 300 instrument. Thin layer chromatography analyses (TLC) were performed with 0.25 mm Polygram silica gel SILG/UV$_{254}$ precoated plates (Macherey–Nagel, (Düres, Germany)). Column chromatographies were performed on silica gel (MN Kieselgel 60, 0.04–0.063 mm) using the flash technique. Radioactive compounds were assayed in a Packard Tri-Carb 1600 TR liquid scintillation analyser. The samples were dissolved in methanol (10 ml), 100 μl of the resulting solution were pipetted and added to Packard Insta-Gel® Plus liquid scintillation cocktail (10 ml). Triplicate samples of each compound were counted under comparable conditions of quenching.

2.2. Chemicals

All chemicals were obtained from Sigma–Aldrich (Bochem, Belgium) and used without further purification, except for $[1-^{14}C]$acetate (250 μCi, 57 mCi/mmol) and $[2-^{14}C]$acetate (250 μCi, 55 mCi/mmol), which were purchased from Amersham (UK).

2.3. Insects

Adults of *A. 2-punctata* were purchased from Horpi Systems (Verlaine, Belgium). Adults of *C. 7-punctata* were field collected in Belgium.

2.3.1. Experimental set up for in vitro incubation assays

Twenty beetles (*A. 2-punctata* or *C. 7-punctata*) were cut in four pieces with a scalpel and then immersed in 1.5 ml of a saline solution (154 mM NaCl, 2.7 mM KCl, 2.7 mM CaCl$_2$, 0.9 mM NaHCO$_3$ and 83 μM NaH$_2$PO$_4$ in bidistilled water) containing phenylmethylsulfonic fluoride (PMSF, 10 mM) and NADPH (1.7 mM). The solution was maintained at 0°C during the time needed for tissue immersion. Then, $[1-^{14}C]$ or $[2-^{14}C]$acetate (81 μCi) was added together with glutamine (50 mM). The solution was kept at room temperature and air was bubbled at a rate of 33 ml/min. After 18h, the aqueous phase was basified with NH$_4$OH, and extracted five times with 10 ml portions of CH$_2$Cl$_2$. The ladybird tissues were also washed three times with 2 ml portions

Table 1
Results of the biosynthesis of adaline with $[1-^{14}C]$- and $[2-^{14}C]$acetate as precursors in A. *2-punctata* tissues, demonstrating the influence of time (entries 1 and 2), of precursor (entries 2 and 3), of nitrogen source (entries 4–8), of the absence of oxygen (entry 9) and of the presence of 2-octynoic acid (entry 10) on the specific activity (SA) and on the specific incorporation rate (SIR)

Entry	Conditions	Total activity (10^{-5} mCi)	Amount of adaline (mg)	SA (mCi/mmol)	SIR (%)
1	6h, $[1-^{14}C]$acetate, Gln, O_2	2.14	2.02	2.2×10^{-3}	3.9×10^{-3}
2	18h, $[1-^{14}C]$acetate, Gln, O_2	4.45	1.48	6.3×10^{-3}	1.1×10^{-2}
3	18h, $[2-^{14}C]$acetate, Gln, O_2	70.50	2.20	6.7×10^{-2}	1.2×10^{-1}
4	18h, $[2-^{14}C]$acetate, without added nitrogen source, O_2	1.28	1.67	1.6×10^{-3}	2.9×10^{-3}
5	18h, $[2-^{14}C]$acetate, Ala, O_2	3.39	2.04	3.5×10^{-3}	6.2×10^{-3}
6	18h, $[2-^{14}C]$acetate, Glu, O_2	0.27	2.16	2.6×10^{-4}	4.6×10^{-4}
7	18h, $[2-^{14}C]$acetate, Lys, O_2	6.27	2.61	5.0×10^{-3}	9.0×10^{-3}
8	18h, $[2-^{14}C]$acetate, NH_4Cl, O_2	0.31	2.05	3.2×10^{-4}	5.6×10^{-4}
9	18h, $[2-^{14}C]$acetate, Gln, N_2	0.075	1.37	1.1×10^{-4}	2.1×10^{-4}
10	18h, $[2-^{14}C]$acetate, Gln, O_2, 2-octynoic acid	0.30	1.71	3.7×10^{-4}	6.6×10^{-4}

of CH_2Cl_2 which were filtered on cotton wool. The combined organic layers were filtered on a WA filter paper, evaporated in vacuo, and the alkaloid purified by successive flash chromatographies on a silica gel column using AcOEt then AcOEt–MeOH–NH_4OH (95:5:1), and CH_2Cl_2 then CH_2Cl_2–MeOH–NH_4OH (95:5:1) as eluent. The purifications were carried out until the radioactivity was constant. The amount of alkaloid isolated in the different experiments (always 20 beetle equivalents) varied from 1.5 to 2.6 mg for adaline (Table 1). For coccinelline this amount was 6.4 mg (Table 2).

2.4. Sources of the nitrogen atom of the alkaloids

Besides the standard experiments with Gln (50 mM) (Table 1, entries 2 and 3), experiments were also carried out under the same conditions but with Ala, Glu, Lys or NH_4Cl as nitrogen source (all at about 50 mM) (Table 1, entries 5, 6, 7 and 8, respectively), or without added nitrogen source (Table 1, entry 4).

2.5. Fatty acid versus polyketide pathway

In one experiment (Table 1, entry 9) standard conditions were used, except that N_2 was bubbled through the solution during the incubation period at a rate of 33 ml/min. In another experiment (Table 1, entry 10), standard conditions were used, except for the addition of 2-octynoic acid (0.90 mM).

2.6. Anatomical localisation of alkaloid biosynthesis

Nineteen adults of *C. 7-punctata* were carefully dissected under a microscope in order to separate as far as possible the fat body from the other tissues. Then, the sample with the pooled fat bodies and the sample with the remaining of the tissues were separately incubated in the usual manner in the presence of $[2-^{14}C]$acetate (81 μCi). The experiment with the fat body yielded 0.23 mg of coccinelline (Table 2, entry 2), whereas that with the other tissues (Table 2, entry 3) yielded 1.08 mg of this alkaloid .

3. Results

3.1. In vitro incubation assay

In vitro production of the pheromone intermediates ipsdienone and ipsenone was recently demonstrated in

Table 2
Results of the biosynthesis of coccinelline with $[2-^{14}C]$acetate as precursor in whole *C. 7-punctata* tissues (entry 1), in *C. 7-punctata* fat body (entry 2), and in *C. 7-punctata* tissues from which the fat body had been removed (entry 3)

Entry	Conditions	Total activity (10^{-5} mCi)	Amount of coccinelline (mg)	SA (mCi/mmol)	SIR (%)
1	18h, $[2-^{14}C]$acetate, Gln, O_2, whole beetles	174.0	6.36	5.7×10^{-2}	1.0×10^{-1}
2	18h, $[2-^{14}C]$acetate, Gln, O_2, fat body	12.9	0.23	1.2×10^{-1}	2.1×10^{-1}
3	18h, $[2-^{14}C]$acetate, Gln, O_2, tissues without fat body	3.29	1.08	6.4×10^{-3}	1.1×10^{-2}

bark beetles, using homogenised or longitudinally cut insects (Ivarsson et al., 1997). These results prompted us to examine whether this method could be adapted to the production of defensive ladybird alkaloids. Thus, 20 adults of the two-spotted ladybird, *Adalia 2-punctata*, were cut in four pieces with a scalpel and immersed in 1.5 ml of the saline solution described by Ivarsson et al. (1997). This solution also contained phenylmethylsulfonic fluoride (PMSF, 10 mM), a protease inhibitor, NADPH (1.7 mM) and $[1-^{14}C]$ or $[2-^{14}C]$acetate (81 μCi). To boost alkaloid biosynthesis, an external source of nitrogen was added. For this purpose, we chose Gln (about 50 mM), as this compound is a well-known biochemical nitrogen donor (Luckner, 1985). Air was then bubbled through the solution at a rate of 33 ml/min and the mixture was incubated for either 6 (Table 1, entry 1) or 18h (Table 1, entries 2 and 3), at room temperature. Then adaline was isolated and repeatedly purified to constant specific activity (SA). The results shown in Table 1 demonstrate that the adaline samples from these experiments were indeed labelled, the highest specific incorporation rate (SIR 0.12%) being measured in the $[2-^{14}C]$acetate experiment after 18h (entry 3). All subsequent experiments were thus conducted for a period of 18h.

The relatively high SIR measured in these in vitro experiments with $[2-^{14}C]$acetate prompted us to test whether $[2-^{13}C]$- and $[1,2-^{13}C_2]$acetate could be suitable precursors for adaline. Unfortunately, the 75.4 MHz ^{13}C NMR spectra of the two samples of adaline isolated from these experiments did not differ significantly from that of a blank, thus precluding the use of this technique for further study of the biosynthesis of this alkaloid by NMR.

3.2. Source of the nitrogen atom of the alkaloids

Having established that the enzymes required for the biosynthesis of adaline were functional under our experimental conditions, we tested the effectiveness of several amino acids (Gln, Glu, Ala, Lys) and of NH_4^+ (all at about 50 mM) as external sources of nitrogen (Table 1, entries 3 and 5–8). These experiments were compared to a control blank carried out without added source of nitrogen (Table 1, entry 4). The results of these incorporation experiments, again using $[2-^{14}C]$acetate as the radioactive precursor clearly show that Gln is the most efficient nitrogen donor of all compounds tested (Table 1, entry 3). The adaline sample from this experiment had a SA 42 times higher than that from the blank experiment. The results of the experiments with other external nitrogen sources are notably inferior to that with Gln (from 13 times for Lys to 260 times for Glu).

3.3. Fatty acid versus polyketide pathway

Having selected what appeared to be the best conditions for the in vitro production of adaline (**2**) (incubation for 18h, Gln as source of nitrogen), we carried out some experiments to distinguish between a fatty acid and a polyketide pathway for the biosynthesis of (**2**). To this aim, we compared the results of incubation experiments in the presence and in the absence of oxygen (Table 1, entries 3 and 9). In the latter case, the SIR measured dropped by a factor of 600. In another experiment, carried out in the presence of oxygen, we added 2-octynoic acid (0.90 mM), a well-known inhibitor of fatty acid biosynthesis (Robinson et al., 1963) (Table 1, entry 10). In this experiment too, the SIR measured for the adaline sample was much lower (200 times) than that of the control (Table 1, entry 3).

3.4. Anatomical localization of alkaloid biosynthesis

Finally, we investigated the anatomical localisation of alkaloid biosynthesis in coccinellids. Since this required dissection of the beetles, we turned to *C. 7-punctata* which is a larger species than *A. 2-punctata*. We first checked that the in vitro conditions devised for *Adalia* could be applied to tissues from whole adults of *C. 7-punctata*. Indeed, the sample of coccinelline (**1**) thus obtained had a SA (5.7×10^{-2} mCi/mmol) and a SIR (0.10%) (Table 2, entry 1), similar to those found for adaline (**2**). Next, 19 adults of *C. 7-punctata* were carefully dissected and the excised fat bodies suspended in the standard incubation medium for 18h (Table 2, entry 2). A parallel incubation experiment was also carried out with the remaining ladybird tissues from which the fat body had been removed (Table 2, entry 3). As shown in Table 2, a comparison of the SIR of these two experiments (0.21 and 0.011%, respectively) and of the values measured with whole ladybird tissues (0.10%) demonstrates that the biosynthesis of coccinelline takes place in the fat body.

4. Discussion

The present work demonstrates that whole ladybird tissues are able to produce defensive alkaloids in vitro. The SA and SIR measured for coccinelline (**1**) (5.7×10^{-2} mCi/mmol; 0.10%) and for adaline (**2**) (6.7×10^{-2} mCi/mmol; 0.12%), using $[2-^{14}C]$acetate as radioactive precursor, were about ten times higher than those measured in feeding experiments (Tursch et al., 1975; Laurent et al., 2001). The values measured with $[2-^{14}C]$ were also about ten times higher than with $[1-^{14}C]$acetate, a phenomenon which has been repeatedly observed in plant and in insect alkaloid biosynthesis (Leete and Olson, 1972; Renson et al., 1994). Unfortunately, our

attempts to employ [2−^{13}C]acetate and [1,2−^{13}C$_2$]acetate as precursors for adaline were unsuccessful. Further work is needed to determine if it will prove possible to increase the SA of adaline or coccinelline in our in vitro assay so that the use of ^{13}C labelled precursors would become practical.

The discovery that, of all the compounds tested, Gln is by far the best nitrogen donor in the biosynthesis of adaline does not come as a surprise. The central role of this amino acid in nitrogen metabolism in plants, microorganisms and animals is well known (Luckner, 1984). The poor results of the experiments with Ala, Glu and Lys appear to rule out the intervention of a transaminase, whereas the result of the NH$_4^+$ experiment rules out a glutaminase which would hydrolyse Gln with the liberation of NH$_4^+$. Accordingly, we propose that the nitrogen atom of adaline originates from Gln under the action of a glutamine amidotransferase (Massière and Badet-Denisot, 1998). The low, but significant, SIR measured in the experiments carried out without external source of nitrogen or with Lys, Ala, Glu, and NH$_4^+$ may be attributed to the small amount of endogenous Gln present in insect tissues.

The subsequent experiments were devised to distinguish between a fatty acid or a polyketide pathway for the biosynthesis of adaline (**2**). As discussed in the Section 1 (Fig. 2, Route A), the production of the putative biosynthetic keto acid intermediate **6** *en route* to adaline through a fatty acid pathway requires several oxydative steps (**4→6**). On the other hand, the production of this intermediate through a polyketide pathway (Fig. 2, Route B), requires one or several reducing steps (**5→6**). Since both the monooxygenases and dioxygenases capable of hydroxylating a CH$_2$ group require molecular oxygen (Luckner, 1984), it was anticipated that, if the fatty acid route is operating, the SA of adaline from the experiments conducted in the absence of oxygen should be much lower than in the reference experiment. This was indeed found to be the case by a factor of 600. This conclusion was reinforced by the result of another experiment carried out under oxygen but in the presence of 2-octynoic acid, a fatty acid biosynthesis
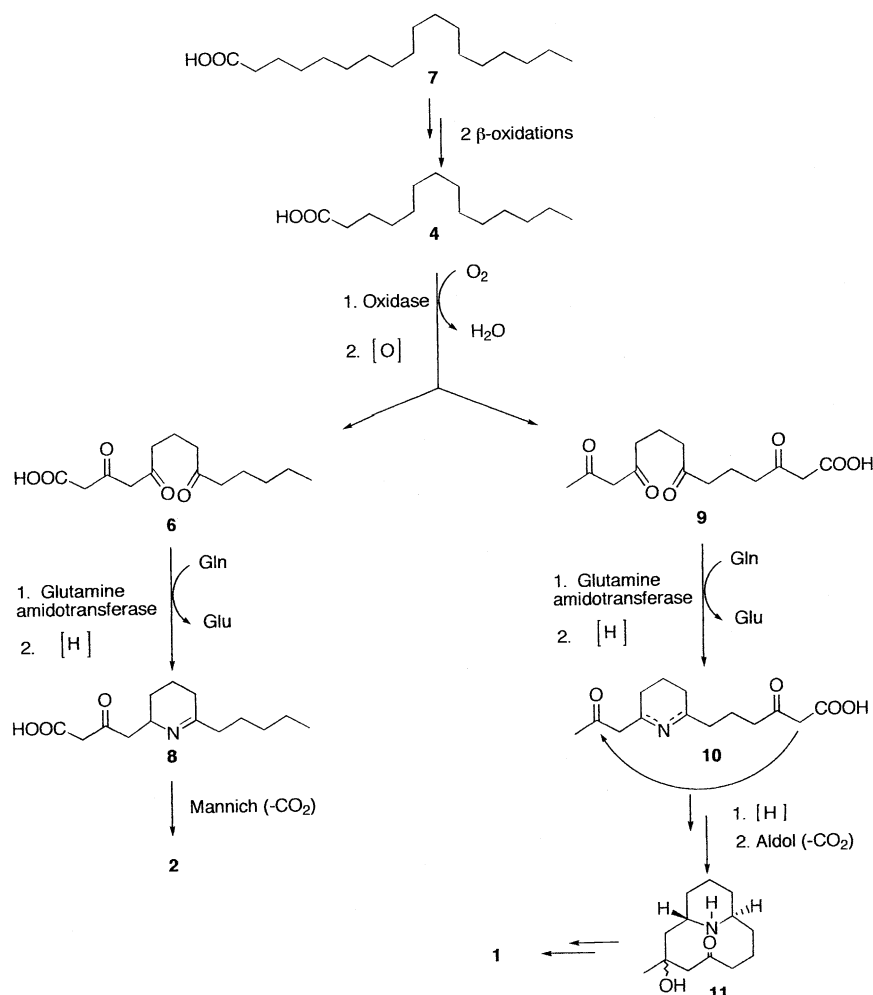

Fig. 3. Biosynthetic schemes proposed for coccinelline (**1**) and adaline (**2**).

inhibitor (Robinson et al., 1963). All these data point to a fatty acid rather than a polyketide pathway for the biosynthesis of adaline (Fig. 2, Route A). However, to unambiguously prove this hypothesis, incorporation experiments with a labelled fatty acid precursor should be performed.

Our results are also consistent with those obtained by Attygalle et al. (1994, 1999) for the biosynthesis of the azamacrocyclic alkaloid epilachnene (**3**), which derives from oleic acid after loss of four carbon atoms, presumably through two β-oxidations. It follows that the true biosynthetic precursor of adaline could be a fatty acid such as stearic acid (**7**). An updated biosynthetic scheme for adaline and the azaphenalene alkaloids, taking into account the results reported herein and in previous papers (Tursch et al., 1975; Laurent et al., 2001), is presented in Fig. 3.

It is highly probable that other ladybird alkaloids, such as calvine (Braekman et al., 1999), and the "dimeric" alkaloids, exochomine (Timmermans et al., 1992) and the chilocorines (McCormick et al., 1994; Shi et al., 1995; Huang et al., 1998) are produced along a similar pathway. Moreover, several other ladybird alkaloids, such as harmonine (Braconnier et al., 1985), signatipennine (Wang et al., 1996), the pyrrolidines (Attygalle et al., 1993) and the pyrrolidinooxazolidines (Radfort et al., 1997), characterised by the presence of a carbon chain bearing two amino groups, could be derived from oleic acid by straightforward oxidation and amination reactions (Wang et al., 1996).

Finally, our data unambiguously show that the fat body is, at least in part, responsible for the biosynthesis of coccinelline in *C. 7-punctata*. This result was also to be expected, considering that the insect fat body is the principal organ of intermediary metabolism, functioning in many aspects of the storage and synthesis of proteins, lipids and carbohydrates (Chapman, 1998). The small SIR (0.011%) measured for the coccinelline sample from the experiment involving the ladybird tissues less the fat body can be interpreted in different ways. It could mean that the biosynthesis of coccinelline takes place in (an)other tissue(s) than the fat body (although much less efficiently). On the other hand, this result may be accounted for by the unwanted presence in that experiment of some residual fat body particles. Further experiments are needed to settle that question.

To the best of our knowledge, this is the first report showing that the fat body plays a major role in alkaloid biosynthesis in insects. It remains to be determined if this finding may be extended to other ladybird species and to other insects as well.

Acknowledgements

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