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# *N*-acylaziridines as potential proinsecticides of carboxylic acids Part VI. Direct HPLC monitoring of the metabolism in insect tissues☆

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**Abstract**

To determine the reversible masking potential of carboxylic acids afforded by the *N*-acyl structure in a proinsecticide perspective, the hydrolysis of monosubstituted *N*-acylaziridines and unsubstituted *N*-acylpyrrolidine was studied by reversed-phase high-performance liquid chromatography (HPLC) during in vitro assays conducted in the presence of insect tissues or of α-chymotrypsin. Chromatographic analysis of unextracted biological samples so-called "the direct injection approach" was simpler and more accurate than the "extraction approach" because it avoids problems associated with extraction. Thus, periodical injections of samples of biological insect tissues or of α-chymotrypsin incubated with *N*-acyl substrates were performed on packings allowing direct injection: a wide-pore column or a monolithic column. Moreover, to allow the simultaneous monitoring of the carboxylic acids and of the parent substrates, ion-pairing was used. In these conditions, it was shown that *N*-acylpyrrolidine is not hydrolyzed whatever the enzymatic conditions or the pH. On the other hand, the unmasking of the carboxylic acid is the preponderant mode of hydrolysis of *N*-acylaziridines in the presence of α-chymotrypsine and the exclusive one in the presence of locust fat-body, which establishes the convenience of this structure in our proinsecticide perspective. Due to the enzymatic character of the unmasking of the carboxylic acid during biological hydrolysis of *N*-acylaziridines, the research of possible chiral recognitions was undertaken. Thus, the enantiomeric composition of these substrates was analysed at the stage of their approximative half hydrolysis using a chiral α-AGP column. It appeared that locust fat-body hydrolyses preferentially the (R)-isomers of *N*-acylaziridines while the reverse is observed when α-chymotrypsine is used.


*Keywords:* *N*-acylaziridine; Proinsecticide; Direct injection; Metabolism; Chirality

## 1. Introduction

For some time, we have been interested in developing proinsecticides masking reversibly acids and/or β-ethanolamines considered as active principles. Concerning the masking structures, we restricted our choice to molecules designed to have hydrolysis as the "activation mode", such as fluorinated esters [1], enol esters [2], oxazolines [3–7] and thiazolines [4,8]. More recently, we have also examined the potential of particular amides represented by the *N*-acylaziridine structure [4,6,8,9].

The study of the "unmasking" of the active principle, or "activation" step, is of most importance in this approach. With fluorinated substrates, $^{19}F[^{1}H]$ NMR method constitutes a very rapid and convenient analytical tool to explore the potential of new structures by in vitro assays [1,2,5,9] or ex vivo assays [2]. On the other hand, chromatography is more suited for non-fluorinated candidate molecules, and the basic method needed for the monitoring of xenobiotics in biological fluids is obviously the reversed-phase partition. Nevertheless,

☆ See Refs. [2,3,5,7,9] for parts I–V, respectively.
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the direct injection of proteinic fluids (plasma, serum, urine, hemolymph, etc.) on classical $C_{18}$ or $C_8$ packings entails the rapid increase of the pressure, the decrease of the performances, especially of the efficiency, with a concomitant peak-broadening, and finally the clogging of the column. The problem is yet increased when eluting lipophilic compounds which need high concentrations of organic solvents in the eluents resulting in the denaturation of the proteins [10]. As these samples matrixes are not directly compatible with LC or LC–MS analysis [11], sample pretreatment using protein precipitation, liquid–liquid extraction or solid-phase extraction (SPE) [12] has been the solution frequently adopted. Nevertheless, as analysis of unextracted biological tissues is simpler, faster and avoids the problems associated with extraction of ionizable compounds, numerous solutions allowing the direct injection have emerged. Most of them can be considered as multidimensional chromatographic techniques (MDC) [13]. Multicolumn techniques represent a first MDC approach and many investigators have demonstrated the advantage of using a precolumn with large particulate alkyl-bonded phase silicas or ion exchangers, placed in-line with high-performance ODS column via switching valve arrangements ([13] and references therein, [14]). Another type of MDC strategy is based on a single packing designed for providing simultaneously several mode of separation which prototype is the Internal Surface Reversed-Phase concept developed by Hagestam and Pinkerton with the GFF packing (Gly-Phe-Phe) [15]. Based on the same or on a similar principle, a second generation of ISRP column GFFII [16] and Restricted Access Media (RAMs) [17–19] have been developed later. With the same objective of "direct injection", Shihabi and co-workers [20,21] have successfully used large-pore packings (300 Å) for the analysis of drugs and their metabolites by direct injection of plasma samples. The inconvenient of the lower number of plates resulting from the larger pores is compensated to some extent by smaller particle size (5 μm and even 2 μm) [22]. Taking into account both the importance of the equipment required for the column switching technique and also the cost of GFFII packings, we have adopted since several years the approach of 300 Å packings for direct injection of various complex matrices using either home-made or commercial $C_1$, $C_4$ or even $C_{18}$ wide-pore columns [4,6,23–28].

The first aim of this work was to rapidly study the eventual unmasking of carboxylic acids triggered by locust tissues for a series of *N*-acylaziridines **1** and for one *N*-acylpyrrolidine **1′**. Consequently, in the "direct monitoring approach", we used commercial $C_4$ 300 Å columns to analyse untreated biological samples. Ion-pairing (IP) was required because of the presence of ionic metabolites as $\mathbf{3}^-$. We have also tested monolithic packings [29] said of the fourth generation of column [30] with $C_{18}$ packings, taking into account recent reports concerning their application for the direct injection of bio-fluids [29–32].

Secondly, for *N*-acylaziridines demonstrated as presenting a significant enzymatic activation, we researched a possible chiral recognition during their hydrolysis. As corresponding enantiomers were not easily available, we indirectly analysed the enantiomeric excess for unchanged *N*-acylaziridines **1** resulting from an incomplete activation of the corresponding racemate. Therefore, after a solid-phase extraction stage, the unchanged *N*-acylaziridines **1** were univocally transformed into their corresponding α-*O*-substituted β-hydroxylamides **4** which enantiomeric excess (e.e.) was determined using a chiral α-AGP column [33,34].

## 2. Experimental

### 2.1. Reagents and chemicals

2-Methylaziridine, dicyclohexylcarbodiimide (DCC) and 2-aminopropanol (racemate or pure enantiomer) were supplied by Aldrich (Sigma–Aldrich Chimie, L'Isle d'Abeau Chesnes, Saint-Quentin Fallavier, France). $CH_2Cl_2$ was supplied by SDS (Solvent Documentation Synthèse, Vitry, France).

*Racemates of N-acyl-2-methylaziridine* ***1a–b*** were obtained by condensation of commercial racemate 2-methylaziridine **2** (1 equiv.) added at 0 °C to corresponding commercial carboxylic acid **3** (1 equiv.) in the presence of DCC (1 equiv.) in $CH_2Cl_2$. After 24 h standing at room temperature, the crude *N*-acylaziridines resulting from filtration of the dicyclohexylurea and evaporation of the solvent were distilled under vacuum.

*N-p-fluoro-phényl-acetyl pyrrolidine* ***1′*** was obtained according to the same protocol.

*Hydroxylamides* **4a–b** were obtained as racemates or as pure enantiomers by condensation of 1 equiv. of 2-aminopropanol (racemate or pure enantiomer) added at 0 °C, with 1 equiv. of carboxylic acid **3a–b** using 1 equiv. of DCC in $CH_2Cl_2$. After 24 h standing at room temperature, and filtering off the dicyclohexylurea and evaporation of the solvent, the crude hydroxylamides were purified by TLC (Silica Merck GF 254, eluent: ethylacetate/*n*-heptane/methanol: 57/41/2). All condensations in the presence of DCC were conducted with reactive and solvents free of water.

Elemental analysis or HRMS, IR and NMR data for **1a–b** and their corresponding hydoxylamides **4a–b** and **1′** agree well with the proposed structures. These new compounds will be completely described elsewhere [35].

### 2.2. Chemicals for chromatography

Acetonitrile and methanol were of HPLC grade (SDS, Solvent Documentation Synthèse, Vitry, France). Deionized water (18 MΩ) obtained with a Milli-Q apparatus (Millipore, Saint-Quentin-en-Yvelines, France) was used for the preparation of mobile phases and phosphate buffers by mixing appropriate volumes of dissolved monobasic and dibasic potassium phosphates which were supplied by Acros (Noisy le Grand, France). Tetrabutylammonium bromide

(TBAB) was supplied by Aldrich (Sigma–Aldrich Chimie, L'Isle d'Abeau Chesnes, Saint-Quentin Fallavier, France). Organic solvents and deionized water were both filtered through GH Polypro filters (0.45 μm, Pall, Ann Arbor, MI, USA).

### 2.3. Biological samples

Locust fat-body and hemolymph were provided by Dr. A. Louveaux, Laboratoire de Biologie évolutive et Dynamique des Populations (Université Paris-Sud) [36]. The African migratory locust *Locusta migratoria* were reared according to the crowded conditions described in reference [9]. After dissection, *locust tissues* were stored at −80 °C in several Eppendorfs until their utilization.

α-Chymotrypsin from bovine pancreas was supplied by Sigma (Sigma–Aldrich Chimie, L'Isle d'Abeau Chesnes, Saint-Quentin Fallavier, France). One unit of the enzyme will hydrolyse 1.0 μmol of *N*-benzoyl-L-tyrosine ethylester per minute at pH 7.8 at 25 °C.

In vitro experiments were performed at room temperature (about 20 °C) using substrate concentrations in the range $5 \times 10^{-5}$ to $10^{-4}$ M. Substrate concentrations are not exceeding $10^{-4}$ M in order to avoid possible enzymatic inhibition by substrate excess. Substrate samples were obtained by diluting a 0.1 M substrate stock solution of **1a–b** or **1′** with the adequate volume of biological medium or phosphate buffer. $CH_3CN$ was used for substrate solubilization in a 5 and 10 percentage (v/v).

*Locust fat-body:* Typically a mixture of 260 mg of this tissue and 400 μl of phosphate buffer (0.1 M, pH 7.4) was ground and centrifuged, resulting in three fractions differing in density, with the bottom one consisting of tissue fragments, upper one consisting of the lipidic components and the intermediate aqueous one being the only fraction used as the biological sample.

*Hemolymph* sampled from adults locusts (male and female, pH 7.4) was centrifuged for 5 min at 300 × *g*, then diluted eight times in a phosphate buffer (0.1 M, pH 7.4) and incubated with the substrate.

*Commercial enzyme:* In situ enzymatic hydrolysis of **1a** and **1b** were performed by adding *α-chymotrypsin* (62.5 units of enzyme) to a 500 μl aliquot of a phosphate buffer solution of the initial substrate **1a** ($10^{-4}$ M), or **1b** ($10^{-4}$ M) at pH 7.8.

### 2.4. HPLC instrumentation

For qualitative analysis and quantification of *N*-acylaziridines **1a–b** and *N*-acylpyrrolidine **1′**, a system consisting of a Waters LC Module I plus (Milford, MA, USA) operating with Millenium 2010 v2.15.01 chromatography manager software was used. The separations were carried out with a 5 μm $C_4$ Uptisphere wide-pore analytical column (300 Å) (50 mm × 4.6 mm I.D.) and 5 μm $C_4$ Uptisphere wide-pore guard column (300 Å) (10 mm × 4 mm I.D.) supplied by Interchim (Montluçon, France) and with a 5 μm Chromolith Speed ROD RP-18 analytical column (50 mm × 4.6 mm I.D.) supplied by Merck (Darmstadt, Germany) and with α-AGP (100 mm × 4 mm I.D.) and a guard column α-AGP (10 mm × 3 mm I.D.). The chiral analyses were carried out with a 5 μm α-AGP (α1 acid glycoprotein) analytical column (100 mm × 4.0 mm I.D.) and a 5 μm α-AGP guard column (10 mm × 4 mm I.D.) both purchased from ChromTech (Stockholm, Sweden). Thermostating (at 20 or 25 ± 0.1 °C) of the chromatographic system (furnace Jet-stream 2+, Jasco) was systematically adopted as a necessary condition for a good reproducibility of the chromatograms.

Table 1
Chromatographic conditions for monitoring the behaviour of *N*-acyl-compounds **1a–b** and **1′** in phosphate buffers and in biological tissues

| Column | Substrate | [TBAB] (mM) | $k_1$ | $k_3$ | $k_4$ |
|---|---|---|---|---|---|
| Uptisphere $C_4$, 300 Å | **1a** | 15 | 11.28 | 5.24 | 2.14 |
| | **1b** | 20 | 8.76 | 3.42 | 1.60 |
| Chromolith $C_{18}$ | **1a** | 7 | 18.82 | 4.22 | 1.98 |
| | **1b** | 10 | 15.36 | 2.54 | 1.32 |

$k_1$: capacity factor of substrates **1a** or **1b**; $k_3$: capacity factor of carboxylates **3a** or **3b**; $k_4$: capacity factor of hydroxylamides **4a** or **4b**.

### 2.5. HPLC monitoring

Standards were prepared by adding known amounts of **1a–b** or **1′** into a solution composed of phosphate buffer (0.1 M, pH 7.4 or 7.8)/$CH_3CN$, 90:10 (v/v). Substrate concentration ranges were $2.5 \times 10^{-5}$ to $10^{-4}$ M for **1a** and $0.5 \times 10^{-5}$ to $10^{-4}$ M for **1b**.

Isocratic separations on the Uptisphere and on the Chromolith columns were achieved at 20 °C with 75% of a phosphate buffer (pH 6.4, 0.01 M) and 25% of $CH_3CN$. Flow rate were optimized at 0.8 ml/min for the Uptispher $C_4$ column and 2 ml/min for the Chromolith column, respectively. Ion-pairing reagent was tetrabutylammonium bromide, at different concentrations (see Table 1). Injections were of 5 μl for each of **1a–b** and **1′** standards or inoculated biological samples. Three replicates of standards at five concentration levels were analysed.

On the chiral analytical column α-AGP, isocratic separations were performed at 25 °C, with 96% of phosphate buffer (pH 6.4–0.01 M) and 4% of $CH_3CN$ (v/v), at a flow rate of 0.45 ml/min for **1a** and with 85% of phosphate buffer (pH 6.4–0.01 M) and 15% of $CH_3CN$ (v/v), at a flow rate of 0.9 ml/min for **1b** ($V_{inj}$ = 6 μl).

The capacity factors, *k*, were calculated by the classical relation between the retention time $t_r$ and the hold-up time $t_0$: $k = (t_r - t_0)/t_0$, $t_0$ being determined by injection of a diluted $NaNO_3$ solution.

In order to optimize the detector response, wavelengths were set at 220 nm for substrates **1b** and **1′** and at 223 nm for **1a**.

Quantification was performed by the external standard method from triplicate injections of standards or samples with five calibration levels. The calibration curves for substrates **1a–b** and **1′** obtained by plotting their concentration $y$ (mol/l) against their peak area $x$ (μV s) are linear within the ranges of concentration of $0.5 \times 10^{-5}$ to $10^{-4}$ mol/l. Substrates **1a–b** and **1′** were directly quantified from chromatograms of the various samples by using their peak area and the calibration curves.

### 2.6. Solid-phase extraction

OASIS HLB (30 mg, Waters) cartridges have been used for the extraction of biological samples. The steps of the extraction were as follows. The cartridges were conditioned with 1 ml methanol and 1 ml deionized water. Then 1 ml of sample was loaded. The washing step consisted in 2 ml of water with 40 and 30% methanol for **1a** and **1b**, respectively. Finally, 0.6 ml of methanol for **1a** and 1.5 ml for **1b** were used for the elution of *N*-acylaziridines **1a–b**. For HPLC analysis, the sample was diluted two times with water.

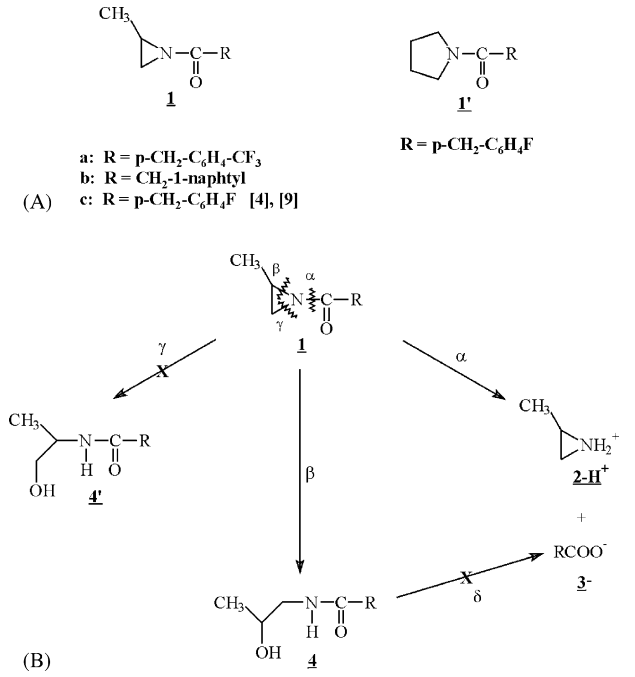


Fig. 1. (A) *N*-acylaziridines **1a–c** and *N*-acylpyrrolidine **1′**. (B) Metabolism and hydrolysis pathways of *N*-acylaziridines **1** according to references [4,9].

## 3. Results and discussion

The behaviours of substrates **1a–b** and **1′** were compared under non-enzymatic conditions and in the presence of locust tissues or of α-chymotrypsin during in vitro assays. To provide rapid analysis of *N*-acyl-compounds, in the "proinsecticides perspective", HPLC monitorings were performed under the isocratic mode according to the "direct injection approach", using either a wide-pore column (Uptisphere $C_4$, 300 Å) or a monolithic packing (Chromolith $C_{18}$). These both columns allow injection of untreated biological samples. Substrates disappearance and carboxylic acids and hydroxylamides formations were followed using UV–vis detection due to the presence of aromatic chromophores in these molecules (see Fig. 1A).

### 3.1. Checking-up of the HPLC eluent

Blanks that were necessary to appreciate the enzymatic effect exercised by diluted locust biological tissues or α-chymotrypsin were conducted in phosphate buffer at pHs corresponding approximately either to the physiological pH of locust tissues (pH 7.4) or to the optimal pH for the catalysis exercised by α-chymotrypsin (pH 7.8). These assays served also to adjust several analytical conditions in order to optimize the retention of substrates and metabolites allowing thus their quantification, and to avoid both the endogenous compounds precipitation and incomplete substrate solubilisation. Due to the relatively high lipophilicity of the substrates (log $P$ [37]: 2.14 for **1a**, 2.27 for **1b** and 1.73 for **1′**), the use of a water soluble organic co-solvent such as acetonitrile was required both for their solubilisation in phosphate buffers or in biological samples, and for their elution with reasonable retentions. On the other hand, the ionic character of the expected metabolites $\mathbf{3}^-$ required to add ion-pairing reagent (IP) (tetrabutylammonium bromide) in the eluents previously optimized for the corresponding substrates to avoid their elution near the void volume.

The checking-up of the HPLC eluent was undertaken at first with the more lipophilic substrate **1b** and its possible hydrolysis compounds $\mathbf{3b}^-$ and **4b** using an Uptisphere $C_4$ 300 Å column. The best eluent was composed of 25% $CH_3CN$ and 20 mM tetrabutylammonium bromide in phosphate buffer. These chromatographic conditions lead to a good separation between the lipophilic substrat **1b** and its corresponding metabolites: carboxylate $\mathbf{3b}^-$ and hydroxylamide **4b** (see Table 1 for $k$ values). This later compound, being the less lipophilic, shows the lowest retention. Carboxylate $\mathbf{3b}^-$ and hydroxylamide **4b** are also well separated from the biological endogeneous components, whose the main part eluted at the void volume of the column. We have checked our chromatographic conditions in order to avoid possible coelution between minor biological components that can be paired with TBAB, and compounds of interest. The optimized eluent was successfully applied to the separation of the less lipophilic substrate **1a** and its metabolites $\mathbf{3a}^-$ and **4a**. Only 15 mM of TBAB were required to pair the carboxylate $\mathbf{3a}^-$ and separate it from the void volume. With these experimental conditions, the Uptispher column can support up to 100 injections of 5 μl without clogging. Nevertheless, in our direct injection approach, we were also interested in using a monolithic column because of its numerous advantages offered by such a packing. Thanks to its dual structure (macroporous and mesoporous), this packing allows the macromolecules

Table 2
Behaviour of *N*-acylaziridines **1a–b** and *N*-acylpyrrolidine **1′** in phosphate buffers at pH 7.4 and 7.8

| Evolution time (h) | **1a** (%) | **4a** (%) | **3a** (%) | **1b** (%) | **4b** (%) | **1′** (%) | **4′** (%) |
|---|---|---|---|---|---|---|---|
| pH 7.4 | | | | | | | |
| 0 | 100[a] | 0.0[a] | 0.0[a] | 100[a] | 0.0[a] | 100[b] | 0.0[b] |
| 0.5 | 87.1[a] | 8.2[a] | 4.7[a] | 97.1[a] | 2.9[a] | 100[b] | 0.0[b] |
| 1 | 80.0[a] | 13.9[a] | 6.1[a] | 93.4[a] | 6.6[a] | 100[b] | 0.0[b] |
| 1.5 | 78.0[a] | 16.7[a] | 5.3[a] | 91.7[a] | 8.3[a] | 100[b] | 0.0[b] |
| 2 | 73.7[a] | 20.8[a] | 5.5[a] | 89.0[a] | 11.0[a] | 100[b] | 0.0[b] |
| 2.5 | 68.2[a] | 25.4[a] | 6.4[a] | 87.3[a] | 12.7[a] | 100[b] | 0.0[b] |
| 3 | 61.2[a] | 31.3[a] | 7.5[a] | 81.5[a] | 18.5[a] | 100[b] | 0.0[b] |
| 24 | – | – | – | – | – | 100[b] | 100[b] |
| pH 7.8[b] | | | | | | | |
| 0 | 99.0 | 1.0 | 0.0 | 100 | 0.0 | – | – |
| 0.5 | 93.2 | 5.1 | 1.7 | 100 | 0.0 | – | – |
| 1 | 90.4 | 7.8 | 1.8 | 99.4 | 0.6 | – | – |
| 1.5 | 88.0 | 10.0 | 2.0 | 98.8 | 1.2 | – | – |
| 2 | 83.0 | 14.3 | 2.7 | 97.8 | 2.2 | – | – |
| 2.5 | 79.4 | 17.3 | 3.3 | 97.2 | 2.8 | – | – |
| 3 | 75.2 | 20.3 | 4.5 | 96.5 | 3.5 | – | – |

[a] Analysis on a Chromolith Speed ROD RP-18 column.
[b] Analysis on a Uptisphere $C_4$, 300 Å column.

to pass through the column via the macropores, while small organic molecules can be retained on the bonded phase in the mesopores [29–32]. Because of a high porosity greater than 80%, the monolithic packing allows to perform chromatography with lower backpressure and higher flow rates (such as 9 ml/min), reducing then the analysis time. Thus, these columns appear to be well suitable for our direct injection approach. Chromolith $C_{18}$ column was used to separate the two substrates **1a** and **1b** from their metabolites and from the biological media. It appeared that the same organic solvent/phosphate buffer eluent previously optimized for the Uptisphere column could be used. However, optimization of the TBAB concentration resulted in values divided by half compared to those needed with the Uptisphere column. The flow rate was set up at 2 ml/min instead of 0.8 ml/min for the wide-pore Uptisphere column, in order to decrease the analysis time. The results presented in Table 1 indicate the same retention order for the hydroxylamide **4**, the carboxylate $\mathbf{3}^-$ and the *N*-acylaziridine **1**.

### 3.2. Behaviour of substrates *1a*, *1b* and *1′* in phosphate buffers

In order to differentiate the possible effect of the pH of biological media from enzymatic catalysis, we studied firstly the stability of substrate **1a**, **1b** and **1′** in buffered solutions at pHs 7.4 and 7.8, using the previously optimized chromatographic conditions. Whatever the pH, we observed over a 3 h period, a slow diminishing of the area of the peak of **1b** (see Table 2). However, the activation process is not implied in the hydrolysis since there is no formation of carboxylate $\mathbf{3b}^-$ (see pathway **α** in Fig. 1B). In contrast, the involvement of the pathway **β** was firstly made evident by the observation of the same capacity factor for a standard of **4b** and the compound resulting from hydrolysis. However, at this stage of the study, the involvement of pathway **γ** was also to consider. Because of the chemical structure similarity of the hydroxylamide **4b** and its regioisomer **4b′**, the eventuality of their coelution was to examine, all the more that hydroxylamide **4b** elutes very early from the column. Structural arguments were necessary to resolve this question. Therefore, a sample of several mg of **4b** was prepared from **1b** by standing in a phosphate buffer at pH 7.4 during 48 h (hydrolysis pathway **β**). $^1$H NMR allowed to demonstrate that the hydrolysis product of **1b** resulting from pathway **β** presents the same structural characteristics as the standard of α-*O*-substituted hydroxylamide **4b** independently synthetized by condensing 1-amino-2-propanol with carboxylic acid **3b**. This result establishes that pathway **γ** leading to α-*N*-substituted hydroxylamide **4b′** is not involved in these hydrolysis conditions of **1b**. This behaviour is in agreement with that previously observed by $^{19}$F NMR for the fluorinated *N*-acylaziridine **1c** [4,9], and with literature data [38,39]. Indeed, for a monosubstituted *N*-acylaziridine, the less stable N–C bond is the more substituted one.

To notice is the better stability observed for **1b** at pH 7.8 than at pH 7.4 (3.5% of hydrolysis instead of 18.5% in 3 h, see Table 2).

Using the same chromatographic approach, we have also established that *N*-acylaziridine **1a** behave in non-enzymatic conditions as *N*-acylaziridine **1b**, the hydrolysis pathway **β** being still slower at pH 7.8 than at pH 7.4. However, the *N*-acylaziridine **1b** exhibits a greater stability than **1a** with 18.5% versus 31.3% of hydroxylamide formed at pH 7.4 in 3 h, and 3.5% versus 20.3% at pH 7.8 (see Table 2). Pathway **α** is involved at a very low level for *N*-acylaziridine **1a**, as it was already observed by $^{19}$F NMR for **1c** [4,9].

In contrast, *N*-acylpyrrolidine **1′**, masking both a saturated carboxylic acid and the pyrrolidine structure, i.e. a five-membered cyclic amine instead of a three-membered one,

appears as very stable in phosphate buffers, since hydrolysis is not observed even after 24 h standing at pH 7.4.

Thus, at this stage, it appears that in solutions buffered at biological pHs, there is no or very slight activation (pathway **α** in Fig. 1B) of the compounds tested. *N*-acylpyrrolidine **1′** is absolutely stable in these conditions, while *N*-acylaziridines **1a–b** hydrolyse slowly at different extent according to the pathway **β** in Fig. 1B.

### *3.3. Behaviour of N-acyl-compounds in enzymatic conditions*

The aims of these assays were three-folds:

(i) To provide a rapid answer about the "activation" of the *N*-acyl-substrates, i.e. is now the pathway **α** in Fig. 1B effectual or not?
(ii) To determine, for the activable compounds, the approximative $t_{1/2}$ in the biological media in order to process later to a research of a possible chiral recognition triggered by the enzymes.
(iii) To try to get an insight into the nature of the implied enzyme(s).

#### *3.3.1. Behaviour of N-acylaziridine **1b** in locust fat-body*

Results of the chromatographic analyses on the wide-pore column of the *N*-acyl-compounds behaviour in presence of diluted locust fat-body (1/3) are presented in Table 3. They show several outstanding effects of this biological tissue on the hydrolysis of *N*-acylaziridines **1a** and **1b**, in comparison to the behaviour observed in phosphate buffers. Indeed, as early as after 30 min, the disappearance of **1b** is complete with the exclusive formation of carboxylate **3b**$^-$ (according to pathway **α**). Therefore, despite its dilution in a phosphate buffer (1/3), locust fat-body triggers a very efficient activation of *N*-acylaziridine **1b**, certainly due to the presence of hydrolases in this tissues [23,40].

Considering a possible recognition from the enzymes present in the locust fat-body, we examined the possibility of a catalysed asymmetric hydrolysis of our chiral *N*-acylaziridines in this tissues. Abundant literature is available concerning the well-known field of enzymatic recognition [41] and especially enantioselectivities [42]. Hydrolases and particularly lipases have mostly been exploited under kinetic conditions because of their remarkable enantioselectivities [43,44]. In the present case, the too rapid unmasking of the carboxylic acids observed for *N*-acylaziridines **1a** and **1b** prevents the research of a possible enantioselectivity during their enzymatic hydrolysis. Therefore, our strategy was to empirically determine the dilution of the locust fat-body allowing the approximative determination of the $t_{1/2}$ of the enzymatic hydrolysis of the *N*-acylaziridines. Consequently, the chiral analysis of the non-transformed substrates remaining after this period of incubation could be easily made.

#### *3.3.2. Behaviour of N-acyl-compounds in very diluted locust fat-body*

It appeared when using 1/50 as a dilution factor of the locust fat-body in a phosphate buffer (pH 7.4) that the disappearance of *N*-acylaziridine **1b** takes more than 3 h. If the hydrolysis of **1b** is still following the expected activation process (pathway **α**), the involvement to a lesser extend of the pathway **β** is now to notice (see Fig. 2). The slowing-down

Table 3
Behaviour of *N*-acylaziridines **1a** and **1b** in the presence of diluted locust fat-body and of α-chymotrypsine

| Evolution time (h) | **1a** (%) | **3a**$^-$ (%) | **4a** (%) | **1b** (%) | **3b**$^-$ (%) | **4b** (%) |
|---|---|---|---|---|---|---|
| A | | | | | | |
| 0 | – | – | – | 6.0 | 94.0 | 0.0 |
| 0.5, 1 | – | – | – | 0.0 | 100 | 0.0 |
| B | | | | | | |
| 0 | 87.7 | 7.9 | 4.4 | 81.3 | 6.6 | 12.1 |
| 0.5 | 62.4 | 32.1 | 5.5 | 57.5 | 24.6 | 17.9 |
| 1 | 31.0 | 57.5 | 11.5 | 39.7 | 38.6 | 21.7 |
| 1.5 | 15.8 | 70.5 | 13.7 | 27.5 | 48.3 | 24.2 |
| 2 | 0.0 | 84.4 | 15.6 | 18.2 | 55.7 | 26.1 |
| 2.5 | 0.0 | 84.7 | 15.3 | 12.1 | 60.7 | 27.2 |
| 3 | 0.0 | 84.8 | 15.2 | 7.6 | 64.2 | 28.2 |
| C | | | | | | |
| 0 | 91.7 | 7.3 | 1.0 | 100 | 0.0 | 0.0 |
| 0.5 | 61.7 | 29.4 | 8.9 | 98.9 | 1.1 | 0.0 |
| 1 | 42.5 | 41.0 | 16.5 | 91.0 | 7.5 | 1.5 |
| 1.5 | 30.1 | 48.7 | 21.2 | 83.2 | 14.3 | 2.5 |
| 2 | 21.9 | 49.6 | 28.5 | 74.4 | 21.3 | 4.3 |
| 2.5 | 17.1 | 53.4 | 29.5 | 66.5 | 28.0 | 5.5 |
| 3 | 14.9 | 53.6 | 31.5 | 56.7 | 36.0 | 7.3 |

(A) Fat-body diluted (1/3) in a phosphate buffer at pH 7.4. (B) Fat-body diluted (1/50) in a phosphate buffer at pH 7.4. (C) α-Chymotrypsine (62.5 units) in a phosphate buffer at pH 7.8.

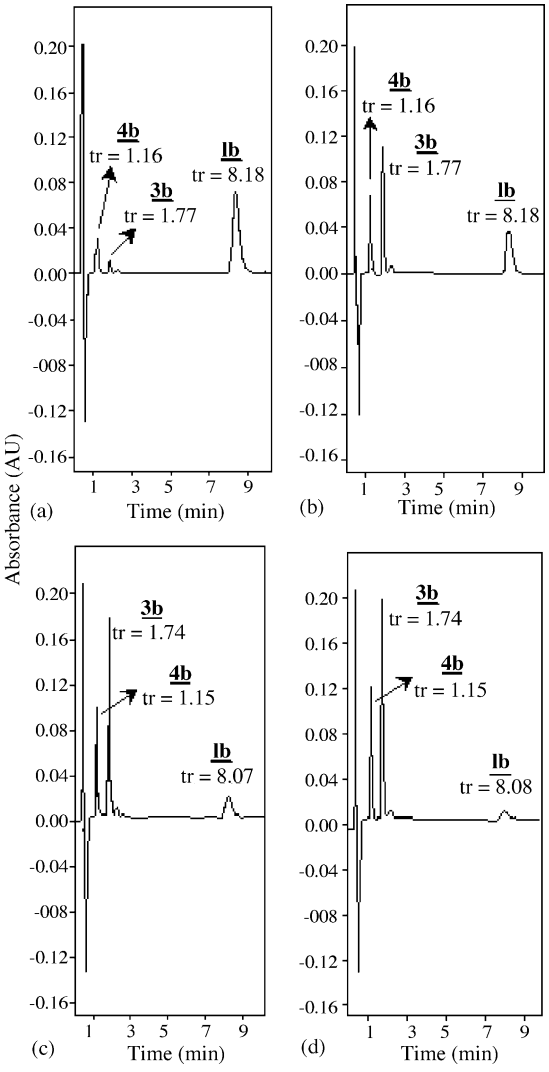


Fig. 2. Chromatograms of *N*-acylaziridine **1b** in the presence of very diluted locust fat-body. (a) $t = 0$; (b) $t = 1$ h; (c) $t = 2$ h; (d) $t = 3$ h.

of the hydrolysis of **1b** allowed to estimate at about 50 min the $t_{1/2}$ of its disappearance in these conditions.

The behaviour of the *N*-acylaziridine **1a** was directly studied in very diluted locust fat-body. In these conditions, the disappearance of **1a** is more rapid than that of **1b** since its complete hydrolysis takes less than 2 h instead of more than 3 h. This trend in the behaviour of **1a–b** in the presence of very diluted locust fat-body is thus the same than that previously observed in phosphate buffers (see Tables 2 and 3). Therefore, it appears that enzymatic catalysis is depending on the structure of the *N*-acylaziridines tested and more precisely on the structure of the masked carboxylic acid. These assays allowed also to estimate at about 40 min the $t_{1/2}$ for the disappearance of **1a**.

In contrast, *N*-acylpyrrolidine **1′** masking the carboxylic acid **3c** appears as a non-activable *N*-acyl-compound, at least in diluted locust hemolymph. Since meanwhile, biological tests against several pests species revealed the inactivity of this compound [8], the study of its behaviour in the presence of more concentrate locust fat-body was not undertaken. *N*-acylpyrrolidine structure seems unusable in a proinsecticide perspective based upon *N*-acyl-compounds.

As a first conclusion, it appears that the enzymatic unmasking of *N*-acylaziridines **1a–b** that was also previously observed for **1c** [4,9], is not only depending on the structure of the masked carboxylic acid but is overall related to the particular cyclic amine that is the aziridine structure.

### 3.3.3. Behaviour of *N*-acyl-compounds in the presence of α-chymotrypsin

We tested this commercial amidase on the *N*-acylaziridines **1a–b** because it is known to hydrolyse peptidic chains and various esters or amides possessing an aromatic group [45].

Adding of α-chymotrypsine in a phosphate buffer at pH 7.8 entails an efficient activation of the two *N*-acylaziridines, with production of about 36% of carboxylate **3b**$^-$ and 53.6% of carboxylate **3a**$^-$ after 3 h of incubation. α-Chymotrypsine triggers a more efficient activation for **1a** than for **1b**. It is to notice that despite its great dilution, the locust fat-body exercises a more efficient activation than α-chymotrypsine used at 62.5 units for both *N*-acylaziridines **1a** and **1b**. Nevertheless, the two enzymatic activations present several analogies. (i) The pathway **β** (see Fig. 1B) which results evidently from a pH effect is now competing with the activation represented by pathway **α** which is the univocal hydrolysis observed at low dilution of the locust fat-body (see also parts A and B in Table 3). (ii) The same structure effect of the masked carboxylic is observed with a more pronounced activation for **1a** than for **1b** (see parts B and C in Table 3). Thus, from the parallel that can be drawn between the effects of locust fat-body and of α-chymotrypsine on the hydrolysis of **1a–b** compared to the blanks, it emerges that:

(i) This commercial enzyme can be used in place of locust tissues for a rapid screening of candidate proinsecticides based on the *N*-acylaziridine structure.
(ii) The activation observed in the presence of locust fat-body seems also enzymatic in nature and is most likely induced by amidases.

### 3.4. Research of a possible chiral recognition during the enzymatic activation of *1a–b*

As the synthesis of enantiomers of *N*-acylaziridines **1** is not easy [8], we projected to analyse indirectly the enantiomeric composition of unchanged substrates **1** resulting from an incomplete activation of the racemate, via their corresponding hydroxylamides **4** subsequently obtained by a non-enzymatic and univocal hydrolysis (see pathway **β** in Fig. 1B).

#### 3.4.1. Effect of very diluted locust fat-body

After incubation in very diluted locust fat-body which duration was previously determined ($t_{1/2} = 40$ and 50 min

Table 4
Comparison of chiral analysis on a α-AGP column of incubated extracts of samples of locust fat-body and of α-chymotrypsine

| Substrate | Tissues | Incubation duration | Hydrolysis (%)[a] | e.e. from chiral analysis of hydroxylamides **4**[b] | e.e. from chiral analysis of *N*-acylaziridines **1**[b] |
|---|---|---|---|---|---|
| **1a** | Fat-body | 40 min | 62 | 15%[c] (see Fig. 3D) | 10.5%[d] (see Fig. 3C) |
| | α-Chymotrypsine | 1 h | 45 | – | 40%[c] (see Fig. 3F) |
| **1b** | Fat-body | 50 min | 60.9 | 9.5%[c] | 4.9%[d] |
| | α-Chymotrypsine | 2.5 h | 33.6 | – | 25.3%[c] |

[a] Uptisphere $C_4$ or Chromolith $C_{18}$ columns.
[b] $|[R]-[S]|/([R]+[S]) \times 100$.
[c] $[R] > [S]$.
[d] $[R] < [S]$.

for **1a** and **1b**, respectively), we proceeded to a solid-phase extraction to obtain the unchanged *N*-acylaziridines **1**. Using an α-AGP chiral column [33,34], we observed that the hydrolysis of *N*-acylaziridines **1a** and **1b** undergoes a chiral recognition (with e.e. = 10.5 and 4.9%, respectively). However, at this stage, it was not possible to state precisely the e.e. sense. Therefore, as enantiomerically pure β-hydroxylamides **4a** and **4b** are easily available from commercial chiral synthons [8], the extracts of **1a** and **1b** were univocally transformed into their corresponding α-*O*-substituted β-hydroxylamides **4** (pathway **β** in Fig. 1B) by standing in a phosphate buffer at pH 7.4. Afterwards, taking into account that the $SN_2$ hydrolysis of the extracts of *N*-acylaziridines **1** implies an inversion of configuration for the chiral center, the e.e. sense of these parent compounds could be deduced from the e.e. determined for the β-hydroxylamides **4** by chiral HPLC on a α-AGP column (see Fig. 3 and Table 4). It results from these chiral analysis that diluted fat-body exercises a significant chiral recognition during the enzymatic hydrolysis of *N*-acylaziridines **1a** and **1b**. Noticeable is the fact that the same sense is observed for the e.e. observed for **1a** and **1b** i.e. that (S)-**1** enantiomers which present the lowest retentions are predominant, establishing therefore that (R)-**1** enantiomers are more efficiently hydrolyzed (see Fig. 3 and Table 4). The chiral recognition is more pronounced for **1a** that has been previously established as a more activable substrate, than **1b**. Therefore, at this stage, we can suppose that undiluted fat-body would exercise a more efficient activation, and consequently, a greater chiral recognition in the hydrolysis of **1a** and **1b**.

### 3.4.2. *Effect of α-chymotrypsine*

As the retention of the enantiomers of *N*-acylaziridines **1a–b** on a α-AGP chiral column was previously established, the chiral recognition exercised by α-chymotrypsine was directly analysed with the same column on the extracts resulting from their incomplete hydrolysis. It appeared unambiguously that this commercial enzyme exercises during the activation of **1a–b** a chiral recognition that is both more pronounced and reversed comparatively to the assays conducted in the presence of very diluted fat-body (see Fig. 3C and F and Table 4).

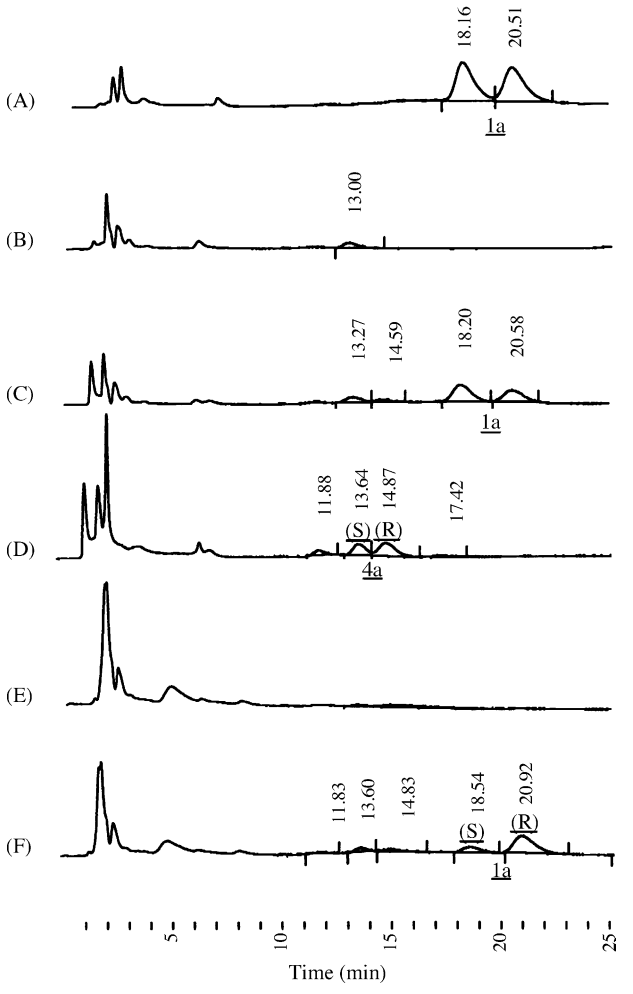


Fig. 3. Chromatograms of the extracts of biological samples of very diluted locust fat-body and α-chymotrypsine incubated with *N*-acylaziridine **1a** on a chiral α-AGP column. (A) Chiral analysis of *N*-acylaziridine **1a** racemate. (B) Chromatogram of the extract of a sample of non-incubated locust fat-body. (C) Chiral analysis of the extract of a sample of locust fat-body incubated with *N*-acylaziridine **1a** racemate during 40 min. (D) Chiral analysis of the previous extract (C) after unequivocal transformation into the corresponding α-*O*-substituted hydroxylamide **4a**. (E) Chromatogram of the extract of a sample of non-incubated α-chymotrypsine. (F) Chiral analysis of the extract of a sample of α-chymotrypsine incubated with *N*-acylaziridine **1a** racemate during 1 h.

## 4. Conclusion

The present results concerning the in vitro behaviour of *N*-acyl substrates in the presence of locust fat-body or α-chymotrypsine highlight the advantage of using either a $C_4$ wide-pore or a $C_{18}$ monolithic column to conduct the HPLC monitoring directly in biological tissues. This approach provides, comparatively to the extraction technique, ease and rapidity without the inconvenience of possible incomplete extraction of ionic species such as carboxylates. By comparing the behaviours of substrates **1** and **1′** with those observed in the blanks, we demonstrated that no activation occurs with *N*-acylpyrrolidine **1′**, while the unmasking of carboxylic acids from *N*-acylaziridines **1** is an unequivocal hydrolysis pathway when elicited by specific insect tissues such as locust fat-body. Therefore, taking into account that *N*-acylaziridines present both an efficient enzymatic activation that can be expected as reinforced in living insects due to the implication of the totality of the undiluted tissues, and a good stability outside of insect tissues, it must be concluded that these particular *N*-acyl-compounds present an ideal structure for developing proinsecticides of carboxylic acids.

The use of an α-AGP column allowed to demonstrate that the enzymatic activation exercised by locust fat-body occurs with a chiral recognition. α-Chymotrypsine triggers also an activation of **1a–b**, however, the resulting chiral recognition is the reverse of that observed in the presence of locust fat-body. Enzymes of this tissue are thus different from those of the commercial amidase.

The work is presently continued with ex vivo monitorings of the behaviour of *N*-acylaziridines in locust fat-body or in other tissues, and by testing the biological properties of pure enantiomers. The aims of these investigations that will be mainly based on the analytical background presented in this work are to ascertain and document the potential afforded by the *N*-acylaziridine structure and particularly to determine the possible advantage to use pure enantiomers in a proinsecticide perspective.

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