Potential proinsecticides of fluorinated carboxylic acids III. Evaluation of the *N*-acylaziridine structure by ^{19}F NMR monitoring of the *in vitro* behaviour in insect tissues†

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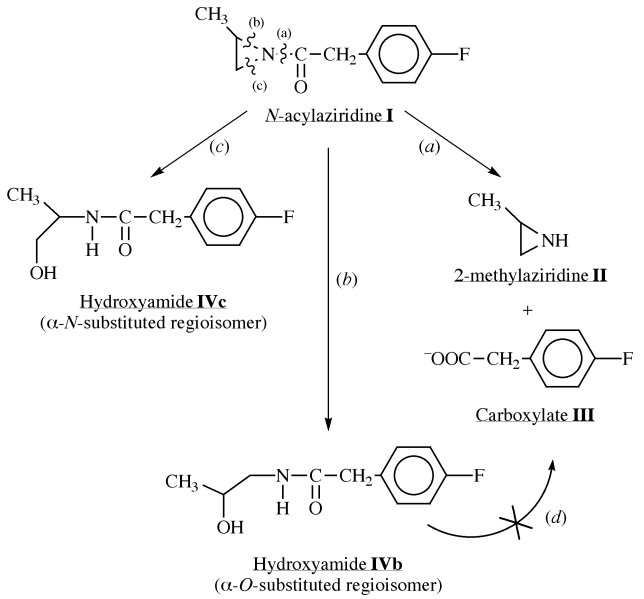
To determine the reversible masking potential of carboxylic acids afforded by the *N*-acylaziridine structure, the hydrolysis of the fluorinated compound **I** was studied in buffered solutions at different pH, in insect tissues during *in vitro* and *ex vivo* assays, and in the presence of α-chymotrypsin. $^{19}F[^{1}H]$ NMR monitoring was used directly with the reacting media, quantifying the fluorinated entities relative to an internal standard. Slightly basic pH (7.8) results in both the hydrolysis of the *N*-acyl function into 2-methylaziridine **II** and the fluorinated carboxylate **III** (pathway *a*), and the regiospecific cleavage of the heterocycle leading exclusively to the α-*O*-substituted regioisomer **IVb** of the corresponding β-hydroxyamide (pathway *b*). Under slightly acidic conditions (pH 6.3) the *N*-acyl function is preserved, but the hydrolysis of the heterocycle is more pronounced and now only regioselective since the minor product, hydroxyamide **IVc** (pathway *c*), is also obtained as well as the predominant regioisomer **IVb**. In the presence of diluted haemolymph, caterpillar or locust, the hydrolysis pathway *a* is accompanied by the hydrolysis pathway *b*. On the other hand, pathway *a* becomes the predominant hydrolysis mode when triggered by α-chymotrypsin, and the very efficient and nearly exclusive one, in the presence of locust fat body and mesenteron. Thus it must be concluded that, in contrast with the β-hydroxyamide **IVb**, the *N*-acylaziridine **I** acts as a proinsecticide by virtue of being a precursor of carboxylate **III**, which is confirmed by several *ex vivo* experiments with locusts.

Proinsecticides potentiels d'acides carboxyliques fluorés III. Evaluation de la structure *N*-acylaziridine par suivi en RMN ^{19}F du comportement *in vitro* dans les tissus d'insectes. Pour déterminer les possibilités de masquage d'acides carboxyliques offertes par la structure *N*-acylaziridine, l'hydrolyse du composé fluoré **I** de cette série est étudiée à différents pH et dans des tissus d'insectes au cours d'expériences *in vitro* et *ex vivo*, ou en présence d'α-chymotrypsine. La RMN $^{19}F[^{1}H]$ a été utilisée comme technique de suivi direct des milieux réactionnels, en quantifiant les différentes entités fluorées relativement à un standard interne. Un milieu faiblement basique (pH 7,8) provoque à la fois l'hydrolyse de la fonction acyle en méthyle-2 aziridine **II** et en carboxylate fluoré **III** (voie *a*) et la rupture régiospécifique de l'hétérocycle conduisant exclusivement au régioisomère α-*O*-substitué du β-hydroxyamide correspondant (voie *b*). En milieu légèrement acide (pH 6,3) la fonction acyle est préservée, cependant que la rupture de l'hérérocycle est seulement régiosélective puisque l'hydroxyamide **IVc** (voie *c*) est également obtenu à côté du régioisomère prépondérant **IVb**. En présence d'hémolymphe diluée de chenille ou de criquet, la voie *a* est concurrencée par la voie d'hydrolyse *b*. En revanche, la voie *a* devient le mode d'hydrolyse très prépondérant avec l'α-chymotrypsine, et le mode exclusif et extrèmement rapide en présence des tissus corps gras ou mésentéron du criquet. On doit donc conclure que la *N*-acylaziridine **I** se comporte bien comme un proinsecticide potentiel en masquant réversiblement le carboxylate **III**, contrairement au β-hydroxyamide **IVb**, ce qui a été confirmé par des expériences *ex vivo* conduites avec le criquet.

Introduction

For some time we have been interested in developing proinsecticides which reversibly mask molecules where acids,[1,2] especially fluorinated acids,[3–6] and/or β-ethanolamines are considered as the active principles.[7] We restricted our choice for the masking structures to proinsecticides designed to have hydrolysis as the "activation mode" (*i.e.*, unmasking of the active principle). The potential of the following structures was studied: fluorinated esters,[5] enol esters[6] and recently Δ^2-oxazolines-1,3.[8] In the latter case, it appeared that, depending on the substituents of the heterocycle, the *in vitro* hydrolysis in

† *Cf.* refs. 5 and 6 for parts I and II, respectively.



Scheme 1 The hydrolysis pathways of *N*-acylaziridine **I**.

locust or caterpillar haemolymph resulted in the formation of either β-hydroxyamides or of the corresponding carboxylates. Moreover, *ex vivo* experiments in locusts indicated, for some oxazolines, a more pronounced unmasking of the carboxylate than *in vitro* experiments in locust haemolymph.[8]

Taking into account the potential of the amide structure as a proinsecticide of the acyl moiety, it seemed interesting to study the particular amide structure represented by *N*-acylaziridines. In fact, the fluorinated *N*-acylaziridine **I** (*cf.* Scheme 1), which was synthesized as the precursor of the corresponding Δ^2-oxazolines-1,3,[8] exhibited noticeable biological activity with respect to some pest or insect species during contact testing.[9] From the "proinsecticide perspective", for a better understanding of the overall behaviour of this compound in insects a study of its metabolization was thus justifiable in order to (*i*) appreciate the extent of the activation and (*ii*) detect possible further metabolization of the active principle(s). Previously, to monitor the behaviour of xenobiotics in biological insect media susceptible to triggering metabolization, such as haemolymph, malpighian tubules or fat body, we focussed on techniques allowing for chromatographic or spectroscopic analysis without any sample pretreatment, other than simple centrifuging. In the case of fluorinated xenobiotics, ^{19}F NMR is a very convenient technique due to the absence of endogenous fluorinated entities in most living tissues. The advantages of this technique have been used for the direct monitoring of fluorinated drugs in mammal tissues,[10–12], of fluorinated pesticide in plants,[13] for the determination of fluorinated pesticide residues in foods[14] and in our laboratory to study the unmasking of fluorinated enol esters,[6] esters[5] and recently Δ^2-oxazolines-1,3.[8]

The aim of this work was to gain insight into what occurs to the fluorinated *N*-acylaziridine **I** when it is applied to the insect integument either by pulverization or by tarsal contact, that is to determine the potential of this substrate as a proinsecticide of fluorinated carboxylates and/or β-ethanolamines. Assuming that the penetration was effective due to the lipophilicity of **I** and its biological properties, we developed a model of the metabolization by *ex vivo* and especially *in vitro* assays with insects selected as models for the relative ease of their tissue sampling: the locust *Locusta migratoria* and the caterpillar *Mamestra brassicae*. We present now our results concerning the behaviour of **I** in locust and caterpillar tissues: haemolymph, fat-body and mesenteron, which are known for their hydrolase content,[15,16] using ^{19}F[^{1}H] NMR monitoring. For comparison, the effect of the commercial amidase α-chymotrypsin was also studied.

Experimental

Biological samples

The caterpillar (*Mamestra brassicae*) was reared in crowded conditions with a light/dark (L/D 16/8) photoperiod cycle and fed with a semi-artificial medium at the Unit of Phytopharmacy and Semi-chemicals (INRA Versailles). The African migratory locust *Locusta migratoria* was reared according to Louveaux *et al.*[17] under crowded conditions with an L/D 12/12 photoperiod cycle and fed on wheat seedlings and bran in the Laboratoire de Biologie Evolutive et Dynamique des Populations (Université de Paris Sud). The mean weight was 922 ± 33 mg for male locusts and 1475 ± 26 mg for the females, with a 76% water ratio.

The α-chymotrypsin from bovine pancreas was supplied by Sigma. One unit of the enzyme will hydrolyse 1.0 μmol min^{-1} of *N*-benzoyl-L-tyrosine ethyl ester at pH 7.8 at 25 °C.

***In vitro* assays.** Haemolymphs sampled from adult locusts (male and female, pH 7.4) or caterpillars at the last larval instar were centrifuged for 1 min, then diluted 8 times with phosphate buffer (0.1 M, pH 7.4) and incubated with the substrate ([substrate] = 5×10^{-4} M). Five percent of DMSO-d_6 was added in order to render the lipophilic substrate soluble and for locking the NMR apparatus.

For the locust fat body 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: the bottom consisting of tissue fragments, the aqueous fraction and the lipidic supernatant. To 475 μl of the intermediate aqueous fraction, an appropriate amount of the *N*-acylaziridine **I** or of β-hydroxyamide **IVb** in phosphate buffer–DMSO-d_6 solution was added, to obtain substrate solutions of 5×10^{-4} M and 5% (v/v) of DMSO-d_6.

To prepare the locust mesenteron samples locust caeca (pH 6.4–7) was ground without any dilution and centrifuged. To 475 μl of the supernatant tissues an appropriate amount of a stock solution of the substrate **I** or **IVb** was added to obtain the same final substrate solutions as previously.

A 500 μl sample of a 5×10^{-4} M solution of substrate **I** in DMSO-d_6 phosphate buffer (5 : 95) was incubated with α-chymotrypsin (62.5 units).

***Ex vivo* assays.** An aliquot (20 μl per insect) of a 10^{-2} M solution of substrate **I** or β-hydroxyamide **IVb** in phosphate buffer–isopropanol 95 : 5 (v/v) was syringed (time t_0) into the haemolymph of living locusts (homogenous set of 8 locusts), through the intersegmental membrane of the abdomen. After 15 min, haemolymph was sampled from the 8 locusts (*ca.* 20 μl per insect) and diluted to a 1 : 4 ratio with phosphate buffer (0.1 M, pH 7.4) containing an initial amount of DMSO-d_6 resulting in a 5% (v/v) proportion in the diluted solution.

Structural characterization

The following instruments (and abbreviations) were used: an IR Bruker IFS 25 IR-FT spectrometer (ν or δ/cm^{-1}, S = strong, M = medium, W = weak, s = sharp, b = broad); a Bruker AC300 spectrometer operating at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C[^{1}H] NMR [the chemical shifts (δ) are reported relative to the TMS resonance for solutions in $CDCl_3$; the symbols for the multiplicities are: t = triplet, d = doublet, m = multiplet, s = singlet]; Cq denotes a quaternary carbon atom.

For the ^{19}F[^{1}H] NMR the Bruker AC300 spectrometer was operated at 282 MHz, the chemical shifts are reported relative to the resonance peak of CF_3COOH (5% in D_2O, v/v) for solutions in DMSO-d_6–phosphate buffer (5 : 95 v/v). Spectra were run with ^{1}H decoupling with the following instrumental conditions: sweep width 2000 Hz, pulse angle 90°, delay 15 s, computer resolution 0.5 Hz per point, number

of scans 104, memory size 8K, line exponential multiplication of the FID 1 Hz. ^{19}F relaxation times measured by the inversion recovery method were 4.25 s for *N*-acylaziridine **I** in phosphate buffer solution and 6.25 s for the *p*-fluorobenzyl alcohol (internal standard **IS**) under the same conditions.

Elemental analysis was performed at the Centre de Microanalyses de l'Université Pierre et Marie Curie. Whenever a satisfactory elemental analysis could not be obtained for a new compound, a high resolution mass spectrum (HR-MS) was obtained at the Laboratoire de Spectrométrie de Masse (Université de Mont-Saint-Aignan, Rouen) on a Jeol AX 500 spectrometer (resolution of 500) equipped with a PDP11 data system, using direct introduction either in the electron impact (EI 70 eV) or in the chemical ionization (CI, isobutane) modes.

Reagents and chemicals

4-Fluorophenylacetic acid **III** and 2-methylaziridine **II** were supplied by Aldrich.

2-Methyl-*N*-(4-fluorophenylacetyl)aziridine I. This compound was obtained by condensation of 1 equiv. of **II** and 1 equiv. of **III** with 1 equiv. of dicyclohexyl carbodiimide (DCC) in CH_2Cl_2. After filtration and evaporation of the solvent, hexane was added to precipitate the remaining DCC and the dicyclohexylurea (DCU) formed. Then, the crude *N*-acylaziridine **I** resulting from filtration and evaporation of the hexane was distilled at $T_{b0.7\ mmHg}$ 95 °C, yield 60% resulting on standing in a white solid, F_{inst} 34 °C. IR (KBr): 3058 (s, M, $\nu_{=C-H}$), 2978, 2933, (s, M, ν_{C-H}), 1686 (s, S, $\nu_{C=O}$), 1513 (s, S, $\nu_{C=C}$), 1223 (s, S, ν_{C-F}), 790 (s, M, γ_{C-H} *p*-disub. arom.). ^1H NMR ($CDCl_3$) δ/TMS: 7.25 (2H, m, *meta* to F in C_6H_4F); 7.00 (2H, m, *ortho* to F in C_6H_4F), 3.68 (2H, s, CH_2Ar), aziridinyl protons: 2.45 (1H, m, CH), 2.34 (1H, d, $J_{cis} = 5.8$ Hz) and 1.91 (1H, d, $J_{trans} = 3.4$ Hz), 1.22 (3H, d, $^3J = 5.4$ Hz, CH_3). ^{13}C NMR ($CDCl_3$) δ/TMS: 183.1 (C=O), 161.8 ($^1J_{CF} = 243.7$ Hz, Cq of C_6H_4F), 130.8 ($^3J_{CF} = 7.8$ Hz, CH of C_6H_4F), 130.3 ($^4J_{CF} = 3.2$ Hz, Cq of C_6H_4F), 115.3 ($^2J_{CF} = 21.3$ Hz, CH of C_6H_4F), aziridinyl carbons: 43.2 (CH_2), 33.2 (CH); 31.5 (CH_2Ar); 17.5 (CH_3). HR-MS (EI): accurate and observed *m/z* for the M^+ ion were respectively 193.0903 and 193.0898 daltons, this concordance ($\Delta M = 0.0005$) agrees with the molecular weight of 193 for compound **I**.

Hydroxyamides IVb and IVc. These were obtained by condensation of 1 equiv. of 1-amino-2-propanol for **IVb** and of 2-amino-1-propanol for **IVc** with 1 equiv. of **III**, using 1 equiv. of DCC in CH_2Cl_2. After filtering off DCU and evaporation of the solvent, the crude hydroxyamides **IVb** and **IVc** were purified by TLC (Silica Merck GF 254; eluent *n*-heptane–ethylacetate 50 : 50, with 5% methanol added).

N-(2-Methyl-2-hydroxyethyl)-(4-fluorophenyl)acetamide ***IVb***. $R_f = 0.125$; 61% yield, white solid, F_{inst} 82 °C. Elem. anal. (% calc., found): C 62.54, 62.57; H 6.63, 6.63; N 6.63, 6.73. IR (KBr): 3307 (b, M, ν_{N-H}), 3093 (s, M, $\nu_{=C-H}$), 2982, 2931, 2885 (s, M, ν_{C-H}), 1642 (s, S, $\nu_{C=O}$ amide I), 1556 (s, S, δ_{N-H} amide II), 1515 (s, S, $\nu_{C=C}$), 1245 (s, S, ν_{C-F}), 828 (s, M, ν_{C-H} *p*-disub. arom.). ^1H NMR ($CDCl_3$) δ/TMS: 7.25 (2H, m, *meta* to F in C_6H_4F), 7.05 (2H, m, *ortho* to F in C_6H_4F), 5.89 (1H, NH), 2.18 (1H, OH), 3.56 (2H, s, CH_2Ar), AMX system ($\Delta\nu/J = 6.1$) complicated for the AM part by coupling with the NH proton: 3.81 (1H, m, H_X), 3.49 (2H, s, CH_2Ar), 3.22 (1H, m, H_M) and 3.09 (1H, m, H_A), 1.10 (3H, d, $^3J = 6.3$ Hz, CH_3). ^{13}C NMR ($CDCl_3$) δ/TMS: 172.0 (C=O), 162.0 ($^1J_{CF} = 244.3$ Hz, Cq of C_6H_4F), 130.8 ($^3J_{CF} = 8$ Hz, CH of C_6H_4F), 130.5 ($^4J_{CF} = 3.3$ Hz, Cq of C_6H_4F), 115.6 ($^2J_{CF} = 21.2$ Hz, CH of C_6H_4F), 66.8 (CH), 47.1 (CH_2), 42.0 (CH_2Ar), 20.6 (CH_3). HR-MS (positive CI, 10^{-5} torr isobutane): accurate and observed *m/z* for the MH^+ ion were respectively 212.1087 and 212.1085 daltons, this concordance ($\Delta M = 0.0002$) agrees with the molecular weight of 211 for compound **IVb**.

N-(1-Methyl-2-hydroxyethyl)-(4-fluorophenyl)acetamide ***IVc***. $R_f = 0.18$; 62% yield, white solid, F_{inst} 48 °C. Elem. anal. (% calc., found): C 62.54, 62.36; N 6.63, 6.70; H 6.63, 6.72. IR (KBr): 3269 (b, M, ν_{N-H}), 3074 (s, M, $\nu_{=C-H}$), 2971, 2939, 2874 (s, M, ν_{C-H}), 1645 (s, S, $\nu_{C=O}$ amide I), 1564 (s, S, δ_{N-H} amide II), 1515 (s, S, $\nu_{C=C}$), 1234 (s, S, ν_{C-F}), 828 (s, M, γ_{C-H} *p*-disub. arom.). ^1H NMR ($CDCl_3$) δ/TMS: 7.25 (2H, m, *meta* to F in C_6H_4F), 7.05 (2H, m, *ortho* to F in C_6H_4F), 5.68 (1H, NH), 2.89 (1H, OH), 3.53 (2H, s, CH_2Ar), ABX system complicated by the coupling of the X part with the methyl protons (not fully resolved): 4.04 (1H, m, CH_X), *ca.* 3.62 (1H, m, H_B) and 3.48 (1H, m, H_A); 1.11 (3H, d, $^3J = 6.6$ Hz, CH_3). ^{13}C NMR ($CDCl_3$) δ/TMS: 171.5 (C=O), 162.1 ($^1J_{CF} = 244.6$ Hz, Cq of C_6H_4F), 130.9 ($^3J_{CF} = 7.9$ Hz, CH of C_6H_4F), 130.4 ($^4J_{CF} = 3.3$ Hz, Cq of C_6H_4F), 115.8 ($^2J_{CF} = 21.2$ Hz, CH of C_6H_4F), 66.9 (CH_2), 47.9 (CH), 42.8 (CH_2Ar), 16.9 (CH_3).

Results and discussion

Feasibility of ^{19}F NMR monitoring of the behaviour of *N*-acylaziridine I and its stability under various pH conditions

From the "proinsecticide perspective" the fluorocarboxylate **III** is expected to arise from the activation of compound **I** (*cf.* Scheme 1). To determine the feasibility of ^{19}F NMR monitoring, a comparison of the chemical shifts of the two compounds **I** and **III** was thus necessary. Moreover, to differentiate the possible effect of the pH of the biological media from enzymatic catalysis, we studied the stability of *N*-acylaziridine **I** over periods of at least 3 h in buffered solutions at pH 7.4, which is the locust physiological pH, and at pH 7.8, which is the optimum pH for α-chymotrypsin function. To represent a slightly acidic medium, pH 6.3 was also tested.

^{19}F NMR protocol. The lipophilicity of substrate **I**, which was deliberately designed as a contact insecticide ($\log P \approx 2.9$[18,19]), requires the use of a water-soluble organic cosolvent for its solubilization in phosphate buffer. We chose 5% DMSO-d_6 (also used for NMR locking) since under such conditions the hydrolase enzymatic activity of locust haemolymph is nearly preserved.[1] Moreover, due to the high sensitivity of ^{19}F chemical shifts to the medium conditions (related to the very wide δ ^{19}F range) we worked at constant temperature, at controlled pH in buffered solution and at comparable substrate concentrations. *p*-Fluorobenzyl alcohol was selected as the internal fluorinated standard **IS** for its structural analogy with the substrate, and in fact we observed similar chemical shifts and T_1 for **I** and **IS** (*cf.* Experimental). The internal standard allowed precise quantification and facilitated signal identification when varying the medium conditions. We used NMR conditions resulting in a spectrum acquired over 30 min, optimized from the standpoints of sensitivity and duration, with a flip angle of 90° and 15 s pulse delay.

With such conditions, the results that are presented in Table 1 demonstrate the feasibility of a simultaneous monitoring of the substrate **I** and its expected hydrolysis product **III** in buffered and diluted biological media, since a $\Delta\delta$ of approximately 1.4 ppm is observed in phosphate buffer. Moreover, the ^{19}F NMR signals for both compounds **I** and **III** are sufficiently different from the selected internal standard **IS** to allow quantification. It also appears that pH 7.8 clearly represents the best stability conditions for **I** since at t_0, which is in fact an average measure over a 30 min period (104 scans), there is no significant formation of carboxylate **III**. In fact, the carboxylate signal appears only in the "4 h" spectrum in a very low proportion (approximately 6%, *cf.* Table 2). It is accompanied by a new signal at −40.17 ppm (17%), which was assigned to the hydroxyamide **IVb** ^{19}F resonance by overloading with an authentic sample. The phosphate buffer at pH 7.4 gives more

Table 1 Feasibility of ^{19}F monitoring of *N*-acylaziridine **I**. ^{19}F chemical shifts[a] of *N*-acylaziridine **I**, carboxylate **III** and the internal standard **IS** in phosphate buffer at various pH

	pH 6.3	pH 7.4	pH 7.8
I[b]	−40.08	−40.07	−40.07
III[b]	−41.48	−41.49	−41.48
IS[c]	−39.39	−39.39	−39.39

[a] The chemical shifts are reported relative to the resonance peak of CF_3COO^- (aqueous solution at 5% v/v) used as an external reference. See Experimental for other NMR conditions. [b] [**I**] = [**III**] = 5×10^{-4} M. [c] [**IS**] = 10^{-3} M.

pronounced changes since some traces of hydroxyamide **IVb** are already observed in the "30 min" spectrum. The substrate **I** is completely transformed over a *ca.* 4 h period in the acidic buffer at pH 6.3, which represents the worst conditions from the stability standpoint. The hydroxyamide **IVb**, which is already formed in significant amounts after a 30 min period, is accompanied in the 4 h spectrum by a signal at −40.26 ppm assigned to the hydroxyamide **IVc** resonance by overloading with an authentic sample synthesized independently.

The behaviour of compound **I** under non-enzymatic hydrolytic conditions can be summarized as follows (*cf.* Scheme 1 and Table 2): (*i*) formation of the carboxylate **III**, which is favoured by basic media, results from the expected cleavage of the *N*-acyl bond of compound **I** (pathway *a*); (*ii*) formation of the hydroxyamides **IVb** and **IVc** requires cleavage of the C^2–N and C^3–N bonds, respectively, of the heterocycle in compound **I** (pathways *b* and *c*). It is noticeable that this behaviour, which is favoured by acidic media, is highly regioselective[20,21] since pathway *b* predominates.

^{19}F NMR monitoring of the *in vitro* behaviour of *N*-acylaziridine I in the presence of natural or commercial biological media

We verified that the ^{19}F NMR spectra of biological media are free of signals, even after 10 000 scans, when performed in the range covering the chemical shifts of both the *N*-acylaziridine **I** and the corresponding carboxylate **III**.

Behaviour of I in caterpillar haemolymph at 23 °C and pH 7.4. The results presented in Table 2 show evident effects of the caterpillar haemolymph, even when very dilute (12.5% in phosphate buffer at pH 7.4), on the ^{19}F NMR characteristics of *N*-acylaziridine **I** and on its behaviour. We notice firstly, in the t_0 spectrum, both a significant enlargement of the ^{19}F NMR signal of substrate **I** (*cf.* $\delta_{1/2}$ presented in Table 3) and a slight variation ($\Delta\delta$) of its chemical shift: −40.05 ppm compared to *ca.* −40.07 ppm (*cf.* Table 3). Conversely, the signal of the internal standard **IS** undergoes only a slight enlargement (*cf.* $\delta_{1/2}$ in Table 3) without significant $\Delta\delta$. We propose as an explanation for the signal enlargement of **I** the same hypothesis as was previously proposed for other substrates and biological media,[5,6] that is substrate association with some endogenous component of the biological medium. A second effect is the catalytic influence of the biological medium, which clearly increases the reaction rate of **I** *via* pathway *b* and, especially, pathway *a*. In fact, in

Table 2 Stability of *N*-acylaziridine **I** under different pH conditions and its behaviour in the presence of various biological media

	Stability of **I** in phosphate buffer				Behaviour of **I** in biological media		
Time/h	Buffer pH 6.3 23 °C	Buffer pH 7.4 23 °C	Buffer pH 7.4 30 °C	Buffer pH 7.8 23 °C	Caterpillar haemolymph 23 °C	Locust haemolymph 30 °C	α-Chymotrypsin 23 °C
% **I**							
t_0	66	93	91	100	81	89	65
2	5	72	48	90	0	30	8
3	0	57	35	86		23	5
4	0	48		77	0		2
x		0 (13 h)					0 (5 h)
% **III**							
t_0	0	0	0	0	0	0	35
2	0	0	7	0	59	23	88
3	0	7	6	0		29	88
4	0	7		6	61		91
x		7 (13 h)					93 (5 h)
% **IVb**							
t_0	34	7	9	0	19	11	0
2	87(8)[a]	28	45	10	41	47	4
3	92(8)[a]	36	59	14		48	7
4	92(8)[a]	45		17	39		7
x		93 (13 h)					7 (5 h)

[a] The number in brackets represents the percentage of hydroxyamide **IVc** formed.

Table 3 Characteristics of ^{19}F NMR signals of the *N*-acylaziridine **I**, carboxylate **III**, the β-hydroxyamide **IVb** and of the internal standard **IS** in different media

	δ ($\delta_{1/2}$/Hz)			
	Phosphate buffer, pH 7.4	Caterpillar haemolymph[a]	Locust haemolymph[a]	α-Chymotrypsin[b]
I	−40.07(2.25)	−40.05(16.92)	−40.11(5.64)	−40.07(2.82)
IS	−39.39(2.25)	−39.40(5.64)	−39.44(5.64)	−39.38(2.25)
III	−41.49(2.25)	−41.49(7.05)	−41.54(2.54)	−41.49(2.54)
IVb	−40.18(2.25)	−40.17(3.95)	−40.21(3.10)	−40.17(2.26)

[a] 12.5% in phosphate buffer at pH 7.4. [b] 62.5 units.

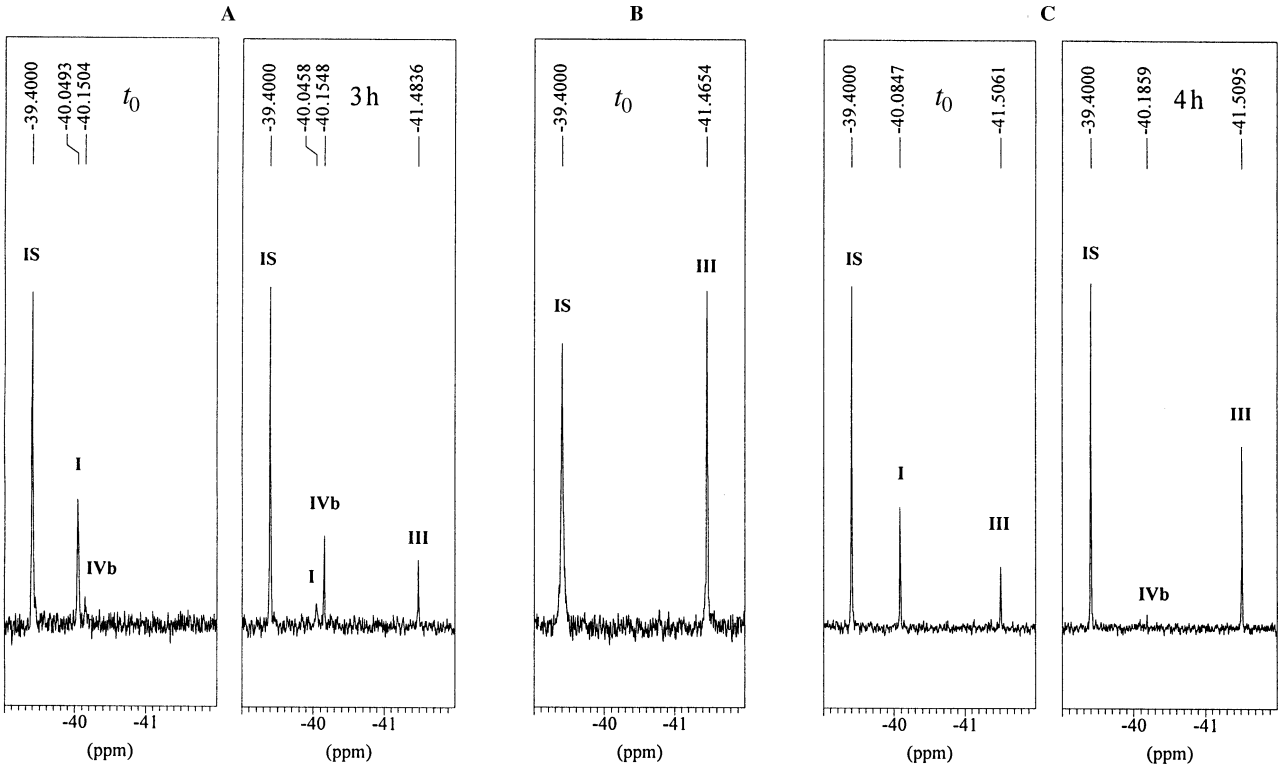


Fig. 1 Comparison of the behaviour of *N*-acylaziridine **I** in the presence of biological media, based upon ^{19}F NMR for *in vitro* assays. (*A*) In diluted locust haemolymph (12.5% in phosphate buffer, pH 7.4, 30 °C). (*B*) In concentrated locust mesenteron (t_0). *Notes*: in concentrated fat-body the same evolution was observed and under the same conditions the β-hydoxyamide **IVb** is stable (t_0 to 7 h). (*C*) In the presence of α-chymotrypsin (62.5 units in phosphate buffer, pH 7.8, 23 °C).

the t_0 spectrum we already observed the signal of the hydroxyamide **IVb** at −40.17 ppm and in the 2 h spectrum the signal of **I** had disappeared, leading to an approximate distribution of 40 : 60 for hydroxyamide **IVb** and carboxylate **III**. The whole transformation requires 13 h in phosphate buffer (*cf.* Table 2). Thus, at this stage it appears that compound **I** acts partly as a proinsecticide of the carboxylate **III**.

***In vitro* monitoring of the behaviour of I and IVb in locust haemolymph at 30 °C and pH 7.4.** A preliminary experiment in phosphate buffer at 30 °C indicated a faster evolution of *N*-acylaziridine **I** (*cf.* Table 2) since over a 3 h period the hydrolytic pathways *a* and *b* represent approximately 6 and 59%, respectively, compared to 7 and 45% over a 4 h period at 23 °C.

The evolution rates of *N*-acylaziridine **I** in locust [*cf.* Fig. 1(*A*)] and caterpillar haemolymphs are nearly comparable except for the enlargement of the signal, which is much less pronounced in the former case (*cf.* Table 3), meaning that there is catalysis for both pathways *a* and *b* (*cf.* Table 2). Formation of carboxylate **III** results from pathway *a* without any contribution from the hydrolysis of hydroxyamide **IVb** [*cf.* pathway *d* in Scheme 1] since during *in vitro* experiments in diluted caterpillar locust haemolymph we observed the stability of **IVb**.

It can be concluded that during *in vitro* experiments in locust haemolymph (and in caterpillar haemolymph) *N*-acylaziridine **I** behaves partly as a proinsecticide of carboxylate **III**, obtained exclusively according to pathway *a* without participation of pathway *d*. But, it cannot be excluded that pathway *a* could be increased *in vitro*, in more concentrated haemolymph (of caterpillar or locust), in the presence of other insect tissues or of commercial enzyme, or lastly, during *ex vivo* conditions in living insects. Similarly, it cannot be excluded that pathway *d* could be triggered under such conditions.

Behaviour of I in the presence of α-chymotrypsin at 23 °C and pH 7.8. The first evidence concerns the width of the signal measured by $\delta_{1/2}$, which appears to be approximately the same as in the phosphate buffer t_0 spectrum and in the other spectra after various times (*cf.* Table 3). The effect of this enzyme, which is known to hydrolyse peptidic chains and also various esters and amides possessing an aromatic group[22] is evidenced by the results presented in Table 2 and Fig. 1(*C*). The hydrolysis of **I**, which is nearly complete after 3 h, follows almost exclusively pathway *a* with the formation of *ca.* 90% **III**. The formation of hydroxyamide **IVb** *via* the hydrolytic pathway *b* is now very inefficient, as observed in the blank experiment in phosphate buffer at pH 7.8 (*cf.* Table 2).

Behaviour of I and IVb in the presence of locust fat body and mesenteron (*in vitro* assays at 23 °C). The enzymatic effects of these concentrated tissues on the behaviour of *N*-acylaziridine **I** are quite similar and spectacular since as early as the first ^{19}F NMR spectra, that is after less than half an hour, the substrate is completely and unequivocally hydrolysed *via* pathway *a* into the fluorinated carboxylate **III** [Fig. 1(*B*)]. Under the same conditions, the hydroxyamide **IVb** remains unchanged even after 7 h. These two results clearly establish that *N*-acylaziridine **I** is a proinsecticide of the carboxylate **III**, acting without any contribution from the hydroxyamide **IVb**. It appears that α-chymotrypsine also exercises a similar effect on the hydrolysis of substrate **I** to these locust tissues, although the reaction is less rapid.

***Ex vivo* monitoring of the behaviour of I in locust haemolymph**

To determine the global effect of the metabolism of *N*-acylaziridine **I**, *ex vivo* experiments were performed by injecting separately this substrate and hydroxyamide **IVb** into different sets of living locusts from which haemolymph was sampled at various time intervals. It appears unambigously that carboxylate **III** is the only fluorinated entity detectable in

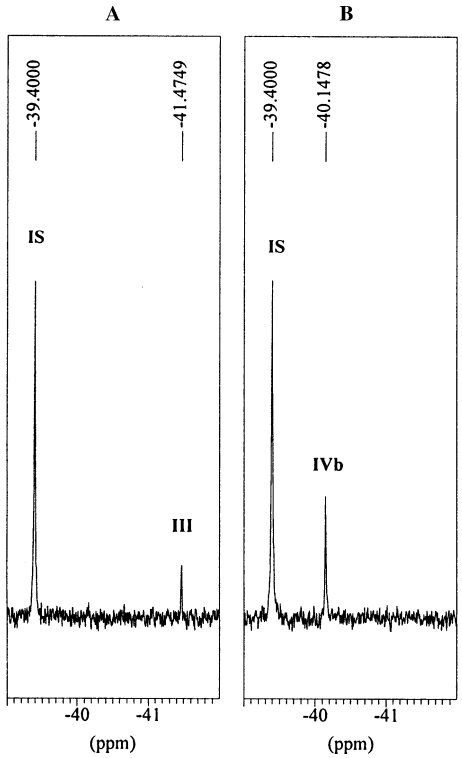


Fig. 2 *Ex vivo* assays concerning the behaviour of β-hydroxyamide **IVb** and of *N*-acylaziridine **I** (haemolymph monitoring). (*A*) *N*-acylaziridine **I**. (*B*) Hydroxyamide **IVb**. Conditions: sets of 8 locusts, incubation time 15 min, then sampling of the haemolymph.

the haemolymph only 15 min after the injection of **I**, while in the case of **IVb** the only signal observed over time corresponds to **IVb** itself (*cf.* Fig. 2). This assay reveals that in living locusts, *N*-acylaziridine **I** behaves as a proinsecticide of carboxylate **III**, which agrees with the previous observations resulting from *in vitro* experiments in locust fat body and mesenteron.

Conclusion

Direct ^{19}F NMR monitoring of insect biological media, used during *in vitro* or *ex vivo* assays, constitutes a very rapid and convenient analytical tool for exploring the potential of new structures for fluorinated proinsecticides. In fact, even a conventional 200–300 MHz NMR apparatus is sufficient to perform the monitoring of the metabolizations.

It was thus demonstrated that the fluorinated *N*-acylaziridine **I** behaves as a proinsecticide of the fluorinated carboxylate **III** as expected. In fact, the efficiency and the importance of pathway *a* corresponding to the "activation" step increases, depending on the biological medium conditions, in the following order: diluted locust haemolymph (*in vitro*) ≈ diluted caterpillar haemolymph (*in vitro*) < α-chymotrypsin ≪ concentrated locust fat body and mesenteron (*in vitro*) ≈ locust haemolymph (*ex vivo*).

In insects, the hydrolytic pathway *a* is probably triggered by hydrolases present in pure fat body and haemolymph and particularly by enzymes similar to α-chymotrypsin[16] in mesenteron, but which act more efficiently on **I** due to their simultaneous intervention. In contrast with **I**, the hydroxyamide **IVb** resulting from pathway *b* cannot be considered as a proinsecticide of the carboxylate **III** due to its great stability under all enzymatic hydrolytic conditions tested (pathway *d* was not observed).

This work is being pursued in order to determine possible chiral catalysis of pathway *a* occurring during *in vitro* or *ex vivo* assays in biological insect media. Moreover, due to the spectacular activation of **I** in locust mesenteron, it would certainly be useful to realize tests on insects by ingestion using treated baits.[4]

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