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Potential proinsecticides of fluorinated carboxylic acids and β-ethanolamines IV. Evaluation of the Δ^2-oxazoline-1,3 structure by ^{19}F NMR monitoring of the in vitro metabolism in locust tissues☆

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Abstract

The enzymatic effect of locust tissues upon hydrolysis of the fluorinated Δ^2-oxazoline-1,3 **Ia** was elucidated using ^{19}F[^{1}H] NMR monitoring.

In a phosphate buffer at pH = 7.4 (mean physiological pH of locust tissues), the substrate **Ia** hydrolyses slowly into the corresponding fluorinated hydroxylamide **VIa**.

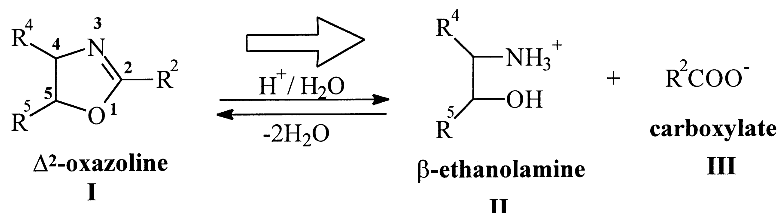
If diluted, locust haemolymph (12.5% in phosphate buffer) catalyses slightly this hydrolytic pathway, it overall triggers the unmasking of carboxylate **IIIa**, corresponding to the expected proinsecticide behaviour of **Ia**. This behaviour is spectacularly almost the unique reaction observed during in vitro assays in concentrated fat body and mesenteron. Inasmuch as β-hydroxylamide **VIa** is not hydrolysed into carboxylate **IIIa** during such conditions, it must be concluded that carboxylate formation exclusively results from hydration and hydrolysis of substrate **Ia** via the aminoester **Va**. The formation of this intermediate aminoester is demonstrated by complementary assays. The enzymes supposed to intervene are of the α-chymotrypsine type for the first step (hydration) and of the esterase type for subsequent hydrolysis of intermediate aminoester **Va**.

Thus, this work constitutes the first example of a Δ^2-oxazoline-1,3 structure exploited for elaborating proinsecticides of carboxylates **III** and/or β-ethanolamines **II** based on enzymatic activation in insects. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Δ^2-oxazoline-1,3; Proinsecticide; Metabolism; ^{19}F NMR monitoring; Insect biological media; African locust *Locusta migratoria*

1. Introduction

From a retrosynthetic standpoint the Δ^2-oxazoline-1,3 structure **I** results from the condensation between a β-ethanolamine **II** and a carboxylic acid **III** by dehydration. So structure **I** can be considered as reversibly masking both moities **II** and **III**, since by acid hydrolysis, the reversed reaction is expected (Equation 1).



Eqn. 1. Retrosynthetic scheme of the Δ^2-oxazoline-1,3 structure.

Meyers and co-workers have intensively exploited this reversible masking for the development of oxazoline mediated asymetric synthesis using these versatile chiral

☆ See [19,35,36] for parts I–III.

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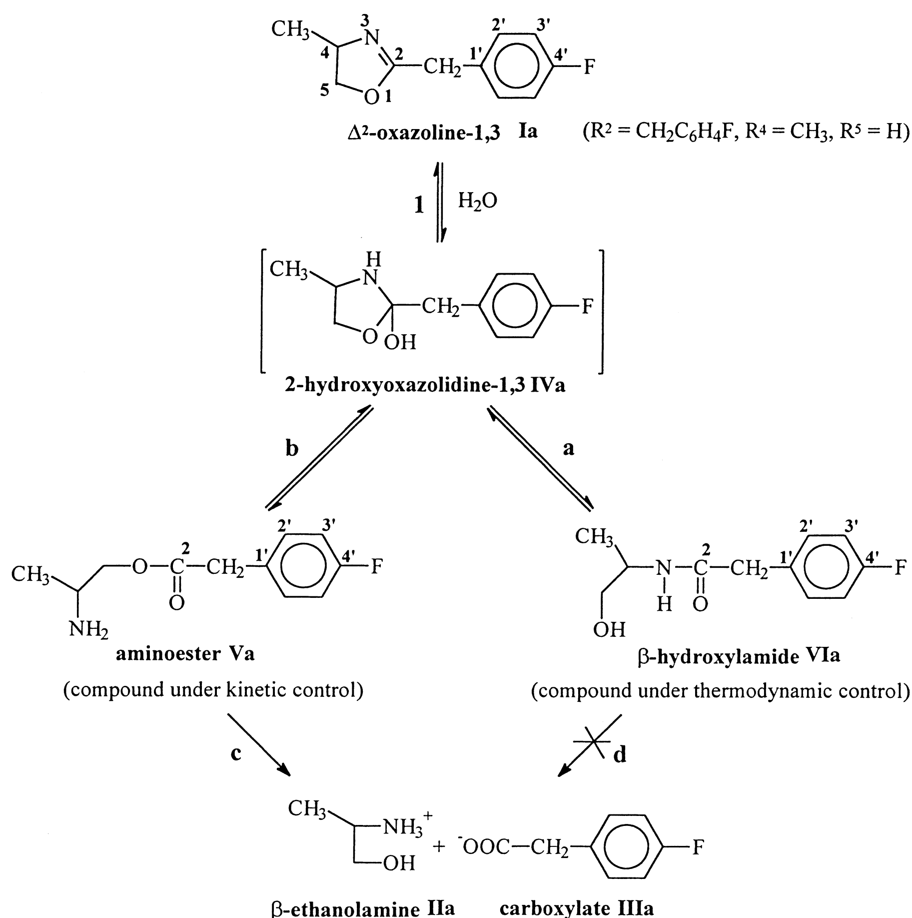
auxiliaries groups [1,2], particularly for the elaboration of α-substituted carboxylic acids in high chemical and optical yields [3,4].

In a "proinsecticide perspective" the metabolisation of simple oxazolines chosen as models has been studied in our laboratory for about 10 years. For monitoring the expected demasking of carboxylates [5,6] and β-ethanolamines [7] selected as active principles, ^{1}H NMR [8,9], HPLC [10] and ^{19}F NMR [11,12] were used as analytical tools during in vitro assays in locust haemolymph.

A few years ago Vorbrüggen and Krolikiewicz developed non steroidal anti-inflammatory drugs (NSAID) based upon Δ^2-oxazolines-1,3 containing such masked carboxylic acids [13]. These derivatives, designed as potential prodrugs [14], hydrolyse gradually in vivo under physiological pH and enzymatic action, into the starting NSAID.

This paper deals with the fluorinated Δ^2-oxazoline-1,3 **Ia**, designed both as a *contact insecticide* due to its pronounced lipophilicity: $\log P = 2.9$ according to the Rekker indexes [15], and as a *proinsecticide* of the corresponding carboxylate **IIIa** and/or β-ethanolamine **IIa** (cf. Scheme 1). In fact, oxazoline **Ia** exhibited noticeable biological activities with respect to some insect or pests species [12]. Thus, it appeared necessary to study its metabolism in insects to appreciate the extent of the unmasking of the active principle(s) and their possible subsequent catabolisation(s). Assuming that the penetration in insects was effective when **Ia** was applied on their integument, we developed in vitro assays in the tissues of the locust *Locusta migratoria* for modelling the true metabolic processes in living insect. The selection of those tissues is connected to their known potentialities to trigger hydrolysis of xenobiotics due to their hydrolases contents: haemolymph [9–12,16,17]; fat body [11,16,17] and mesenteron [18,19].

For monitoring the behaviour of fluorinated xenobiotics in biological tissues, ^{19}F NMR is a very convenient tool, having inherent advantages. First, most biological systems are devoid of NMR-detectable fluorinated endogenous compounds. This implies that all resonances observed can be unambiguously ascribed to the studied xenobiotic and its biodegradation products, without interference from endogenous or solvent nuclei. Second, fluorine 19 has a



Scheme 1. Hydrolysis pathways for Δ^2-oxazoline-1,3 **Ia**. Entities labelled $\mathbf{F_1}$ and $\mathbf{F_2}$ in the ^{19}F spectrum presented in Fig. 1 were, respectively, identified to intermediate aminoesters **Va** and **VIa**.

sensitivity to NMR detection that is 83% that of the sensitivity of ^{1}H, which sensitivity is obviously an important factor because of the usually low concentrations of xenobiotic compounds and their biodegradation products. Third, due to a chemical shift range of about 500 ppm, the ^{19}F nucleus is highly sensitive to its molecular surroundings, resulting in relatively large changes in chemical shifts as a result of biochemical modifications of a xenobiotic with few chances of peak overlap.

Numerous advantages of the fluorine spin labelling for NMR studies of biological systems have been recognised for more than 30 years [20], and related reviews have appeared [21–23]. Briefly, ^{19}F NMR spectroscopy has been applied to: monitoring of drugs in mammalian tissues developed by Malet-Martino and Martino [24,25]; pH measurement using fluoropyridoxol [26]; complexation study of carbonic anhydrase with a fluorosulfonamide [27] and also for agrochemical and environmental research [28–31], for the determination of fluorinated pesticides in foods [32], study of a fungicide in a pathogenic fungus [33], and as structural probe for internal amino acid packing in proteins [34]. In the "proinsecticide perspective", ^{19}F NMR was also used in our laboratory, to clarify the behaviour of fluorinated enol esters [35], esters [36] and recently, *N*-acylaziridines in insect tissues [12,19].

This work highlights the convenience of direct $^{19}F[^{1}H]$ monitoring to study the behaviour of the Δ^2-oxazoline-1,3 **Ia** during in vitro assays in locust haemolymph, fat body and mesenteron, and demonstrates that this nitrogen heterocycle is an effective structure for the elaboration of proinsecticides of carboxylic acids. As a comparison, the effects of commercial enzymes, α-chymotrypsine and a supported esterase were also studied.

2. Results and discussion

2.1. ^{19}F NMR monitoring of the behaviour of the Δ^2-oxazoline-1,3 Ia in a phosphate buffer at different pH

2.1.1. Feasibility of the monitoring

The feasibility of the ^{19}F monitoring was clear from the recording (cf. following protocol) of the ^{19}F spectra of the substrate **Ia** synthesised according to the Vorbrüggen method [13], and **IIIa** in a phosphate buffer at pH 7.4. Indeed, the chemical shifts of the two compounds were, respectively, −40.06 and −41.49 ppm relative to external reference CF_3COOH (cf. Table 1), i.e. sufficiently different to allow their quantitative measurement.

2.1.2. Detailed ^{19}F NMR protocol

The lipophilicity of the substrate ($\log P \sim 2.9$) required the use of a water-soluble organic co-solvent for its solubilisation in the phosphate buffer.

We chose 5% of DMSO-d_6, since in such conditions the hydrolases enzymatic activity of locust tissues is well preserved [9,11,19]. Due to the high sensitivity of ^{19}F chemical shifts to the medium conditions, we have been working under controlled temperature (25°C) and pH (buffered solution) and with comparable substrate concentration. *p*-Fluorobenzylalcohol was selected as the internal fluorinated standard **IS** for its structural analogy with the substrate. Effectively, we observed similar chemical shifts for **Ia** and **IS** and similar relaxation times T_1 (cf. Table 1). This authorises the use of peak integration for the quantitative following of the balance-sheet of the fluorinated entities. In addition, the internal standard facilitated signal identification when varying media conditions.

Table 1
^{19}F monitoring of the behaviour of Δ^2-oxazoline-1,3 **Ia** in phosphate buffer[a]

δ ppm [T_1] (s)	**Ia**[b] [4.94]	**IIIa** [4.97]	**IS**[c] [6.25]	F_1 = **Va**	F_2 = **VIa** [4.76]
At pH 7.4					
t_0 (%)	−40.06 (89)	−41.49 (0)	−39.40 (100)	−39.81 (4)	−40.27 (7)
1 h	(88)	(0)	(100)	(5)	(7)
2 h	(81)	(0)	(100)	(9)	(10)
3 h	(78)	(0)	(100)	(10)	(12)
4 h	(73)	(0)	(100)	(11)	(16)
10, 12 h	(35)	(3)	(100)	(18)	(44)
At pH 6.3					
t_0	(81)	(0)	(100)	(10)	(9)
1 h	(60)	(0)	(100)	(31)	(9)
2 h	(41)	(0)	(100)	(50)	(9)
3 h	(30)	(0)	(100)	(61)	(9)
4 h	(23)	(0)	(100)	(68)	(9)
12 h	(0)	(0)	(100)	(90)	(10)

[a] ^{19}F chemical shifts (δ are reported relative to the resonance peak of $CFCOO^-$ (5% v/v aqueous solution) used as an external reference. See Section 4 for NMR conditions and T_1 of **Ia**, carboxylate **IIIa**, internal standard **IS** and fluorinated entities F_1 and F_2. Evolution with time within the limits of the integration precision, the balance ([**Ia**] + [**IIIa**] + [**Va** = F_1] + [**VIa** = F_2]) = [**IS**]) = 100%.

[b] [**Ia**] = 10^{-3} M.

[c] [**IS**] = 10^{-3} M.

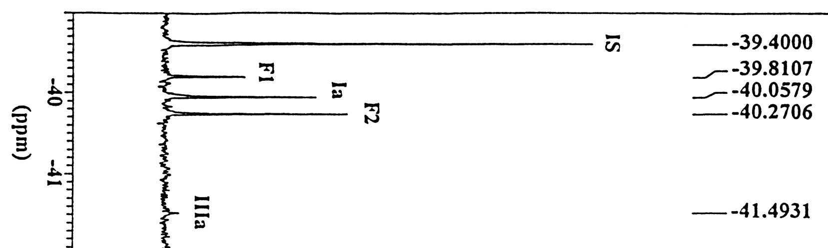


Fig. 1. ^{19}F NMR spectrum of a solution of Δ^2-oxazoline-1,3 **Ia** in phosphate buffer at pH 7.4 after 10 h (cf. also Table 1).

Optimised NMR conditions from the standpoints of sensitivity and duration resulted in spectrum acquisition over 30 min (cf. Section 4).

2.1.3. Behaviour of *Ia* in phosphate buffer at pH 7.4 (physiological) and 6.3

To appreciate subsequently the enzymatic effect of the biological media on the hydrolysis of the substrate **Ia**, we have first studied a possible pH effect for these media, using the same NMR conditions, in the phosphate buffer at pH = 7.4.

As early as the first spectrum at "t_0" time, which represents in fact an averaged evolution over a 30 min period, there is formation of two fluorinated entities labelled $\mathbf{F_1}$ at −39.81 ppm and $\mathbf{F_2}$ at −40.27 ppm, differing from **Ia**, **IS** and **IIIa**. The intensities of $\mathbf{F_1}$ and $\mathbf{F_2}$ increase slowly with time, cf. Table 1. On the "10 h" spectrum (cf. Fig. 1), there is also to notice the appearance of a very small signal at −41.4 ppm corresponding to the carboxylate **IIIa**. $\mathbf{F_2}$ was assigned to the β-hydroxylamide **VIa** by overloading with an authentic sample. Its formation can be rationalised according to pathway **1,a** in the Scheme 1, which implies the orthoimidate intermediate 2-hydroxy-oxazolidine-1,3 **IVa**, as generally assumed in the literature [37–42]. Both the formation of $\mathbf{F_1}$, which appears as a transient species, and of carboxylate **IIIa**, can be explained by the other hydrolytic cleavage possibility of the heterocycle **Ia** described in pathway **1,b** (Scheme 1). Thus, $\mathbf{F_1}$ would be the aminoester **Va**. Since this pathway **1,b** is known to be rather favoured by acidic conditions [39,41], where **Va** would be the kinetic product, we performed other assays with **Ia** at pH 6.3 (i.e. in about 10-fold more acidic solution than previously) in order to facilitate $\mathbf{F_1}$structure elucidation.

2.1.4. Structure of the fluorinated intermediate $\mathbf{F_1}$

*2.1.4.1. ^{13}C NMR monitoring of the evolution of **Ia** (c = 0.08 M) at pH 6.3.* Comparison of the ^{19}F NMR monitorings of the evolution of **Ia** in phosphate buffer at pH 7.4 and 6.3 (cf. Table 1) indicates an important increase in the formation of $\mathbf{F_1}$ at the lower pH after t_0 spectrum, with a very significant difference after 2 h: 50% at pH 6.3 compared to only 9% at pH 7.4. Thus, after 2 h standing at pH about ~6.3 (the buffer pH was doubtless modified by the high ratio of DMSO-d$_6$), the ^{13}C[^{1}H] spectrum of a 8×10^{-2} M solution of **Ia** in phosphate buffer–DMSO-d$_6$ (80–20%) solution was recorded in 12 000 scans. Beside the signals of **Ia** and **VIa** the spectrum showed a series of signals assignable to $\mathbf{F_1}$ (cf. Table 2). In the expanded region at about 175 ppm, it is of great importance to notice the presence of two Cq resonances both corresponding clearly to carbonyls, of amidic or ester character, which were assigned to the hydroxylamide **VIa** (= $\mathbf{F_2}$) and the aminoester **Va** (= $\mathbf{F_1}$).

*2.1.4.2. Esterase and α-chymotrypsine effects on **Va** observed by ^{19}F monitoring.* The hypothetical ester structure **Va** previously considered for $\mathbf{F_1}$, was confirmed by adding a commercial esterase to the diluted solution of **Ia** in a phosphate buffer at pH ~ 6.3, since after passage of 12 h transformation of **Ia** into **Va** (= $\mathbf{F_1}$) (90%) and **VIa** (= $\mathbf{F_2}$) (10%) was achieved, (cf. Table 1). The rapid increase in carboxylate **IIIa** observed (cf. Fig. 2C) correlates with the

Table 2
^{13}C chemical shifts of Δ^2-oxazoline-1,3 **Ia**, **VIa** (= $\mathbf{F_2}$) and **Va** (= $\mathbf{F_1}$) observed in the ^{13}C[^{1}H] spectrum of the mixture resulting from standing of **Ia** for 2 h in a phosphate buffer at pH ~ 6.3, DMSO-d6 (80/20)[a]

	CH_3	CH_2–O	CH–N	CH_2Ar	C_2	C'_1	C'_2	C'_3	C'_4
Ia	21.8	76.0	61.7	34.5	169.1	132.7	132.2	117.1	163.2
VIa = $\mathbf{F_2}$	17.5	65.9	48.8	40.6	175.2	132.7	132.3	117.0	163.3
Va = $\mathbf{F_1}$	16.1	67.4	47.9	43.1	175.1	131.1	132.9	117.0	163.2

[a] The chemical shifts δ (see Scheme 1 for carbon numbering) are reported relative to TMS as internal reference. N.S. = 12 000 scans, see Section 4 for other details.

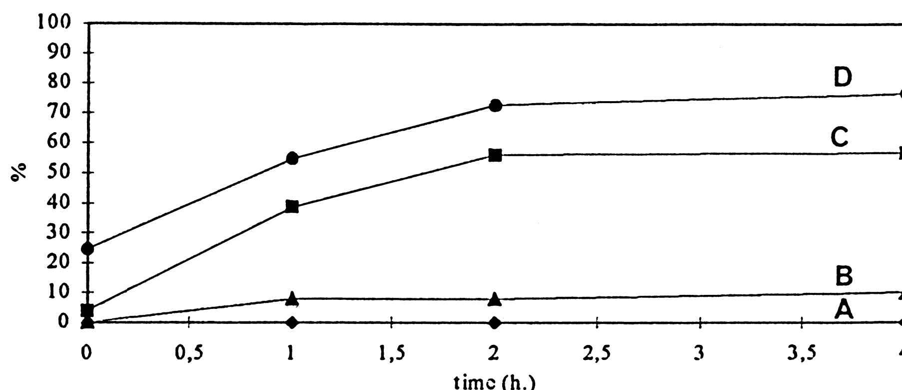


Fig. 2. ^{19}F NMR monitoring of carboxylate **IIIa** formation from **Ia** and from the intermediate $\mathbf{F_1} = \mathbf{Va}$ in the presence of an esterase or of α-chymotrypsine: A (◆) esterase on **Ia** (at pH 7.4); B (▲) α-chymotrypsine on **Ia** (at pH 7.8); C (■) esterase on **Va** (obtained from **Ia** after 12 h standing at pH 6.3); D (●) α-chymotrypsine on **Va** (at pH 6.3).

decrease of **Va** since the hydroxylamide **VIa** ratio remained essentially constant with time at this pH (cf. Table 1). This result thus confirmed the aminoester **Va** structure for $\mathbf{F_1}$. α-Chymotrypsine exercised an even more pronounced hydrolytic effect on **Va** (cf. Fig. 2D).

To summarise, under non-enzymatic hydrolytic conditions, the substrate **Ia** is slowly converted at the physiological pH 7.4 into the hydroxylamide **VIa** (pathway **1,a**) without very significant evolution of intermediate **Va** (formed according the pathway **1,b**) towards the carboxylate **IIIa** (pathway **c**). This pathway is made efficient by the presence of hydrolase: esterase or α-chymotrypsine.

2.2. *^{19}F NMR monitoring of the in vitro behaviour of Δ^2-oxazoline-1,3* **Ia** *in the presence of biological media: locust tissues, esterase and α-chymotrypsine*

It was previously shown that locust tissues such as haemolymph, fat body and mesenteron are free of detectable ^{19}F resonance over the range covering the signals of **Ia**, **IIIa** and **IS** [8,9,19,35,36] and in this work, we observed the same result with esterase and α-chymotrypsine samples.

As early as the "t_0" spectra evident effects of the biological media are observed, contrary to the phosphate buffer media with: (i) noisy baselines and (ii) peak broadening of some of the signals (particularly for **Ia** and **Va**) in caterpillar and locust diluted haemolymphs, and mostly in concentrated locust fat body and mesenteron (cf. Fig. 3A–C). For such ^{19}F NMR signal broadening, we put forward the explanation previously proposed for other fluorinated substrates interacting with locust [19,35,36] and caterpillar tissues [19] that is substrate association with some endogenous component of the biological media. It was also often observed that substrate association with macromolecules resulted in variation of the chemical shifts $\Delta\delta$ and enlargement $\delta_{1/2}$ of the signals. For instance, the association of human carbonic anhydrase with 3,5-difluorobenzenesulfonamide showed a dramatic decrease of longitudinal relaxation time T_1 [27]. In the present case, the T_1 determination was impossible because of substrate **Ia** reaction in the presence of biological media, thus the signal-enlargements where defined by the $\delta_{1/2}$ (Hz), but we did not observe very significant $\Delta\delta$ (cf. Table 3).

The main effects of the biological media are catalysis of the hydrolytic pathways **1,a**; **1,b** and **c** to varying extents.

2.2.1. *In diluted locust haemolymph (12.5%) at 25°C and pH 7.4*

A clear effect of this medium is catalysis of pathway **1,a**, since the ratio of **VIa** is 44% instead of 16% in a phosphate buffer after 4 h and also catalysis of pathway **c**, since **IIIa** is formed in 10% after 10 h (cf. Fig. 3A) instead of 3% (Table 1). The behaviour of **Ia** in diluted caterpillar haemolymph is almost similar for both catalysis and the peak broadening. At this stage, it can be concluded that **Ia** acts partly as a proinsecticide of the carboxylate **IIIa**.

Table 3
Variations of ^{19}F NMR chemical shifts $\Delta\delta$ and peak width ($\delta_{1/2}$) of the fluorinated entities **Ia**, **IIIa**, **IS**, $\mathbf{F_1} = \mathbf{Va}$ and $\mathbf{F_2} = \mathbf{VIa}$ according to the media: insect tissues, α-chymotrypsine and phosphate buffer

δ ($\delta_{1/2}$) ppm (Hz)	Ia	IIIa	IS	VIa = F$_2$	Va = F$_1$
Phosphate buffer 7.4	−40.06 (2)	−41.49 (2)	−39.40 (2)	−40.27 (2)	−39.81 (2)
Locust haemolymph	−40.00 (6)	−41.48 (4.5)	−39.40 (4.5)	−40.24 (4.5)	−39.78 (4.5)
Caterpillar haemolymph	−40.03 (7.2)	−41.48 (2)	−39.39 (2)	−40.25 (2.5)	−39.77 (9)
Locust fat body	−40.06 (30)	−41.46 (3)	−39.40 (3)	40.28 (3)	–
Locust mesenteron	−39.99 (130)	−41.46 (3)	−39.40 (3)	−40.21 (2.5)	–
α-Chymotrypsine	−40.06 (2)	−41.51 (2)	−39.40 (2)	−40.28 (2)	–

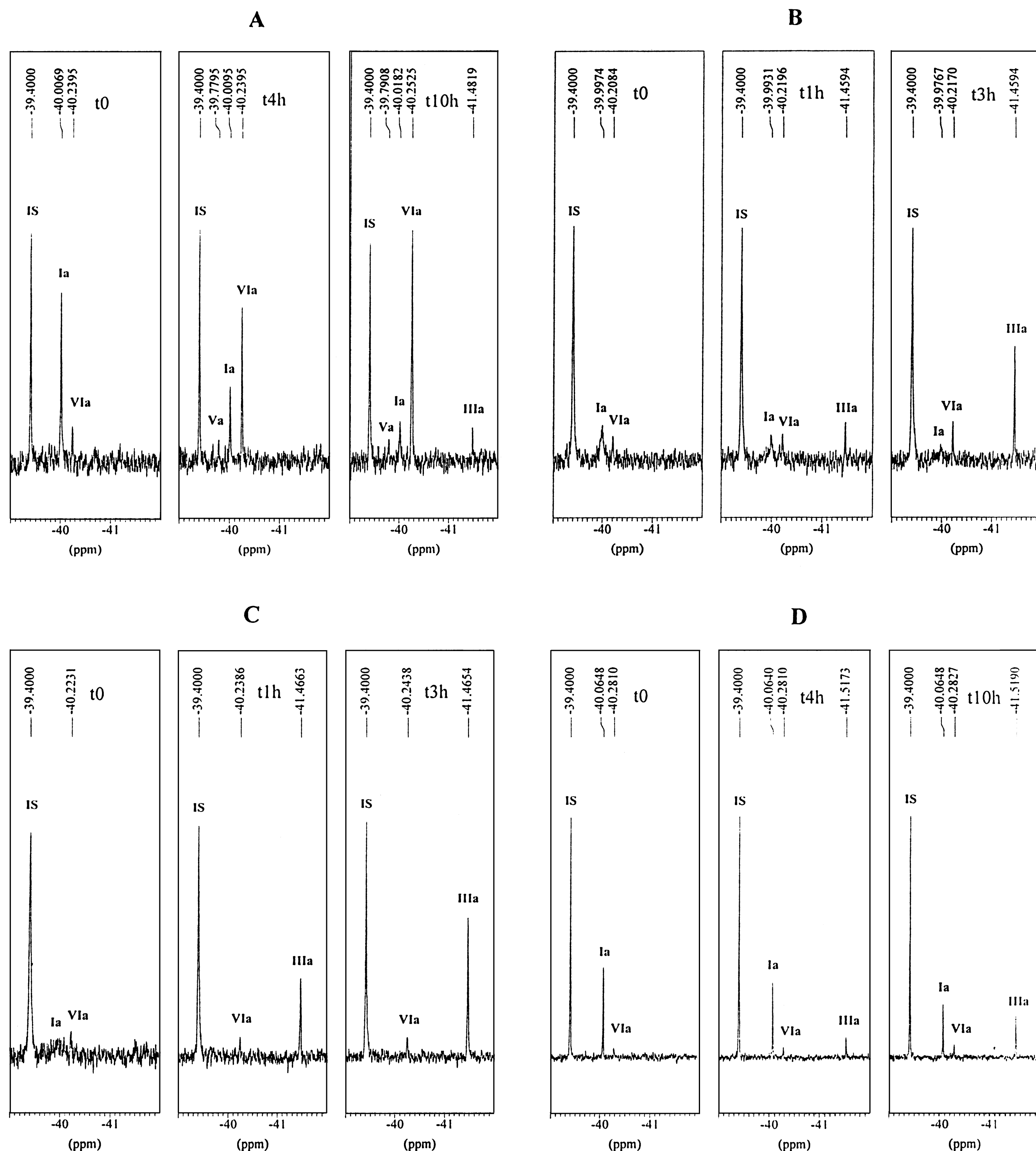


Fig. 3. Behaviour of Δ^2-oxazoline-1,3 **Ia** in insect tissues or in the presence of α-chymotrypsine. (A) [**Ia**] = 10^{-3} M in diluted locust haemolymph (12.5% in phosphate buffer); balance-sheet = 100% at $t = t_0$, 4 and 10 h. (B) [**Ia**] = 5×10^{-4} M in locust fat body; balance-sheet = 100% at $t = t_0$, 1 and 3 h. (C) [**Ia**] = 5×10^{-4} M in locust mesenteron; balance-sheet = 63, 55 and 100% at $t = t_0$, 1 and 3 h, respectively. (D) [**Ia**] = 5×10^{-4} M in the presence of α-chymotrypsine; balance-sheet = 100% at $t = t_0$, 4 and 10 h.

2.2.2. *In concentrated locust fat body and mesenteron at 25°C*

The unmasking of carboxylate **IIIa** (pathways **1,b** and **c**) is the preponderant development in the fat body, cf. spectrum "3 h" in Fig. 3B, where **IIIa** integrates to ~60%, **VIa** and unchanged **Ia** for ~20%, and spectacularly **IIIa** is almost the unique product of evolution in the mesenteron, since in 3 h **Ia** has completely disappeared (cf. Fig. 3C: 87% **IIIa** and

13% **VIa**). In both tissues, intermediate **Va** (= $\mathbf{F_1}$) was not detected, which result confirms a metabolic correlation between **Va** and **IIIa**, the only difference between the two media being the spectacularly more pronounced enlargement of the resonance of **Ia** in the mesenteron sample.

We have previously demonstrated that pathway **d** is inefficient in locust tissues [19], which result is now confirmed by the nearly constant amount of β-hydroxylamide **VIa** observed in Fig. 3C.

From these independant in vitro assays into concentrated locust tissues it can be concluded that the Δ^2-oxazoline-1,3 **Ia** acts mainly as a proinsecticide of the carboxylate **IIIa**. The hydrolases content of these tissues are likely to be different from the haemolymph media which, even diluted, catalysed mainly the formation of β-hydroxylamide **VIa**. Fat body and mesenteron are known to contain hydrolytic enzymes of esterase [35,36] and α-chymotrypsine [18,19] types, likely to trigger catalysis of the pathways **1,b** and **c**.

In this work, during assays using the aminoester **Va**, we have made evident the role of a commercial esterase and of α-chymotrypsine in pathway **c** catalysis. Thus, a possible effect of these commercial enzymes was tested directly against **Ia**.

2.2.3. In the presence of esterase

This diluted biological medium (see Section 4) did not show a significant catalytic effect on the hydrolysis of **Ia** at pH 7.4, which clearly means that this esterase has no effect on pathway **1,b** (cf. Fig. 2A and Table 4).

2.2.4. In the presence of α-chymotrypsine

By contrast with the esterase, this dilute biological medium (see Section 4) exercised a noticeable catalytic effect in the hydrolysis of **Ia** leading to **IIIa** (cf. Fig. 2B), but did not show significant $\Delta\delta$ nor increase in $\delta_{1/2}$ (cf. Table 3). It must be concluded that α-chymotrypsine catalyses both pathways **1,b** and **c** and thus presents some analogies with the locust fat body and mesenteron actions (cf. Table 4).

Table 4
Summary of the different catalytic effects exercised by pH, and biological media on the evolution of **Ia**, **Va** (= $\mathbf{F_1}$) **VIa** (= $\mathbf{F_2}$) (cf. Figs. 2 and 3)[a]

Pathway/catalysis (cf. Scheme 1)	Proinsecticide behaviour		Hydroxyamide formation	
	1,b	**c**	**1,a**	**d**
pH < 7/**Ia**	**yes**	–	ε	–
pH > 7/**Ia**	no	yes	**yes**	–
Esterase/**Ia**	no	no	no	–
Esterase/**Va**	–	**yes**	–	–
α-Chymotrypsine/**Ia**	**yes**	**yes**	no	–
α- Chymotrypsine/**Va**	–	**yes**	–	–
α- Chymotrypsine/**Via**	–	–	–	no
Haemolymph diluted/**Ia**	yes	yes	yes	–
Haemolymph diluted/**VIa**	–	–	–	no
Mesenteron*/**Ia**	yes	**yes**	no	–
Mesenteron*/**VIa**	–	–	–	no

[a] Important, weak, very weak and non-significant effects are indicated, respectively, by **yes**, yes, ε and no.

3. Conclusion

Direct in vitro $^{19}F[^{1}H]$ monitoring in insect tissues provides a very suitable and efficient tool for evaluating the potentiality of chemical structures in the masking of fluorinated active principles.

This work demonstrates that Δ^2-oxazoline-1,3 is a good model nitrogen heterocyclic structure for developing proinsecticides based upon carboxylic acids and/or β-ethanolamines, as active principles, fluorinated or not. In fact, their hydrolytic unmasking is nearly the only reaction observed during in vitro assays in concentrated insect tissues. The different assays performed with isolated locust tissues or with commercial enzyme showed both catalysis of the pathway **1,b** and the hydrolysis of intermediate $\mathbf{F_1}$ identified as aminoester **Va** via enzymes of the α-chymotrypsine and/or of the esterase types. There is no doubt that in a living insect the unmasking would be even more pronounced due to the simultaneous action of all the undiluted tissues susceptible to trigger enzymatic hydrolysis. This study is now developed in order: to confirm this hypothesis, performing ex vivo assays in locust [19,35], and to determine possible chiral catalytic effect of insect biological media on the pathways **1,a**, **1,b** and **c**. Moreover, due to the spectacular activation of **Ia** in the locust mesenteron, it would be certainly useful to carry out tests by ingestion against insects, using baits [43] treated with **Ia** and more polar oxazolines, i.e. those not designed as contact insecticides. Finally, taking into account that intermediate **Va** resulting from **Ia** is efficiently activated by esterases, it would be also interesting to test the effects of **Ia** against insecticide-resistant aphids, where metabolic resistance results from the increased production of esterases [6].

4. Experimental

4.1. Biological samples

The caterpillar (*Mamestra brassicae*) and the african migratory locust *Locusta migratoria* were reared according to the crowded conditions described in [19].

The α-chymotrypsin from bovine pancreas was supplied by Sigma (Sigma Aldrich Chimie L'Isle d'Abeau Chesnes B.P. 701 38297, Saint-Quentin Fallavier, France). One unit of the enzyme will hydrolyse 1.0 μmole of *N*-benzoyl-L-tyrosine ethylester per minute at pH 7.8 at 25°C.

The esterase from hog liver immobilised on Eupergit was supplied by Fluka (L'Isle d'Abeau Chesnes B.P. 701, 38297, Saint-Quentin Fallavier, France). One unit of the enzyme

will hydrolyse 1.0 μmole of ethylvalerate per minute at pH 8 and 25.

4.2. In vitro assays

Haemolymph sampled from adults of locusts (male and female, pH 7.4 were centrifuged for 5 min at 3000 g, then diluted eight times with phosphate buffer (0.1 M, pH 7.4) and incubated with the substrate **Ia** ([substrate] $= 10^{-3}$ M). Five percent of DMSO-d_6 was added in order to dissolve the lipophilic substrate and for locking the NMR apparatus.

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, 25 μl of a 10^{-2} M solution of Δ^2-oxazoline-1,3 **Ia** in DMSO-d_6 was added, giving a 5×10^{-4} M substrate solution with 5% proportion (v/v) of DMSO-d_6.

Locust mesenteron: the sample of locust caeca (pH 6.4–7) was ground without dilution and centrifuged. To 475 μl of the supernatant tissues, 25 μl a stock solution of a 10^{-2} M Δ^2-oxazoline-1,3 **Ia** in DMSO-d_6 was added to obtain the same final substrate solutions as previously.

α-Chymotrypsin and esterase effects on ***Ia***: a 500 μl sample of a 5×10^{-4} M solution of the substrate **Ia** in DMSO-d_6 and phosphate buffer (5/95) was incubated with α-chymotrypsin (62.5 units), or with 60 mg of immobilised esterase, and the evolution of the mixtures were monitored according the classic ^{19}F NMR protocol.

α-Chymotrypsin and esterase effects on intermediate ***Va***: a 10^{-3} M solution of **Ia** in DMSO-d_6 and phosphate buffer 0.1 M and pH 6.3 (5/95) was left to develop at room temperature over 12 h. Then α-chymotrypsin (62.5 units) or immobilised esterase (60 mg) were added to an aliquot of 600 μl, and the evolution of the mixtures were monitored with time according the classic ^{19}F NMR protocol.

4.3. Structural characterisation

The following instruments and abbreviations were used:

- IR spectra were taken on *Bruker IFS 25 IR-FT spectrometer* and are described with the following abbreviations: for the vibrations ν, δ and γ, the wavenumbers are expressed in cm^{-1}, S: strong, M: medium, W: weak, s: sharp, b: broad.
- ^{1}H NMR, ^{13}C[^{1}H] NMR, ^{19}F[^{1}H] NMR spectra were recorded on *Bruker AC300 spectrometer*, respectively at 300, 75 and 282 MHz. The ^{1}H and ^{13}C chemical shifts δ are reported in ppm relative to TMS for solutions in $CDCl_3$ The symbols for the proton multiplicities are as follows: t: triplet; d: doublet; m: multiplet and s: singlet. For ^{13}C NMR, Cq is the symbol for quaternary carbon. ^{19}F chemical shifts are reported in ppm relative to CF_3COO^- (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 in the following instrumental conditions: sweep width, 2000 Hz; pulse angle, 90°; delay, 15 s; computer resolution, 0.5 Hz/point; number of scans, 104; memory size, 8 K; line exponential multiplication of FID, 1 Hz.
  ^{19}F relaxation times measured by the inversion recovery method are 4.94 s for Δ^2-oxazoline-1,3 **Ia** in solution in phosphate buffer, 4.76 s for β-hydroxylamide **VIa** [19], 4.97 s for carboxylate **IIIa** and 6.25 s for *p*-fluorobenzylalcohol **IS** (cf. Table 1).
- High-resolution mass spectrometry (HRMS) was performed at the ''Laboratoire de Spectrométrie de Masse, Université de Mont-Saint-Aignan'', Rouen, France using a Jeol AX 500 spectrometer (resolution 500) equipped with a PDP11 data system.

4.4. Reagents and chemicals

4-Fluorophenylacetic acid **IIIa** and 2-amino-1-propanol **IIa** were supplied by Aldrich (Sigma Aldrich Chimie L'Isle d'Abeau Chesnes B.P. 701, 38297, Saint-Quentin Fallavier, France).

2-(4-Fluorobenzyl)-4-methyl-Δ^2-oxazoline-1,3 **Ia** was obtained according to the Vorbrüggen protocol [13]. An equimolecular mixture (1.623 mmoles) of **IIa** and **IIIa** dissolved in 3.57 mmoles of hexachloroethane and 5 ml of dichloromethane (dried over 3 Å molecular sieves) was cooled at −2°C under nitrogen atmosphere. Then a mixture of 3.57 mmoles of triphenylphosphine and 7.14 mmoles of triethylamine in 4 ml of dry dichloromethane was slowly added. After reflux (3 h) and standing at room temperature (24 h), the mixture was washed with dilute NaOH and extracted with dichloromethane. After precipitation of Ph_3PO with hexane, filtration and evaporation of the solvent, the resulting crude Δ^2-oxazoline-1,3 **Ia** was distilled $Eb_{0.5\ mmHg}$, 84°C; yield, 35%.

IR (KBr): 2971 (s, M, $\nu_{=C-H}$), 2927, 2883, (s, M, ν_{C-H}), 1666 (s, S, $\nu_{C=N}$), 1510 (s, S, $\nu_{C=C}$), 1222 (s, S, ν_{C-F}), 826 (s, M, ν_{C-H}, *p*-disubstituted aromatic).

^{1}H NMR ($CDCl_3$) δ/TMS: 7.27 (2H, m, *meta* F in C_6H_4F); 7.00 (2H, m, *ortho* F in C_6H_4F); 3.58 (2H, s, CH_2Ar); the heterocyclic protons (H_4, H_5, H'_5) constitute an ABX system at the limits of an AMX system, complicated by the $C_4\underline{H}/C\underline{H}_2Ar$ coupling: 4.33 (^{1}H, dd, H_5), 4.18 (^{1}H, m, H_4), 3.78 (^{1}H, ~t, H'_5); J H_4–H_5 = 9.4 Hz, J H_5–H'_5 = −7.9 Hz, J H_4–H'_5 = 7.4 Hz.

^{13}C NMR ($CDCl_3$) δ/TMS: 165.5 (C=N), 161.9 ($^1J_{CF}$ = 243.4 Hz, Cq of C_6H_4F), 130.4 ($^3J_{CF}$ = 7.9 Hz, CH of C_6H_4F), 130.9 ($^4J_{CF}$ = 3.2 Hz, Cq of C_6H_4F), 115.4 ($^2J_{CF}$ = 21.2 Hz, CH of C_6H_4F), heterocyclic carbons: 74.2 (CH_2), 61.4 (CH); 34.0 (CH_2–Ar); 21.4 (CH_3).

HRMS was performed in the electron impact mode (EI) using direct introduction. Accurate and observed *M*/*e* for M^+ ion were, respectively, 193.0903 and 193.0901 Da which

concordance ($\Delta M = 0.0002$) agrees with a molecular weight of 193 for the compound **Ia**.

N-(1-methyl-2-hydroxyethyl)-(4-fluorophenyl)-acetamide **VIa** was prepared as previously described [19].

^{19}F monitorings were performed with 5×10^{-4} or 10^{-3} M solutions of **Ia** (or **Va**, or **VIa**) in a phosphate buffer 0.1 M at pH 7.4 (or at pH 6.3, cf. Table 1, Figs. 1 and 2), or in the presence of biological media with 5% DMSO-d_6, using the optimised ^{19}F NMR conditions described before. The **IS** concentration was 10^{-3} M. For monitoring at pH 6.3, the concentrations of **Ia** and **IS** were both 10^{-3} M.

^{13}C spectrum of the reaction mixture resulting from the evolution of **Ia** at pH 6.3: a 8.8×10^{-2} M solution of substrate in DMSO-d_6 phosphate buffer 0.1 M at pH $\sim$ 6.3 (20/80) was left to develop for 2 h, then the ^{13}C[^{1}H] spectrum recorded in 12 000 scans (cf. Table 2).

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