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Catalyzed asymmetric hydrolysis of fluorinated chiral esters in locust haemolymph. *In vitro* ^{19}F NMR monitoring

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Abstract. ^{19}F NMR is used for monitoring the *in vitro* behaviour in locust organs or tissues of an ester with a fluorinated alkoxy structure and a chiral acyl moiety. In carrying out analysis directly in the biological media, the problematic extraction of polar metabolites is avoided, contributing to an easier and more reliable monitoring. Thus, in diluted haemolymph a remarkable enantioselectivity of the transformation is evidenced. Over a four hour period the ester R remains unalterated, while the S enantiomer is completely hydrolyzed. This enantioselectivity is the reverse of the trend which is generally observed in the hydrolysis of esters by lipases.

1. Introduction

For some years, we have been interested in developing proinsecticides, masking reversibly ω-fluorinated fatty acids or aldehydes [1] and/or β-ethanolamines considered as active principles [2]. We restricted our choice, among numerous possibilities, to proinsecticides designed upon hydrolysis as the activation mode[1]. The selection of the structures was further achieved studying the activation of enol esters, oxazolidines, oxazolines, thiazolines, dihydrooxazines, etc., using simple available molecules as models.

For this purpose analytical techniques such as ^{1}H NMR or HPLC allowing a direct monitoring within the biological media of insects, namely the locust [2–5], were used in organs or tissue samples. In fact, such an approach avoids, before analysis, the tedious and often problematic prepurification step which can entail preferential extraction and poor reliability of the monitoring.

From our earlier 'direct monitoring' studies, it appeared that the oxazolidine structure is too rapidly hydrolysed while thiazoline is a very stable heterocycle. Thus, we focussed on enol ester and oxazoline structures for the masking of ω-fluorinated fatty acids. Taking into account the advantages of ^{19}F NMR for the direct monitoring of fluorinated drugs into mammal tissues [6,7], we confirmed that the previous

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[1]Activation: unmasking of the active principle.

structures are effectively 'activated' in locust haemolymph or fat-body during *in vitro* and *ex vivo* assays [5]. The enzymatic character of the hydrolysis was also evidenced.

Then, considering a possible recognition from the enzymes present in the locust biological media, the need of a study of the activation step of substituted oxazolines or esters from structural standpoints; i.e., lipophilic character, regioisomerism, chirality, etc., became evident. In fact, abundant literature is available concerning the well-known and documented field of enzymatic recognition [8] and especially enantioselectivities [9]. Hydrolases and particularly lipases have mostly been exploited under kinetic conditions due to remarkable enantioselectivity [10,11].

The present work concerns the ^{19}F NMR study of the behaviour of fluorinated chiral esters chosen as proinsecticide models, in locust organs or tissues, compared to their behaviour in presence of a commercial esterase.

2. Experimental

2.1. Biological samples

Esterase from porcine liver, in 3.2 M $(NH_4)_2SO_4$ solution, pH 8, was supplied by Sigma (St Quentin Fallavier, France). One unit hydrolyzes 1.0 μmole of ethyl butyrate to butyric acid and ethanol per min at pH 8.0 at 25°C.

The mature African migratory locust *L. migratoria migratorioides* was grown in crowded conditions with L/D (12/12) photoperiod cycle and fed on wheat seeds and bran in the 'Laboratoire de Biologie Evolutive et Dynamique des Populations', Université de Paris-Sud.

2.2. In vitro assays

Esterase from porcine liver (0.4 units) was incubated with 500 μl of a 2×10^{-3} M solution of each enantiomer R or S of the ester **1** in DMSO-d_6/phosphate buffer.

Haemolymph sampled from 18 mature locusts (ca. 20–30 μl per locust), according to [12] was pooled in 500 μl of phosphate buffer (0.1 M, pH 7.4) and centrifuged for 5 min. Then, a volume of 250 μl of diluted haemolymph was added into the NMR tube to an equal volume of a 2×10^{-3} M solution of each enantiomer R or S of the ester **1** in DMSO-d_6/phosphate buffer (10 : 90, v/v).

The sample (100 mg) of locust fat-body was added to 1.5 ml of phosphate buffer (0.1 M, pH 7.4), stirred and centrifuged. A volume of 250 μl of the aqueous fraction of intermediate density between the lipidic supernatant (top) and the tissues fragments (bottom) was added into the NMR tube to respectively 250 μl of a 10^{-3} M solution of each enantiomer of the ester **1** in DMSO-d_6/phosphate buffer (10 : 90, v/v).

2.3. Structural studies

The following instruments and abbreviations were used: IR Bruker IFS25 IR-FT spectrometer (ν or δ (cm^{-1}) (S = strong, M = medium, W = weak, s = sharp, b = broad)); Bruker AC 300 spectrometer for ^{1}H NMR at 300 MHz (chemical shifts, δ, in $CDCl_3$ (multiplicity: t = triplet, m = multiplet)); Bruker AC 300 spectrometer for both decoupled ^{13}C NMR and ^{19}F NMR spectra, recorded respectively at 75 MHz in $CDCl_3$ and at 282 MHz in DMSO-d_6/phosphate buffer (10 : 90, v/v).

Table 1
Feasibility of the direct ^{19}F NMR monitoring of the ester **1**

	Chemical shifts of ester **1** and alcohol **3**[1]			
	DMSO + buffer	DMSO + buffer + esterase (0,4U)	DMSO + buffer + haemolymph (20–25%)	DMSO + buffer + fat-body (16.6 mg)
[Ester **1**]		2×10^{-3} M	10^{-3} M	0.5×10^{-3} M
δ ^{19}F Ester **1**	–38.0	–38.0	–37.7[2]	–37.9
δ ^{19}F Alcohol **3**	–39.2	–39.2	–39.2	–39.15

[1] The chemical shifts are reported relatively to the resonance peak of CF_3COOH (aqueous solution at 5% v/v) used as an external reference.
[2] When haemolymph is diluted at 5%, a supplementary and stretcher signal is observed at -38.0 ppm, cf. Fig. 2.

Concerning the ^{19}F NMR spectra, the chemical shifts were reported relatively to the resonance peak of CF_3COOH (5% aqueous solution v/v) used as an external reference [6]. Spectra were run with ^1H decoupling in the following instrumental conditions: sweep width 31,250 Hz, pulse width 7μs (flip angle 90°), computer resolution 1 Hz/point, number of scans: 64–256, acquisition time 1.05 s, memory size 64 K, line exponential multiplication of the FID: 1 Hz.

The optical rotation $[\alpha]_D^{19}$ and $[\alpha]_{546}^{19}$ of each of the two enantiomers R and S of the ester **1** were measured with a Perkin–Elmer 241 polarimeter.

2.4. Reagents and chemicals

2-phenylbutyric acid (racemate and enantiomers) and 4-fluorobenzylalcohol were Aldrich products (St Quentin Fallavier, France).

The esters obtained in $\sim$90% yields by H_2SO_4 catalyzed esterification were purified by thin-layer chromatography on silica gel Merck GF 254 by using a heptane/ethyl acetate (90 : 10) mixture as eluent; formula $C_{17}H_{17}FO_2$ according to the centesimal composition. $[\alpha]_D^{19} = +27°$ for ester S (ethyl acetate; c = 0.97) and $-27°$ for ester R; $[\alpha]_{546}^{19} = +32°$ (ethyl acetate; c = 0.97) for ester S and $-32°$ for ester R. IR (NaCl): 3031 (s and W, ν H$-$C=C), 2966 (M, ν CH), 1735 (b and S, ν C=O), 1605 (M, ν C=C arom), 1511 (M, ν C=C arom), 1454 (M, δ CH_2), 1379 (M, δ CH_3), 1224 (M, ν C$-$O), 824 (M, δ H$-$C=C). ^1H NMR ($CDCl_3$) δ/TMS: 7.3 (7H, m, meta Ar$-$F and ϕ), 7.0 (2H, m, ortho Ar$-$F), 5.1 (2H, AB: –COO$\underline{CH_2}$–Ar–F), 3.5 (1H, t, –$CH_2\underline{CH}$–), 2.15 (1H, m, $CH_3\underline{CH_2}CH$–), 1.85 (1H, m, $CH_3\underline{CH_2}CH$–) 0.9 (3H, t, $\underline{CH_3}CH_2$–). ^{13}C NMR ($CDCl_3$) δ/TMS: 173.7 (–$\underline{C}$OO–), 162.4 ($^1J_{CF} = 240$ Hz, Cq of ArF), 138.8(Cq of ϕ), 131.8 ($^4J_{CF} = 2.8$ Hz, Cq of ArF), 129.75 ($^3J_{CF} = 8.4$ Hz, CH of ArF), 128.5 (CH meta of ϕ), 127.9 (CH ortho of ϕ), 127.2 (CH para of ϕ), 115.3 ($^2J_{CF} =$ 21.4 Hz, CH of ArF), 65.5 (–COO$\underline{C}H_2$–), 53.4 ($CH_3CH_2\underline{C}H$–), 26.5 ($CH_3\underline{C}H_2CH$–), 12.0 ($\underline{C}H_3$–).

The chemical shifts observed by ^{19}F NMR under different conditions are given in Table 1.

3. Results and discussion

The chosen esters **1** (R and S) correspond to 2-phenyl butyric acid **2** (R and S) and 4-fluorobenzyl alcohol **3**. In our 'proinsecticide purpose', this choice is based upon the lipophilic character of such model molecules (calculated $\log P = 4.71$ using Reckker indexes [13]) requisited for 'contact' insecticides. Moreover, taking into account the previous results obtained with enol esters [2,3], one

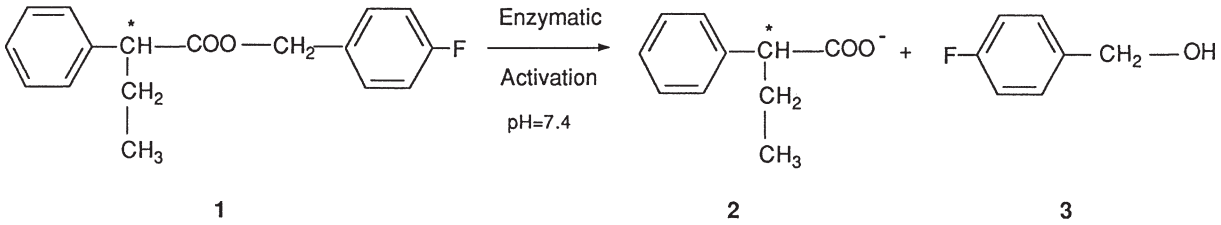

Fig. 1. Fluorinated chiral esters chosen as proinsecticide models. Catalyzed enzymatic hydrolysis (activation).

could expect an efficient 'activation' of these esters in locust haemolymph or in fat-body according to Fig. 1. At last, this choice allows HPLC monitoring of the three entities **1**, **2** and **3** by UV-visible detection and also, *a priori*, a possible ^{19}F monitoring based on the entities **1** and **3**.

3.1. Feasibility and check up of the monitoring

Previous works with lipophilic enol esters $(\log P = 2.24)$ required approximately {10% of CD_3OD + 5% of DMSO-d_6} as cosolvent to solubilize the substrate in phosphate buffer [5]. However, even in the presence of relatively important amounts of cosolvent, haemolymph or fat-body hydrolases remained active towards the substrate.

^{19}F NMR spectra of the biological media performed in the range covering the chemical shifts of both the ester **1** and the corresponding alcohol **3** are free of signal even after 10,000 scans. Moreover, due to the large ^{19}F chemical shift range, remote structural modification can entail sufficient chemical shift differences allowing a quantitative analysis as observed with fluorinated enol esters [5]. ^{19}F NMR spectra of compounds **1** (racemate) and **3**, recorded in buffered solution, either in presence of locust haemolymph or fat-body, or in presence of a commercial esterase, show clearly the feasibility of the monitoring of these entities directly in these media. Indeed, whatever the conditions, ester **1** and alcohol **3** exhibit sufficiently separated signals ($\Delta\delta$ = 1.5–1.2 ppm, Table 1). To be noted is that the chemical shift of alcohol **3** is not significantly affected by changing the solution conditions: it is nearly constant at $\delta = -39.2$ ppm either in phosphate buffer solution or in different biological media. On the contrary, the spectrum of ester **1** is sensitive to the nature of the solution. In phosphate buffer with and without commercial esterase, and in presence of fat-body, a unique signal is observed at ca. -38.0 ppm (Table 1), while in haemolymph moderately diluted (20–25%) the chemical shift is –37.7 ppm (Fig. 2b). Moreover, in more diluted haemolymph, besides this peak which is relatively broad, a stretcher peak is observed at –38.0 ppm (Fig. 2a). This behaviour can be explained assuming an association phenomenon of the substrate with some haemolymph component leading to a unique entity (signal at –37.7 ppm) at low dilution of the biological medium.

As presented in Table 1, the spectra were recorded using substrate **1** concentrations ranging from 0.5 to 2×10^{-3} M in 0.1 M phosphate buffer (pH 7.4) solutions with 10% DMSO-d_6. Under these conditions, the stability of ester **1** was verified with a buffered racemate solution: no peak corresponding to alcohol **3** was observed after one night. The experiments have been carried out at room temperature except in the case of haemolymph where the temperature was raised to 33°C.

3.2. Behaviour of esters 1R and 1S in presence of a commercial esterase

The monitoring of R and S esters **1** were performed in duplicate using the previous conditions, with a commercial esterase from porcine liver. In both cases, we observed a decrease in the ester peak

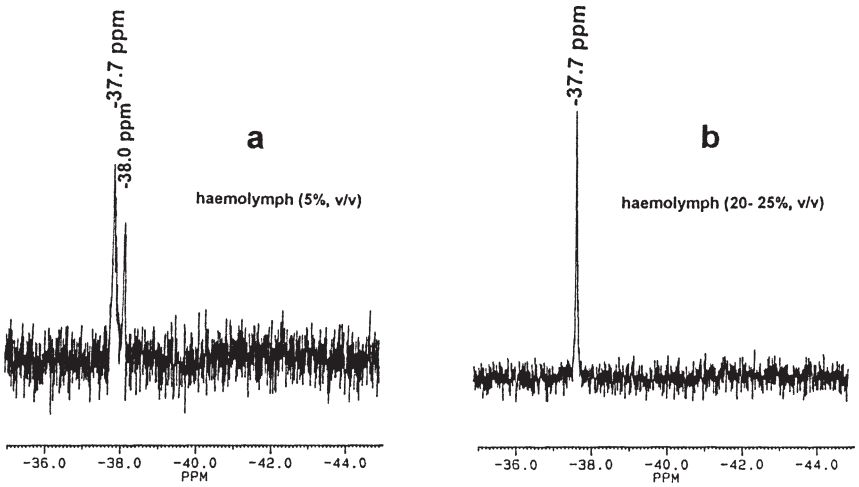

Fig. 2. ^{19}F NMR spectra of ester **1** in locust haemolymph at different dilutions.

(at –38.0 ppm) and a concomitant increase of a new signal at (–39.2 ppm) assignable to alcohol **3** by overload. However, the hydrolysis presented different rates according to the enantiomers. The enantioselectivity of the hydrolysis is clearly evidenced indicating a faster reaction in the case of enantiomer R. For instance after about one hour, assuming that in diluted haemolymph the longitudinal relaxation times T_1 are similar for the fluorine nucleus of **1** and **3**, the hydrolysis ratio can be estimated to $\sim$ 45–50% for enantiomer R and only to $\sim$ 20–25% for enantiomer S. Our esters **1** seem to follow the general trend observed for the lipase catalysis of asymmetric esters, which is in favour of the R enantiomer reaction [10].

3.3. Behaviour of 1R and 1S in presence of fat-body

In presence of fat-body, the enantiomers R and S are hydrolyzed roughly at the same rate. Under the conditions described in the experimental section, the hydrolysis is completed within two hours for both esters.

3.4. Behaviour of esters 1R and 1S in locust haemolymph

In diluted haemolymph (20% v/v), over a five hours period, the ester R remains unalterated while ca. 60% of S enantiomer is hydrolyzed. A second experiment with 25% of haemolymph confirmed the enantiospecific hydrolysis of the S ester with 100% of hydrolysis over a shorter period of four hours while the ester R remains unalterated (Fig. 3).

The enantioselectivity displayed by haemolymph hydrolases is remarkable with regard to: (i) the reverse trend generally observed and mentioned above for the hydrolysis of esters by lipases, and (ii) the complexity of this biological medium which contains certainly several hydrolases more or less specific of such esters.

4. Conclusion

Performing ^{19}F NMR directly in locust organs or tissues during *in vitro* assays is a very convenient approach for monitoring the behaviour of potential proinsecticides.

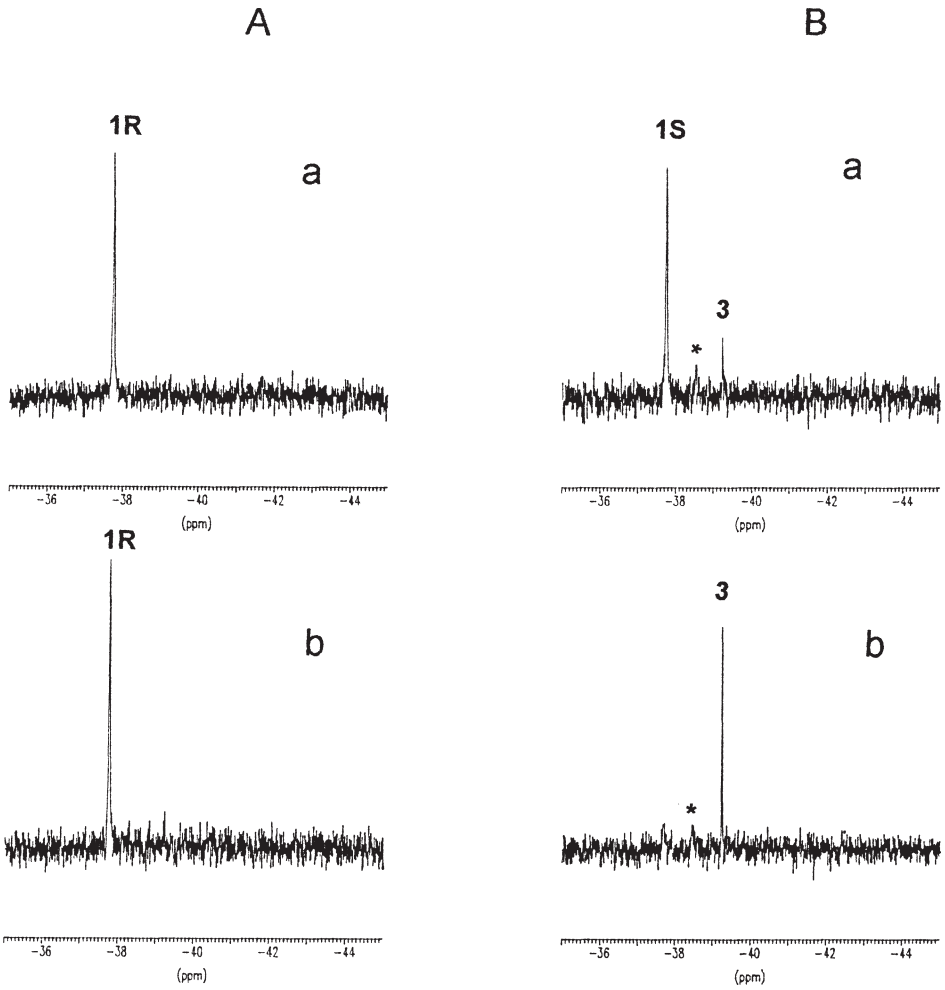

Fig. 3. Compared *in vitro* hydrolysis of esters **1**(R) and **1**(S) by direct ^{19}F NMR monitoring in locust haemolymph. Haemolymph diluted at 25% in {DMSO-d_6/0.1 M phosphate buffer pH 7.4} (10 : 90, v/v) at 33°C. [ester **1**] = 10^{-3} M; A = enantiomer R; B = enantiomer S; a: $t = 1$ h; b: $t = 4$ h.
* This impurity was identified to di 4-fluorobenzyl oxyde.

It appeared that during *in vitro* assays haemolymph displays a remarkable enantiospecificity in the hydrolysis of ester **1** whereas locust fat-body acts as a random hydrolase.

So, *ex vivo* assays will be undertaken to obtain informations about the enantioselectivity in living locust. At the same time the racemate will be examined by using chiral chromatographic techniques and the work will be developed by studying the behaviour of more sophisticated structures than the ester one, such as chiral Δ^2 oxazolines 1–3, for masking ω-fluorinated fatty acids as active principles.

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References

[1] G.D. Prestwich, J.K. Mauldin, J.B. Engstrom, J.F. Carvalho and D.J. Cupo, *J. Econ. Entomol.* **76** (1983), 690.
[2] J.-C. Cherton, D. Davoust, P. Escoubas, R. Oumeddour, J.-L. Clément, N. Ronzani and J.-J. Basselier, *Analusis* **16** (1988), 440.
[3] J.-C. Cherton, M. Amm, R. Oumeddour, I. Piccinin and D. Ladjama, *Analusis* **18** (1990), 37i.
[4] J.-C. Cherton, D. Ladjama and D. Soyez, *Analusis* **18** (1990), 71.
[5] A. Cavellec, Thèse de Doctorat de l'Université Pierre et Marie Curie, Paris, 1995.
[6] M.-C. Malet-Martino and R. Martino, *Biochimie* **74** (1992), 785.
[7] M.-C. Malet-Martino and R. Martino, *Xenobiotica* **19** (1989), 583.
[8] C.G. Rabiller, K. Königsberger, K. Faberand and H. Griengl, *Tetrahedron* **46** (1990), 4231.
[9] M. Kloosterman, E.W.J. Mosmuller, E.H. Schoemaker and E.M. Meijer, *Tetrahedron Letters* **28** (1987), 2989.
[10] K. Mori and R. Bernotas, *Tetrahedron: Asymmetry* **1** (1990), 87.
[11] F. Monot, *Revue de l'Institut Français du Pétrole* **49** (1994), 187.
[12] P. Cassier, *Ann. Sci. Nat. Zool., Paris 12ème Série* **VII** (1965), 213.
[13] R.F. Reckker, *The Hydrophobic Fragmental Constant*, Elsevier, New York, 1977.