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Thermostable derivatives of mesocarb and its p-hydroxy metabolite

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Introduction

Mesocarb (MB: I_a; 3-(1-methyl-2-phenylethyl)-*N*-(phenylaminocarbonyl)-1,2,3-oxadiazolium-5-aminide; Figure 1) belongs to the group of *N*-carbamylsydnone imines, which are relative stable meso-ionic heterocycles [1]. The structure of this type of heterocycle can only be drawn as a resonance hybrid of several dipolar, e.g. II-IV, and tetrapolar forms. These meso-ionic molecules may be represented by formulae of a general type (V-VI) and of which type V is no longer favored and type VI is preferred [1]. The large full circle and the positive sign in structure VI symbolize a cyclic delocalization of the pi-electrons of the meso-ionic ring in association with a partial positive charge. The exocylic group is associated with the corresponding partial negative charge.

The main urinary metabolite of MB in humans is the sulfate conjugate of *p*-hydroxymesocarb (*p*-OH-MB: I_b; 3-(1-methyl-2-phenylethyl)-*N*-(*p*-hydroxy-phenylaminocarbonyl)-1,2,3-oxadiazolium-5-aminide; Figure 1) [2]. An important drawback of the gas chromatographic analysis of MB and its main metabolite is the thermolability of the respective compounds [2]. The decomposition may be explained by the equilibrium between the ring (I) and ketene form (VII) (Figure 2). The ketene form in is thermolabile and leads to pyrolysis products (VIII), when heated.

However, during the described analytical procedures of MB and the sulfate conjugate of *p*-OH-MB in doping analysis, it can be assumed that the compound of interest already undergoes several conversions [1]. A summary of the most probable conversions is also given in Figure 2. First hot aqueous hydrochloric acid may hydrolyse the MB or its metabolite to 5-amino-1,2,3-oxadiazolium (IX). During acid extraction this

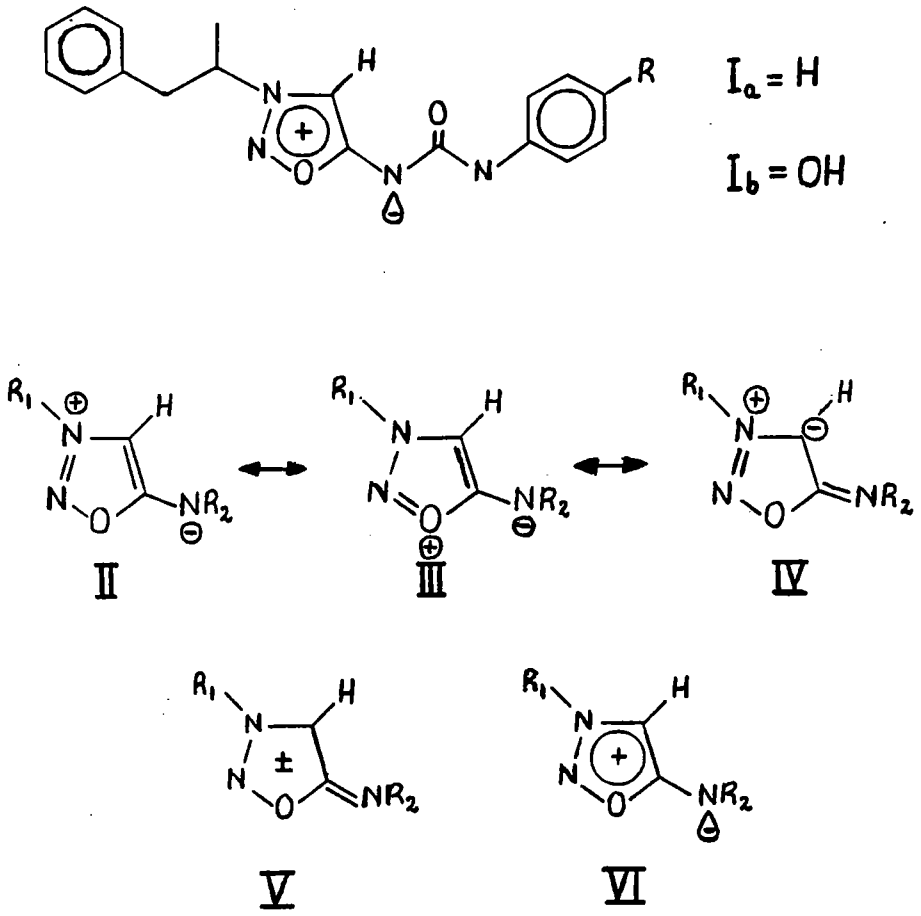

Figure 1: Structures of mesocarb (I_a) and *p*-hydroxy mesocarb (II_b) in particular and dipolar forms (II-IV) and general formulae (V-VI) of meso-ionic heterocycles in general

product remains in the aqueous layer. It is converted into *N*-(1-methyl-2-phenylethyl)-*N*-nitroso-α-aminoacematide (X) after treatment with sodium hydroxide. Derivatization with trifluoroacetic anhydride results in cyclodehydration. The end-product is again a meso-ionic compound, 3-(1-methyl-2-phenylethyl)-*N*-(trifluoroacetyl)-1,2,3-oxadiazolium-5-aminide (XI). The last reaction is analogous to the classical preparation of sydnones; cyclodehydration of *N*-nitroso-α-amino acids with acetic anhydride. Direct derivatization of I also results in XI.

Depending on the procedure, the end-product, which is detected, is thus either the deconjugated metabolite (I_b), a pyrolysis product (VIII) or a *N*-trifluoroacetyl derivative (XI). Some of these products indeed have been described and identified, although the *N*-trifluoroacetyl derivative has been interpreted as the pyrolysis product. In this paper, a GC/MS evaluation of different *N*-acyl and *N*-fluoroacylsydnone imine derivatives of MB

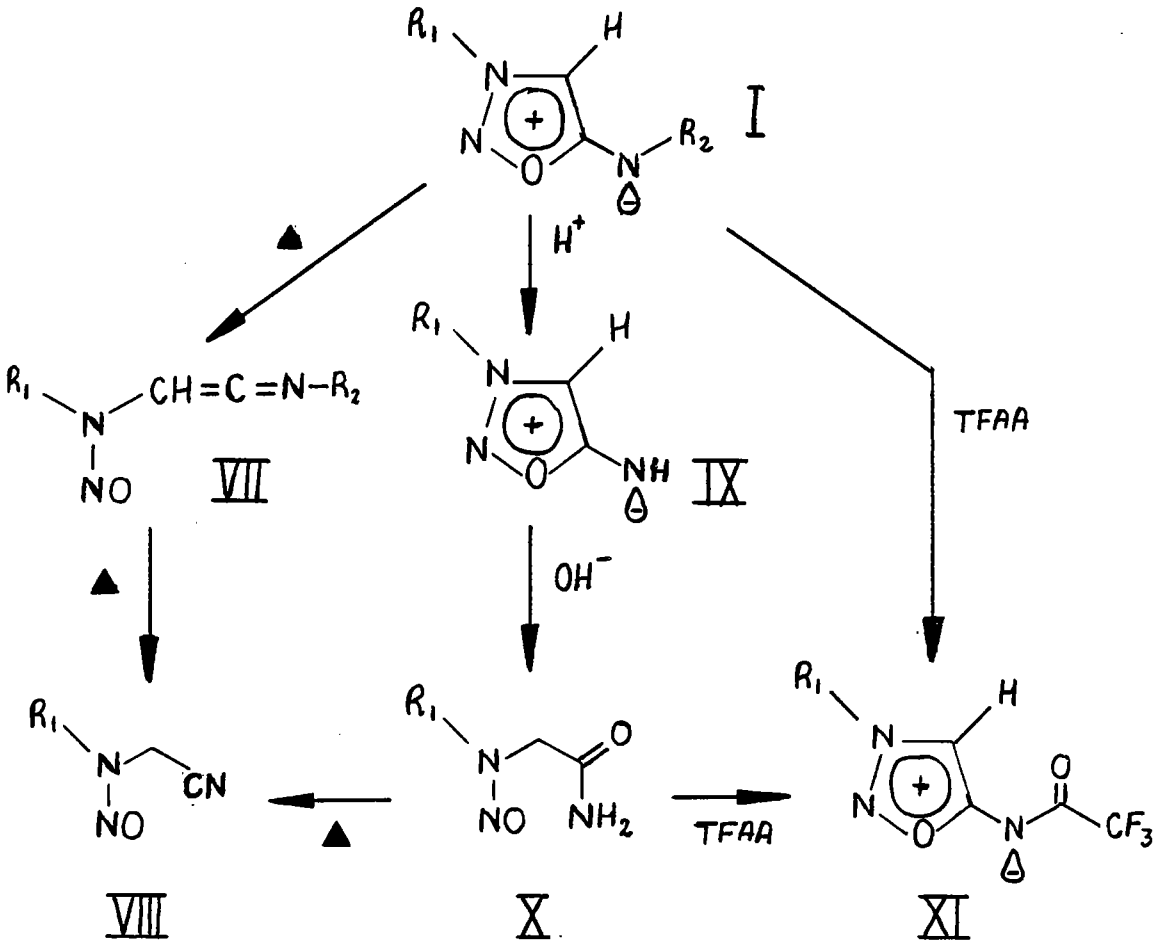

Figure 2: Possible conversions of the *p*-hydroxy mesocarb during isolation and GC/MS analysis

and *p*-OH-MB is presented.

Experimental

Reference compound

A methanolic stock solution of MB was prepared by dissolving a tablet of Sydnocarb®, containing each 5 mg of MB, in 5 ml of methanol.

Sample collection

Two tablets of Sydnocarb® were administered to a male volunteer. Urine samples were collected on spontaneous voiding of the bladder in the first 12 h period after administration.

Sample preparation

Method 1: Acidic hydrolysis and basic extraction according to the MB procedure as recommended by Donike *et al.*

Method 2: Two ml of urine were applied to XAD-2 extraction columns, the retained compounds were eluted with methanol and the organic phase was evaporated under nitrogen. To the dry residue 1 ml of 0.2 M sodium acetate buffer pH 5.2 and 50 µl of the *Helix pomatia* enzymatic solution were added. The mixture was incubated at 55°C for 3 h. The samples were made basic and extracted with 4 ml of ethyl acetate. After shaking and centrifugation, the organic layer was separated and evaporated under nitrogen to dryness.

Derivatization

To the dry residue 50 µl of anhydride reagent and 50 µl of ethyl acetate were added and the mixture was heated for 30 min at 60°C. Excess of derivatization reagents was removed under nitrogen and the residue was dissolved in 100 µl of ethyl acetate for GC/MS analysis.

GC/MS analysis

GC/MS analysis of the 3-(1-methyl-2-phenylethyl)-*N*-fluoroacylsydnone imines was performed by the GC/TSQ (Triple Stage Quadrupole 45 of Finnigan MAT) in the Electron Impact (EI), Positive Chemical Ionization (PCI) and Negative Chemical Ionization (NCI) mode, respectively.

Results and discussion

EI and PCI mass spectra

The GC/MS evaluation of the different *N*-acyl and *N*-fluoroacylsydnone imine derivatives was based on direct derivatization of MB. The EI mass spectra the studied *N*-acyl derivatives studied are non-characteristic and are dominated by the tropylium ion $[C_7H_7]^{+\cdot}$ (Table 1). This fragment, together with $[C_6H_5\text{-}CH_2\text{-}CH{=}CH]^{+\cdot}$ at *m/z* 117, $[C_6H_5\text{-}CH_2\text{-}CH{=}CH_2]^{+}$ at *m/z* 118 or $[C_6H_5\text{-}CH_2\text{-}CH_2\text{-}CH_2]^{+\cdot}$ at *m/z* 119, results from the 3-(1-methyl-2-phenylethyl) side chain. In contrast *N*-acyl derivative and although at low abundances, the *N*-fluoroacyl derivatives show their molecular ion at the respective *m/z* values. The data of the PCI spectra confirm the molecular weights of the *N*-fluoroacyl derivatives (Table 2). In the EI as well as in the PCI mode the ion at *m/z* 230 corresponds to the loss of the *N*-side chain. The relative large number of EI-like fragmentations in the PCI mode, points out the relative instability of the derivatives of the sydnones imines studied under the reported mass spectrometric conditions. The *N*-fluoroacyl derivatives are however more stable than the *N*-acyl derivatives. It has already been known, that depending on the substituent, sydnones are largely present in the gaseous phase as in a valence isomeric *N*-nitroso ketene form [3,4] or in the ring form. Also it has been reported that electron attracting groups favor the ring form [4], therefore stabilizing the molecule, while electron donating groups favor the ketene form and thus the formation of pyrolysis products. For that reason, the *N*-fluoroacyl derivatives thus are stable under high temperatures and MB and the *N*-acyl derivatives are not.

NCI mass spectra

Besides stability, the advantage of *N*-fluoroacyl-3-(1-methyl-2-phenylethyl)-sydnone imines is the possibility of performing NCI mass spectrometry. The molecular ions $M^{-\cdot}$ and/or $[M - H]^-$ in the spectra of the *N*-fluoroacyl-3-(1-methyl-2-phenylethyl)-sydnone imines were observed at low abundance (Table 3). More prominent were the ions $[M - C_6H_5\text{-}CH_2\text{-}CH_2\text{-}CH_2]^-$ and $[HN\text{-}CO\text{-}C_nX_{2n+1}]^-$, but those are not so characteristic.

The ring fragments A,B and C provided more structural information. Ring fragment A was observed for all *N*-fluoroacyl derivatives studied (Figure 3). The formation of ring fragment B is probably favored by the possibility of arranging an aromatic six-membered heterocyclic ion. Ring fragment C is an analogous fragment of B, resulting from the

transfer of three hydrogens instead of one.

GC/MS analysis of p-OH-MB

The metabolite *p*-OH-MB was deconjugated by acidic hydrolysis and isolated by a basic extraction (method 1) or by enzymatic hydrolysis with *Helix pomatia* solution and a basic extraction (method 2). Method 2 resulted in largest recovery, supporting the unwanted conversions as described in Figure 2. The XAD-2 extraction prior to enzymatic hydrolysis was essential, indicating inhibition of the enzymatic hydrolysis by anorganic compounds. In an example we selected the derivatization with trifluoroacetic anhydride in order to obtain the 3-(1-methyl-2-phenylethyl)-*N*-(trifluoroacetyl)-1,2,3-oxadiazolium-5-aminide. GC/MS analysis in the NCI-mode easily demonstrated the presence of the derivative in a urine sample obtained from a excretion study (Figure 4).

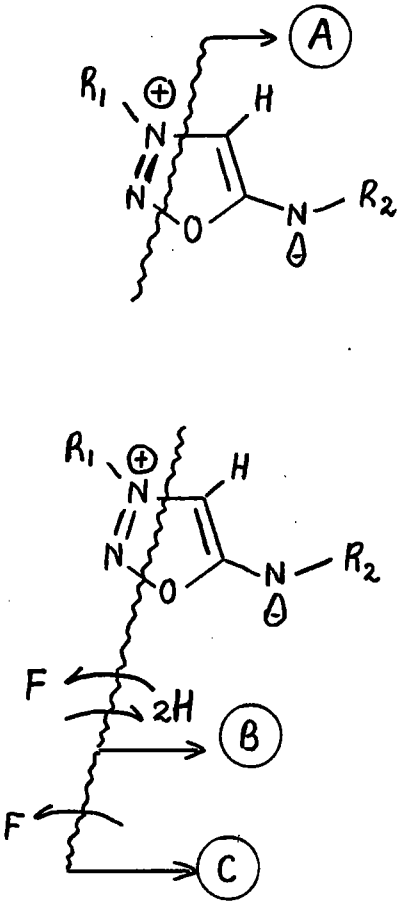

Figure 3 Ring fragmentation of *N*-fluoroacyl derivatives of 3-(1-methyl-2-phenylethyl)-sydnone imine

Conclusion

It can be concluded that the identification of *p*-OH-MB can be achieved by a relative thermostable *N*-fluoroacyl derivative. Compared to identification by means of the GC/MS analysis of the pyrolysis product, the GC/MS analysis of the *N*-pentafluoropropionyl derivative in the NCI mode is an significant improvement.

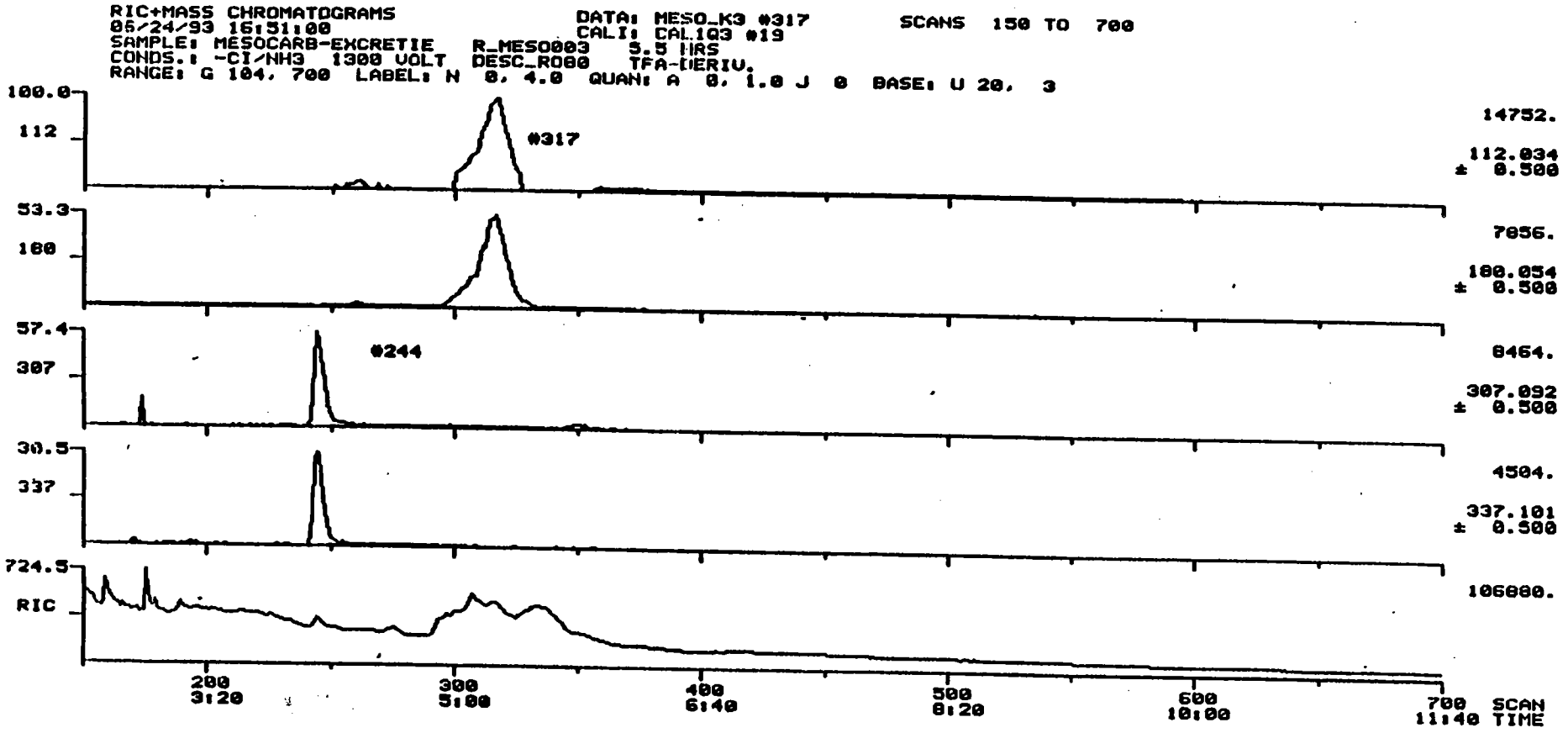

Figure 4 Characteristic mass chromatograms as obtained by the GC/MS analysis in the NCI-mode of *p*-OH-MB after isolation using method 2 and conversion into the *N*-trifluroacetyl derivative

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Table 1 Partial mass spectrometric data of *N*-acyl- and *N*-fluoroacyl-3-(1-methyl-2-phenylethyl)-sydnonimine derivatives in the EI mode

derivate	MW	*m/z* values of characteristic ions (normalized on the base peak)						
		M+·	[M - R_2]+	[C_6H_5-CH_2-CH=CH]+·	[C_6H_5-CH_2-CH=CH_2]+	[C_6H_5-CH_2-CH_2-CH_2]+·	[C_7H_7]+·	miscellaneous
N-ACYL								
acetyl	245	n.d.	230(1)	117(5)	118(4)	119(1)	91(100)	173(7)
propyl	259	n.d.	230(1)	117(3)	118(4)	119(1)	91(100)	173(7)
butyryl	273	n.d.	n.d.	117(3)	118(5)	119(1)	91(100)	173(6)
N-FLUOROACYL								
trifluoroacetyl	299	299(1)	230(7)	n.d.	118(14)	119(22)	91(100)	
pentafluoropropionyl	349	349(1)	230(7)	n.d.	118(9)	119(30)	91(100)	
heptafluorobutyryl	399	399(1)	230(8)	n.d.	118(11)	119(35)	91(100)	

n.d. = not detected; R_2 = *N*-side chain

Table 2A Partial mass spectrometric data of *N*-acyl- and *N*-fluoroacyl-3-(1-methyl-2-phenylethyl)-sydnonémine derivatives in the PCI mode

derivate	MW	*m/z* values of characteristic ions (normalized on the base peak)					
		MH+	[M - R_2]+	[C_6H_5-CH_2-CH_2-CH_3]+	[C_6H_5-CH_2-CH_2-CH_2]+·	[C_7H_7]+·	miscellaneous
N-ACYL							
acetyl	245	n.d.	n.d.	120(10)	119(100)	91(22)	204(15) 177(10) 148(12)
propionyl	259	n.d.	n.d.	120(10)	119(100)	91(27)	204(12) 177(11) 148(13)
butyryl	373	n.d.	n.d.	120(10)	119(100)	91(31)	204(13) 177(12) 148(12)
N-FLUOROACYL							
trifluoroacetyl	299	300(21)	230(6)	120(10)	119(100)	91(22)	see Table 2B
pentafluoropropionyl	349	350(27)	230(7)	120(10)	119(100)	91(15)	see Table 2B
heptafluorobutyryl	399	400(35)	230(6)	120(10)	119(100)	91(14)	see Table 2B

n.d. = not detected; R_2 = *N*-side chain

Table 2B Partial mass spectrometric data of *N*-fluoroacyl-3-(1-methyl-2-phenylethyl)-sydnonimine derivatives in the PCI mode

derivate	MW	*m/z* values of characteristic ions (normalized on the base peak)			
		$[M - CO - F]^{+\cdot}$	$[M - C_6H_5\text{-}CH_2\text{-}CH_2\text{-}CH_2 + 2H]^{+\cdot}$	ring fragment d	$[H_3N\text{-}CO\text{-}C_nF_{2n+1}]^+$
trifluoroacetyl (n = 1)	299	252(15)	182(4)	154(15)	114(16)
pentafluoropropionyl (n = 2)	349	302(1)	232(4)	204(11)	164(9)
heptafluorobutyryl (n = 3)	399	352(1)	282(4)	254(14)	214(6)

n.d. = not detected

Table 3 Partial mass spectrometric data of *N*-fluoroacyl-3-(1-methyl-2-phenylethyl)-sydnonimine derivatives in the NCI mode

derivate	MW	*m/z* values of characteristic ions (normalized on the base peak)				
		M-·	[M-H]-	[M - C_7H_7]-	[M - C_7H_7-CH_2-CH_2-CH_2]-	ring A
trifluoroacetyl (n = 1)	299	n.d.	298(1)	208(3)	180(85)	152(1)
pentafluoropropionyl (n = 2)	349	n.d.	348(2)	258(4)	230(100)	202(4)
heptafluorobutyryl (n = 3)	399	n.d.	398(3)	308(3)	280(100)	252(1)
		[M - C_7H_7 - CF_3]-·	ring B	ring C	[M - C_7H_7-CH_2 - CF_3]-·	[HN-CO-C_nF_{2n+1}]-·
trifluoroacetyl (n = 1)	299	139(4)	n.f.	n.f.	125(17)	112(100)
pentafluoropropionyl (n = 2)	349	189(4)	185(5)	183(23)	n.d.	162(50)
heptafluorobutyryl (n = 3)	399	239(2)	235(1)	233(11)	n.d.	212(30)

n.d. = not detected

n.f = not formed