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Biological Implications of the Reaction Possibilities of the Proximate Carcinogenic Compound, *N*-Hydroxy-2-fluorenylacetamide

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Abstract—The biological implications, the reaction possibilities and the structure of different derivatives of *N*-hydroxy-2-fluorenylacetamide have been described.

Introduction

MOST carcinogenic compounds are active because they are metabolized to a highly reactive electrophilic intermediate which reacts with macromolecular cellular constituents to initiate the carcinogenic event.[1,2]

N-2-fluorenylacetamide, a potent hepatocarcinogen, is both ring- and *N*-hydroxylated in many animal species, including man. Whereas the ring-hydroxylated products are noncarcinogenic, the *N*-hydroxy derivative is considerably more carcinogenic than *N*-2-fluorenylacetamide and is considered as being the proximate carcinogenic product. *N*-hydroxy-2-fluorenylacetamide has been shown to undergo a further metabolic conversion into a sulphuric acid ester presumed to be the ultimate carcinogen.[3–5]

In the course of a study undertaken to measure the influence of environmental factors on the rates of *in vitro* and *in vivo* transformation of *N*-2-fluorenylacetamide in active and inactive metabolites, we have endeavoured to develop a method enabling us to determine the ring as well as the *N*-hydroxy derivatives of 2-fluorenylacetamide at very low concentrations in different biological fluids. During the development of this method, which will be described elsewhere, some interesting derivatives were obtained and identified by g.c.m.s. The present paper is devoted to the description and the possible biological significance of such derivatives.

Experimental

MATERIALS

All solvents used were analytical grade solvents (Merck). The silylating and acetylating reagents were purchased from Macherey-Nagel. *N*-methyl *N'*-nitro-*N*-nitrosoguanidine (Aldrich) was used as a source of diazomethane.

A pure sample of *N*-hydroxy-2-fluorenylacetamide was supplied by J. H. Weisburger (National Cancer Institute, Bethesda, Maryland 20014, U.S.A.).

METHODS

N-Hydroxy-2-fluorenylacetamide (50 μg) was silylated for 30 min at 40 °C with Trisil, which is a mixture of hexamethyldisilazane (1 ml), trimethylchlorosilane (0.5 ml) and pyridine (2 ml). The reaction mixture was evaporated to dryness and the residue dissolved in 50 μl of acetonitrile. The acetonitrile solution was submitted to g.c.m.s. analysis. Two peaks with a retention time of 4 min (derivative **1**) and 6 min (derivative **2**), respectively, were recorded and magnetically scanned under the conditions described in the instrumentation section.

N-hydroxy-2-fluorenylacetamide (50 μg) was methylated for 30 min at room temperature with diazomethane in ethereal solution. The ethereal solution was evaporated to dryness and the residue dissolved in 50 μl of chloroform. The reaction mixture was submitted to g.c.m.s. analysis. Two peaks with a retention time of 1.5 min (derivative **3**) and 2.5 min (derivative **4**), were recorded and scanned under the conditions described in the instrumentation section.

N-hydroxy-2-fluorenylacetamide (50 μg) was trifluoroacetylated for 30 min with 30 μl of trifluoroacetic anhydride at 50 °C. The reaction mixture was evaporated to dryness and the residue dissolved in 50 μl of chloroform.

The resulting solution was submitted to g.c.m.s. analysis. One peak with a retention time of 1.5 min was recorded and scanned under the conditions described in the instrumentation section.

N-hydroxy-2-fluorenylacetamide (100 μg) was allowed to react at 60 °C for 30 min in 6 N hydrochloric acid. The reaction mixture was basified by NH_4OH to pH 8 and extracted twice with 1 ml of chloroform. The chloroformic extracts were evaporated to dryness and the residue redissolved in 100 μl of chloroform. Fifty μl of the resulting solution was submitted to g.c.m.s. analysis, and one peak with a retention time of 55 s was recorded and scanned under the conditions described below. The remaining 50 μl was evaporated to dryness and the residue trifluoroacetylated for 30 min with

30 μl of trifluoroacetic anhydride at 50 °C. The reaction mixture was evaporated to dryness and the residue dissolved in 50 μl of chloroform. The resulting solution was submitted to g.c.m.s. analysis and one peak with a retention time of 1 min was recorded and scanned under the following conditions.

INSTRUMENTATION

Mass spectrometric analysis was carried out with a LKB 9000 S instrument. All the derivatives were injected into the gas chromatograph with a flash heater temperature of 250 °C, a helium carrier gas flow rate at 30 ml per min and an oven temperature of 220 °C.

The column was a standard LKB coiled glass gas chromatographic column 2.2 m long by 0.5 cm o.d packed with 1% OV_1 on 60/80 mesh chromosorb W. The Ryhage type of molecular separator was maintained at 270 °C. All mass spectra were recorded at 70 eV electron energy with 3500 V accelerating voltage, trap current 60 μA and ion source temperature 270 °C.

Chromatograms were recorded from the total ion current monitor located between the electrostatic and magnetic sectors.

Results and discussion

MASS SPECTRA OF THE SILYLATED DERIVATIVES

The mass spectra of the two silylated derivatives **1** and **2** are illustrated in Fig. 1. The mass spectrum of **1** reveals a series of characteristic ions at *m/e* 311, 296, 295, 281, 269, 253, 237, 223, 206, 195, 181, 165, 152, which are nearly all present in the mass spectrum of **2** but with significant differences in intensities.

Thus the ions *m/e* 237 and 195 are almost absent in the mass spectrum of **1** and are present in that of **2**, the ions *m/e* 223 and 181 are more intense for **1** than for **2**, and the ion of *m/e* 206 is present in the mass spectrum of **1** and absent in that of **2**.

In conclusion, **1** and **2** have the same molecular weights and fragmentation patterns, but display differences in ion intensities. The only reasonable explanation for this fact is that *N*-hydroxy-2-fluorenylacetamide may undergo different tautomeric equilibria similar to those illustrated in Scheme 1.

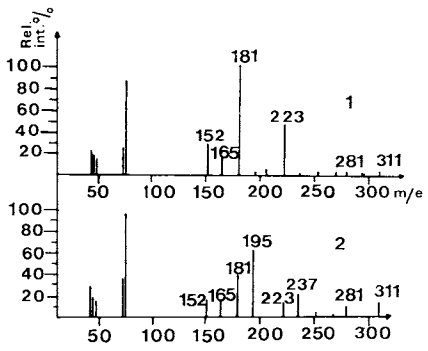


FIG. 1. Mass spectra of the silylated derivatives **1** and **2** of *N*-hydroxy-2-fluorenylacetamide.

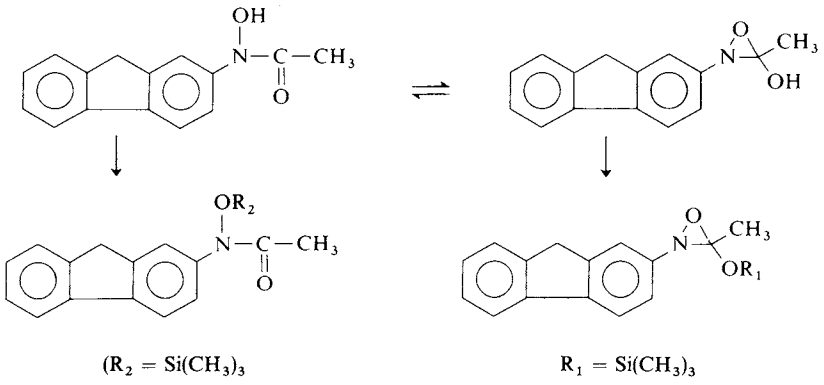
The possible and comparative fragmentation patterns for the two derivatives **1** and **2** are illustrated in Scheme 2.

Derivative **1**, which contains an oxaziridine ring, may lose oxygen[6] and a C_3H_8Si molecule to give rise to *m/e* 223. The ion of *m/e* 223 may give rise to other ionic entities such as *m/e* 181, 165 by loss of ketene or an acetamido radical. By loss of ketene derivative **2** may give rise to *m/e* 269, which may be followed by further loss of trimethylsilane to form *m/e* 195. Derivative **2** may lose a C_3H_8Si molecule to give *N*-hydroxy-2-fluorenylacetamide which in turn may lose oxygen to produce *m/e* 223 as already noted in other studies.[5] Loss of a C_2H_6SiO molecule from derivative **2** gives the ion of *m/e* 237 which in turn may lose ketene to form *m/e* 195.

MASS SPECTRA OF THE METHYLATED DERIVATIVES

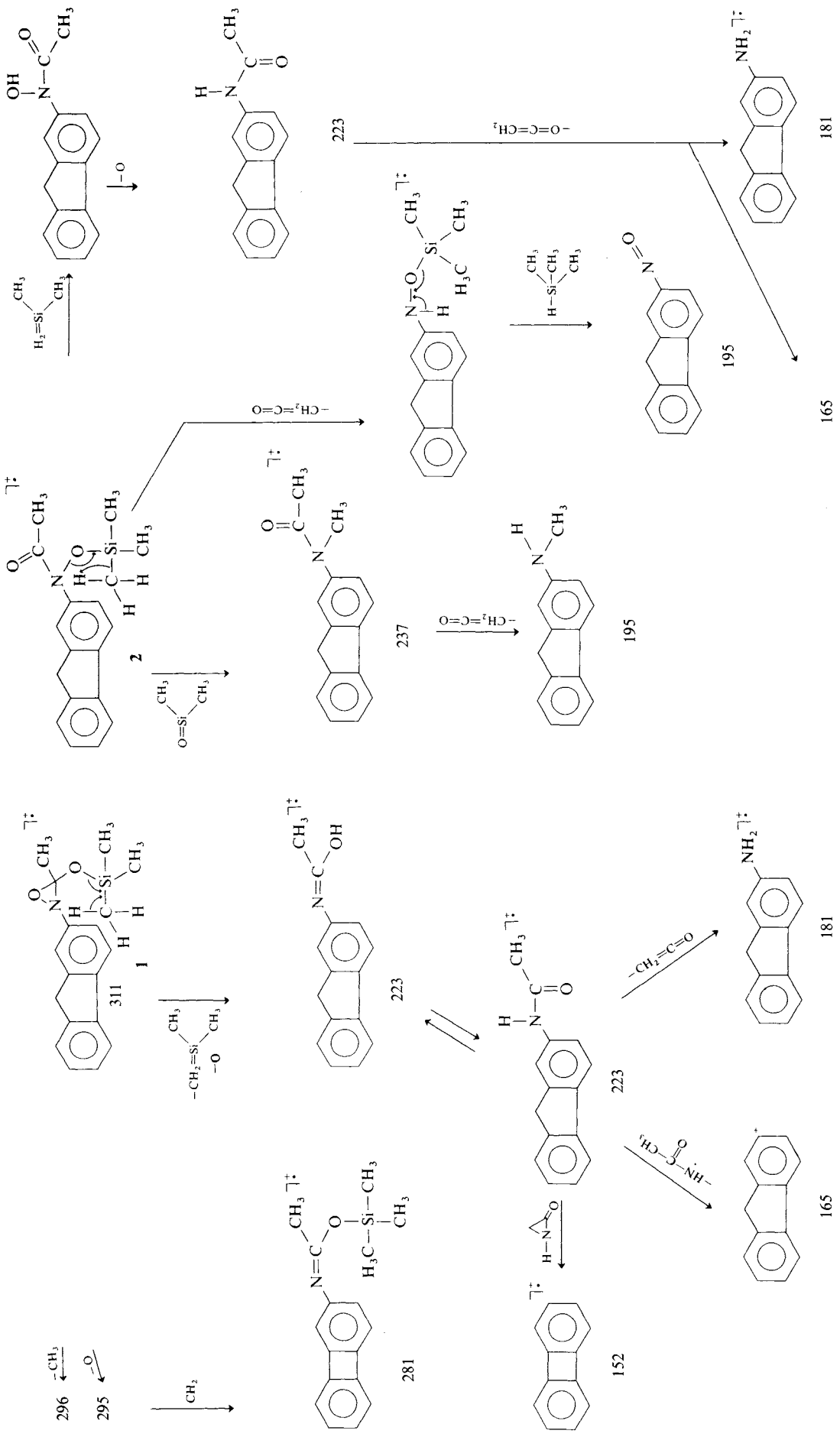
The same situation may be observed in the mass spectra (Fig. 2) of the methylated compounds **3** and **4**. The mass spectrum of compound **3** shows a series of characteristic ions at *m/e* 253, 223, 211, 196, 181, 180, 165, 152 which are nearly all present in the mass spectrum of **4** but with different intensities. Ions of appreciable abundance such as *m/e* 237 (base peak) and *m/e* 206 in the mass spectrum of **4** are absent in that of **3**.

In contrast, ions such as *m/e* 223 and 211 are absent in the mass spectrum of **4** and are present in that of **3**.



(R_2 = $Si(CH_3)_3$ R_1 = $Si(CH_3)_3$

SCHEME 1. Tautomers of *N*-hydroxy-2-fluorenylacetamide and their silylated derivatives.



SCHEME 2. Possible fragmentation pattern of the silylated derivatives.

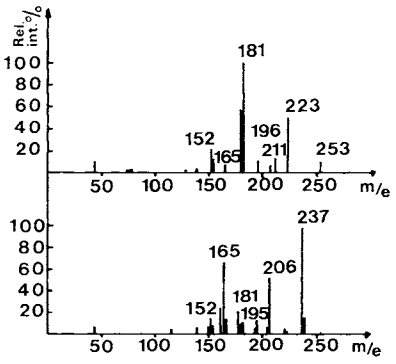


FIG. 2. Mass spectra of the methylated compounds **3** and **4**.

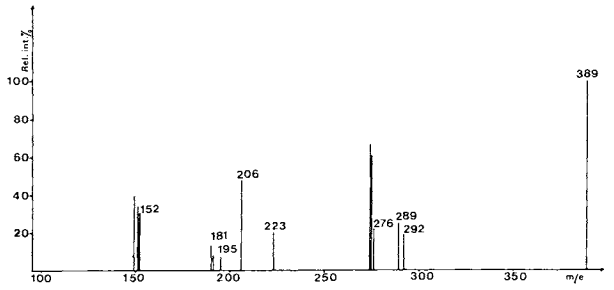


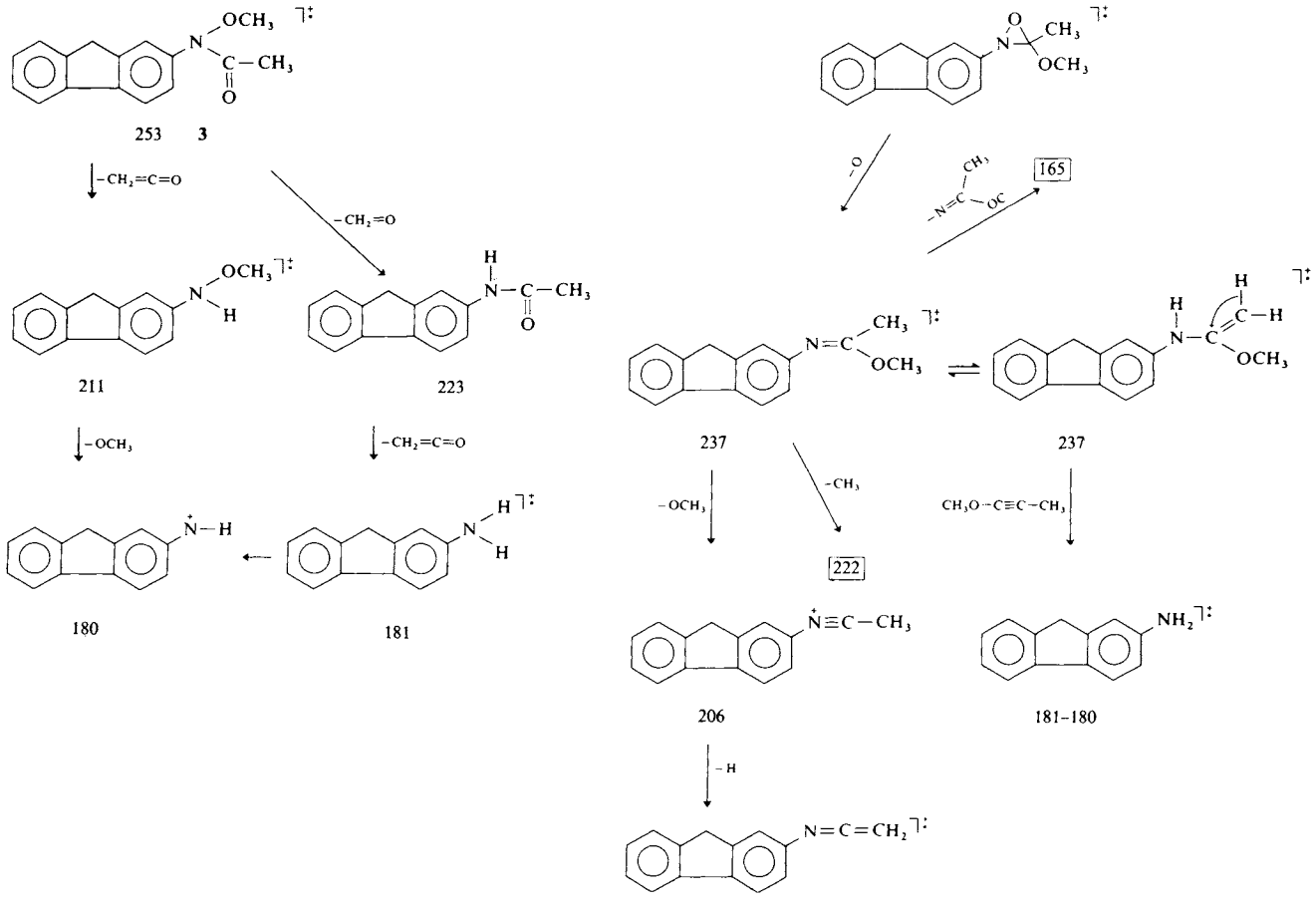
FIG. 3. Mass spectrum of the trifluoroacetylated derivative of *N*-hydroxy-2-fluorenylacetamide.

These observations may be explained easily if the different tautomeric forms of *N*-hydroxy-2-fluorenylacetamide are reconsidered. The possible fragmentation patterns of the methylated compounds **3** and **4** are illustrated in Scheme 3. It can be seen that the derivative **3** may lose formaldehyde from the ion of *m/e* 253 to give rise to *m/e* 223, which in turn may lose ketene to produce *m/e* 181.

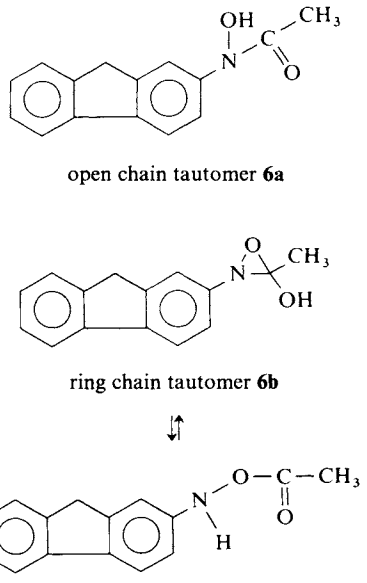
Successive losses of ketene and a methoxy group give rise to the ions *m/e* 211 and 180. The absence of ions *m/e* 223 and 211 in the mass spectrum of derivative **4**, must certainly be attributed to the structure of derivative **4** that contains an oxaziridine ring and may lose oxygen to give *m/e* 237, which in turn may give rise to *m/e* 206 by loss of a methoxy group and to *m/e* 222 by loss of a methyl group.

MASS SPECTRA OF THE TRIFLUOROACETYLATED DERIVATIVE

The mass spectrum (Fig. 3) of the trifluoroacetylated derivative **5** of *N*-hydroxy-2-fluorenylacetamide shows

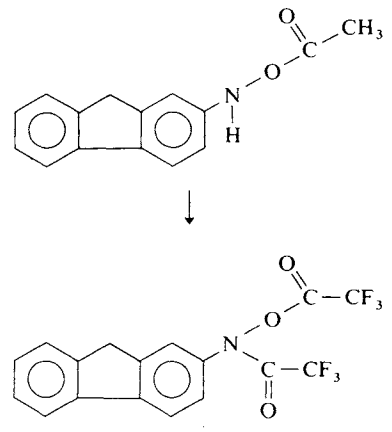


SCHEME 3. Possible fragmentation pattern of the methylated compounds **3** and **4**.



SCHEME 4. Open chain and ring chain tautomers of *N*-hydroxy-2-fluorenylacetamide.

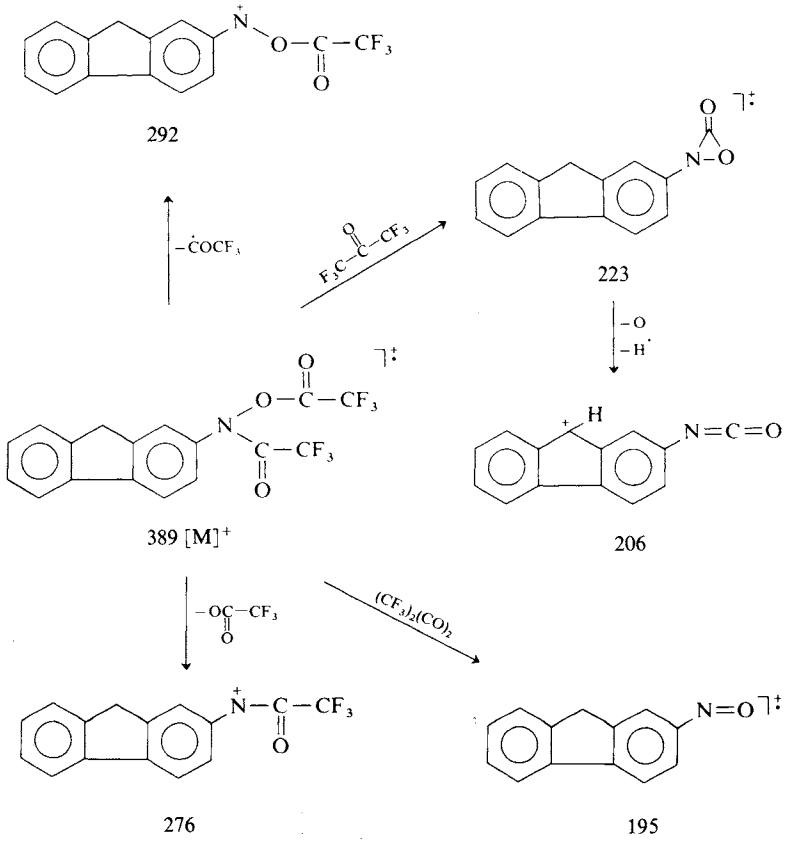
a series of ions at *m/e* 389, 292, 289, 276, 275, 274, 223, 206, 195, 181, 180. The existence of these ions may be explained easily if the following open chain and ring chain tautomers (Scheme 4) of *N*-hydroxy-2-fluorenylacetamide are considered.



SCHEME 5. Formation of derivative **5** from tautomer **6c**.

The trifluoroacetylation of the open chain tautomer **6c** (Scheme 5) gives rise to two subsequent reactions, including the trifluoroacetylation of the NH group and the transesterification of the acetate group leading to derivative **5**.

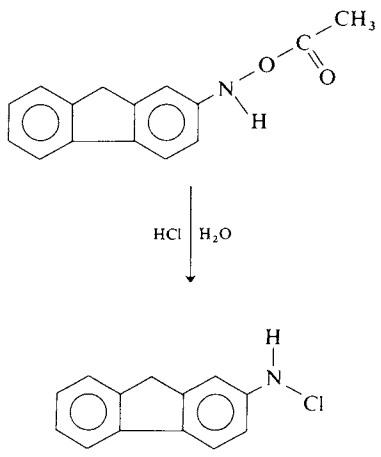
The possible fragmentation pattern of substance **5** is shown in Scheme 6. It may be seen that the most important ions in the mass spectrum of **5** are easily derived from the molecular ion by simple breakdowns or rearrangement mechanisms.



SCHEME 6. Possible fragmentation pattern of substance **5**.

FORMATION OF *N*-CHLORO-2-FLUORENAMINE

When submitted to 6 N hydrochloric acid, *N*-hydroxy-2-fluorenylacetamide gives rise to a derivative (*N*-chloro-2-fluorenamine) which is detected by g.c.m.s. analysis. The formation of this substance (Scheme 7) may be explained if we consider the same tautomer **6c** of *N*-hydroxy-2-fluorenylacetamide previously used to explain the formation of the trifluoroacetylated derivative **5**.

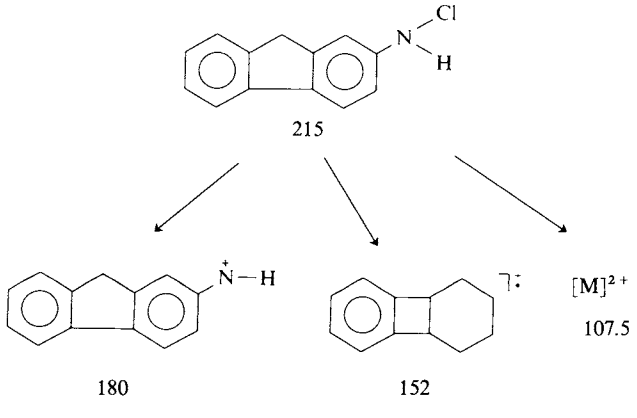


N-chloro-2-fluorenamine

SCHEME 7. Formation of *N*-chloro-2-fluorenamine.

The mass spectrum (Fig. 4) of *N*-chloro-2-fluorenamine shows a molecular ion at *m/e* 215 accompanied by an isotopic peak at *m/e* 217. The other main fragmentation peaks (Scheme 8) are found at *m/e* 180 due to the loss of the chlorine atom and at *m/e* 152. A doubly charged ion may also be observed at *m/e* 107.5 corresponding to $[M]^{2+}$. Fragment ions, where chlorine atoms are retained on the aromatic part of the molecule are not observed. When trifluoroacetylated, *N*-chloro-2-fluorenamine gives *N*-chloro-2-fluorenyltrifluoroacetamide.

This is confirmed by the mass spectrum (Fig. 5) of *N*-chloro-2-fluorenyltrifluoroacetamide, which shows a molecular ion at *m/e* 311 accompanied by an



SCHEME 8. Possible fragmentation pattern of *N*-chloro-2-fluorenamine

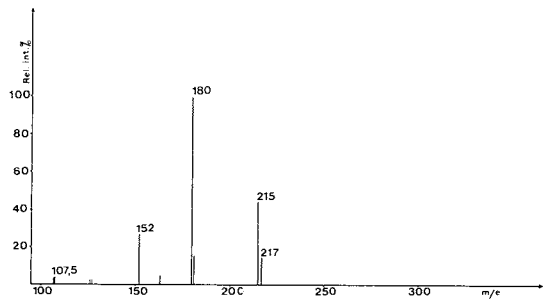


FIG. 4. Mass spectrum of *N*-chloro-2-fluorenamine.

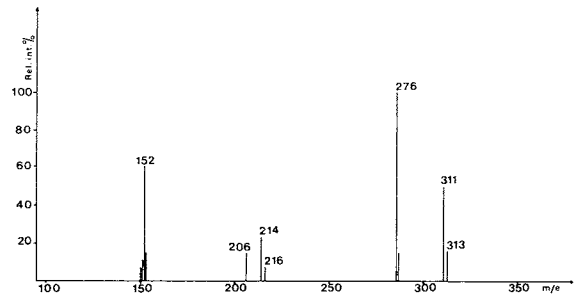
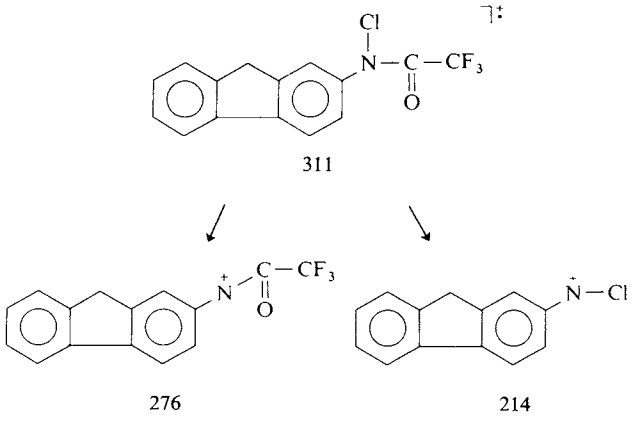


FIG. 5. Mass spectrum of *N*-chloro-2-fluorenyltrifluoroacetamide.

isotopic peak at *m/e* 313. The presence and the origin of other fragmentation peaks (Scheme 9) at *m/e* 276, 216, 214, 152, are explained in the same way as for *N*-chlorofluorenamine.



SCHEME 9. Possible fragmentation pattern of *N*-chlorofluorenyltrifluoroacetamide.

It may be concluded from the series of silylated, methylated, acetylated and chlorinated derivatives obtained from *N*-hydroxy-2-fluorenylacetamide, that the chemistry of this compound is closely related to the existence of ring chain tautomers such as those shown in Scheme 4. The fact that all these reactions are taking place in the aliphatic moiety of the molecule is particularly evident in the case of *N*-chloro-2-fluorenamine.

It could be believed that *N*-chloro-2-fluorenamine may be formed from *N*-hydroxy-2-fluorenamine, consequently decreasing the need to invoke the existence

of a ring chain tautomerism effect. It is a known fact that in diluted hydrochloric acid, arylhydroxylamines give the corresponding *o*- and *p*-substituted chloroanilines[5] and, as already mentioned, fragment ions in the mass spectrum of *N*-chloro-2-fluorenamine, where the chlorine atom would be attached to the aromatic ring, are not observed. Examples, where the existence of ring chain tautomers may orientate a particular reaction, are not uncommon[7–10] but not mentioned in the literature for *N*-hydroxy-2-fluorenylacetamide.

From a biochemical point of view and as is generally recognized, if the complex biological effects of *N*-2-fluorenylacetamide rests first on *N*-hydroxylation and second on esterification of the *N*-hydroxy derivative with sulphate, then it could be suggested that all the tautomers of *N*-hydroxy-2-fluorenylacetamide as such may be considered to be active intermediates which play an important rôle in the biochemical reactions of this compound and consequently in its carcinogenic properties.

Moreover, the formation of *N*-chloro-2-fluorenyltrifluoroacetamide leaves the door open for the specific determination of *N*-hydroxy-2-fluorenylacetamide in different biological fluids.

ACKNOWLEDGEMENTS

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