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Hydroxyproline reaction with free radicals generated during benzoyl peroxide catalytic decomposition of carbon tetrachloride Structure of reaction products formed

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Summary. Benzoyl peroxide catalytic decomposition of carbon tetrachloride in a model system produces trichloromethyl and trichloromethylperoxyl free radicals. These radicals are also produced by CCl_4 bioactivation in liver and are considered to be responsible for the deleterious effects of this hepatotoxin. In this study, it is attempted to learn about how the $.CCl_3$ and $CCl_3O_2.$ tend to react with hydroxyproline in a model system. Hydroxyproline was selected because of its role in collagen metabolism. During the interaction of both radicals with hydroxyproline a total of 16 reaction products were isolated and identified by gas chromatographic-mass spectrometric analysis. All of them were hydroxyproline analogs, no single one contained C from CCl_4 and only three contained chlorine. Consequently, most adducts would be missed in experiments where formation of reaction products are studied by formation of ^{14}C or ^{36}Cl labeled adducts (e.g. covalent binding studies used by toxicologists). If similar hydroxyproline analog reaction products were observed during CCl_4 intoxication it might be reasonably expected that they interfered with collagen metabolism and participate in cirrhogenic effects of CCl_4 on the liver.

Keywords: Amino acids – Carbon tetrachloride – Hydroxyproline – Free radicals – Collagen – Cirrhosis

Introduction

CCl_4 is a very well known hepatotoxin producing in that organ necrosis, fat accumulation, cirrhosis and cancer (IARC, 1997; USEPA, 1983). Deleterious effects of this haloalkane on the liver are believed to require the prior biotransformation of it in a cytochrome P450 catalyzed reaction to $.CCl_3$ and $CCl_3O_2.$ free radicals (Slater, 1982; Castro, 1984, 1990; Dianzani, 1987; Recknagel, 1967; Recknagel et al., 1986). These reactive moieties would later

interact with lipids, proteins, nucleic acids, heme and other cellular components to initiate a chain of alterations ending in the undesirable effects described above. The chemistry of those interactions has been very well studied in the case of lipids (Frank et al., 1989; Ansari et al., 1982). Less is known in the case of nucleic acids (Castro et al., 1989a; Castro and Castro, 1993; Castro et al., 1994a); Díaz Gómez and Castro, 1980, 1981; proteins (de Castro et al., 1984; Castro et al., 1989a,b, 1991, 1994b; Villarruel and castro, 1980) or other molecules (Fernández et al., 1982). In a recent work from our laboratory we provided evidence that the amino acid proline is a very sensitive target molecule for the reaction with the $\cdot CCl_3$ and $CCl_3O_2\cdot$ free radicals (Castro et al., 1995). We also envisaged the possibility that this type or equivalent interactions between those radicals and that amino acid somehow were involved in the dissorders of collagen-metabolism sparked by CCl_4 and ending in cirrhosis (Castro et al., 1995). In this work the interactions between $\cdot CCl_3$ and $CCl_3O_2\cdot$ with other amino acid relevant to collagen metabolism, 4-hydroxyproline (Darnell et al., 1986) are studied in a chemical model system. The mass spectrometric analysis of reaction products formed is performed to elucidate their structure.

Materials and methods

Chemicals

CCl_4 was purchased from Mallinkrodt and distilled before use. Benzoyl peroxide was purchased from Sigma Chemical Co. Benzoyl peroxide contains about 30% water as stabilizer. N-acetylhydroxy-l-proline ethyl ester (OHPROAE) was synthesized from N-acethlhydroxy-l-proline (Sigma Chemical Co.) by heating for two hours with 4% hydrogen chloride in absolute ethanol under nitrogen. Purity of the product was checked by GC/MS (as the silylated derivative). No detectable amounts of the reaction products were originally present in the OHPROAE itself.

Procedures

The study of the in vitro chemical interaction of CCl_4 with OHPROAE was carried out as follows: 0.2 mg of the amino acid derivative in 3 ml of CCl_4 was heated at 80°C for 5 hours with benzoyl peroxide (1 mg or 0.1 mg as indicated) in an ampoule. Reaction mixture and ampoule were purged with either nitrogen or air, as indicated in each experiment before sealing. At the end of this period, the solvent was evaporated under nitrogen at 40°C. The residue was then silylated with a mixture of BSTFA: acetonitrile (1:1), 0.2 ml at 100°C for 30 min. Blanks without benzoyl peroxide or without OHPROAE were run simultaneously. It is not possible to run blanks without CCl_4 because it is the only solvent of reaction mixture. Further, it is not possible to run the reaction in solvents other than CCl_4 (eg. methanol, glyme, acetone and others) because they generate themselves free radicals which obscure identification by production of many artifacts.

Total ion current (TIC) chromatographic analysis and mass spectrometric identification of reaction products were performed in a Hewlett Packard Model 5970 B mass selective detector interfaced to a HP 5890 gas chromatograph.

Chromatographic conditions were as follows: injection port temperature 250°C, in the splitless injection mode. Separation was carried out in a fused silica capillary column (12 m × 0.2 mm id) crosslinked with 5% phenylmethylsilicone (0.33 um film thickness) (Hewlett Packard, Palo Alto, CA) carrier gas helium (column head pressure, 100 kPa).

Column temperature was maintained at 100°C for 1 min and then increased to 280°C at a ramp velocity of 10°C/min. GC/MS interface temperature was 280°C and ion source was ca. 200°C. Spectra was taken at 70 eV scanning quadrupole from 40 to 750 amu, at 0.77 sec/scan.

It is important to emphasize that we had to rely entirely on MS for identification because the very low product yield from the reaction mixture under experimental conditions employed prevented the use of additional NMR methods to further elucidate the structures.

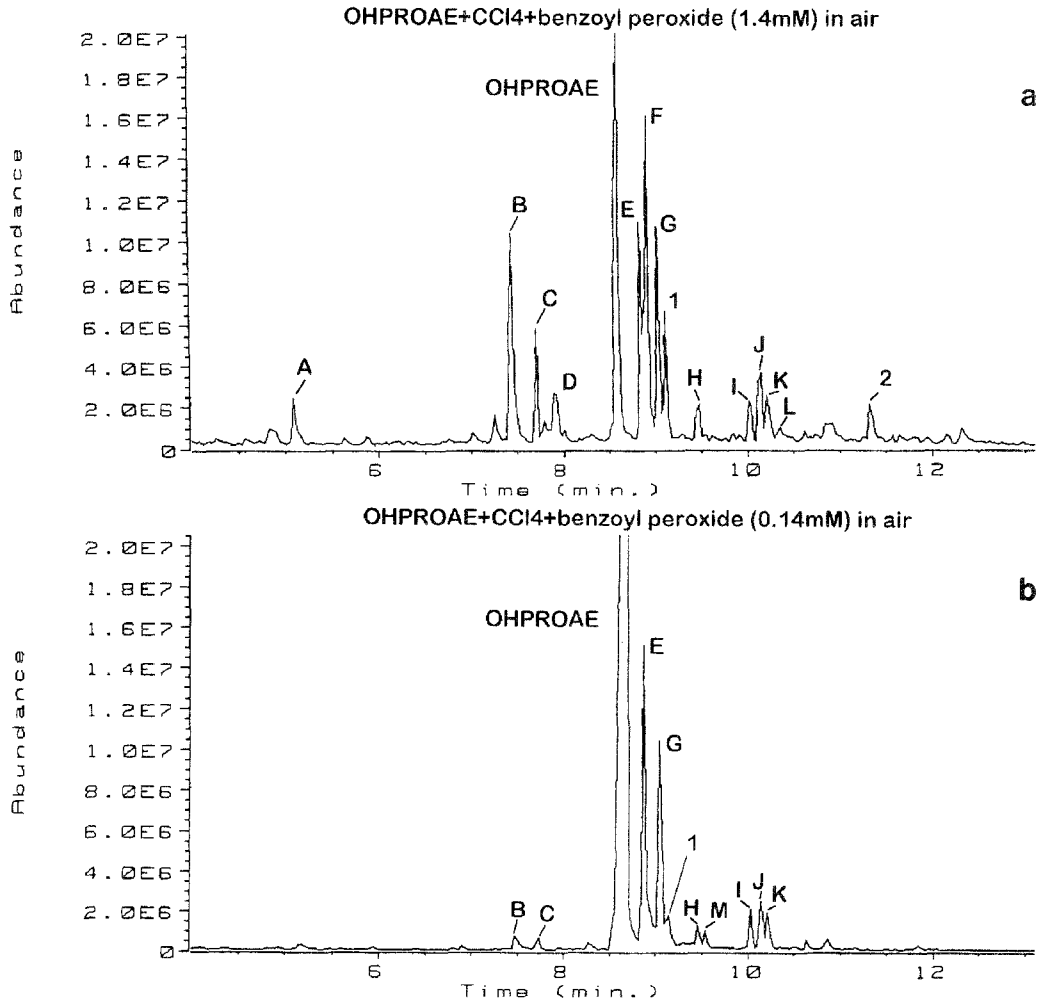

Fig. 1. Gas chromatogram obtained from a sample of the reaction between OHPROAE and CCl_4 in the presence of benzoyl peroxide and under air atmosphere, after trimethylsilylation: **a** With 1 mg (1.4 mM) benzoyl peroxide. Column, fused silica capillary crosslinked with 5% phenylmethyl silicone, programmed at 10°C/min, from 100°C to 280°C. See Methods for datails. Peaks: *A* an N-acetyl-4-hydroxypyrrolidone; *B* opened ring OHPROAE derivatives; *C* and *D* carbonylic OHPROAE; *E* N-acetyl-hydroxyproline trimethylsilyl ester; *F* unesterified analog of peak C; *G* and *J* hydroxy OHPROAE; *H* and *L* unesterified analogs of peak G; *I* and *K* chlorohydroxy OHPROAE. Peaks *1* and *2* denote side products derived from the peroxide, phenyl benzoic acids. **b** With 0.1 mg (0.14 mM) benzoyl peroxide. Peak M corresponds to an isomer of G (hydroxy OHPROAE)

Results

Interaction of CCl_4 with OHPROAE in the presence of benzoyl peroxide under aerobic conditions

The capillary GC analysis with TIC detection of reaction products arising when CCl_4 derived decomposition products attack OHPROAE in a benzoyl peroxide catalyzed reaction under aerobic conditions, is shown in Fig. 1a and b, corresponding to the higher and lower concentration of the peroxide respectively. In the first case eleven reaction products were shown (Fig. 1a). Peak A corresponded to a carbonyl containing derivative of OHPROAE, probably with a 2-pyrrolidone structure. Spectrum (Fig. 2) indicated the absence of the carboxy ethyl group and the loss of 42 amu, (additional to the one corresponding to the acetyl group in the amino acid), supporting the idea of existence of a new carbonyl group. Such kind of proline derivative was observed by Uchida and col. (Uchida et al., 1990) in a Fenton type hydroxyl generation system.

Peak B would be a compound presenting an opened ring structure. Some spectrum features (Fig. 3), such as the loss of the methylamino extreme, suggested this hypothesis.

Next peak (C in Fig. 4) showed to be a carbonyl compound too but in contrast with compound A, the ester group remained intact. Peak D showed essentially the same spectrum.

Peak E corresponded to the trimethylsilyl ester of the acetylated amino acid. Its presence involves the desesterification of OHPROAE carboxyethyl group leading to the free acid. We checked it was not an artifact of

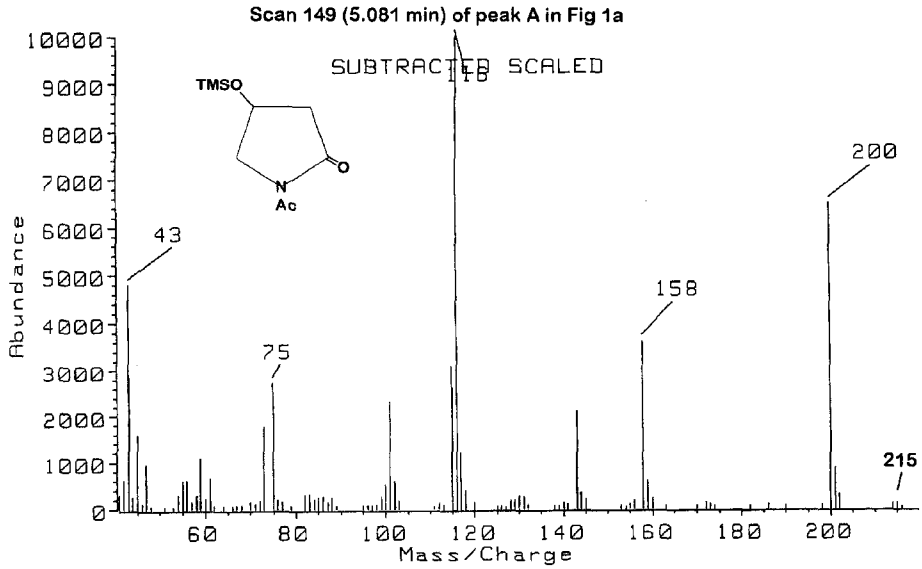

Fig. 2. Mass spectrum taken from peak A in Fig. 1a. M^+. and $(M-15)^+$ were found at m/z 215 and m/z 200 respectively. Loss of CH_2CO from $(M-15)^+$ lead to a fragment at m/z 158 and the base peak at m/z 116 corresponded to the release of 42 amu (ketene or NCO)

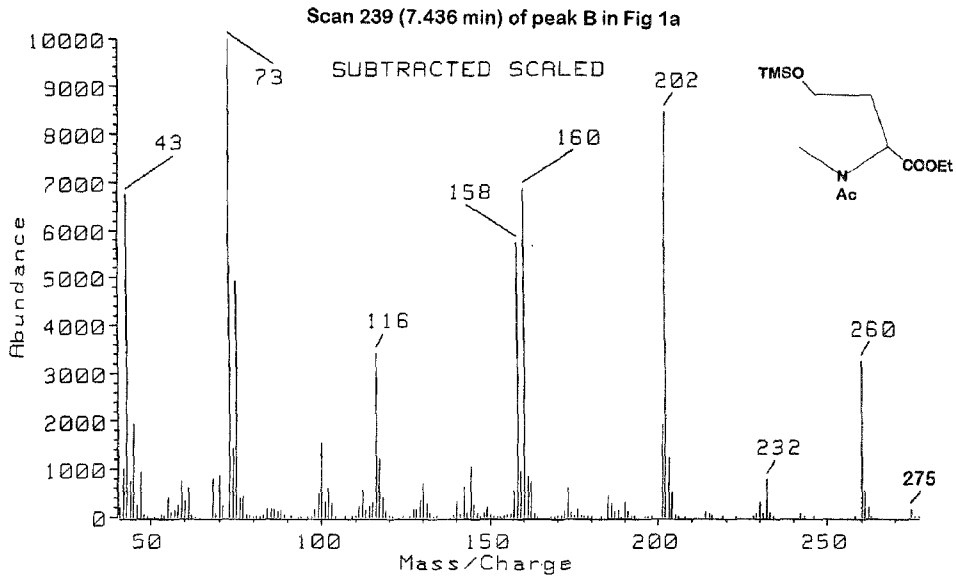

Fig. 3. Mass spectrum taken from peak B in Fig. 1a. M^+. and $(M-15)^+$ were found at m/z 275 and m/z 260 respectively. Loss of .COOEt from the molecular ion lead to a fragment at m/z 202. Other relevant fragments were: m/z 160 (loss of ketene m/z 202), m/z 116 (loss of C_2H_3N from m/z 160), m/z 232 (loss of acetyl from M^+.), and m/z 158 (loss of C_2H_3N from m/z 202)

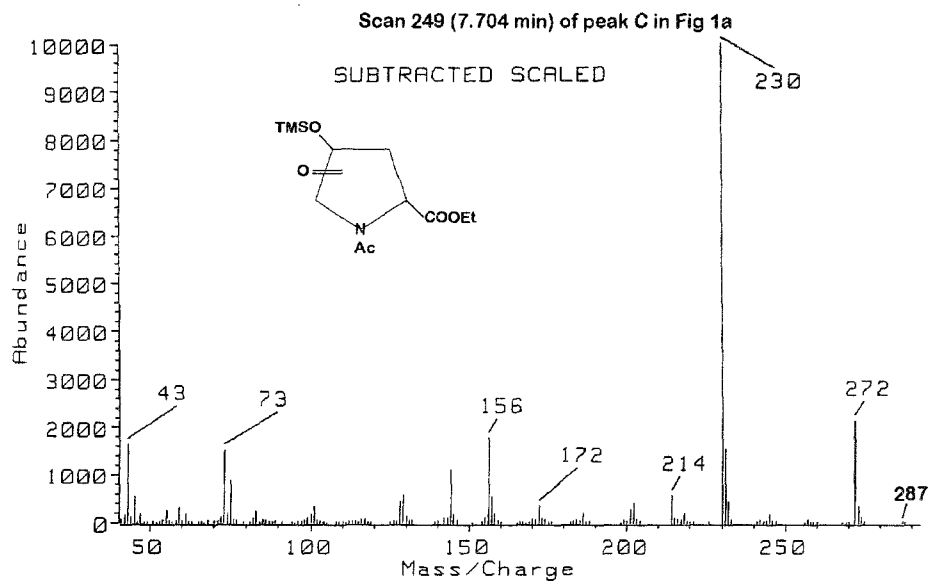

Fig. 4. Mass spectrum taken from peak C in Fig. 1a. M^+. was found at m/287. Loss of ketene from $(M-15)^+$ lead to m/z 230 (base peak) at m/z 156 corresponding to the loss of TMSOH from m/z 230. Other fragments were: m/z 214 (loss of .COOEt from M^+.) and m/z 172 (loss of ketene from m/z 214)

derivatization process with a pure sample of OHPROAE. In a previous work concerning the reaction of radicals with proline (Castro et al., 1995) we observed a similar product.

Peak F corresponded to the unesterified (trimethylsilylated carboxyl group) analog of peak C.

Next peak G (Fig. 5) resulted to be a hydroxy OHPROAE derivative. Spectrum was in accordance with the product observed for the case of proline

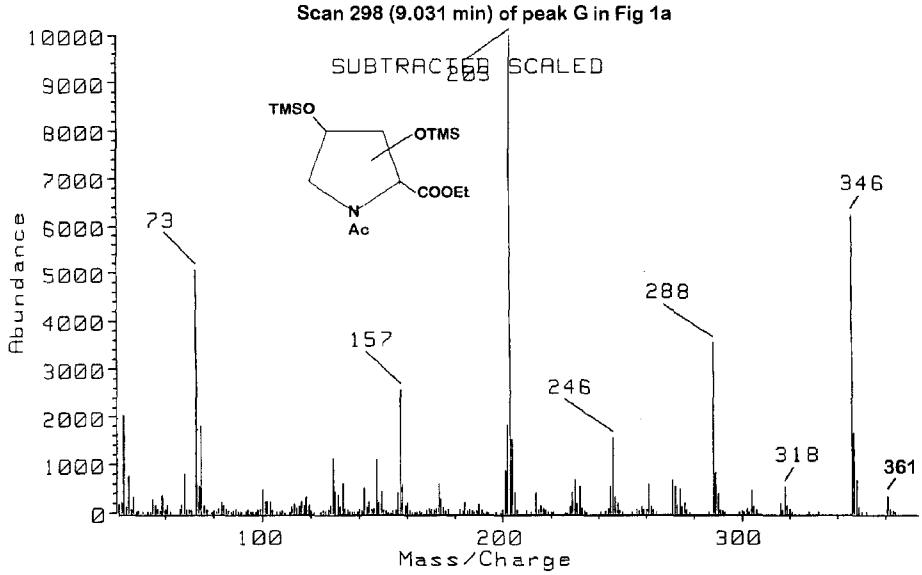

Fig. 5. Mass spectrum taken from peak G in Fig. 1a. M^+. appeared at m/z 361 and was confirmed by m/z 346 (M-15)+). Other relevant fragments were m/z 288 (loss of .COOEt), m/z 246 (loss of ketene from m/z 288) and m/z 157 (loss of TMSO. from m/z 246). The base peak corresponded to m/z 203, a difference of 158amu with M^+. but it could not be accounted for

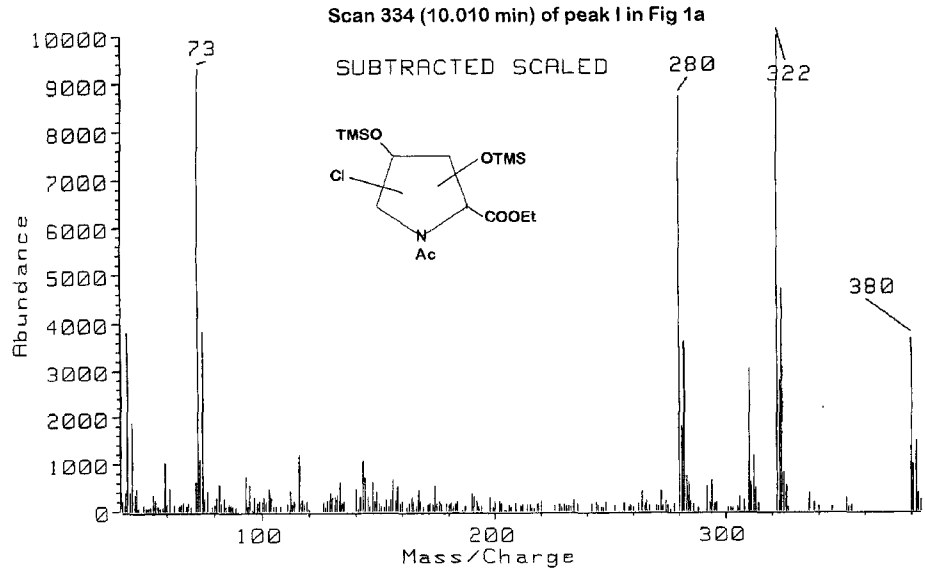

Fig. 6. Mass spectrum taken from peak I in Fig. 1a. M^+. was not present in the spectrum but was inferred from $(M-15)^+$ (m/z 380/382) and m/z 322/324 (loss of .COOEt from M^+.). Other fragment was m/z 280/282 (loss of ketene from m/z 322/324)

with CCl_4 (Castro et al., 1995). Peak J was an isomer of G. Peaks H and L were unesterified analogs of peak G.

Peaks I (spectrum of peak I in Fig. 6) and K corresponded to chloro hydroxy adducts of OHPROAE. Similar products were observed previously by us (Castro et al., 1995).

As the peroxide concentration was decreased, reaction was less intense (Fig. 1b). Unesterified amino acid was still present, as the principal product. Other adducts observed were peaks B, C, G, H, I, J and K. An additional hydroxy adduct isomer of G was observed (peak M).

Interaction of CCl_4 with OHPROAE in the presence of benzoyl peroxide under anaerobic conditions

When oxygen was withdrawn from the previously described reaction a different pattern of reaction products was obtained as revealed by capillary GC analysis (Fig. 7a and b). In the case of the higher concentration of the peroxide, some of the products reported above for the case of reaction proceeding under air appeared again (peaks B, C, E, F, G, H and J). In addition, new peaks were identified.

Peak N (spectrum in Fig. 8) corresponding to a dihydroxy adduct of OHPROAE. At higher retention times two isomeric dimer derivatives appeared (peaks O and P). Spectrum (Fig. 9) from peak O indicated a compound having two OHPROAE moieties linked by a single bond. We were not able to determine to specific place of the linkage. A trace quantity of peak O was also present in the chromatogram corresponding to the reaction mixture proceeding under air and for the lower concentration of peroxide.

When the reaction was allowed to proceed in the presence of a lower concentration of benzoyl peroxide, no significant changes were observed (Fig. 7b) (peaks B, C, E, G, H, I, J, K and M remained present and dimers were present).

Controls in the absence of the peroxide showed only little reaction. Either under air or under nitrogen we observed peaks E and G as the only products, in a lower yield.

Discussion

The present results evidence that this lipid soluble hydroxyproline derivative is able to interact with free radicals arisen during the catalytic decomposition of CCl_4 (presumably CCl_3 and CCl_3O_2.) to give numerous hydroxyproline analogous molecules. No single adduct formed contained C from CCl_4 and only three of them contained chlorine from it. This means that most reaction products would be missed in studies employing isotopic ^{14}C or ^{36}Cl and that they are generated via hydrogen atom abstraction and not by addition reactions of the free radicals on the amino acid.

It is not possible at present to attribute a given relevance to the here described reactions in relation to some aspects of CCl_4 hepatotoxicity. The

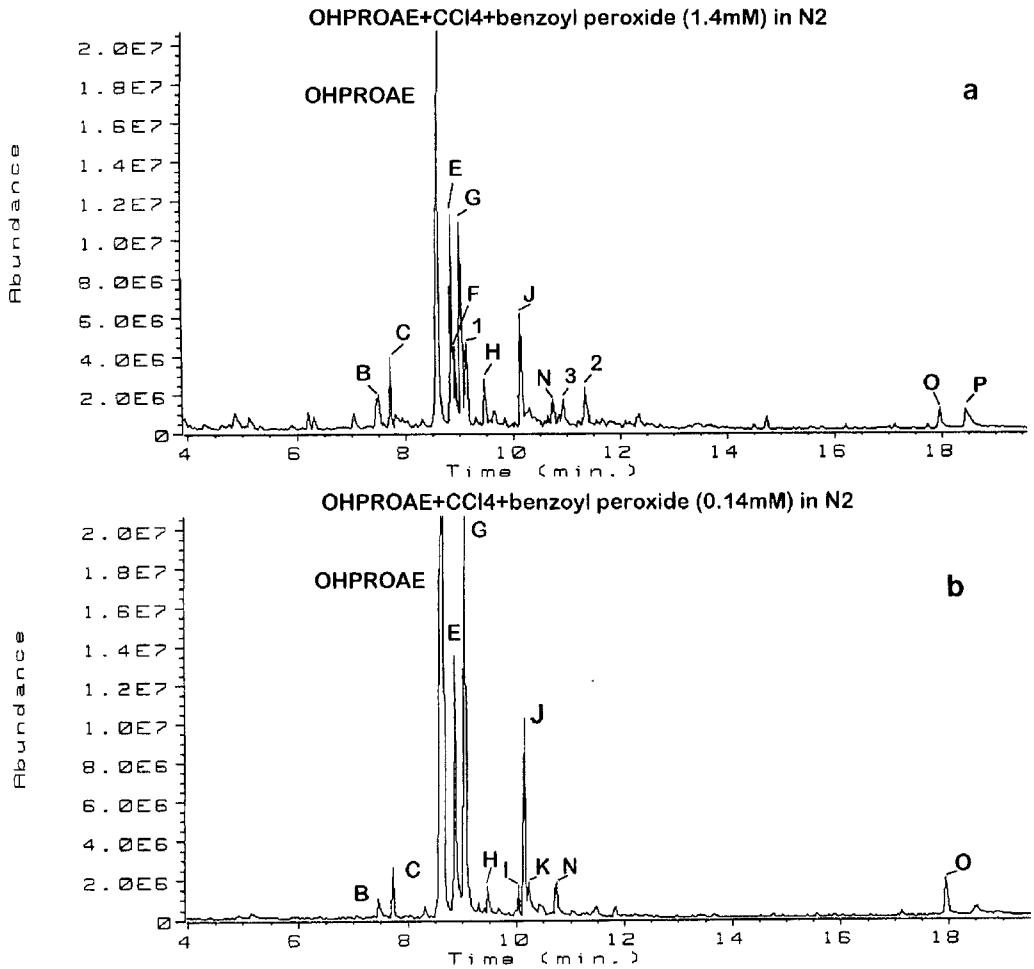

Fig. 7. Gas chromatogram obtained from a sample of the reaction between OHPROAE and CCl_4 in the presence of benzoyl peroxide and under nitrogen atmosphere, after trimethylsilylation. **a** With 1 mg (1.4 mM) benzoyl peroxide. See Methods and Fig. 1 for details. Peaks: *N* dihydroxy OHPROAE; *O* and *P* dimeric OHPROAE. Peaks *1*, *2* and *3* correspond to phenyl benzoic acids. **b** With 0,1 mg (0.14 mM) benzoyl peroxide

formation of at least some of the here reported adducts in vivo should be evidenced prior to hypothesizing about it. The potential significance of these interactions of CCl_4 derived free radicals with hydroxyproline and those previously reported for the case of proline, derives from the role these amino acids have in collagen metabolism and, in relation to the well known cirrhogenic effects of CCl_4 on the liver (IARC, 1979; USEPA, 1983; Darnell et al., 1986; Rojkind, 1982a,b). Several alterations in proline; hydroxyproline and collagen metabolism were reported to occur in the course of CCl_4-induced cirrhosis (Rojkind, 1982a,b; McGee et al., 1973; Montfort and Perez-Tamayo, 1978; Okazaki and Mamyama, 1974; Perez-Tamayo, 1979; Siegel et al., 1978; Galligani et al., 1979; Ehrinpreis et al., 1980; Bolarin et al., 1987; Chofkier et al., 1988; Martinez-Hernandez, 1985; Lindblad and Fuller, 1989; Myara et al., 1987; Camps et al., 1992; Sakaida et al., 1994; Murawaki et al., 1994; Seigert et

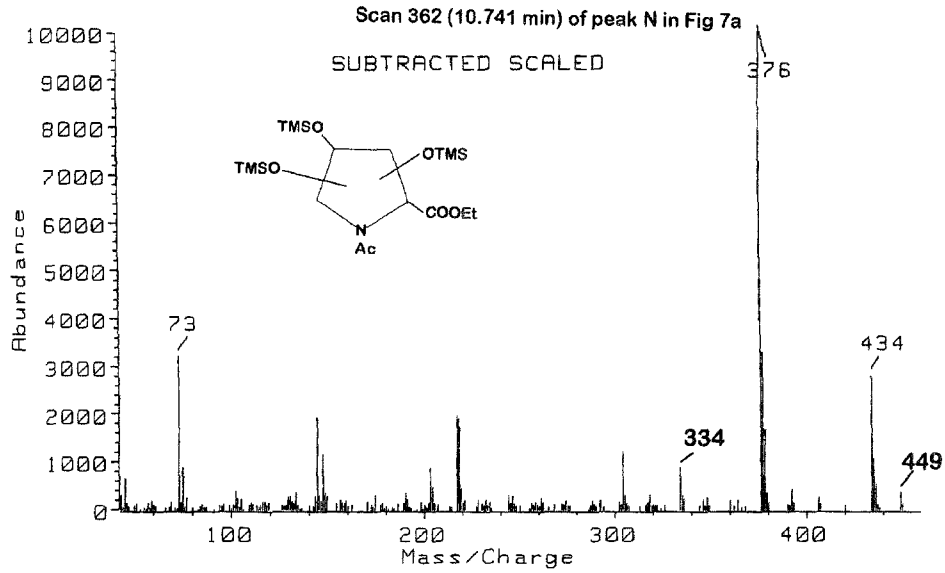

Fig. 8. Mass spectrum taken from peak N in Fig. 7a. M^+. and $(M-15)^+$ were found at m/z 449 and m/z 434 respectively. Loss of .COOEt from the molecular ion lead to a fragment at m/z 376 (base peak) and m/z 334 corresponded to the release of ketene from it

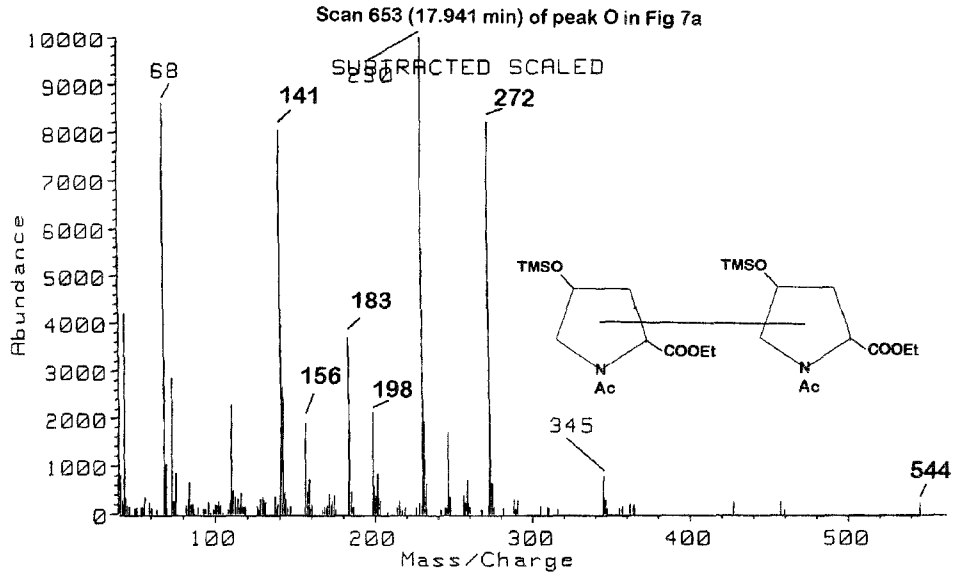

Fig. 9. Mass spectrum taken from peak O in Fig. 7a. M^+. was found at m/z 544. Fragmentation pattern corresponds essentially to the half molecule: m/z 272 (M^+./2), m/z 230 (loss of ketene from M^+./2), m/z 198 (loss of HCOOEt from M^+ ./2), m/z 183 (loss of .Me from m/z 198) and m/z 156 (loss of ketene from m/z 198). Fragment at m/z 345 would correspond to a half molecule plus a carboxyethyl group

al., 1994; Yamane et al., 1993). They include increases in the hydroxyproline pool; inhibition of proline oxidase, enhancement of lysine oxidase activity and production in animals poisoned with CCl_4 of factors stimulating collagen synthesis and enhancing prolyl hydroxylase activity.

Further, alterations in collagenase activity and in hepatic galactosyl hydroxylysyl glucosyl transferase and hydroxylysyl galactosyl transferase were reported. The possibility remains that some of the here reported hydroxy proline adducts of CCl_4 derived free radicals as well as those previously informed by our laboratory for the case of proline (Castro et al., 1995) might somehow interfere as antimetabolite analogs of the respective amino acids in collagen metabolism to explain, at least in part, some of the observed alterations. It is interesting in this respect that free radicals' formation is involved in the cirrhogenic effects of CCl_4; that proline is a relevant target for other free radicals attack, e.g. $\cdot OH$ and that some antioxidants prevent CCl_4 induced cirrhosis (Dean et al., 1989; Bedossa et al., 1994; Vanaja and Gajalakshmi, 1993; Gaudio et al., 1993; Mourelle et al., 1989; Sakaida et al., 1994). All those interesting possibilities are being challenged in our laboratory and they still remain to be proved.

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