In vivo reaction between [60] fullerene and vitamin A in mouse liver

Fathi Moussa,*[,a] Stéphane Roux,[b] Monique Pressac,[c] Eric Génin,[d] Michelle Hadchouel,[e] François Trivin,[f] André Rassat,[b] René Céolin[c] and Henri Szwarc[g]

[a] *Laboratoire de Chimie Analytique, (JE 1990), Faculté de Pharmacie, 21, rue Monge, 37 200 Tours, France*
[b] *Laboratoire de Processus d'Activation Moléculaire (CNRS URA 1110), ENS, 24, rue Lhomond, 75 006 Paris, France*
[c] *Faculté de Pharmacie, 4, avenue de l'Observatoire, 75 006 Paris, France*
[d] *ThermoQuest, 12, avenue des Tropiques, 91944 Les Ulis, France*
[e] *Génétique et Mécanismes des Maladies du Foie de l'Enfant, (INSERM U 347), 80, rue du Général Leclerc, 94 276 Le Kremlin-Bicêtre, France*
[f] *Hôpital St Joseph, 7, rue Pierre Larousse, 75 014 Paris, France*
[g] *Laboratoire de Chimie Physique des Matériaux Amorphes (CNRS URA 1104), Bâtiment 490, Université Paris XI, 91405 Orsay, France*

An *in vivo* biotransformation of [60] fullerene is observed that does not follow a usual redox metabolic pathway. Following the administration of a single dose of micronized C_{60} to Swiss mice, C_{60}-retinol and retinyl palmitate adducts were identified in liver by UV/VIS spectroscopy and mass spectrometry after high performance liquid chromatography. NMR investigations of the main biotransformed compound, after *in vitro* synthesis, show cycloaddition of retinol to C_{60}. The observed biotransformation, which proves that C_{60} does not remain unchanged in the liver as believed previously, also shows that Diels–Alder-like reactions can occur *in vivo*.

Over the past few years, many research groups have begun to seek biological uses of [60] fullerenes.[1] Little is known about the toxicity and metabolism of C_{60} itself. While micronized C_{60} has no acute toxic effects on mice,[2] C_{60} solubilized with polyvinylpyrrolidone in water severely disrupts the yolk sac and embryonic morphogenesis in mouse embryos.[3] Preliminary investigations of the metabolism of C_{60} and of one of its derivatives show that they are not oxidized by rat liver microsomes under conditions known to oxidize polynuclear aromatic hydrocarbons.[4] They accumulate mainly in the liver and apparently remain unchanged, suggesting that the C_{60} moiety is rather stable towards metabolic attack.[1e] The use of C_{60}, or its derivatives that could be cleaved back to the parent C_{60} *in vivo*, would likely lead to long-term fullerene accumulation in the liver.[4] Although no acute toxic effects of this accumulation have been reported, deposition of C_{60} is a negative factor in potential biomedical applications.[1e]

The present paper, in contrast, provides definitive evidence of a biotransformation of C_{60} *in vivo*. Electronic absorption spectroscopy between 290 and 500 nm, mass spectrometry and NMR investigations show that a Diels–Alder cycloaddition is involved that, to our knowledge, has never been described *in vivo* before,[5] although the existence of a Diels-Alderase has been hypothesised.[6] The synthesis of a main biotransformed adduct is also described.

Results

Chromatography with photodiode array detection

Hepatic extracts obtained from C_{60}-treated mice contain several additional compounds less hydrophobic than C_{60}, which elute before C_{60} when analysed by means of reversed phase high performance liquid chromatography (HPLC) [Fig. 1(*A*)]. These compounds do not form in hepatic extracts of control mice when C_{60} is added to liver homogenates, indicating that they are not formed during the extraction procedure [Fig. 1(*B*)]. Absorption spectra of these metabolites [Fig. 1(*C*)] obtained from chromatograms reproduce spectra of C_{60} derivatives of the 1,9-dihydrofullerene type, including $C_{60}H_2$.[7] Moreover, all these spectra exhibit a sharp band in the 410–430 nm region [Fig. 1(*C*), insert] characteristic of methanofullerene compounds, di- and tetrahydrofullerenes and pseudo-dihydrofullerene derivatives resulting from cycloadditions.[7]

HPLC–MS

Mass spectrometric detection of the main compounds, coupled with HPLC, using both atmospheric pressure chemical (APCI-MS) and electrospray (ESI-MS) ionization sources, shows that they are C_{60} derivatives (Fig. 2). APCI-MS spectra obtained from the major peaks (from 1 to 7) all exhibit the usual negative ion peak at *m*/*z* 720 accompanied by satellites at 721, 722, and 723 [Fig. 2(*A*)] corresponding to ^{13}C-containing C_{60}.[8] Furthermore, the mass spectra of peaks 3 [Fig. 2(*B*)] and x_5 each exhibit an additional ion peak at *m*/*z* 1006 and 791, respectively, accompanied by satellites featuring the same isotopic profile as that of C_{60}.

Using an ESI source that is less energetic but less sensitive than the APCI one under these conditions, the negative ion peak at *m*/*z* 720 is detected only in the case of C_{60}. Nevertheless, ESI-MS spectra of peaks 1, 3, x_5 and 7 show several ion peaks at *m*/*z* higher than 720 and exhibiting characteristic C_{60}-derivative profiles. Peaks 1 and 3 exhibit the same parent ion peaks at *m*/*z* 1006, indicating that they correspond to isomers of the same C_{60} derivative. The first isomer is less stable than the second one as its parent ion peak is not observed in the APCI mode. ESI-MS of peak x_5 [Fig. 2(*C*)] gives the same parent ion at *m*/*z* 791 as with APCI-MS, while peak 7 exhibits an ion peak at *m*/*z* 1244 with satellites characteristic of a C_{60} derivative [Fig. 2(*D*)].

MS/MS fragmentation

HPLC–APCI-MS/MS fragmentations of the ion peaks at *m*/*z* 1006 and 791 show that they correspond to C_{60} derivatives since they lead to the ion peak at *m*/*z* 720. The fragmentation

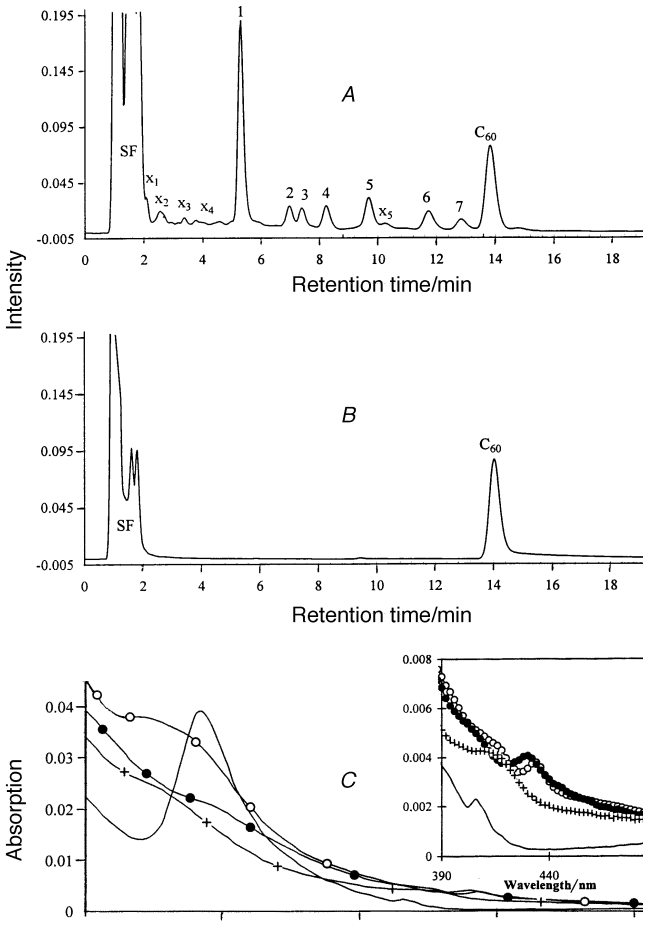


Fig. 1 Chromatograms [(*A*) and (*B*), UV detection at 330 nm] and corresponding visible spectra (*C*) of hepatic extracts. (*A*) From mice treated with mC_{60}, at day 7 (SF = solvent front). (*B*) From untreated mice spiked with mC_{60}. (*C*) UV/VIS spectra of (—) C_{60}, (○) peak 1, (●) peak 3 and (+) peak x_1, obtained from *A*. The spectra of peaks 2, 4, 5, 6 and 7, not shown here, are identical with that of peak 1 and those of peaks x_n are identical with that of peak x_1

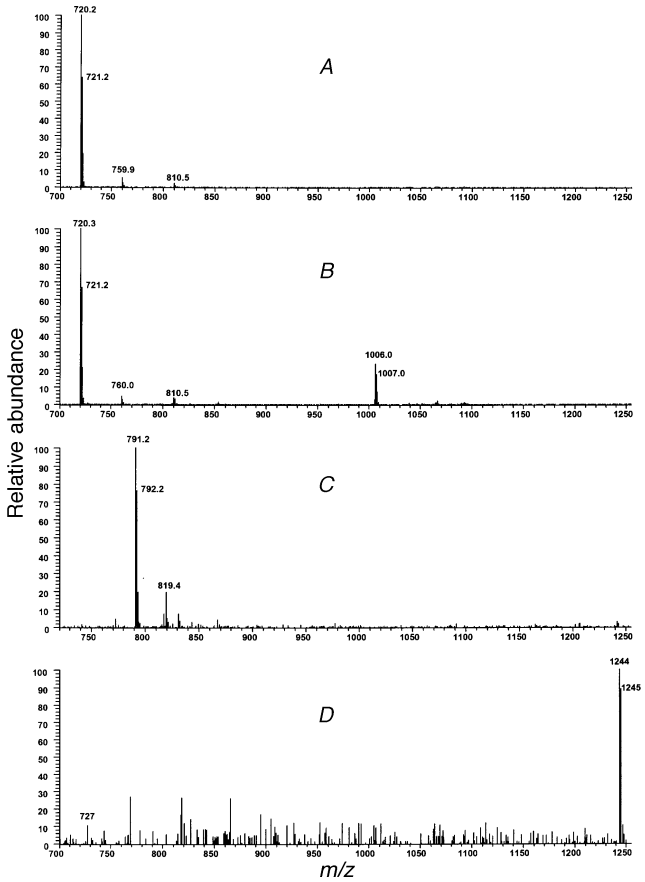


Fig. 2 Mass spectra obtained from chromatograms (see Fig. 1 for chromatographic conditions). (*A*) APCI-MS spectrum of peak 1 (spectra of peaks 2, 4, 5, 6, 7 and C_{60}, not shown here, are identical with that of peak 1). (*B*) APCI-MS spectrum of peak 3. (*C*) ESI-MS spectrum of peak x_5. (*D*) ESI-MS spectrum of peak 7

analysis of the ion peak at *m/z* 1006 (Fig. 3) suggests that it is formed by Diels–Alder cycloaddition of retinol on C_{60} (Scheme 1). The most significant fragmentation is that occurring at the C^5 site, leading to the ion peak at *m/z* 817.

In vitro synthesis of a 'main metabolite'

In order to check these findings, we have put ethanolic solutions of retinol and retinyl palmitate in contact with mC_{60}. When using concentrations of retinoids ten times higher than those occurring in normal mouse livers[9] and amounts of C_{60} equivalent to the mean concentration observed at day 7 in liver extracts of treated mice,[8] the expected reactions occur with yields of about 1.0% and 0.1% conversion for retinol and retinyl palmitate, respectively. The absorption chromatograms (UV/VIS detection) of the reaction mixture, extracted under the conditions used to extract liver samples, exhibit additional peaks with retention times and UV/VIS and MS spectra identical with those of peaks 1 and 3, for retinol, and peaks 2, 5 and 7, for retinyl palmitate, obtained from the hepatic extracts.

The C_{60}–retinol adduct was then synthesised in order to try to confirm the proposed reaction by NMR spectrometry. When using dichlorobenzene as solvent and a C_{60}-to-retinol ratio equal to two, complete conversion was reached as shown by HPLC analysis. Once purified, the solubilized C_{60}–retinol adduct was prone to a retro-Diels–Alder reaction. The freed retinol can react again with C_{60} or C_{60}–retinol, leading to the production of multiadducts. To prevent this cycloreversion, which is a drawback for NMR analysis, excess C_{60} was needed.

NMR investigations

^1H NMR spectrometry of the C_{60}–retinol adduct shows 14 signals, appearing between 0.98 and 6.525 ppm. The most significant signals are those corresponding to hydrogens of the ethylenic system (one signal at 6.525 ppm corresponds to the C^4 proton and a broad one at 6.1 ppm corresponds to the C^6, C^8 and C^9 protons), the hydrogens of the ring linked to C_{60} (a very broad signal at 5.075 ppm for the hydrogen on C^5 and a broad one at 4.359 ppm for C^2) and to the diastereotopic hydrogens of C^1 [one at 5.075 ppm and a triplet centred at 4.74 ppm (4.76, 4.73, 4.71 ppm)]. These results reveal the

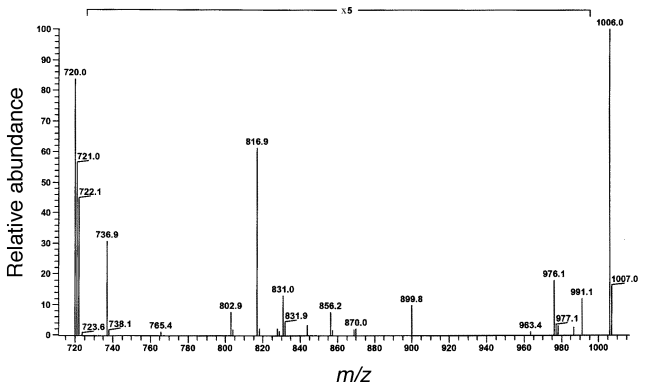
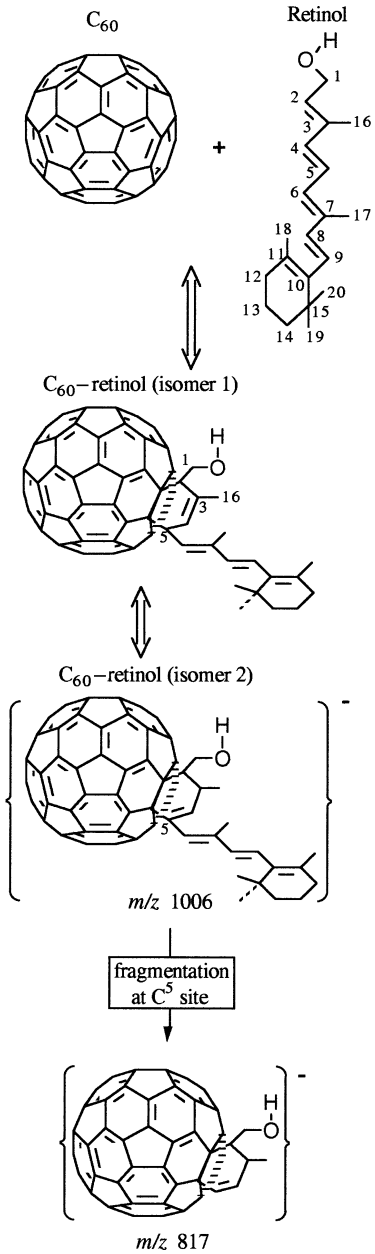


Fig. 3 HPLC–APCI-MS/MS spectrum of the ion peak at *m/z* 1006 obtained from peak 3 (collision energy at 50% of the scale)



Scheme 1 Proposed reaction between C_{60} and retinol and proposed fragmentation at the C^5 site of the ion peak at *m/z* 1006

expected structure, which confirms the proposed reaction product (Scheme 1). ^{13}C NMR spectrometry shows only 69 out of the expected 80 signals due to overlap of the excess C_{60} signal appearing between 155.547 and 13.106 ppm (results available from author on request).

Retinoid liver contents of C_{60}-treated mice

In order to investigate the effects of C_{60} on retinoid storage in C_{60}-treated mice, their liver retinol and retinyl ester contents were analysed by HPLC. As compared with chromatograms of control livers, those obtained from C_{60}-treated mice show a decrease of the peak areas of retinol and retinyl esters eluting before retinyl palmitate, containing either polyunsaturated chains or shorter chains than retinyl palmitate,[9] which suggests that they reacted also with C_{60}.

Discussion

In animals, including humans, lipophilic xenobiotics must be converted to polar derivatives before they can be excreted in urine or bile.[10] Redox reactions are a common route for introducing more polar groups and are often the first step in the detoxification of xenobiotics.[10] Alternatively, the initial step in the metabolism of xenobiotics may consist simply of the hydrolysis of a functional group, which leads directly to subsequent excretion.[11]

The C_{60} biotransformation we report here does not follow a usual redox metabolic path. Although unusual, this *in vivo* Diels–Alder reaction could have been expected because inside the liver C_{60} mainly accumulates in fat-storing (Ito) cells[2] that are specialized towards retinoid storage.[12] In normal liver, fat-storing cells represent 5%–8% of all liver cells and contain about 75% of the total retinoids.[12] Within these cells, C_{60} is in close contact with high concentrations of retinoids providing optimal reaction conditions. Consequently, the negative ion peak at *m/z* 1244 may be ascribed to the deprotonated negative ion of C_{60}–retinyl palmitate (720 + 524,85). In the same way, the ion peak at *m/z* 791 probably corresponds to a triply charged adduct resulting from the addition of three retinyl oleates (MW = 550.89) to $C_{60}\{[720 + (550.89 \times 3)]/3 = 790.89\}$.

The isomerisation of the C_{60}–retinol adduct may proceed through a base-induced rearrangement. Indeed, triethylamine treatment of the silica gel used to purify the synthesised C_{60}–retinol adduct by flash chromatography led to partial isomerisation of the adduct corresponding to peak 3 of Fig. 1 as shown by HPLC analysis. Triethylamine can promote the departure of the C^5 proton and the resulting anion can then be protonated at C^5 or C^3. The protonation at the C^3 site introduces conjugation with the side chain (Scheme 1), which might explain the observed differences between the electronic absorption spectra of these isomers [Fig. 1(*C*)] and the relative stability of the second one during APCI-MS analyses.

Peaks 4 and 6, which exhibit UV/VIS spectra identical with those of peaks 1, 2, 5 and 7 (Fig. 1), could then be ascribed to the addition of these retinyl esters to C_{60}. In the same way, as the other shorter time peaks (from x_1 to x_4) exhibit UV/VIS spectra identical to that of the x_5 peak (Fig. 1), they were ascribed to compounds formed from the multiple addition of retinol and retinyl esters to C_{60}.

Conclusions

The experimental results reported here lead to an important conclusion: C_{60} does not remain unchanged *in vivo*, since it can react with retinoids. It now remains to investigate whether these C_{60} biotransformations are followed by elimination of these 'metabolites'. In any case, the observed additions show that C_{60} can react outside the usual redox metabolic pathways and that the Diels–Alder reaction can occur *in vivo*.

Experimental

We used the same reagents and materials as described previously for C_{60} quantitation in tissues.[8] Retinol, retinyl acetate and retinyl palmitate were provided by Sigma (Sigma-Aldrich Chimie, France).

Animal and sample preparation

After intraperitoneal administration of a micronized C_{60} suspension (mC_{60} at 100 mg ml^{-1}) into Swiss mice, liver samples were collected at day 7 and stored as described previously.[8]

About 100 mg of liver (cut from the right lobe) was placed in a tissue pulverizer and 1 ml of an aqueous 0.9% NaCl solution added. After homogenization, the pulverizer content was transferred into a 5 ml glass tube. At this stage, the homogenate obtained from the control liver sample (untreated mice) was spiked with 100 µl of the mC_{60} diluted in aqueous 0.9% NaCl (1 : 9, v/v). Then, the resulting homogenate was mixed with 2 ml of acetone and 1 ml of toluene and the mixture

agitated for 30 s at room temperature in the dark. After centrifugation at 3000 g and −4 °C for 10 min, the supernatant was collected and evaporated at 38 °C under a stream of nitrogen. Finally, the dry residue was dissolved in 0.2 ml of the mobile phase for hepatic extracts of treated mice or 1.0 ml for control samples, before injection of 0.1 ml into the chromatograph.

For retinol and retinyl ester analyses we used the same mixture of acetone–toluene; 15 min of agitation allowed lipid fractions to be extracted. The dry residue obtained after evaporation of the supernatant was then dissolved in 0.5 ml of methanol before injection of 0.1 ml into the chromatograph.

Chromatography

Chromatographic analyses of C60 and its derivatives were performed at ambient temperature as described previously:[8] isocratic elution (acetonitrile–toluene, 58 : 42, v : v) at 0.8 ml min^{-1}.

Analyses of retinol and retinyl esters in hepatic extracts were achieved using a gradient elution (100% methanol during 10 min, then from 0 to 30% toluene in methanol within 10 min and 30% toluene in methanol during 10 min) at ambient temperature and 0.8 ml min^{-1}.

MS detection

Analyses were performed in the negative ionization mode on the same instruments as described previously.[8] We used both ESI and APCI sources with the following instrumental settings: needle voltage −4.0 kV, heated capillary temperature 220 °C, flow of the N2 sheath gas 80 units and flow of the N2 auxiliary gas 2 units, for ESI mode and source current 4.5 mA, heated capillary temperature 220 °C, vaporizer temperature 450 °C, flow of the N2 sheath gas 20 units and flow of the N2 auxiliary gas 2 units for APCI mode. Full scan spectra (300–1800) were acquired with a maximum ion injection time of 200 ms.

In vitro synthesis of the C60–retinol adduct

We used argon-saturated 1,2-dichlorobenzene as solvent and a C60-to-retinol ratio equal to 2. After 24 h stirring at 30 °C in the dark under argon, the reaction mixture was poured slowly into argon-saturated acetonitile under stirring. The resulting brown suspension was filtered on a sintered glass under a stream of argon and the precipitate dried in the dark under vacuum. HPLC analysis of this residue showed that it consisted of 54% C_{60} and 46% C_{60}–retinol adduct (based on the relative areas of the two peaks) exhibiting identical retention times and spectra (UV and ESI-MS) as the isomer corresponding to peak 1 obtained from the hepatic extract. For NMR investigations, this residue was used without further purification. The C_{60}–retinol adduct could be purified by flash chromatography on silica gel deactivated with 15% sodium acetate using a mixture of hexane–toluene (50 : 50, v : v) as the mobile phase.

^{1}H NMR (400 MHz, CS_2, 25 °C, $CDCl_3$) δ: 6.525 (d, $^3J = 1.5$ Hz, 1H, H^4), 6.1 (m, 3H, $CH^{8.10.11}$), 5.075 (m, 2H, $CH_2{}^1$ and CH^5), 4.74 (dd appearing as a t, 1H, $CH_2{}^1$), 4.359 (large pic, 1H, CH^2), 2.486 [s, 3H, $(CH_3)^{22}$], 2.03 [s, 3H, $(CH_3)^{21}$], 2.00 [t, $J = 6.2$ Hz, 2H, $(CH_2)^{14}$], 1.719 (b, 1H, OH), 1.63 [m, 2H, $(CH_2)^{15}$], 1.618 [s, 3H, $(CH_3)^{20}$], 1.48 [m, 2H, $(CH_2)^{16}$], 1.026 [s, 3H, $(CH_3)^{18}$], 0.98 [s, 3H, $(CH_3)^{19}$]. ^{13}C NMR (130 MHz, CS_2, 25 °C, $CDCl_3$) δ 155.547, 155.339, 153.649, 153.268, 147.135, 146.967, 146.901, 146.546, 146.200, 146.112 (2C), 145.888, 145.816, 145.774 (2C), 145.615, 145.523 (2C), 145.317, 145.124, 145.055 (2C), 144.964 (3C), 144.892, 144.364 (2C)144.247, 144.124, 143.(C_{60}), 142.436, 142.390, 142.335, 142.279, 141.965, 141.947, 141.863, 141.827, 141.809, 141.621, 141.566, 141.489, 141.147, 141.120, 141.069, 141.033, 140.35, 138.756, 138.673, 138.437, 138.311, 138.130, 137.181, 136.881 (CH), 130.487, 1300.161 (ODCB = *o*-dichlorobenzene), 128.638, 127.215 (ODCB), 126.537 (CH), 74.193, 69.5721, 62.792, 50.676, 45.764, 39.322, 33.745, 32.902, 28.61, 28.561, 21.482, 19.507, 13.106.

Acknowledgements

We thank Drs R. V. Bensasson (CNRS, Laboratoire de Biophysique, Muséum National d'Historie Naturelle, Paris), M. Czok (Faculté de Pharmacie, Paris, France), E. J. Land (Christie Hospital, Manchester, UK) and S. Leach (DAMAP, Meudon, France), and Professor B. C. Das (ICSN, CNRS, Gif-sur-Yvette, France) for valuable discussions and critical review of the manuscript.

References

1 (*a*) S. H. Friedman, D. L. DeCamp, R. Sijbsma, G. Srdanov, F. Wudl and J. L. Kenyon, *J. Am. Chem. Soc.*, 1993, **115**, 6506. (*b*) R. Sijbsma, G. Srdanov, F. Wudl, J. A. Castro, C. Wilkins, S. H. Friedman, D. L. DeCamp and J. L. Kenyon, *J. Am. Chem. Soc.*, 1993, **115**, 6510. (*c*) H. Tokuyama, S. Yamago, E. Nakamura, T. Shiraki and Y. Sugiura, *J. Am. Chem. Soc.*, 1993, **115**, 7918. (*d*) A. S. Boutorine, H. Tokuyama, M. Takasugi, H. Isobe, E. Nakamura and C. Helene, *Angew. Chem., Int. Ed. Engl.*, 1994, **33**, 2462. (*e*) A. W. Jensen, S. R. Wilson and D. I. Schuster, *Biorg. Med. Chem.*, 1996, **4**, 767. (*f*) Y. N. Yamakoshi, T. Yagami, S. Sueyoshi and N. Miyata, *J. Org. Chem.*, 1996, **61**, 7236. (*g*) M. Satoh, K. Matsuo, H. Kiriya, T. Mashino, T. Nagano, M. Hirobe and I. Takayanagi, *Eur. J. Pharmacol.*, 1997, **327**, 175. (*h*) M. C. Tsai, Y. H. Chen and L. Y. Chiang, *J. Pharm. Pharmacol.*, 1997, **49**, 438. (*i*) L. L. Dugan, D. M. Turetsky, C. Du, D. Lobner, M. Wheeler, C. R. Almli, C. K. F. Chen, T. Y. Luh, D. W. Choi and T. S. Lin, *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 9434. (*j*) Y. L. Lai and L. Y. Chiang, *J. Autonomic. Pharmacol.*, 1997, **17**, 229. (*k*) T. H. Ueng, J. J. Kang, H. W. Wang, Y. W. Cheng and L. Y. Chiang, *Toxicol. Lett.*, 1997, **93**, 29.

2 F. Moussa, F. Trivin, R. Céolin, M. Hadchouel, P. Y. Sizaret, V. Greugny, C. Fabre, A. Rassat and H. Szwarc, *Fullerene Sci. Technol.*, 1996, **4**, 21.

3 T. Tsuchiya, I. Oguri, Y. N. Yamakoshi and N. Miyata, *FEBS Lett.*, 1996, **393**, 139.

4 R. Bullarddillard, K. E. Creek, W. A. Scrivens and J. M. Tour, *Bioorg. Chem.*, 1996, **24**, 376.

5 F. E. Romesberg, B. Spiller, P. G. Schultz and R. C. Stevens, *Science*, 1998, **279**, 1929.

6 H. Oikawa, K. Katayama, Y. Susuki and A. Ichihara, *J. Chem. Soc., Chem. Commun.*, 1995, 1321.

7 (*a*) C. C. Henderson and P. A. Cahill, *Science*, 1993, **259**, 1885. (*b*) Y. Z. An, J. L. Anderson and Y. Rubin, *J. Org. Chem.*, 1993, **58**, 4799. (*c*) A. Hirsch, *The Chemistry of the Fullerenes*, Thieme, Stuttgart, 1994, pp.80–99. (*d*) Y. Nakamura, T. Minowa, S. Tobita, H. Shizuka and J. Nishimura, *J. Chem. Soc. Perkin Trans. 2*, 1995, 2351. (*e*) R. V. Bensasson, E. Bienvenue, J. M. Janot, S. Leach, P. Seta, D. I. Schuster, S. R. Wilson and H. Zaho, *Chem. Phys. Lett.*, 1995, **245**, 566.

8 F. Moussa, M. Pressac, E. Genin, S. Roux, F. Trivin, A. Rassat, R. Céolin and H. Szwarc, *J. Chromatogr. B*, 1997, **696**, 153.

9 H. E. May and S. I. Koo, *J. Liq. Chromatogr.*, 1989, **7**, 1261.

10 D. M. Ziegler, in *The Liver: Biology and Pathobiology*, 3rd edn., ed. I. M. Arias, J. L. Boyer, N. Fausto, W. B. Jakoby, D. A. Schater and D. A. Shafritz, Raven Press, New York, 1994, pp. 415–427.

11 W. B. Jakoby, in *The Liver: Biology and Pathobiology*, 3rd edn., ed. I. M. Arias, J. L. Boyer, N. Fausto, W. B. Jakoby, D. A. Schater and D. A. Shafritz, Raven Press, New York, 1994, pp. 429–442.

12 A. Geerts, P. De Bleser, M. L. Hautekeete, T. Niki and E. Wisse, in *The Liver: Biology and Pathobiology*, ed. I. M. Arias, J. L. Boyer, N. Fausto, W. B. Jakoby, D. A. Schater and D. A. Shafritz, Raven Press, New York, 1994, pp. 819–839.

Received in Montpellier, France, 14th April, 1998;
Paper 8/03120G