Oxidation of [60]Fullerene by Cytochrome P450 Chemical Models

Takeshi Hamano, Tadahiko Mashino and Masaaki Hirobe*

Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

Reaction of [60]fullerene in cytochrome P450 (P450) chemical model systems gives several oxidation products; sequential epoxidation occurs, and the second and third oxygen atoms are each introduced at a double bond adjacent to an existing epoxide.

Since the discovery of a large-scale production method of fullerene-containing soot in 1990,[1] these intriguing molecules, particularly [60]fullerene, have attracted much attention. However, little is known about their biological properties.[2] As many researchers are handling these molecules, the biological action of fullerenes must be of interest. Since [60]fullerene has many condensed six- and five-membered rings in its structure, it can be regarded as a derivative of polycyclic aromatic hydrocarbons (PAHs). Many PAHs, especially benzo[*a*]pyrene, have been reported to have potential carcinogenic activity,[3] and they are converted to their ultimate form, diol epoxides, by P450 and epoxide hydrolase.[4]

In studies of drug metabolism, chemical model systems have many advantages, such as the large-scale and single-step synthesis of unstable, minor or unexpected metabolites, and detection of compounds that would not be observed in *ex vivo* experiments because of binding to biological polymers. We[5] and other groups[6] have applied P450 chemical models to studies on drug metabolism and have so far found several novel putative metabolic pathways. Here, as a first step in the analysis of the metabolic transformations of [60]fullerene, we report the characterization of the oxidation products of [60]fullerene by several P450 chemical model systems.

In the tetraphenylporphinatoiron(III) chloride [Fe^{III}TPPCl]–iodosylbenzene system, a typical P450 chemical model, [60]fullerene was mixed with 1 equiv. of oxidant and 0.01 equiv. of catalyst in benzene at room temp. The reaction was completed within 60 min, and the reaction product was purified by preparative HPLC [octadecylsilica (ODS) column; toluene–methanol, 55 : 45]. The ^{13}C NMR and IR spectra of this product were identical with those of 1,2-epoxy[60]fullerene.[7,8]† Quantitative analysis of the production of 1,2-epoxy[60]fullerene was carried out in various P450 chemical model systems by HPLC. Table 1 shows that most systems gave 1,2-epoxy[60]fullerene as a product in moderate yield. Slight 1,2-epoxy[60]fullerene formation was observed in the systems without catalysts, but the reaction was greatly accelerated in the presence of a catalyst.

The tetraphenylporphinatoruthenium(II) carbonyl [RuTPP(CO)]–2,6-dichloropyridine-*N*-oxide–HBr system[9] is recognized as one of the most efficient P450 model systems developed so far. [60]Fullerene was mixed with 3 equiv. of oxidant and 0.01 equiv. of catalyst in benzene, and then molecular sieve 4A (MS4A) and a few drops of 47% HBr aqueous solution were added to the mixture. The reaction was carried out at room temperature. In this system, in addition to 1,2-epoxy[60]fullerene, many other oxidation products were observed on the HPLC chromatogram. The analysis of reaction progress with time showed that the yield of each product reached a maximum, and then decreased owing to conversion to a more oxidized form. The reaction mixture at 4 h was separated and 1,2-epoxy[60]fullerene, product 2 and product 3 were isolated by silica gel column chromatography (eluent CS_2).

Product 2 gave a signal at *m/z* 753 for the $[M + H]^+$ ion with a strong fragment peak at *m/z* 721, corresponding to $[C_{60} + H]^+$, in the FAB mass spectrum using *m*-nitrobenzyl alcohol as a matrix. The ^{13}C NMR spectrum showed 30 signals (4 signals integrate as one carbon atom each, and 26 signals as two carbon atoms each) between δ 140 and 150, as well as 2 signals (each two carbon atoms) at δ 82.40 and 78.46. This suggests that this compound is a diepoxy[60]fullerene with a C_s symmetrical structure. There are three possible structures that would satisfy the spectroscopic data (Fig. 1). In the ^{13}C NMR spectrum, product 2 showed its sp^3 carbon signals at higher field (δ 82.40 and 78.46) than those of the 1,2-epoxy[60]fullerene (δ 90.02). This high-field shift may be caused by the addition of an epoxide functional group for an alkenic group at the β-position of an sp^3 carbon (one connected directly with oxygen) in 1,2-epoxy[60]fullerene. While both types of sp^3 carbons of **1a**

Table 1 $C_{60}O$ formation by various cytochrome P450 chemical models[a]

Catalyst	Oxidant[b] PhIO	No oxidant
FeTPPCl	22.01	0.35
FeTDFPPCl	21.31	0.32
MnTPPCl	20.62	0.38
MnTDFPPCl	9.44	0.22
None	0.68	—

[a] *Conditions*: $[C_{60}]$ = 1 mmol dm^{-3}, [catalyst] = 10 μmol dm^{-3} [oxidant] = 1 mmol dm^{-3}, total volume 1.0 ml. These reactions were carried out in benzene at room temp. for 45 min. FeTPPCl = tetraphenylporphinatoiron(III) chloride; FeTDFPPCl = tetrakis(2,6-difluorophenyl)porphinatoiron(III) chloride; MnTPPCl = tetraphenylporphinatomanganese(III) chloride; MnTDFPPCl = tetrakis(2,6-difluorophenyl)porphinatomanganese(III) chloride; PhIO = iodosylbenzene. [b] % Based on substrate.

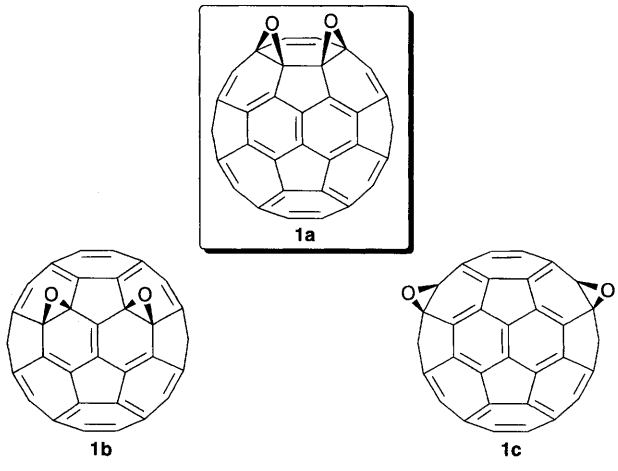

Fig. 1 Possible C_S symmetrical structures of diepoxy[60]fullerene

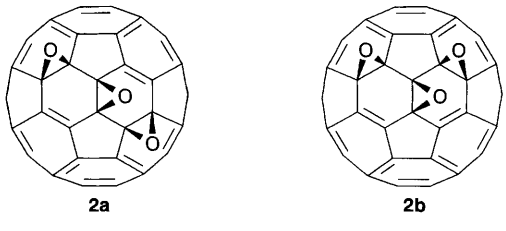

Fig. 2 Proposed structures of triepoxy[60]fullerene

in Fig. 1 have an epoxide carbon at the β-position, **1b** in Fig. 1 has only one type, and **1c** in Fig. 1 has none. This suggested that product 2 was the 1,2;3,4-diepoxy[60]fullerene (**1a**).‡ In support of this conclusion, Hirsch *et al.* showed experimentally, in the reaction of *tert*-butyllithium and [60]fullerene followed by hydrolysis, that the α-position of the carbon connected directly to the *tert*-butyl addend has increased reactivity.[10] Moreover, they confirmed the result by AM1 calculations. Ishida *et al.* reported that a second addend was introduced at a double bond adjacent to an existing addend, when the steric hindrance by the existing addend was relatively weak (epoxide or adduct of dichlorocarbene).[11] Avent *et al.* reported that 1,2,3,4-tetrahydro[60]fullerene was the main product from reduction of [60]fullerene with diimide from the detailed analyses by ^{1}H and ^{13}C NMR spectroscopy.[12] These findings also favour the **1a** type structure for product 2.

Product 3 showed a signal at *m/z* 769 for the $[M + H]^+$ ion in the FAB MS. Its ^{13}C NMR spectrum was more complicated than that of product 2 (1,2;3,4-diepoxy[60]fullerene). We measured the ^{13}C NMR spectrum of product 3 which was synthesized from ^{13}C-enriched [60]fullerene (8% enrichment). Carbons in the sp^2 region showed a very complicated spectrum, but the sp^3 carbons gave seven characteristic peaks. These seven peaks could be classified into two groups. One group consisted of δ 82.40, 75.04, and 68.94 (peak height ratio, 1 : 1 : 1), and the other, δ 80.05, 76.70, 72.98 and 65.75 (peak height ratio, 2 : 2 : 1 : 1). These results suggested a C_2 symmetrical structure for the former component and a C_s symmetrical structure for the latter. Since product 3 was formed even when product 2 was employed as a substrate, it was concluded that the two components are 1,2;3,4;9,10-triepoxy[60]fullerene **2a** and 1,2;3,4;11,12-triepoxy[60]fullerene **2b** (Fig. 2). This conclusion is consistent with the fact that a double bond adjacent to an existing epoxide is more reactive than the others. The production ratio of **2a** : **2b** was about 3 : 2 as judged from their peak heights.

There have been several reports on oxidation products of [60]fullerene, where the structures have been determined by spectroscopic methods. For example, $C_{60}O$ was produced by UV irradiation in the presence of benzil as a photosensitizer in benzene solution,[7] or by reaction of [60]fullerene with dimethyldioxirane.[8] Ishida *et al.* have reported the diepoxy[60]fullerene,[11] which was produced by reaction with *m*-chloroperbenzoic acid for 2 d, and they proposed the structure **1a** (1,2;3,4-diepoxy[60]fullerene) in Fig. 1 for the product. But no example has been reported of triepoxy[60]fullerene or higher oxidation forms of [60]fullerene. The UV-VIS spectra suggest that these oxidation products of [60]fullerene retain the essential electronic character of their parent compound, [60]fullerene. Although there are several small peaks beside the main peaks in the HPLC chromatogram, they have not yet been isolated and identified. They may be regioisomers of the identified products. It is noteworthy that these oxidation products gradually decomposed at room temperature when left in air, probably by autoxidation. These findings indicate that the oxidation products are more unstable to molecular oxygen than [60]fullerene.

In conclusion, we have identified the structures of $C_{60}O$, $C_{60}O_2$ and $C_{60}O_3$ formed in P450 chemical model systems. It is strongly suggested that the oxygen atom transferred from the active species was introduced at a double bond adjacent to an existing epoxide. We are currently examining whether this oxidation is catalysed by liver microsomes *in vitro* or by P450 *in vivo*. We are also investigating singlet oxygen production, which has already been reported in the case of [60]fullerene itself,[13,14] upon visible light irradiation of these epoxides in the presence of O_2.

Received, 7th February 1995; Com. 5/00732A

Footnotes

† *Selected data* for $C_{60}O$: ^{13}C NMR (100 MHz, *o*-dichlorobenzene–C_6D_6 2 : 1): δ 145.33(4), 145.29(4), 145.21(4), 145.08(2), 144.39(4), 144.35(4), 144.01(4), 143.95(4), 143.60(2), 143.12(8), 142.59(2), 142.41(4), 142.24(4), 142.01(4), 140.89(4), 90.02(2); FAB-MS: *m/z* 737 $[M + H]^+$, 721 $[M + H - O]^+$

‡ *Selected data* for **1a**: ^{13}C NMR (100 MHz, *o*-dichlorobenzene–C_6D_6 2 : 1): δ 149.57(2), 147.75(1), 147.11(2), 146.67(2), 146.15(2), 146.09(2), 145.96(2), 145.87(2), 145.50(2), 145.08(2), 144.85(4), 144.29(4), 144.00(2), 143.48(2), 143.32(1), 143.18(2), 142.07(2), 142.56(2), 142.48(4), 142.42(4), 141.80(2), 141.53(2), 141.39(1), 140.77(2), 140.08(2), 139.19(2), 82.40(2), 78.46(2); FAB-MS: *m/z* 753 $[M + H]^+$, 737 $[M + H - O]^+$, 721 $[M + H - O_2]^+$

References

1 W. Krätschmer, L. D. Lamb, K. Fostiropoulos and D. R. Huffman, *Nature*, 1990, **347**, 354.

2 S. H. Friedman, D. L. DeCamp, R. P. Sijbesma, G. Srdanov, F. Wudl and G. I. Kenyon, *J. Am. Chem. Soc.*, 1993, **115**, 6506; R. F. Schinazi, R. Sijbesma, G. Srdanov, C. L. Hill and F. Wudl, *Antimicrob. Agents Chemother.*, 1993, **37**, 1707; H. Tokuyama, S. Yamago, E. Nakamura, T. Shiraki and Y. Sugiura, *J. Am. Chem. Soc.*, 1993, **115**, 7918; M. A. Nelson, F. E. Domann, G. T. Bowden, S. B. Hooser, Q. Fernando and D. E. Carter, *Toxicology and Industrial Health*, 1993, **9**, 623.

3 P. L. Grover, *Chemical Carcinogens and DNA*, CRC Press, Boca Raton, 1979, vols. 1 and 2.

4 C. Heidelberger, *Ann. Rev. Biochem.*, 1975, **44**, 79; E. C. Miller, *Cancer Res.*, 1978, **38**, 1479; P. Sims, *Br. Med. Bull.*, 1980, **36**, 11; P. L. Grover, *Xenobiotica*, 1986, **16**, 915.

5 H. Masumoto, K. Takeuchi, S. Ohta and M. Hirobe, *Chem. Pharm. Bull.*, 1989, **37**, 1788; Y. Nagatsu, T. Higuchi and M. Hirobe, *Chem. Pharm. Bull.*, 1989, **37**, 1410; Y. Nagatsu, T. Higuchi and M. Hirobe, *Chem. Pharm. Bull.*, 1990, **38**, 400; H. Masumoto, S. Ohta and M. Hirobe, *Drug Metab. Dispos.*, 1991, **19**, 768; M. Komuro, Y. Nagatsu, T. Higuchi and M. Hirobe, *Tetrahedron Lett.*, 1992, **33**, 4949; T. Doi, T. Mori, T. Mashino and M. Hirobe, *Biochem. Biophys. Res. Commun.*, 1993, **191**, 737; T. Ohe, T. Mashino and M. Hirobe, *Arch. Biochem. Biophys.*, 1994, **310**, 402; M. Komuro, T. Higuchi and M. Hirobe, *Biomed. Chem.*, 1995, **3**, 55.

6 B. Meunier, *Chem. Rev.*, 1992, **92**, 1411; J. Bernadou, M. Bonnafous, G. Labat, P. Loiseau and B. Meunier, *Drug Metab. Dispos*, 1991, **19**, 360; D. Mansuy, P. Valadon, I. Erdelmeier, P. Lopez-Garcia, C. Amer, J-P. Girault and P. M. Dansette, *J. Am. Chem. Soc.*, 1991, **113**, 7825.

7 K. M. Creegan, J. L. Robbins, W. K. Robbins, J. M. Millar, R. D. Sherwood, P. J. Tindall, D. M. Cox, A. B. Smith, III, J. P. McCauley, Jr., D. R. Jones and R. T. Gallagher, *J. Am. Chem. Soc.*, 1992, **114**, 1103.

8 Y. Elemes, S. K. Silverman, C. Sheu, M. Kao, C. S. Foote, M. M. Alvarez and R. L. Whetten, *Angew. Chem., Int. Ed. Engl.*, 1992, **31**, 351.

9 H. Ohtake, T. Higuchi and M. Hirobe, *J. Am. Chem. Soc.*, 1992, **114**, 10660.

10 A. Hirsch, A. Soi and H. R. Karfunkel, *Angew. Chem., Int. Ed. Engl.*, 1992, **31**, 766.

11 N. Ishida, K. Tanaka, T. Furutachi, T. Nogami, S. Kurono and M. Ohashi, *Abstract of 6th C_{60} Symposium*, Tokyo, 1994, p. 154.

12 A. G. Avent, A. D. Darwish, D. K. Heimbach, H. W. Kroto, M. F. Meidine, J. P. Parsons, C. Remars, R. Roers, O. Ohashi, R. Taylor and D. R. M. Walton, *J. Chem. Soc., Perkin Trans. 2*, 1994, 15.

13 J. W. Arbogast, A. P. Darmanyan, C. S. Foote, Y. Rubin, F. N. Diederich, M. M. Alvarez, S. J. Anz and R. L. Whetten, *J. Phys. Chem.*, 1991, **95**, 11; A. A. Krasnovsky, Jr. and C. S. Foote, *J. Am. Chem. Soc.*, 1993, **115**, 6013.

14 T. Nagano, K. Arakane, A. Ryu, T. Masunaga, K. Shinmoto, S. Mashiko and M. Hirobe, *Chem. Pharm. Bull.*, 1994, **42**(11), 2291.