Title	Synthesis of novel amino acid-type fullerenes and their inhibition activity of HIV reverse transcriptase and HCV RNA polymerase
Sub Title	
Author	中村, 成夫(Nakamura, Shigeo) Ikegami, Noriko(Shimotohno, Kumiko) Harada, Manami(Takahashi, Kyoko) 下遠野, 久美子(Mashino, Tadahiko) 高橋, 恭子 増野, 匡彦
Publisher	共立薬科大学
Publication year	2006
Jtitle	共立薬科大学雑誌 (The journal of Kyoritsu University of Pharmacy). Vol.1, (2006. 3) ,p.77- 84
JaLC DOI	
Abstract	We synthesized novel fullerene derivatives with amino, carboxylic, and ethyl ester groups. The HIV reverse transcriptase (HIV-RT) inhibition activities of these derivatives were higher than those of nevirapine, a clinically used HIV-RT inhibitor. However, they were less effective than those of the tricarboxylic derivative previously reported by our group, and the numbers of carboxylic groups did not affect the HIV-RT inhibition activity. Some derivatives have HCV RNA polymerase (HCV-RP) inhibition activities as well. Further optimization of the structure of the fullerene derivatives is required.
Notes	
Genre	Departmental Bulletin Paper
URL	https://koara.lib.keio.ac.jp/xoonips/modules/xoonips/detail.php?koara_id=jkup2006_1_077

Synthesis of Novel Amino Acid-Type Fullerenes and Their Inhibition Activity of HIV Reverse Transcriptase and HCV RNA Polymerase

Shigeo Nakamura,[1] Noriko Ikegami,[1] Manami Harada,[1] Kumiko Shimotohno,[2] Kyoko Takahashi,[1] Tadahiko Mashino[1*]

[1]Department of Bioorganic and Medicinal Chemistry, Kyoritsu University of Pharmacy
[2]Department of Basic Biological Sciences, Kyoritsu University of Pharmacy

Summary

We synthesized novel fullerene derivatives with amino, carboxylic, and ethyl ester groups. The HIV reverse transcriptase (HIV-RT) inhibition activities of these derivatives were higher than those of nevirapine, a clinically used HIV-RT inhibitor. However, they were less effective than those of the tricarboxylic derivative previously reported by our group, and the numbers of carboxylic groups did not affect the HIV-RT inhibition activity. Some derivatives have HCV RNA polymerase (HCV-RP) inhibition activities as well. Further optimization of the structure of the fullerene derivatives is required.

Introduction

HIV infection is one of the major causes of morbidity and mortality in the world. There are many anti-HIV agents, but their efficiency is not very high. Moreover, the emergence of drug-resistant mutant forms of HIV requires the development of effective and well-tolerated new remedies.

HCV is the major etiological virus of non-A and non-B hepatitis. An estimated 2–3% of the world population is chronically infected with HCV. HCV infection causes severe liver disease and can lead to the development of hepatocellular carcinoma. Currently, interferon and rebavirin are used for HCV therapy, but their therapeutic ratio is low. HCV RNA-dependent RNA polymerase, protease, and helicase, which are essential for the replication of the virus, are new targets for an anti-HCV drug. These threats provide the motivation to search for new types of lead compounds to be used as medicine against HIV and HCV infections.

Both the human immunodeficiency virus (HIV) and the hepatitis C virus (HCV) are RNA viruses and have similar enzymes. For example, HIV-reverse transcriptase (HIV-RT) and HCV-RNA-dependent RNA polymerase (HCV-RP) are RNA-dependent polymerases which are essential for the replication of the virus. Indeed, HIV-RT is one of the major targets for anti-HIV agents.

Fullerene, a condensed aromatic ring compound with an extended π-conjugated system, is a new type of organic compound that was discovered in 1985.[1] This compound has a cage-like shape, and a great deal of attention has been focused on its unique properties.

Some biological activities that are dependent on its unique physical properties and chemical reactivities have been reported.[2] For example, DNA scissions[3,4] and

Corresponding author: Tadahiko Mashino, Department of Bioorganic and Medicinal Chemistry, Kyoritsu University of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan

the oxidation of biological materials[5] depend on photo-induced active oxygen production by fullerene.[6] We intend to develop fullerene derivatives as a new type of lead compound to be used as medicine and have reported that the anionic fullerene derivatives, carboxy fullerenes, show interesting antioxidant activities, and the cationic derivatives, C_{60}-bis(N,N-dimethylpyrrolidinium iodide) and its alkylated derivatives, have excellent antibacterial and antiproliferative activities.[7]

We have also previously reported that aminotricarboxylic acid derivative **3** has a very strong activity on HIV-RT inhibition.[8] Thus, we started to synthesize novel compounds by modifying **3** as a lead compound. In this report, we examine the HIV-RT and HCV-RP inhibition activities of amino acid-type fullerene derivatives (Fig. 1).
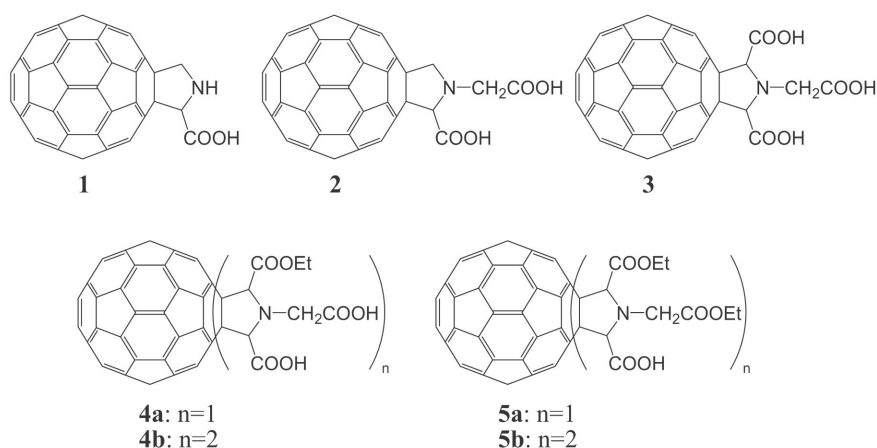

Figure 1. Structures of fullerene derivatives **1 – 5**.

Materials and Methods
Chemicals

Tert-butyl glyoxylate was synthesized by a known procedure.[9] The preparation of **2** and **3** has been previously reported.[10] Other chemicals are commercially available.

2-*tert*-Butoxycarbonylpyrrolidino[60]fullerene (6)

One hundred and fifty milligrams of C_{60} (0.21 mmol) was dissolved in toluene (300 mL), then, a methanol solution (30 mL) of glycine *tert*-butyl ester hydrochloride (200 mg, 11.9 mmol) and sodium hydroxide (473 mg, 11.8 mmol) was added to the C_{60} solution. After the addition of paraformaldehyde (342 mg, 11.4 mmol), the mixture was stirred at 120°C for 8 hr. The reaction mixture was washed with water and brine, dried, and concentrated. The crude product was chromatographed on a silica-gel column (toluene, toluene/AcOEt=100/1) to give 87 mg of **6** as a brown powder (yield 48%).

^{1}H-NMR (CDCl$_3$, 500 MHz): δ 1.49 (s, 9H, -C(CH$_3$)$_3$), 4.75 (d, 1H, *J*=11.5 Hz, C$_{60}$-CH$_2$-), 5.05 (d, 1H, *J*=12.5 Hz, C$_{60}$-CH$_2$-), 5.38 (s, 1H, C$_{60}$-CH-).

2-Carboxypyrrolidino[60]fullerene (1)

Trifluoromethanesulfonic acid (103 mg, 0.68 mmol) was added to a solution of **6** (30.0 mg, 34.2 μmol) in toluene (30 mL), and the mixture was stirred at room temperature for 15 min. Precipitates were collected by filtration and washed with toluene, 1 M sodium hydrogen carbonate, 1 M HCl, and water to give 17 mg of **1** as a brown powder (yield 60%). ^{1}H-NMR (DMSO-*d$_6$*, 500MHz): δ 4.94 (d, 1H, *J*=11.5 Hz, C$_{60}$-CH$_2$-), 5.16 (d, 1H, *J*=12.0 Hz, C$_{60}$-CH$_2$-), 5.91 (s, 1H, C$_{60}$-CH-).

2-*tert*-Butoxycarbonyl-*N*-*tert*-butoxycarbonylmethyl-5-ethoxycarbonylpyrrolidino[60]fullerene (7a)

Di-*tert*-butyl iminodiacetate (344 mg, 1.4 mmol) and

glyoxylic acid ethyl ester 750 µl (1.3 mmol, 50% content) were added to a solution of C_{60} (348 mg, 480 µmol) in toluene (400 mL). The mixture was refluxed for 30 min. The reaction mixture was washed with water and brine, dried, and concentrated. The crude product was chromatographed on a silica-gel column (n-hexane/toluene=1:1, toluene/AcOEt=20/1) to give 148 mg of **7a** as a brown powder (yield 29%). ^{1}H-NMR(CDCl_3, 500 MHz): δ 1.25(t, 3H, *J*=7 Hz, -CH_2C*H*_3), 1.50(s, 9H, -C(CH_3)_3), 1.61(s, 9H, -C(CH_3)_3), 4.02(d, 1H, *J*=16.5 Hz, N-CH_2-), 4.21(d, 1H, *J*=16.5 Hz, N-CH_2-), 4.34(q, 2H, *J*=3.0 Hz, -C*H*_2CH_3), 6.04(s, 1H, C_60-CH-), 6.14(s, 1H, C_60-CH-).

Bis(2-*tert*-butoxycarbonyl-*N*-tert-butoxycarbonylmethyl-5-ethoxycarbonylpyrrolidino)[60]fullerene (7b)

The reaction conditions were almost the same as those for the preparation of **7a** except for a 50-min reaction period. **7b** (469 mg) was obtained as a brown powder (yield 71%). ^{1}H-NMR(CDCl_3, 500 MHz): δ 0.88-1.72 (m, 42H, -C(CH_3)_3, -CH_2CH_3), 3.74-4.66 (m, 8H, N-CH_2-, -CH_2CH_3), 5.50-6.38 (m, 4H, C_60-CH-).

2-Carboxy-*N*-carboxymethyl-5-ethoxycarbonylpyrrolidino[60]fullerene (4a)

Trifluoromethanesulfonic acid (43 mg, 286 µmol) was added to a solution of **7a** (30 mg, 28.6 µmol) in toluene (30 mL), and the mixture was stirred at room temperature for 5 min. Precipitates were collected by filtration and washed with 1 M sodium hydrogen carbonate, water, and 1 M HCl to give 31 mg of **4a** as a brown powder (yield 116%). ^{1}H-NMR (C_5D_5N, 500 MHz): δ 1.20 (t, 3H, *J*=7.0 Hz, -CH_2C*H*_3), 4.40 (q, 2H, *J*=7.0 Hz, -C*H*_2CH_3), 4.76 (d, 1H, *J*=16.5 Hz, N-CH_2-), 5.04 (d, 1H, *J*=16.5 Hz, N-CH_2-), 6.89 (s, 1H, C_60-CH-), 6.90 (s, 1H, C_60-CH-); TOF-MS: *m/z* 847 ([M]^+–2COOH), 720.

Bis(2-carboxy-*N*-carboxymethyl-5-ethoxycarbonylpyrrolidino)[60]fullerene (4b)

Trifluoromethanesulfonic acid (164 mg, 1.09 mmol) was added to a solution of **7b** (150 mg, 109 µmol) in toluene (150 mL), and the mixture was stirred at room temperature for 5 min. Precipitates were collected by

filtration and washed with toluene and n-hexane to give 180 mg of **4b** as a brown powder (yield 143%). ^{1}H-NMR (C_5D_5N, 500 MHz): δ 0.78-1.61 (m, 6H, -CH_2C*H*_3), 4.20-4.94 (m, 8H, -C*H*_2CH_3, N-C*H*_2-), 6.21-6.94 (m, 4H, C_60-CH-); TOF-MS: *m/z* 974 ([M]^+–4COOH), 720.

2-*tert*-Butoxycarbonyl-*N*-ethoxycarbonylmethyl-5-ethoxycarbonylpyrrolidino[60]fullerene (8a) and Bis(2-*tert*-butoxycarbonyl-*N*-ethoxycarbonylmethyl-5-ethoxycarbonylpyrrolidino)- [60]fullerene (8b)

Iminodiacetic acid diethyl ester (86 mg, 467 µmol) and *tert*-butyl glyoxylate[9] (1.0 g) were added to a solution of C_{60} (200 mg, 278 µmol) in toluene (200 mL). The mixture was refluxed for 30 min. The reaction mixture was washed with water and brine, dried, and concentrated. The crude product was chromatographed on a silica-gel column (toluene, toluene/AcOEt=100/15) to give 137 mg of **8a** (yield 49%) and 69 mg of **8b** (yield 19%).

8a, ^{1}H-NMR (CDCl_3, 500 MHz): δ 1.24 (t, 3H, *J*=7.0 Hz, -CH_2C*H*_3), 1.41 (t, 3H, *J*=7.0 Hz, -CH_2C*H*_3), 1.49 (s, 9H, -C(CH_3)_3), 4.14 (d, 1H, *J*=16.5 Hz, N-CH_2-), 4.26 (d, 1H, *J*=17 Hz, N-CH_2-), 4.35 (q, 4H, *J*=6.5 Hz, -C*H*_2CH_3), 6.01 (s, 1H, C_60-CH-), 6.14 (s, 1H, C_60-CH-); TOF-MS: *m/z* 1021 (M)^+, 720. **8b**, ^{1}H-NMR (CDCl_3, 500 MHz): δ 0.98-1.72 (m, 30H, -C(CH_3)_3, -CH_2C*H*_3), 3.91-4.61 (m, 12H, N-CH_2-, -C*H*_2CH_3), 5.51-6.38 (m, 4H, C_60-CH-).

2-Carboxy-*N*-ethoxycarbonylmethyl-5-ethoxycarbonylpyrrolidino[60]fullerene (5a)

Trifluoromethanesulfonic acid (177 mg, 1.18 mmol) was added to a solution of **8a** (150 mg, 147 µmol) in toluene (150mL), and the mixture was stirred at room temperature for 5 min. Precipitates were collected by filtration and washed with 1 M sodium hydrogen carbonate, water, and 1 M HCl to give 116 mg of **5a** as a brown powder (yield 82%). ^{1}H-NMR (C_5D_5N, 500 MHz): δ 1.19 (t, 3H, *J*=7.0 Hz, -CH_2C*H*_3), 1.20 (t, 3H, *J*=7.0 Hz, -CH_2C*H*_3), 4.27 (q, 2H, *J*=7.0 Hz, -C*H*_2CH_3), 4.39 (q, 2H, *J*=7.0 Hz, -C*H*_2CH_3), 4.65 (d, 1H, *J*=16.5 Hz, N-CH_2-), 4.86 (d, 1H, *J*=16.5 Hz, N-CH_2-), 6.72 (s,

1H, C_{60}-CH-), 6.77 (s, 1H, C_{60}-CH-); FAB-MS: *m/z* 966(M^++1), 720.

Bis(2-carboxy-*N*-ethoxycarbonylmethyl-5-ethoxycarbonylpyrrolidino)[60]fullerene (5b)

Trifluoromethanesulfonic acid (144 mg, 956 µmol) was added to a solution of **8b** (158 mg, 120 µmol) in toluene (150 mL), and the mixture was stirred at room temperature for 5 min. Precipitates were collected by filtration and washed with 1 M sodium hydrogen carbonate, water, and 1 M HCl to give 68 mg of **5b** as a brown powder (yield 48%). ^{1}H-NMR (C_5D_5N, 500 MHz): δ 0.75-1.58 (m, 20H), 3.82-4.97 (m, 18H), 6.19-7.01 (m, 4H).

HIV-RT inhibition activity

The HIV-RT inhibition activities were examined according to Mizrahi et al.[11] One µL of a DMSO sample solution and 1 µL of HIV-RT (0.01 U/mL) were added to a 18 µL reaction mixture containing 50 mM Tris-HCl (pH 8.3), 30 mM NaCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.125 mg/ml poly(rA)・oligo(dT)$_{12-18}$, and 2.5 µM dTTP including ^{32}P-dTTP. The mixture was incubated for 1 h at 37°C. Then, 10 µL of the reaction mixture was placed on a sheet of Whatman® DE81 chromatography paper. After the chromatography paper had dried, it was washed three times with a 0.5 M NaH_2PO_4 buffer (pH 7.0), 70% ethanol, and ethanol. The radioactivity of the dried chromatography paper was counted with a liquid scintillator, and the HIV-RT activity was measured.

HCV-RP inhibition activity

The HCV-RP inhibition activities were examined according to Yamashita et al.[12] The assay was preformed in a total volume of 40 µL containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 20 units of an RNase inhibitor, 0.125 mg/mL poly(rA)・oligo(dU)$_{12-18}$, and 2.5 µM dUTP including ^{32}P-dUTP. It was then incubated for 2 h at 30°C.

Results and Discussion

We have previously reported that aminotricarboxylic acid derivative **3** has a very strong activity on HIV-RT inhibition.[8] However, it is possible that, because of its high hydrophilicity, **3** may not be able to penetrate the biomembrane. We chose ethyl ester derivatives because they can be hydrolyzed after permeating into a cell.

Three carboxylic groups of **3** seem to be important for the HIV-RT inhibition activity. We have already prepared **2** with two carboxylic groups. To further investigate the effect of the number of carboxyl groups, we prepared **1** with one carboxyl group. Fullerene derivative **6** was synthesized from C_{60} with glycine *tert*-butyl ester and paraformaldehyde by 1,3-dipole addition. Then, *tert*-butyl ester was hydrolyzed by trifluoromethanesulfonic acid to give **1** (Scheme 1).
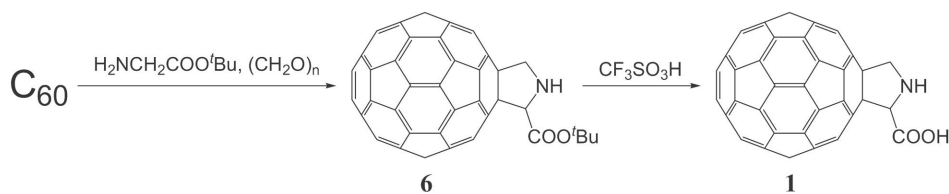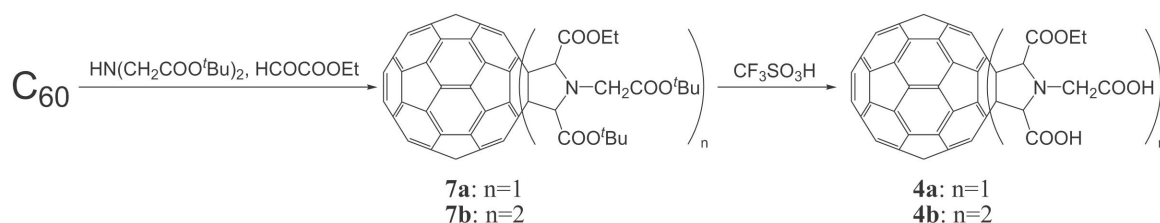

Scheme 1. Synthesis of fullerene derivative **1**

Prato et al. have also reported that C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide) was comparatively effective on HIV-RT inhibition.[13,14] We speculated that bis-adducts tend to inhibit HIV-RT more successfully than mono-adducts; therefore, we designed both mono- and bis-adducts. **4a** and **5a** are mono-adducts with one or two ethyl ester groups, respectively. **4b** and **5b** are bis-adducts with two or four ethyl ester groups, respectively. We also tried to synthesize the bis-adduct of **3**; however, the hydrolysis

of corresponding ethyl ester was difficult. **7a** and **7b** were synthesized from C_{60} with di-*tert*-butyl iminoacetate and ethyl glyoxylate. Then, the *tert*-butyl ester was hydrolyzed by trifluoromethanesulfonic acid to give **4a** and **4b** (Scheme 2). **5a** and **5b** were also synthesized with diethyl iminodiacetate and *tert*-butyl glyoxylate, followed by acid-catalyzed hydrolysis (Scheme 3).

Scheme 2. Synthesis of fullerene derivatives **4a** and **4b**
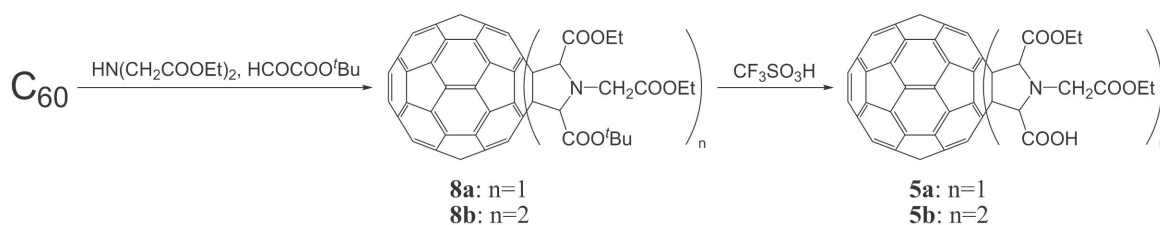
Scheme 3. Synthesis of fullerene derivatives **5a** and **5b**.

The main components of **3**, **4a**, and **5a** were *trans*-isomers with a 5–10% amount of *cis*-isomers (δ 5.79, s, C_{60}-CH-). **1** and **2** were racemic, and **4b** and **5b** were mixtures of a regio-isomer. The fullerene derivatives were dissolved in DMSO to apply biological assay systems. Table 1 shows the HIV-RT inhibition activity of the fullerene derivatives. All examined fullerene derivatives were more effective than the non-nucleoside analog of the HIV-RT inhibitor nevirapine,[15] which is clinically used for HIV infection.

Table 1. HIV-RT inhibition of fullerene derivatives

Compound	IC$_{50}$ (μM)	Compound	IC$_{50}$ (μM)
Nevirapine	3.0	**4a**	0.156
1	0.150	**4b**	0.209
2	0.073	**5a**	0.163
3	0.029	**5b**	0.160

The newly synthesized fullerene derivatives were less effective than the lead compound **3**. There were no differences between mono-adducts (**4a** and **5a**) and bis-adducts (**4b** and **5b**). The numbers of the carboxylic groups did not affect the HIV-RT inhibition, either; however, **4a** and **5a** may be easily hydrolyzed to **3** by esterases after incorporation into the cell. Thus, these derivatives may have anti-HIV effects in an *in vivo* assay.

In the case of HCV-RP inhibition, among the examined fullerene derivatives, **4b** and **5b** were more effective (Table 2). The HCV-RP inhibition effects of mono-adducts and bis-adducts were significantly different from the HIV-RT inhibition results. However, the IC_{50} values of the fullerene derivatives were higher than those of the benzo-1,2,4-thiadiazine analog.[16] **4b** and **5b** were also less effective than C_{60}-bis(*N,N*-dimethylpyrrolidinium iodide) (IC_{50}, 0.3 µM), as we previously reported. Further optimization of the structure of the fullerene derivatives is required.

For other interesting enzymes, for example, glutathione transferase, glutathione reductase, and nitric oxide synthase, the inhibition activities of fullerene derivatives have also been reported.[10,17,18] These activities depend on the properties of the fullerene core, while the substituents on the fullerene core control and modify the biological activities of fullerene derivatives. We are now investigating the mechanisms of HIV-RT inhibition and HCV-RP inhibition.

Table 2. HCV-RP inhibition of fullerene derivatives

Compound	IC_{50} (µM)	Compound	IC_{50} (µM)
Thiadiazine analog	0.10	**4a**	>10
1	–[a]	**4b**	1.17
2	>10	**5a**	>10
3	2.16	**5b**	0.87

a; not examined

Conclusion

We successfully synthesized novel fullerene derivatives **1**, **4a**, **4b**, **5a**, and **5b**. Their HIV-RT inhibition activities were higher than those of nevirapine, a clinically used HIV-RT inhibitor. However, they were less effective than those of **3**, which we have previously reported. The ethyl esters of **4a** and **5a** could be easily converted to **3** after incorporation into the cell; therefore, these derivatives may have anti-HIV effects in an *in vivo* assay. **4b** and **5b** also have HCV-RP inhibition activities, although they are not as effective.

Acknowledgement

References

1. Kroto H W, Heath J R, O'Brien S C, Curl R F, Smally R E. C_{60}: Buckminsterfullerene. *Nature* **318**, 162 (1985).

2. Tagmatarchis N, Shinohara H. Fullerenes in medicinal chemistry and their biological applications. *Mini Rev Med Chem* **1**, 339-348 (2001).

3. Boutorine A S, Tokuyama H, Takasugi M, Isobe H,

Nakamura E, Helene C. Fullerene-oligonucleotide conjugates: Photo-induced sequence-specific DNA cleavage. *Angew Chem Int Ed Engl* **33**, 2462-2465 (1994).

4. Yamakoshi Y N, Endo T, Sueyoshi S, Miyata N. Acridine adduct of [60]fullerene with enhanced DNA-cleaving activity. *J Org Chem* **61**, 7236-7237 (1996).

5. Sera N, Tokiwa H, Miyata N. Mutagenicity of the fullerene C_{60}-generated singlet oxygen dependent formation of lipid peroxides. *Carcinogenesis* **17**, 2163-2169 (1996).

6. Hamano T, Okuda K, Mashino T, Hirobe M, Arakane K, Ryu A, Mashiko S, Nagano T. Singlet oxygen production from fullerene derivatives: Effect of sequential functionalization of the fullerene core. *Chem Commun* 21-22 (1997).

7. Mashino T, Nishikawa D, Takahashi K, Usui N, Yamori T, Seki M, Endo T, Mochizuki M. Antibacterial and antiproliferative activity of cationic fullerene derivatives. *Bioorg Med Chem Lett* **13**, 4395-4397 (2003).

8. Mashino T, Shimotohno K, Ikegami N, Nishikawa D, Okuda K, Takahashi K, Nakamura S, Mochizuki M. Human immunodeficiency virus-reverse transcriptase inhibition and hepatitis C virus RNA-dependent RNA polymerase inhibition activities of fullerene derivatives. *Bioorg Med Chem Lett* **15**, 1107-1109 (2005).

9. Våbenø J, Brisander M, Lejon T, Luthman K. Diastereoselective reduction of a chiral *N*-Boc-protected δ-amino-α,β-unsaturated γ-keto ester Phe-Gly dipeptidomimetic. *J Org Chem* **67**, 9186-9191 (2002).

10. Mashino T, Okuda K, Hirota T, Hirobe M, Nagano T, Mochizuki M. Inhibitory effect of fullerene derivatives on glutathione reductase. *Fullerene Sci Technol* **9**, 191-196 (2001).

11. Mizrahi V, Lazarus G M, Miles L M, Meyers C A, Debouck C. Recombinant HIV-1 reverse transcriptase: Purification, primary structure, and polymerase/ribonuclease H activities. *Arch Biochem Biophys* **273**, 347-358 (1989).

12. Yamashita T, Kaneko S, Shirota Y, Qin W, Nomura T, Kobayashi K, Murakami S. RNA-dependent RNA polymerase activity of the soluble recombinant hepatitis C virus NS5B protein truncated at the C-terminal region. *J Biol Chem* **273**, 15479-15486 (1998).

13. Bosi S, Da Ros T, Spalluto G, Balzarini J, Prato M. Synthesis and anti-HIV properties of new water-soluble bis-functionalized[60]fullerene derivatives. *Bioorg Med Chem Lett* **13**, 4437-4440 (2003).

14. Marchesan S, Da Ros T, Spalluto G, Balzarini J, Prato M. Anti-HIV properties of cationic fullerene derivatives. *Bioorg Med Chem Lett* **15**, 3615-3618 (2005).

15. Merluzzi V J, Hargrave K D, Labadia M J, Grozinger K, Skoog M, Wu J C, Shinh C, Shinh C K, Eckner K, Hattooy S, Sullivan L L. Inhibition of HIV-1 replication by a non-nucleoside reverse transcriptase inhibitor. *Science* **250**, 1411-1413 (1990).

16. Dhanak D, Duffy K J, Johnston V K, Lin-Goerke J, Darcy M, Shaw A N, Gu B, Silverman C, Gates A T, Nonnemacher M R, Earnshaw D L, Casper D J, Kaura A, Baker A, Greenwood C, Gutshall L L, Maley D, DelVecchio A, Macarron R, Hofmann G A, Alnoah Z, Cheng H -Y, Chan G, Khandekar S, Keenan R M, Sarisky R T. Identification and biological characterization of heterocyclic inhibitors of the hepatitis C virus RNA-dependent RNA polymerase. *J Biol Chem* **277**, 38322-38327 (2002).

17. Nakamura E, Tokuyama S, Yamago S, Shiraki T, Sugiura Y. Biological activity of water-soluble fullerenes. Structure dependence of DNA cleavage, cytotoxicity, and enzyme inhibitory activities including HIV-protease inhibition. *Bull Chem Soc Jpn* **69**, 2143-2151 (1996).

18. Wolff D J, Mialkowski K, Richardson C F, Wilson

S R. C_{60}-Fullerene monomalonate adducts selectively inactivate neuronal nitric oxide synthase by uncoupling the formation of reactive oxygen intermediates from nitric oxide production. *Biochemistry* **40**, 37-45 (2001).