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

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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 scissions³ and
the oxidation of biological materials depend on photo-induced active oxygen production by fullerene. 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, carboxyfullerenes, show interesting antioxidant activities, and the cationic derivatives, C60-bis(N,N-dimethylpyrrolidinium iodide) and its alkylated derivatives, have excellent antibacterial and antiproliferative activities.

We have also previously reported that aminotricarboxylic acid derivative 3 has a very strong activity on HIV-RT inhibition. 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.](image-url)

**Materials and Methods**

**Chemicals**

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

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

One hundred and fifty milligrams of C60 (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 C60 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%).

\[ ^1H-NMR \text{ (CDCl}_3, \text{ 500 MHz}): \delta \text{ 1.49 (s, 9H, -C(CH}_3)_3}, \text{ 4.75 (d, 1H, J=11.5 Hz, C}_6\text{O-CH2-}}, \text{ 5.05 (d, 1H, J=12.5 Hz, C}_6\text{O-CH2-}}, \text{ 5.38 (s, 1H, C}_6\text{O-CH-).} \]

**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%).

\[ ^1H-NMR \text{ (DMSO-d}_6, \text{ 500 MHz}): \delta \text{ 4.94 (d, 1H, J=11.5 Hz, C}_6\text{O-CH2-}}, \text{ 5.16 (d, 1H, J=12.0 Hz, C}_6\text{O-CH2-}}, \text{ 5.91 (s, 1H, C}_6\text{O-CH-).} \]

**2-tert-Butoxycarbonyl-N-tert-butoxy carbonylmethyl-5-ethoxycar bonylpyrrolidino[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 C₆₀ (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%).

1H-NMR(CDC1₃, 500 MHz): δ 1.25 (t, 3H, J=7 Hz, -CH₂CH₃), 1.50 (s, 9H, -C(CH₃)₃), 1.61 (s, 9H, -C(CH₃)₃), 4.02 (d, 1H, J=16.5 Hz, N-CH₂-), 4.21 (d, 1H, J=16.5 Hz, N-CH₂-), 4.34 (q, 2H, J=3.0 Hz, -CH₂CH₃), 6.04 (s, 1H, C₆₀-CH-), 6.14 (s, 1H, C₆₀-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%). 1H-NMR(CDC1₃, 500 MHz): δ 0.88-1.72 (m, 42H, -C(CH₃)₃, -CH₂CH₃), 3.74-4.66 (m, 8H, N-CH₂-, -CH₂CH₃), 5.50-6.38 (m, 4H, C₆₀-CH-).

2-Carboxy-N-carboxymethyl-5-ethoxycarbonylpyrrolidino[60]fullerene (8a)

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 М HCl to give 31 mg of 8a as a brown powder (yield 116%). 1H-NMR(C13D₄, 500 MHz): δ 0.88-1.72 (m, 42H, -C(CH₃)₃, -CH₂CH₃), 3.74-4.66 (m, 8H, N-CH₂-, -CH₂CH₃), 5.50-6.38 (m, 4H, C₆₀-CH-); TOF-MS: m/z 847 ([M]+-2COOH), 720.

Bis[2-carboxy-N-carboxymethyl-5-ethoxycarbonylpyrrolidino][60]fullerene (8b)

Trifluoromethanesulfonic acid (164 mg, 1.09 mmol) was added to a solution of 8a (150 mg, 147 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 8b as a brown powder (yield 143%). 1H-NMR (C13D₄N, 500 MHz): δ 0.78-1.61 (m, 6H, -CH₂CH₃), 4.20-4.94 (m, 8H, -CH₂CH₃, N-CH₂-), 6.21-6.94 (m, 4H, C₆₀-CH-); TOF-MS: m/z 974 ([M]+-4COOH), 720.

2-tert-Butoxycarbonyl-N-ethoxycarbonylmethyl-5-ethoxycarbonylpyrrolidino[60]fullerene (8a)

Imidodiacetic acid diethyl ester (86 mg, 467 µmol) and tert-butyl glyoxylate (1.0 g) were added to a solution of C₆₀ (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%).

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 (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 116 mg of 5a as a brown powder (yield 82%). 1H-NMR (C13D₄N, 500 MHz): δ 1.24 (t, 3H, J=7.0 Hz, -CH₂CH₃), 1.41 (t, 3H, J=7.0 Hz, -CH₂CH₃), 1.49 (s, 9H, -C(CH₃)₃), 4.14 (d, 1H, J=16.5 Hz, N-CH₂-), 4.26 (d, 1H, J=17 Hz, N-CH₂-), 4.35 (q, 4H, J=6.5 Hz, -CH₂CH₃), 6.01 (s, 1H, C₆₀-CH-), 6.14 (s, 1H, C₆₀-CH-); TOF-MS: m/z 1021 (M)⁺, 720. 8b, 1H-NMR (C13D₄, 500 MHz): δ 0.98-1.72 (m, 30H, -C(CH₃)₃, -CH₂CH₃), 3.91-4.61 (m, 12H, N-CH₂-, -CH₂CH₃), 5.51-6.38 (m, 4H, C₆₀-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 (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 116 mg of 5a as a brown powder (yield 82%). 1H-NMR (C13D₄N, 500 MHz): δ 1.19 (t, 3H, J=7.0 Hz, -CH₂CH₃), 1.20 (t, 3H, J=7.0 Hz, -CH₂CH₃), 4.27 (q, 2H, J=7.0 Hz, -CH₂CH₃), 4.39 (q, 2H, J=7.0 Hz, -CH₂CH₃), 4.65 (d, 1H, J=16.5 Hz, N-CH₂-), 4.86 (d, 1H, J=16.5 Hz, N-CH₂-), 6.72 (s,
1H, C60-CH-), 6.77 (s, 1H, C60-CH-); FAB-MS: m/z 966(M⁺+1), 720.

Mullerene (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%). 1H-NMR (C5D5N, 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. 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 MgCl₂, 5 mM dithiothreitol, 0.125 mg/ml poly(rA·oligo(dT)12-18, and 2.5 μM dTTP including 32P-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 NaH₂PO₄ 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. The assay was performed in a total volume of 40 μL containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 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 32P-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. 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 C60 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 C60-bis(N,N-dimethylpyrrolidinium iodide) was comparatively effective on HIV-RT inhibition. 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 C₆₀ 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](image1)

![Scheme 3. Synthesis of fullerene derivatives 5a and 5b.](image2)

The main components of 3, 4a, and 5a were trans-isomers with a 5–10% amount of cis-isomers (δ 5.79, s, C₆₀-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, which is clinically used for HIV infection.

**Table 1. HIV-RT inhibition of fullerene derivatives**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (μM)</th>
<th>Compound</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nevirapine</td>
<td>3.0</td>
<td>4a</td>
<td>0.156</td>
</tr>
<tr>
<td>1</td>
<td>0.150</td>
<td>4b</td>
<td>0.209</td>
</tr>
<tr>
<td>2</td>
<td>0.073</td>
<td>5a</td>
<td>0.163</td>
</tr>
<tr>
<td>3</td>
<td>0.029</td>
<td>5b</td>
<td>0.160</td>
</tr>
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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 IC50 values of the fullerene derivatives were higher than those of the benzo-1,2,4-thiadiazine analog.4b and 5b were also less effective than C60-bis(N,N-dimethylpyrrolidinium iodide) (IC50, 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>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
<th>Compound</th>
<th>IC50 (μM)</th>
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</thead>
<tbody>
<tr>
<td>Thiadiazine analog</td>
<td>0.10</td>
<td>4a</td>
<td>&gt;10</td>
</tr>
<tr>
<td>1</td>
<td>a</td>
<td>4b</td>
<td>1.17</td>
</tr>
<tr>
<td>2</td>
<td>&gt;10</td>
<td>5a</td>
<td>&gt;10</td>
</tr>
<tr>
<td>3</td>
<td>2.16</td>
<td>5b</td>
<td>0.87</td>
</tr>
</tbody>
</table>

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**

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**References**

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


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