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Fullerene derivatives as dual inhibitors of HIV-1 reverse transcriptase and protease

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ABSTRACT

In the present study, we newly synthesized three types of novel fullerene derivatives: pyridinium-type derivatives *trans*-**3a** and **4a**-**5b**, piperidinium-type derivative **9**, and proline-type derivatives **10a**-**12**. Among the assessed compounds, **5a**, **10e**, **10f**, **10i**, **11a**-**d**, and **12** were found to inhibit both HIV reverse transcriptase and HIV protease (HIV-PR), with IC_{50} values in the low micromolar range being observed. Regarding HIV-PR inhibition activity, proline-type derivatives **11a**-**11d** and **12**, bearing an alkyl chain between the hydroxylmethylcarbonyl (HMC) moiety and pyrrolidine ring, were more potent than other derivatives. This result might indicate that connecting HMC moieties with proline-type fullerene derivatives through properly sized alkyl chain leads to improved HIV-PR inhibitory activity.

The human immunodeficiency virus (HIV), which is a retrovirus with a positive-sense single-stranded RNA genome, causes acquired immunodeficiency syndrome (AIDS). Globally, more than 37 million people infected HIV, and approximately 35 million people died due to HIV to date.[1] HIV infection leads to the destruction of immune cells, including CD4-positive *T*-cells and macrophages.

Antiretroviral therapy (ART), the standard regimen for people living with HIV, provides maintained suppression of viral replication and the maintenance of an acceptable level of immune reconstitution. The ART regimen is recommended to include two nucleoside reverse transcriptase inhibitors (NRTI) in combination with a nonnucleoside reverse-transcriptase inhibitor (NNRTI), one protease inhibitor or an integrase inhibitor.[2] In spite of the spread of ART, the emergence of multidrug-resistant HIV is still a serious problem in anti-HIV therapy. For this reason, it is an urgent challenge to develop a novel anti-HIV drug that is structurally distinct from the pharmaceutical agents currently used. Recently, single tablet regimens (STRs), e.g., Biktarvy® and Genvoya®, were approved by the FDA. These STRs contain an HIV integrase inhibitor, two NRTIs and a CYP3A-specific inhibitor, contributing to the improvement of medication adherence and simplification of the regimen.

In addition to STRs, multitarget drugs have also been a focus over the past few years. It was reported that multitarget drugs, interacting with multiple disease-relevant targets, are able to improve therapeutic efficacy, prevent drug resistance and reduce unwanted side effects.[3] For instance, several multikinase inhibitors, e.g., sorafenib, ponatinib and lenvatinib, have been approved to date. With regard to anti-HIV agents, **KNI-1039**[4] was reported to inhibit both HIV reverse transcriptase (HIV-RT) and HIV protease (HIV-PR). The HIV-RT/PR dual inhibitor **KNI-1039**, a conjugant of the peptide mimic HIV-PR inhibitor and 3′-azido-3′-deoxythymidine, improved HIV-RT/PR inhibition activity compared with parental compounds. Despite the high therapeutic potential of multitarget drugs, their rational drug discovery remains a challenge, and it is particularly difficult to create compounds exhibiting well-balanced potency against each target.

Fullerene (C_{60}), discovered by Kroto et al.,[5] is a carbon allotrope and is expected to be pharmaceutically useful[6] on account of its three-dimensional structure and physical properties. However, evaluation of

Abbreviations: AIDS, acquired immunodeficiency syndrome; ART, antiretroviral therapy; HIV, human immunodeficiency virus; HIV-PR, HIV protease; HIV-RT, HIV reverse transcriptase; HMC, hydroxylmethylcarbonyl; NNRTI, nonnucleoside reverse-transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitors; SAR, structure-activity relationship; STRs, single tablet regimens.

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the bioactivity of fullerene has been restricted due to its insolubility in water. To overcome this issue, a variety of fullerene derivatives with hydrophilic substituents have been studied. Water-soluble fullerene derivatives have been reported to possess a range of pharmacological properties, including antitumor,[7] antiproliferative[8,9] and antibacterial [9–11] activities, as well as inhibitory effects against various enzymes, such as protein tyrosine phosphatase,[12] cysteine/serine protease,[13] HCV NS3/4A protease,[14] HCV NS5B polymerase,[14,15] HIV-RT [15–17] and HIV-PR.[18]

We previously reported that several types of fullerene derivatives exhibit HIV-RT inhibitory activity: pyrrolidinium-type derivative **1**, proline-type derivatives **2a** and **2b**, and pyridinium-type *cis*-**3a** and **3b** (Fig. 1).[15–17] The HIV-RT inhibitory activities of pyridinium-type *cis*-**3a** and **3b** were comparable to, or slightly less potent than, proline-type derivatives **2a** and **2b**, while they were found to be stronger than that of pyrrolidinium-type derivative **1**.[17] Furthermore, we also reported that proline-type derivative **2a** showed HCV NS3/4A protease/NS5B polymerase dual inhibition activity,[14] suggesting that **2a** has potency as a multitarget agent.

Regarding the HIV-PR inhibition activity of fullerene derivatives, there are few precedents for synthesis, while a number of groups have reported on 3D QSAR, molecular docking and molecular dynamics of fullerene-based HIV-PR inhibitors.[19–22] HIV-PR is one of the significant enzymes that leads to HIV proliferation. The active site of HIV-PR consists of hydrophobic amino acid residues, except the two hydrophilic aspartic acids. HIV-PR inhibitors occupy the substrate binding site and prevent catalytic cleavage of large polyprotein precursors. Ibrahim and Saleh et al. reported that fullerene derivatives bearing a hydroxymethylcarbonyl (HMC) moiety, the transition-state mimic isostere of substrate processing, might have high HIV-PR inhibitory activity due to the effective interaction between the HMC moiety and aspartic acid residues of HIV-PR on the docking simulation.[21,22]

On the basis of these reports, we hypothesized that it is possible to create fullerene-based HIV-RT/PR dual inhibitors. Thus, we strategically designed three types of fullerene derivatives: pyridinium-type derivatives *trans*-**3a** and **4a**-**5b**, piperidinium-type derivative **9**, and proline-type derivatives **10a**-**12** (Fig. 1). Then, we evaluated HIV-RT and HIV-PR inhibition activities of these novel fullerene derivatives as well as the derivatives which were already reported as HIV-RT inhibitors (**1**, **2a**, **2b**, *cis*-**3a**, **3b**) or antitumor agents (**3c**, **6a**, **6b**, **7a**-**c**, **8**) in our previous work,[7,15–17] and we examined their structure–activity relationship (SAR).

We previously reported that the formation of the *trans* isomer may be explained by a mechanistic hypothesis in which the (*E*, *Z*) or (*Z*, *E*) azomethine ylide intermediate (which leads to the *trans* product) generated from the corresponding secondary amine and ketone or aldehyde is more dominant than the other configurations due to its steric stability.[14] On the basis of this finding, derivative *trans*-**3a"** was synthesized by the 1,3-dipolar cycloaddition reaction of 3-pyridinecarboxaldehyde and *N*-(4-methoxybenzyl)-glycine ethyl ester (**15**), as illustrated in Scheme 1. In fact, the methine proton on the C-5 of the pyrrolidine ring in the *trans* isomer was clearly downfield-shifted

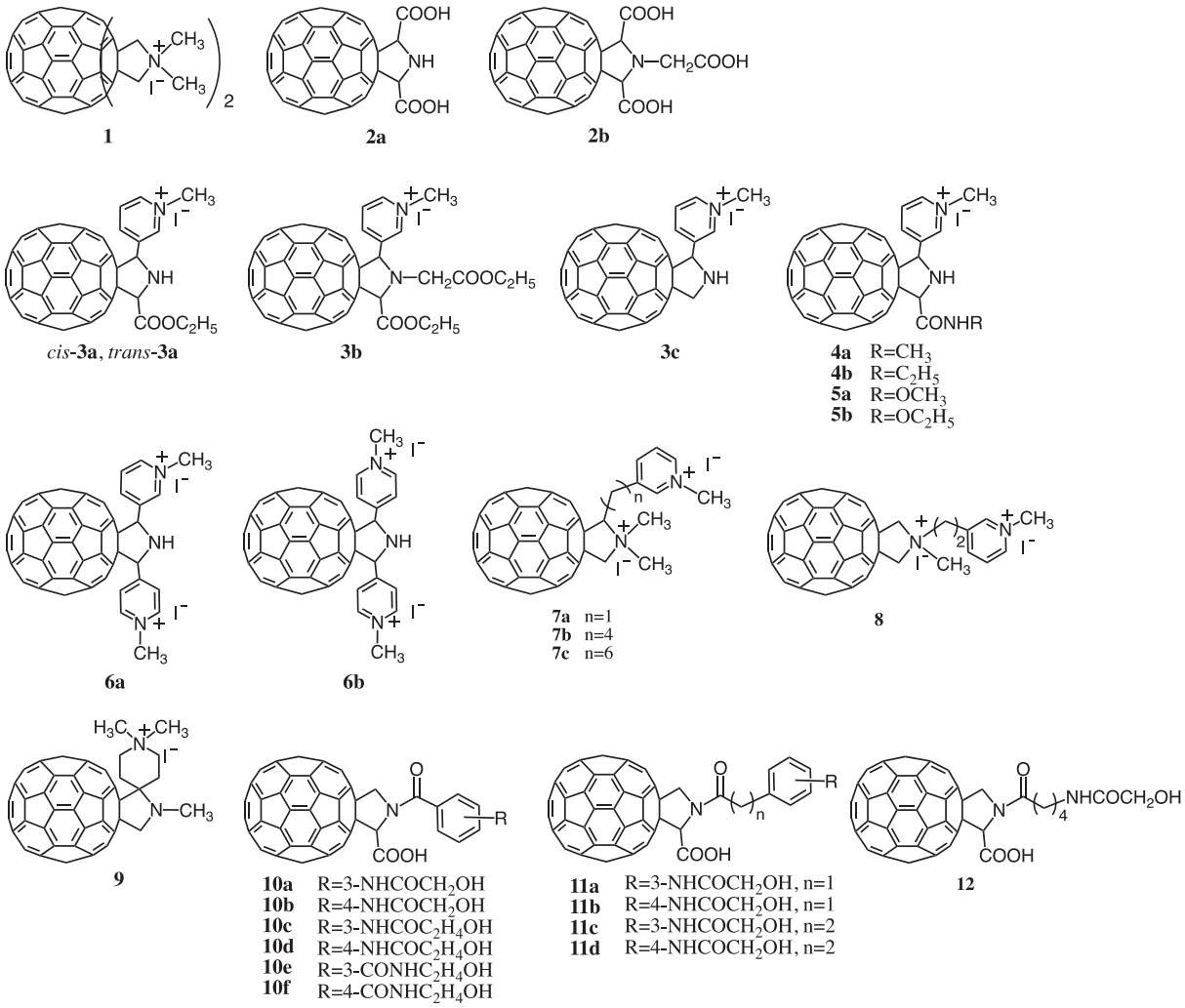
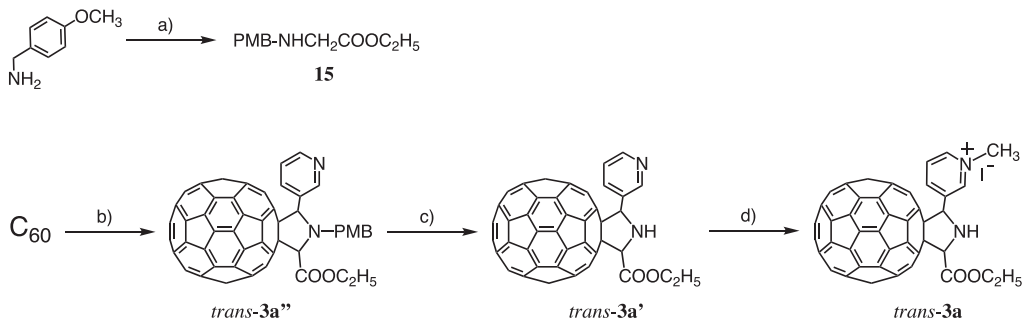


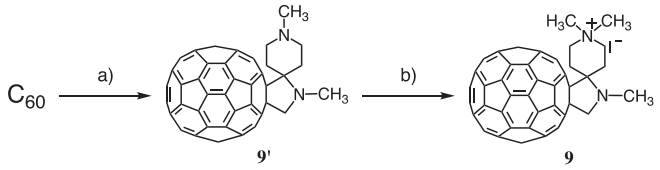
Fig. 1. Structures of fullerene derivatives.



Scheme 1. Synthesis of *trans*-**3a**. (a) ethyl chloroacetate, triethylamine, CH_2Cl_2, 0 °C to r.t, 44%; (b) **15**, 3-pyridinecarboxaldehyde, toluene, reflux, 11%; (c) Trifluoroacetic acid, $CHCl_3$, r.t, 61%; (d) CH_3I, r.t, 65%.

compared to the *cis* isomer, which was consistent with the NMR-analysis reported by Filippone et al.[23] Derivative *trans*-**3a** was subsequently synthesized through PMB deprotection of *trans*-**3a''** and methylation of *trans*-**3a'** with methyl iodide. In the case of **4a-5b**, the precursor **13** was synthesized in the same manner as our previous report.[17] Amidation with corresponding amine followed by methylation with methyl iodide produced **4a-5b** (Scheme 2). The introduction of methyl groups onto the pyridine nitrogen was confirmed by the downfield shift of the ^{1}H NMR signals for the aromatic protons and the observation of a signal on the 1-position of the pyrrolidine ring. For the synthesis of piperidinium derivative **9**, precursor **9′** were synthesized by 1,3-dipolar cycloaddition reaction of the corresponding ketone and glycine derivatives. Subsequent methylation with methyl iodide produced compound **9** (Scheme 3). In the case of **9**, the introduction of methyl group onto the piperidine nitrogen was confirmed by the downfield shift of the ^{1}H NMR signals for the piperidine-ring protons and the observation of an unchanged signal on the 1-position of the pyrrolidine ring.

The preparation of proline derivatives **10a-f**, **11a-d** is illustrated in Scheme 4. Compound **14** was synthesized by 1,3-dipolar cycloaddition reaction of C_{60}, paraformaldehyde and glycine *tert*-butyl ester. Regarding the benzoic acid analogues **18a**, **18b** and **18e-h**, ethyl glycolate was used as the starting material. Protection of the hydroxyl group with TBDPS-Cl produced **16a**, which was hydrolyzed with NaOH to yield **17a**. After the treatment of **17a** with oxalyl chloride, amidation using the corresponding carboxylic acids produced **18a**, **18b** and **18e-h**. In the case of **18c** and **18d**, amidation of the corresponding amino benzoic acids with **17b**, prepared stepwise from methyl 3-hydroxypropanoate, gave **18c** and **18d**. For the synthesis of compounds **21a** and **21b**, 2-aminoethan-1-ol was used as the starting material. Protection of the hydroxyl group in a similar manner to **17a**/**17b** followed by amidation with 3-/4-formylbenzoic acid and Pinnick oxidation of carbonyl group produced **21a**/**21b**. After the treatment of **18a-h**, **21a** and **21b** with oxalyl chloride, amidation of the corresponding acid chlorides with **14**. Subsequent deprotection with trifluoromethanesulfonic acid produced the proline derivatives **10a-f** and **11a-d**. With respect to **12**, condensation of **17b** with 5-aminovaleric acid gave **22**. After the treatment of **22** with oxalyl chloride, amidation with **14** and the following deprotection produced proline derivative **12** (Scheme 5).

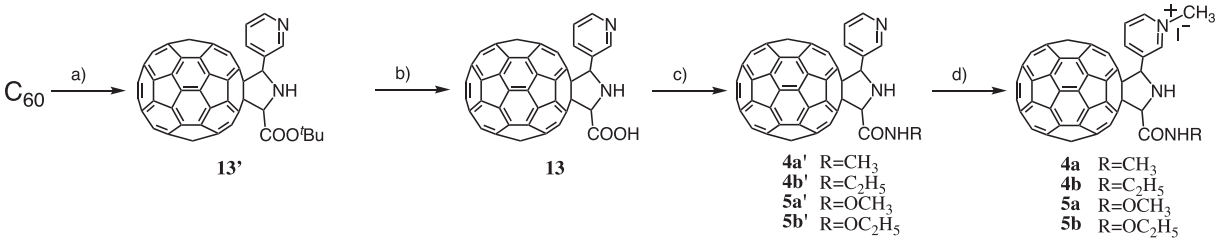


Scheme 3. Synthesis of **9**. (a) sarcosine, 1-methyl-4-pyrrolidone, toluene, reflux, 27%; (b) CH_3I, DMF, r.t. to 60 °C, 76%.

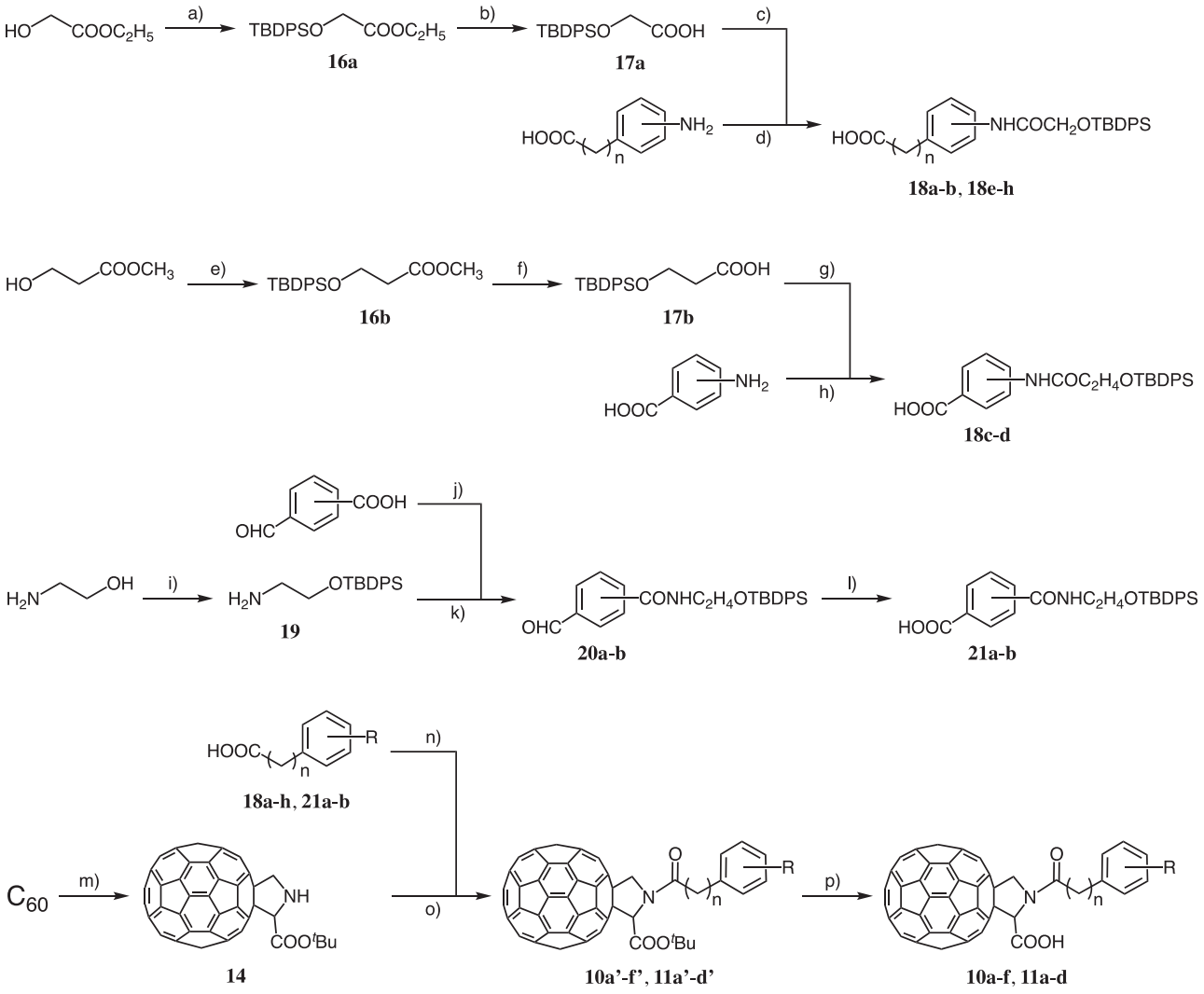
Compound **23**, the *exo*-substituent of **10b**, was synthesized by deprotection of **18b** with trifluoromethanesulfonic acid (Scheme 6). The identification and the purity of the compounds synthesized in the present study were confirmed by NMR as well as by mass spectrometry and in some cases by high-resolution mass spectrometry.

We first investigated the HIV-RT inhibition activities of fullerene derivatives using recombinant RT derived from HIV-1. The HIV-RT inhibition activities were examined in a similar manner as O'Meara et al.[24] The HIV-RT inhibition activities of all of the novel fullerene derivatives synthesized in the present study were more potent than nevirapine (Table 1). Regarding the HIV-RT inhibition activities of C_{60}-based compounds, pyrrolidinium-type derivative **1**, proline-type derivatives **2a** and **2b**, and pyridinium-type *cis*-**3a** and **3b** were comparable to previous data (IC_{50} = 1.4–1.7 μM (**1**), 0.032 μM (**2a**), 0.029 μM (**2b**), 0.094 μM (*cis*-**3a**), 0.25 μM (**3b**), respectively).[15–17] In the case of pyridinium-type and piperidinium-type derivatives, there was no remarkable difference in the inhibitory activities of *trans*-**3a**, **4a-5b**, **7a-c**, **8** and **9**, whereas **3c**, **6a** and **6b** inhibited HIV-RT with slightly lower potency than *cis*-**3a**. These results suggest that it is favorable to bear an ester or amide moiety on the pyrrolidine ring in terms of the HIV-RT inhibition of pyridinium-type derivatives. Among the proline-type derivatives **10a-12**, **10c** was equally potent relative to **2a** and **2b**, while the HIV-RT inhibition activities of the others were slightly weaker than those of **2a** and **2b**.

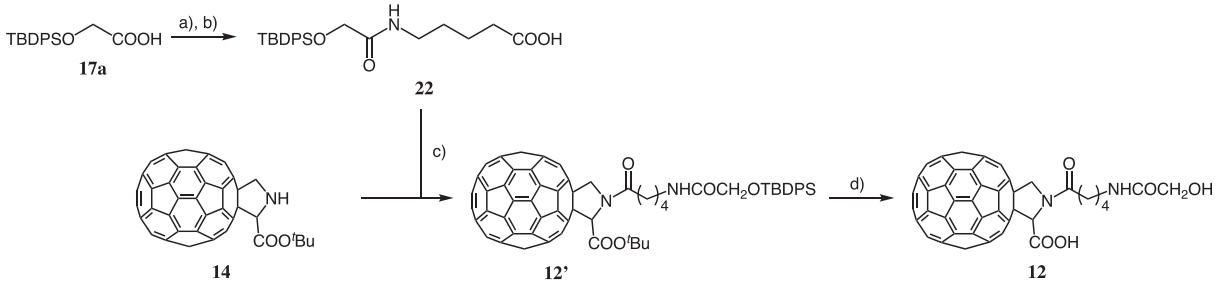
We next examined HIV-PR inhibitory activity using recombinant PR derived from HIV-1 in a similar manner to Friedman et al.[18] Pyridinium-type and piperidinium-type derivatives *trans*-**3a**, **4a**, **4b**, **5a**, **5b** and **9** exhibited potency of HIV-PR inhibition, whereas most cationic



Scheme 2. Synthesis of **4a-b** and **5a-b**. (a) glycine *tert*-butyl ester hydrochloride, 3-pyridinecarboxaldehyde, toluene, reflux, 29%; (b) trifluoromethanesulfonic acid, CS_2, r.t, 91%; (c) corresponding amine hydrochloride, HOBt, EDC hydrochloride, *N*-methylmorpholine, CH_2Cl_2, r.t, 10–54%; (d) CH_3I, r.t, 45–92%.

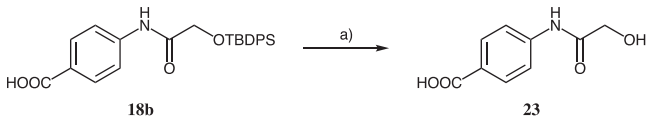


Scheme 4. Synthesis of **10a-f** and **11a-d**. (a) TBDPS-Cl, imidazole, DMF, 0 °C to r.t, quant; (b) NaOH*aq*, EtOH, 0 °C to r.t, 72%; (c) $(COCl)_2$, DMF, Et_2O, 0 °C to r.t; (d) pyridine, Et_2O or THF or CH_2Cl_2, 0 °C to r.t, 12–67%; (e) TBDPS-Cl, imidazole, CH_2Cl_2, 0 °C to r.t, 77%; (f) NaOH*aq*, MeOH, 0 °C to r.t, 78%; (g) $(COCl)_2$, DMF, Et_2O, 0 °C to r.t; (h) pyridine, Et_2O, 0 °C to r.t, 80%-quant; (i) TBDPS-Cl, imidazole, CH_2Cl_2, 0 °C to r.t, 94%; (j) $(COCl)_2$, DMF, acetonitrile, 0 °C to r.t; (k) pyridine, THF, 0 °C to r.t, 18–63%; (l) 2-methyl-2-butene, $NaClO_2$, NaH_2PO_4, ^{t}BuOH/water, r.t, 12%-quant; (m) glycine *tert*-butyl ester hydrochloride, paraformaldehyde, $LiClO_4$, triethylamine, toluene, reflux, 42%; (n) $(COCl)_2$, DMF, toluene or CH_2Cl_2, 0 °C to r.t; (o) pyridine, toluene or CH_2Cl_2, 0 °C to r.t, 15–70%; (p) trifluoromethanesulfonic acid, toluene or CH_2Cl_2, 0 °C or 0 °C to r.t, 21–96%.



Scheme 5. Synthesis of **12**. (a) EDC hydrochloride, CH_2Cl_2, r.t; (b) 5-aminovaleric acid, CH_2Cl_2, r.t, 30%; (c) EDC hydrochloride, CH_2Cl_2, r.t, 14%; (d) trifluoromethanesulfonic acid, toluene, 0 °C to r.t, 52%.

derivatives lack HIV-PR inhibitory activity. Among the pyridinium-type derivatives *cis*-**3a**, *trans*-**3a** and **3c**, only *trans*-**3a** showed HIV-PR inhibitory activity, indicating that the stereochemistry of ethyl ester might contribute to the inhibition of HIV-PR. In addition, **4a-5b** showed HIV-PR inhibition activity. This result suggests that an amide moiety of the pyrrolidine ring significantly contributes to the enhancement of HIV-PR inhibition. Furthermore, the comparison between HIV-PR inhibition activities of **10a-12** with those of **2a** and **2b** indicated that fullerene derivatives bearing hydroxylmethylcarbonyl (HMC) moieties are more likely to have higher inhibition activity. In the case of **10a-f**, there was



18b 23

Scheme 6. Synthesis of **23**. (a) trifluoromethanesulfonic acid, toluene, 0 °C, 88%.

Table 1
HIV-RT/PR inhibition activities of fullerene derivatives.

Compound	HIV-RT Inhibitory Activity IC50 (μM)	HIV-PR Inhibitory Activity IC50 (μM)
1	1.0	>10
2a	0.06	6.4
2b	0.05	4.3
cis-**3a**	0.4	>10
trans-**3a**	0.4	7.7
3b	0.4	>10
3c	1.0	>10
4a	0.6	6.2
4b	0.3	6.4
5a	0.6	1.9
5b	0.3	8.7
6a	1.5	>10
6b	1.6	>10
7a	0.3	>10
7b	0.2	>10
7c	0.1	>10
8	0.5	>10
9	0.5	5.7
10a	0.4	1.4
10b	0.2	2.0
10c	0.05	2.1
10d	0.7	4.9
10e	0.1	1.9
10f	0.6	3.0
11a	1.0	1.3
11b	0.5	1.3
11c	0.6	0.6
11d	0.2	0.7
12	0.8	1.0
23	N. T.	>10
Nevirapine	2.1	N. T.
Ritonavir	N. T.	1.7 nM

N.T.: Not Tested.

no remarkable difference in HIV-PR inhibitory activity, suggesting that it is not effective to replace an HMC moiety (**10a** and **10b**) into a hydroxyethylcarbonyl unit (**10c** and **10d**) or reversed amide (**10e** and **10f**). In contrast, it should be noted that proline-type derivatives **11a-d** and **12**, bearing an alkyl spacer between the HMC moiety and pyrrolidine ring, tended to show more potency than **10a** and **10b**. Furthermore, HMC analogue **23**, the *exo*-substituent of **10b**, indicated a loss of HIV-PR inhibition activity. These results might imply that connecting the HMC moiety with proline-type fullerene derivatives through properly sized alkyl chain leads to improved HIV-PR inhibitory activity. Among the proline-type derivatives, compound **11c** and **11d** showed the best HIV-PR inhibition activity.

Table 2
Membrane permeability and cytotoxicity of **11c** and **11d.**

Compound	PAMPA P_{app} (10^{-6} cm/s)	Cell growth Inhibition CC50 (μM)	
		NIH3T3	HepG2
11c	16	>30	>30
11d	9.7	>30	>30
Propranolol	8.1	N.T.	N.T.
Furosemide	0.52	N.T.	N.T.
Saquinavir	1.2	N.T.	N.T.

N.T.: Not Tested.

The membrane permeability and cytotoxicity of fullerene derivatives **11c** and **11d** were evaluated (Table 2). The P_{app} values of the fullerene derivatives in parallel artificial membrane permeability assay (PAMPA) were higher than saquinavir, a clinically used HIV-PR inhibitor. It should be noted that the permeability of **11c** and **11d** was comparable to propranolol, a highly membrane permeable drug. In addition, the cytotoxicity of **11c** and **11d** on normal cells (mouse fibroblast NIH3T3) and human cells (human hepatoma HepG2) were assessed using a conventional trypan blue dye exclusion test. The CC_{50} values on both cell lines were over 30 μM. These results indicate that **11c** and **11d** are able to passively permeate across the cell membrane and inhibit HIV-RT and HIV-PR without serious toxicity.

In summary, this is the first report to indicate that cationic-type and proline-type fullerene derivatives show potent HIV-RT and HIV-PR dual inhibition. The inhibition activities against HIV-PR were weaker than a clinically used HIV-PR inhibitor, ritonavir. However, the newly synthesized fullerene derivatives **5a**, **10a**, **10b, 10e**, **11a-d** and **12** showed HIV-PR as well as HIV-RT inhibition activities. Of these, proline-type derivatives **11c** and **11d** showed the best-balanced activity in the current work, namely, a similar level of inhibitory activities against HIV-RT and HIV-PR with submicromolar IC_{50} values, suggesting that **11c** and **11d** may strongly inhibit HIV replication by the synergistic effect as an HIV-RT/PR dual inhibitor. The effects of these derivatives in HIV-infected cells are under investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127675.

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