Communications to the Editor

A New Candidate for an Anti-HIV-1 Agent: Modified Cyclodextrin Sulfate (mCDS71)

Tamon Moriya,*,† Kiyosi Saito,† Hironori Kurita,‡ Kazuo Matsumoto,† Toru Otake,§ Haruyo Mori,§ Motoko Morimoto,§ Noboru Ueba,§ and Nobuharu Kunita§

Research Laboratory of Applied Biochemistry, Tanabe Seiyaku Co., Ltd., Osaka 532, Japan, Organic Chemistry Research Laboratory, Tanabe Seiyaku Co., Ltd., Toda 335, Japan, and Osaka Prefectural Institute of Public Health, Osaka 537, Japan

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The pandemic of acquired immunodeficiency syndrome (AIDS) is continuing to expand worldwide at an exponential rate, as a sexually transmitted disease. Numerous compounds with various mechanisms of action against the causative human immunodeficiency virus (HIV) of AIDS[1] are under development, but at present the only drugs approved by the US FDA for the treatment of AIDS are nucleoside derivatives: 3′-azido-3′-deoxythimidine (AZT), 2′,3′-dideoxyinosine (DDI), and 2′,3′-dideoxycytidine (DDC). Although these drugs have a potent inhibitory activity on the reverse transcriptase of HIV, serious side effects of the drugs (e.g. myelosuppression, neuropathy, and pancreatitis) and the emergence of drug-resistant strains of HIV have been reported.[1,2]

Considerable attention is currently being focused on polyanionic compounds[3] that show highly potent inhibitory activity on the replication of HIV in vitro because of their synergistic activity[4] with the nucleoside drugs and their anti-HIV action mechanism that is entirely different from that of the nucleoside drugs, i.e., they inhibit virus binding to the cell membrane resulting in the marked inhibition of cell fusion (syncytium formation). Among the polyanionic compounds, sulfated polysaccharides such as dextran sulfate (DS), pentosan sulfate (HOE/BAY-946), curdran sulfate, and others, have been investigated most actively as potentially useful agents for the treatment of AIDS. However, their effectiveness in vivo has not been established as yet[5] because of their poor absorbability owing to their large molecular size, short half-life time in the body by metabolic hydrolysis, and unfavorable anticoagulant activity in blood. Additionally, a variety of modes of action of the sulfated polysaccharides have recently been proposed.[6] This suggests manifold and complicated interaction of virus and targeted cells, and such variety could also be associated with the indefinable molecular structures showing broad ranges of molecular weight and undefined numbers of the sulfate groups and the sulfated positions.

In a previous paper,[7] we proposed a sort of guiding-star of research to overcome the above problems for development of polyanionic compounds as oral anti-HIV agents. Thus, it is necessary to simplify the molecular structure

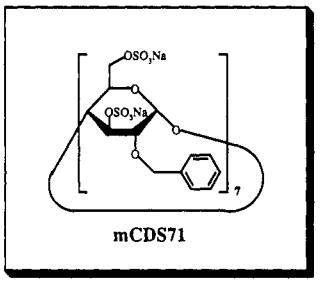

Figure 1.

for a rational drug design based on structure–activity relationships. Additionally, down-sizing and introduction of lipophilic groups to the molecule should improve both absorbability in the gut and the separation of anticoagulant activity. In accordance with the guide, we synthesized a series of modified cyclodextrin sulfates (C-6 mCDSs) with various functional groups on the C-6 positions of the cyclodextrin (CD) frame. We found a candidate compound mCDS11 which was modified with three benzylthio groups as the lipophilic moiety, along with 16 anionic sulfate groups, as shown Figure 1. Enhancement of anti-HIV activity and separation of the defective anticoagulant activity were well established in mCDS11. The absorption of mCDS11 from the gut was confirmed by the anti-HIV activity of the plasma obtained from rats given the compound per os.

In this paper, we report an advanced investigation of mCDS and a new candidate mCDS71. A series of C-2 modified cyclodextrin sulfates (C-2 mCDS) were synthesized instead of C-6 modified analogs for the development of a more satisfactory harmless oral anti-HIV agent with high activity. The introduction of lipophilic substituents to the C-2 position of CD was carried out according to the method of Takeo et al.[8] with slight modification, as shown in Scheme I. The most reactive seven C-6 hydroxyl groups of CD were protected with *tert*-butyldimethylsilyl (TBS) groups before modification of the C-2 position hydroxyl groups. The resultant C-6 silyl CD derivative (**I**) was reacted with various alkyl halides, using a barium oxide–barium hydroxide mixture as a base in dimethyl formamide (DMF), and the silyl groups of the formed 6-silyl-2-alkylated-cyclodextrin derivative (**II**) were then removed by tetrabutylammonium fluoride. The C-2 modified cyclodextrin (**III**) thus obtained was sulfated by sulfur trioxide–pyridine complex in pyridine to afford the objective C-2 mCDS. The compound mCDS78, in which the C-6 hydroxyl groups are not sulfated, was prepared by direct sulfation of **II** and by successive deprotection under acidic conditions.

The C-2 mCDS thus prepared were screened in the same way as reported previously.[7] Anti-HIV-1 activity which was estimated by determining the inhibition of the cytopathic effect (CPE) in MT-4 cells, using two strains of LAV-1 (a well-established cultured strain in laboratories) and KK-1_{AIDS} (a clinically isolated HIV-1 strain from a Japanese AIDS patient); anticoagulant activity was examined in human serum, and cytotoxicity was investigated in MT-4 cells.

Three of four mCDS compounds had roughly equal inhibitory activity against the two strains of HIV-1 tested.

† Tanabe Seiyaku Co., Ltd., Osaka.
‡ Tanabe Seiyaku Co., Ltd., Toda.
§ Osaka Prefectural Institute of Public Health.

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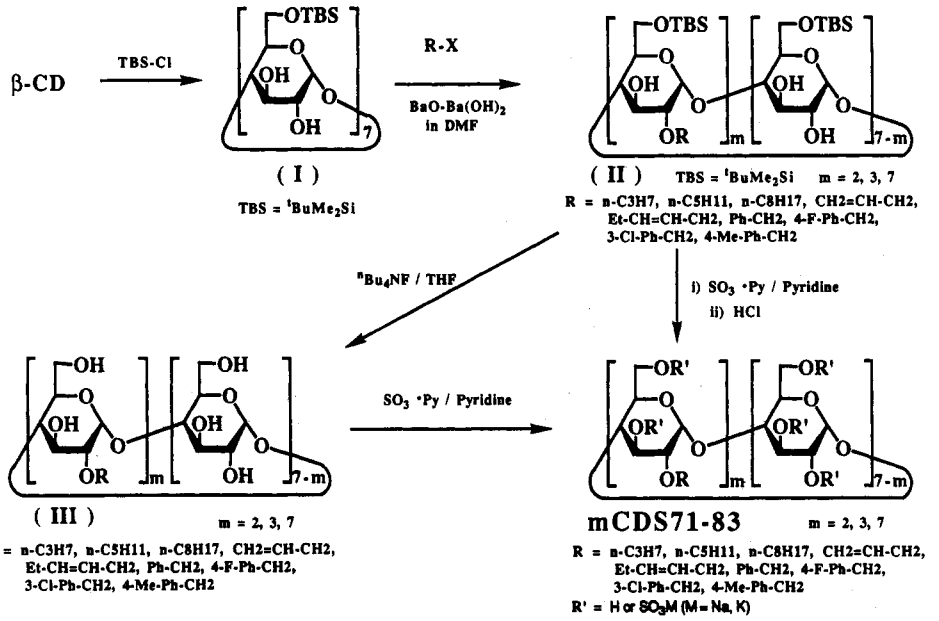
Table I. Anticoagulant Activity and Inhibitory Effect of mCDSs on HIV-1 Replication and Cell Toxicity

compound [abbreviated formula][a]	anti-HIV-1[b] (IC_{100}, μg/mL) LAV-1	anti-HIV-1[b] (IC_{100}, μg/mL) $KK\text{-}1_{AIDS}$	APTT[c] (μg/mL)	cytotox[d] (TD_{50}, μg/mL)
mCDS71 [β-CD(2-*O*-CH_2Ph)$_7$(SO_3Na)$_{14}$]	0.98	0.98	14.8	1000
mCDS77 [β-CD(2-*O*-CH_2Ph)$_3$(SO_3K)$_{16}$]	1.95	3.90	4.0	>1000
mCDS78 [β-CD(2-*O*-CH_2Ph)$_3$(SO_3K)$_9$]	7.80	15.60	13.5	>1000
mCDS11 [β-CD(6-*S*-CH_2Ph)$_3$(SO_3K)$_{16}$]	0.98	1.95	7.1	>1000
CDS [β-CD(SO_3K)$_{14}$]	31.26	125	2.8	>1000
DS [DS8000]	3.90	250	3.3	>1000

[a] mCDS71: Tetradecasodium heptakis(2-*O*-benzyl)-β-cyclodextrin tetradecasulfate. mCDS77: Hexadecasodium tris(2-*O*-benzyl)-β-cyclodextrin hexadecasulfate. mCDS78: Nonasodium tris(2-*O*-benzyl)-β-cyclodextrin nona(2- and 3-sulfate). mCDS11: Hexadecapotassium tris(6-(benzylthio)-6-deoxy)-β-cyclodextrin hexadecasulfate. CDS: Tetradecapotassium β-cyclodextrintetradecasulfate. DS: Dextran sulfate purchased from Sigma Chemical Co. prepared from average molecular weight of 8000. [b] The minimum concentration for complete inhibition of HIV-1-induced CPE in MT-4 cells (IC_{100}): MT-4 cells were infected with 0.001 $TCID_{50}$ (determined by MT-4 cells on day 5 after infection) on HIV-1 (strain LAV-1 or $KK\text{-}1_{AIDS}$, a strain clinically isolated from an AIDS patient) per cell for 1 h, and nonadsorbed virus was removed by washing. After 5 days of incubation with various concentrations (12 doses, 0.49–1000 μg/mL) of the test compound, the number of viable cells in both the HIV-1- and mock-infected cell cultures was determined by trypan blue staining. [c] Anticoagulation effect: Zuchker's activated partial thromboplastin time (APTT) method[11] was used. The value is indicated by the concentration (μg/mL) required to obtain 2-fold APTT. [d] Minimum concentration (μg/mL) for appearance of MT-4 cell toxicity after 5 days of incubation with the test compound. All data represent median values of two or three experiments.

Scheme I. Synthesis of 2-O-Modified β-Cyclodextrin Sulfates



All mCDS compounds had enhanced potency when compared to CDS. These findings strongly suggest that the introduction of lipophilic groups to the polyanionic compound CDS particularly enhanced anti-HIV-1 activity.

The anticoagulant activity of mCDS71 and 78 was very weak: this was mainly attributed to the location of the sulfate groups at one side of the CD ring (in the case of mCDS78) or to masking of the C-3 sulfate groups by C-2 lipophilic groups (in the case of mCDS71). Thus, the sulfate groups located at top and bottom of the CD ring appear to behave as the site corresponding to the two binding sites of heparinoides to antithrombin III.[9]

The most potent compound among the C-2 mCDS, mCDS71 [tetradecasodium heptakis(2-*O*-benzyl)-β-cyclodextrin tetradecasulfate, $C_{91}H_{98}O_{77}S_{14}Na_{14}$, MW 3194.71], has seven uniformly modified glucose units bearing a benzyloxy group at the C-2 position and sodium sulfate groups at the C-3 and C-6 positions as shown in Figure 1. Therefore, it has a C_7 symmetry axis at the center of the doughnut-like β-cyclodextrin molecular frame. The extremely orderly constructed structure was suggested from the sharp and well-assignable NMR spectra. The uniformity of mCDS71 should serve to elucidate the mechanisms of action of a desirable polyanionic anti-HIV agent and should also help in the development of such agents. mCDS71 exhibited anti-HIV-1 activity at 0.98 μg/mL [the minimum concentration for complete inhibition (IC_{100}) of both the $HIV_{LAV\text{-}1}$- and $HIV_{KK\text{-}1/AIDS}$-induced cytopathic effect (CPE) in MT-4 cells]. The anticoagulant activity was exerted at 14.80 μg/mL [the concentration for doubling of the activated partial thrombin time (APTT)], which corresonded to only half and one-fourth of the anticoagulant activity of mCDS11 and DS, respectively. The cytotoxicity of mCDS71 as low as 1000 μg/mL did not affect MT-4 cells.

To determine which compound, mCDS71 or -11, is a more suitable development candidate as an AIDS treatment, the biological studies of each were further conducted. Anti-HIV-1 activity was reexamined in the presence of 50% fresh human serum (HS) in the medium to reflect the *in vivo* situation by the experimental conditions and also to account for the suppressive effect of HS, which had been reported to reduce the activity of DS.[5d] As shown in Table II, excellent results for mCDS71 were documented in all the anti-HIV-1 activity assay systems tested. That is, the undesirable influence of HS on mCDS71 was negligible; high activity (IC_{50} = 0.87 μg/mL) was shown on the CPE assay system constructed with a conventional combination of MT-4 cells and the LAV-1 strain, whereas

Table II. Inhibitory Effect of mCDS71 and -11 on HIV-1 Replication and Giant Cell Formation in the Presence of Human Serum

	anti-HIV-1 (IC_{50}, μg/mL)			
	LAV-1	$KK\text{-}1_{AIDS}$		suppression of G-cell formation[c] (IC_{50}, μg/mL)
compound [abbreviated formula]	MT-4[a]	MT-4[a]	PBMC[b]	MOLT-4/MOLT-$4_{LAV\text{-}1}$
mCDS71 [β-CD(2-*O*-$CH_2Ph)_7(SO_3Na)_{14}$]	0.87	5.80	11.50 (3.6)	0.81 (0.46)
mCDS11 [β-CD(6-*S*-$CH_2Ph)_3(SO_3K)_{16}$]	4.50	11.00	27.50 (6.5)	12.70 (2.00)
DS8000	6.40	350	>500 (500)	21.00 (6.60)

[a] Concentration that causes 50% inhibition of the CPE, estimated in the same way as described in Table I, in the presence of 50% human serum (HS) in the culture medium. [b] Inhibition of HIV-1 replication in peripheral blood mononuclear cells (PBMC) is expressed as the inhibitory concentration that reduces the RT activity of the culture supernatant by 50% (IC_{50}): PBMC, obtained by the Ficoll-Hypaque technique from a healthy donor, were stimulated with 0.1% phytohemagglutinin (PHA, Difco) for 3 days. The PBMC were infected with 0.001 $TCID_{50}$ (determined by PBMC on day 10 after infection) of HIV-1 (strain $KK\text{-}1_{AIDS}$ from a patient) per cell for 3 h. After removal of nonadsorbed virus by washing, HIV-1-infected or mock-infected PBMC were cultured in the presence of 200 units/mL recombinant interleuken-2 (Shionogi Laboratories) and the test compounds, in various concentrations (6 doses, 2.1–500 μg/mL), for 6 days. Half of the cells and culture medium were then removed. The remaining half was further incubated with the same concentrations of the compounds and the PHA-stimulated fresh PBMC in fresh medium for 4 days. HIV-1 reverse transcriptase (RT) activity of each culture supernatant was evaluated by the method of Lee et al.[13] with poly(rA)oligo(dT) used as the template primer. Mean RT activity (cpm) of the positive control (not treated with compound) was 1.2×10^6 cpm; the negative control (not exposed to HIV-1 and not treated with compound) was 1.1×10^4 cpm. The values in parentheses were obtained in the presence of 20% FCS instead of HS. [c] Suppressive effect on giant-cell formation (IC_{50}): Via the modified method described by Nakashima et al.,[13] MOLT-4 and MOLT-4/$HIV_{LAV\text{-}1}$ cells were mixed at a ratio of 1:1 (total cell number of 5×10^5 cells/mL). The mixture was cultured for 24 h with medium containing the test compounds and 50% HS. The number of viable cells was counted by the trypan blue exclusion method, and the fusion index (FI) was calculated as follows: FI = 1 − [no. of cells in test well (MOLT-4 + MOLT4/HIV-1)]/[no. of cells in control (MOLT-4 cells)]. The values in parentheses were obtained in the presence of 10% FCS instead of HS.

the activity of mCDS11 was reduced, being shown at 4.50 μg/mL. The excellence of mCDS71 was also shown in the natural isolated HIV-1 strain: the cytopathogenicity of the clinically prepared $HIV_{KK\text{-}1/AIDS}$ was well inhibited by mCDS71 in MT4 cells at 5.80 μg/mL and the replication of $HIV_{KK\text{-}1/AIDS}$ in PBMC was blocked at 11.50 μg/mL. These activities were approximately 2-fold the corresponding activities of mCDS11. (As a reference, the inhibition concentrations of the replication of $HIV_{KK\text{-}1/AIDS}$ in PBMC in 20% FCS are shown in parentheses.) The most remarkable superiority of mCDS71 to mCDS11 was shown in the inhibition of giant cell formation. mCDS71 inhibited syncytium formation at 0.81 μg/mL, and its activity was more than 10 times that of mCDS11, due to the smaller reduction of this activity by HS. (Compare the effective concentrations in 10% FCS given in parentheses.)

To elucidate these different undesirable influences of HS on the anti-HIV activity of polyanionic compounds, binding to serum proteins was estimated by determining the anti-HIV-1 activity of an ultrafiltered HS solution of the agents after removal of the formed agent–protein complexes. Surprisingly, the most striking binding, 98.4%, was observed in mCDS71, with the binding magnitudes of mCDS11 and DS being 93.8% and 87.4%, respectively, indicating a reciprocal relation to the influence of HS. These findings suggested that mCDS71 binds nonselectively to serum proteins in plasma and then rebinds specifically to the surface of HIV virions and/or HIV infected cells, if present, thereby exerting potent inhibition of viral replication and especially of syncytium formation.

In addition, the synergistic antiviral effect of mCDS71 with AZT was detected in terms of an inhibitory effect on the replication of clinically isolated $HIV\text{-}1_{KK\text{-}1/AIDS}$ in PBMC cultures in medium containing 50% HS. Elion's[10] fractional inhibitory concentration (FIC) values were between 0.5 and 1.0.

From the viewpoint of therapy, conservation of potent anti-HIV activity *in vivo* and the bioavailability of the agent after oral administration (oral bioavailability) are the most important aspects to be focused on in the development of polysulfated compounds such as DS and HOE/BAY-946. The oral absorbability of mCDS71 was estimated to be about 3-fold that of mCDS11. The plasma level of mCDS71, determined by the inhibition of 320-fold diluted plasma on $HIV_{LAV\text{-}1}$-induced CPE in MT-4 cells, was 320 μg/mL at 2–3 h after oral administration of 1 g/kg in male rats.

On the basis of the above result, the hydrophobicity of mCDS71 increased by the seven benzyloxy groups, the rigid cyclic skeleton, and the relatively small molecular size are considered to facilitate penetration of mCSD71 through the intestinal wall and prevent the hydrolytic destruction of the molecule in the body.

In an oral toxicity test of mCDS71 in male mice, no toxicity was exhibited at 2.0 g/kg/day for 5 days.

The elucidation and characterization of the mechanisms of action and toxicity of mCDS71 are still in progress. Of particular importance will be to determine if the biological diversity of HIV-1 affects the inhibitory potency of mCDS71. We are presently in the process of testing this compound against a panel of reference strains of HIV isolates and will report this data in a full paper.

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Supplementary Material Available: Experimental details for the synthesis of tris(2-*O*-benzyl)heptakis(6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin (**IIb**), tris(2-*O*-benzyl)-β-cyclodextrin (**IIIb**), tetradecasodium heptakis(2-*O*-benzyl)-β-cyclodextrin tetradecasulfate (mCDS71), and nonasodium tris(2-*O*-benzyl)-β-cyclodextrin nonasulfate (mCDS78). (4 pages). Ordering information in given on any current masthead page.

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