Potent Inhibitory Effect of a Series of Modified Cyclodextrin Sulfates (mCDS) on the Replication of HIV-1 in Vitro

According to the urgent demand for an effective and safe agent for acquired immunodeficiency syndrome (AIDS), there are numerous compounds in development with individual action mechanisms against the causative human immunodeficiency virus (HIV) of AIDS.[1] Among them, polysulfated compounds, such as dextran sulfate (DS), pentosan polysulfate (HOE/BAY-946), etc.,[2] are some of the most potent and selective inhibitors of HIV type 1 and 2 in vitro through blocking the viral adsorption to cell membrane and cell fusion (syncytium formation). However, the effectiveness in vivo has not been clarified as yet[3] because of their poor absorbability, owing to the large molecular size and unfavorable anticoagulant activity of blood.

To overcome these problems and to get a clue to rational drug design based on a structure-activity relationships, it is necessary to simplify the molecular structure to a moderate size and a more rigid skeleton, because most of the polyanionic agents with high activity that have been reported so far have a long-chained main frame, a large molecular weight (more than 5000 Da), and a high density of sulfate groups.[2f,4] Therefore to begin with, we studied and reported previously[5] that cyclodextrin sulfates (CDS)[6] which are constructed with a doughnut-like cyclodextrin frame (CD, Figure 1a), and with a pair of anionic circular moieties as shown in Figure 1b, have effective anti-HIV-1 activities. The activity of CDS increased from α- to γ-derivatives along with an increase of the constructed glucose unit. In addition to this, partially chlorinated cyclodextrin phosphate (CDP) while it was somewhat cytotoxic, showed anti-HIV-1 activity and lower anticoagulant activity than DS and CDS.

Here, we wish to report the further investigation of the separation of the anti-HIV and anticoagulant activities and the improvement of the absorbability in the gut by the introduction of hydrophobic substituents (XR) to one of the anionic moiety of CDS as illustrated in Figure 1c. More than 50 modified β-cyclodextrin sulfates (mCDS) having various sulfonate, sulfide, and amino groups on the 6-position of the cyclodextrin skeleton were synthesized according to the method as shown in Scheme I. The 6-position hydroxyl groups of β-cyclodextrin were selectively mesitylenesulfonated to 6-*O*-mesitylenesulfonylated β-cyclodextrins (I).[7] The sulfonyl groups of I were substituted for sulfide or amino groups to 6-deoxy-6-thio or 6-amino-6-deoxy β-cyclodextrins (II or III) by a reaction with thiols or amines, respectively. The hydroxy groups of these modified β-cyclodextrins (I-III) were sulfated by sulfur trioxide-pyridine complex in pyridine solution to the corresponding mCDS.[8] In the case of I, a partial replacement of the sulfonyl group to a quaternary pyridinium group occurred. The mCDSs thus formed were screened by the anti-HIV-1 activity, anticoagulant activity, and also cytotoxicity. All of the representative mCDSs (**3**, **11**, **38**) bearing different hydrophobic substituents showed superior results to the positive controls [CDS, CDP, and DS, as listed in the Table I (columns HIV-1; LAV-1/MT-4, APTT, and cytotox)]. Thus, the advantage of the introduction of hydrophobic moieties to the mCDS was clearly revealed in the potentiated anti-HIV-1 activity and the reduced unfavorable anticoagulant activity.

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(7) Heptakis(6-*O*-mesitylenesulfonyl)-β-cyclodextrin (**I**; $m = 7$, $n = 0$) was prepared according to a previous report: Tsujihara, K.; Kurita, H.; Kawazu, M. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 1567-1571. Partially 6-*O*-mesitylenesulfonylated β-cyclodextrins (I; $m = 1$-4, $n = 3$-6) were similarly synthesized and purified by CHP-20 (a high-polar resin supplied by Mitsubishi Kasei Co., Ltd.) column chromatography with aqueous methanol used as an eluent and obtained as mixtures of regioisomers.

(8) The number of sulfate groups was calculated from the elemental analysis.

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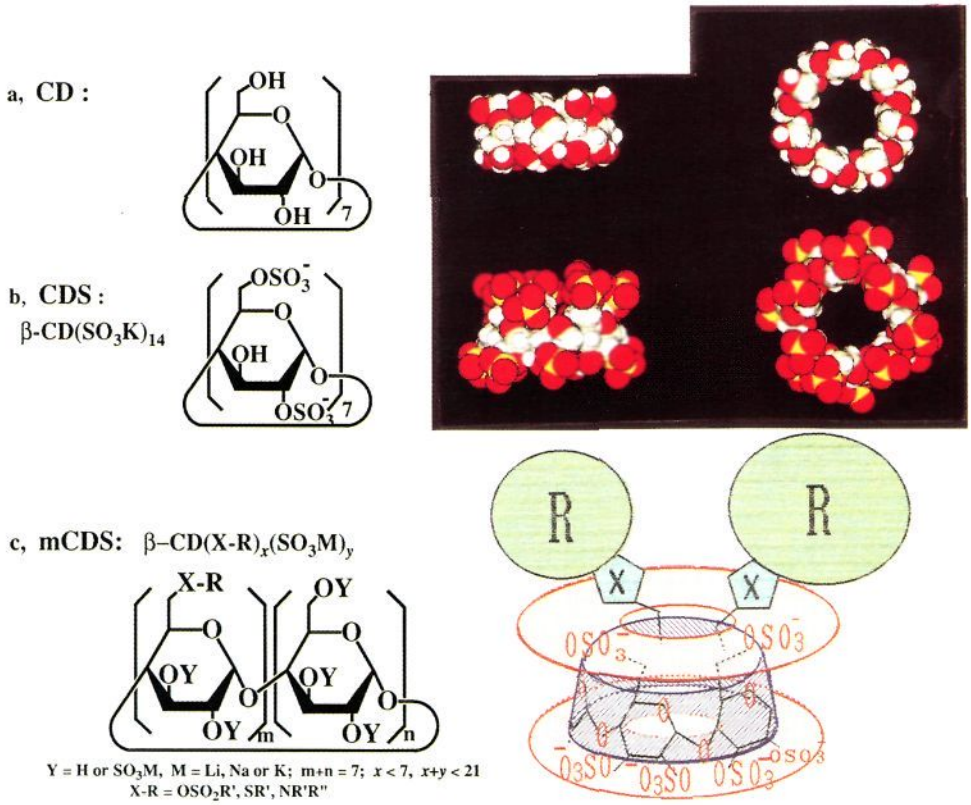

Figure 1. Side and top views of β-cyclodextrin (a) and β-cyclodextrin sulfate (b) molecules illustrated as a space field model. The color of the balls indicates the kind of atoms: white, hydrogen; gray, carbon; red, oxygen; yellow, sulfur. Part c shows illustrative structures of the β-cyclodextrin sulfate molecule which was modified with hydrophobic substituents (X–R).

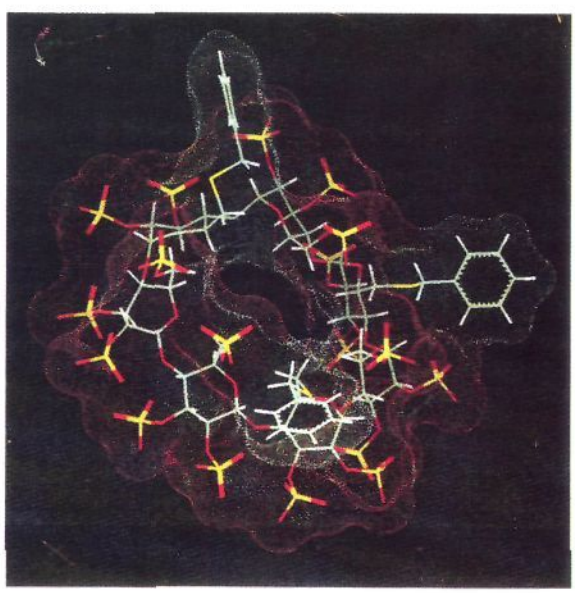

Figure 2. Computer graphics image of the one of the isomers of the mCDS 11 molecule constructed by the X-ray crystallographic data of β-cyclodextrin and the SYBIL fragment library. The red dotted regions represent the surface of the anionic part of the molecule and the others are the hydrophobic parts.

The most potent compound, mCDS 11, having three benzylthio substituents exhibited the anti-HIV-1 activity on the HIV-1_{LAV-1}-induced cytopathic effect (CPE) in MT-4 cells at 0.98 μg/mL (Table I, column LAV-1/MT-4), inhibition of syncytia formation (G-cell) in a coculture system of MOLT-4 with persistently HIV-1_{LAV-1}-infected MOLT-4 cells at 1.4 μg/mL or with persistently HIV-2_{GH-1}-infected MOLT-4 cells at 1.7 μg/mL, and anticoagulant activity expressed by duplication of the activated partial antithrombin time (APTT) at 7.0 μg/mL. It is notable that the inhibitory activity of mCDS 11 is very strong in the syncytium formation in both the HIV-1 and HIV-2 infected cells. The cytotoxicity of mCDS 11 was above 1000 μg/mL, while the inhibition of reverse transcriptase activity (RT) was rather weak, 630 μg/mL.

It is surmised that the conventional assay system for anti-HIV-1 activity using a combination of the strain LAV-1 which has been cultured for a long time in laboratories and the targeted MT-4 cells carrying HTLV-I does not reflect the natural infection in human body. Therefore, we designed an assay system using freshly isolated HIV-1 strains (KK-1_{AIDS} isolated from an AIDS patient and KK-5_{AC} from an asymptomatic virus carrier) and peripheral blood mononuclear cells (PBMC) from a healthy donor. After preincubation of the PHA-stimulated PBMC with the either strain of HIV-1 for 3 h, mCDS effectively inhibited the replications of the HIV-1, while DS was ineffective (Table I, column KK-1_{AIDS}/PBMC and KK-5_{AC}/PBMC). This remarkable difference between mCDS and DS was also observed in an assay system which used a combination of HIV-1 (strain KK-1_{AIDS}) and MT-4 cells. Thus, the main factor which caused the differences was not due to the cell lines but due to the virus strains. Contrary to this, when the test compounds were presented at the initial infection period, both mCDS 11 and DS inhibited the replication completely (Table I, column KK-1_{AIDS}/PBMC, data in parentheses).

From the foregoing facts, the main action mechanisms of mCDS suggest that mCDS does not only inhibit the

Scheme I. Synthesis of Modified Cyclodextrin Sulfates (mCDS)

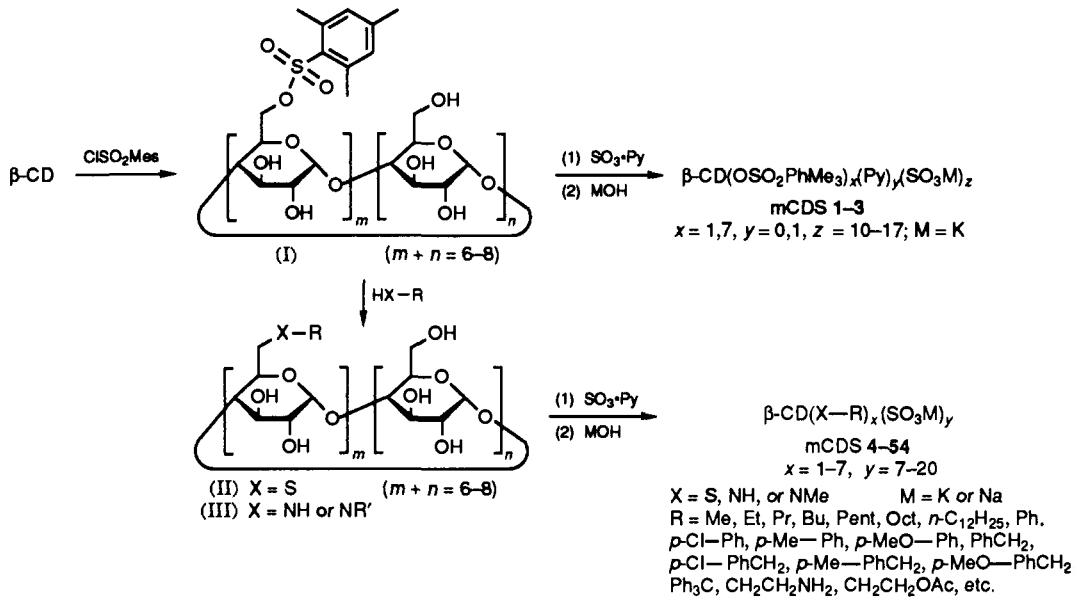

Table I. Anticoagulant Activity and Inhibitory Effect of mCDS on HIV-1 Replication, Reverse Transcriptase, Giant Cell Formation, and Cell Viability

		HIV-1					G-cell[e]			
compd	abbreviated formula[a]	LAV_{-1}[b] MT-4	$KK\text{-}1_{AIDS}$[b] MT-4	$KK\text{-}1_{AIDS}$[c] PBMC	$KK\text{-}5_{AC}$[c] PBMC	RT[d]	$HIV\text{-}1_{LAV\text{-}1}$ MOLT-4	$HIV\text{-}2_{GH\text{-}1}$ MOLT-4	APTT[f]	cytotox[g]
mCDS 3	β-CD$(OSO_2PhMe_3)(SO_3K)_{16}$-$(SO_3^-)(Py^+)$	1.95	62.5	382	180	>1000	42.0	64.4	4.15	>1000
mCDS 11	β-CD$(SCH_2Ph)_3(SO_3K)_{16}$	0.98	1.95	6.5 (0.60)	19.6	630	1.4	1.7	7.00	>1000
mCDS 38	β-CD$(NHPh\text{-}4\text{-}OMe)_7(SO_3Na)_{12}$	0.98	31.2	86	72.5	706	5.5	40.2	4.30	>1000
CDS	β-CD$(SO_3K)_{14}$	31.20	125	330		>1000	60.0	519	2.83	>1000
CDP	β-CD$(PO_3HK)_8Cl_5$	31.20	40			200			47.20	>1000
DS	(8000, SIGMA)	3.90	500	>500 (1.52)	500	>1000	9.9	172	3.30	>1000

[a] mCDS 3: Potassium 6-deoxy-6′-*O*-(mesitylenesulfonyl)-6-pyridinio-β-cyclodextrin heptadecasulfate. mCDS 11: Potassium tris(6-benzylthio-6-deoxy)-β-cyclodextrin hexadecasulfate. mCDS 38: Sodium heptakis[6-deoxy-6-(4-methoxyanilino)-β-cyclodextrin. [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) of HIV-1 (strain LAV-1 or $KK\text{-}1_{AIDS}$ from 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] Inhibition of HIV-1 replication in peripheral blood mononuclear cells (PBMC) is expressed as the inhibitory concentration, which reduces by 50% the RT activity of the culture supernatant (IC_{50}): PBMC obtained by the Ficoll–Hypaque technique from healthy donor were stimulated with 0.1% phytohemagglutinin (PHA, Difco) for 3 days. The PBMC and freshly isolated HIV-1 (strain $KK\text{-}1_{AIDS}$ or $KK\text{-}5_{AC}$ from an asymptomatic virus carrier) were incubated for 3 h with or without the test compounds. After removal of nonadsorbed virus by washing, HIV-1 infected or mock-infected PBMC was cultured in the presence of 200 unit/mL recombinant interleukin-2 (Shionogi Laboratories) and the test compounds of various concentrations (6 doses, 0.49–500 μg/mL) for 6 days. Half of the cells and culture medium were then removed and 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.[14] 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, and 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 when the test compounds were presented at the initial infection period. [d] The IC_{50} for inhibitory effect on reverse transcriptase of HIV-1: The direct effect of the compounds on cell-free RT activity of HIV-1(LAV-1) was determined with poly(rA)oligo(dT) as the template primer, as described by Lee et al.[14] [e] Suppressive effect on giant-cell formation: By following a modified method described by Nakashima et al.,[15] MOLT-4 and MOLT-4/$HIV\text{-}1_{LAV\text{-}1}$ or MOLT-4/$HIV\text{-}2_{GH\text{-}1}$ cells were mixed at a ratio of 1:1 (total cell number of 5×10^5 cells/mL) and the mixture was cultured for 24 h with the medium containing the test compounds. 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 + MOLT-4/HIV-1 or 2)]/[no. of cells in control (MOLT-4 cells)]. [f] Anticoagulation effect: Zuchker's activated partial thromboplastin time (APTT) method[16] was used. The value is indicated by the concentration (μg/mL) required to obtain 2-fold APTT. [g] 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 2 or 3 experiments.

initial adsorption of HIV to the target cells and cell to cell infections, like DS, but it also has an additional effect. This additional effect might play a significant role in inhibiting the replication of the freshly isolated HIV-1 strains. The structure of mCDS 11 is unique in having a hydrophobic cavity surrounded by a cloudy ring of swarmed anionic sulfate groups and lipophilic substituents which are like tentacles, as illustrated in the image of the molecule in Figure 2. It is conceivable that the cavity and the tentacles include either the nonpolar binding site on gp-120 or CD-4 such as Phe^{324} on the epitope β of gp-120,[9] Trp^{432} on gp-120 for CD4 binding,[10] and Phe^{43} on the domain D1 of CD4 molecule.[11]

From the viewpoint of therapy, conservation of the potent anti-HIV activity in vivo and oral absorbability were the most important problems that preceded developing

polysulfated compounds such as the DS and HOE/BAY946.[3,4a,12] Effectiveness of oral administration of mCDS 11 was suggested from the following ex vivo test. The HIV-1_{LAV-1}-induced CPE in MT-4 cells was completely inhibited by 50- and 160-fold diluted plasma which were prepared 2 h after giving 1 and 2 g/kg per os of mCDS 11 to male rats, respectively. On the basis of this result, the hydrophobic benzylthio groups, rigid cyclic skeleton, and the relatively small molecular size[13] of mCDS 11 are being considered to facilitate the penetration to the intestinal membrane and prevent the hydrolytic destruction of the molecule in body.

The acute toxicity of mCDS 11 was not observed at 3 g/kg per os in mice.

The elucidation and characterization of the action mechanisms of mCDS and selection of the most suitable candidate for the treatment of AIDS patients and asymptomatic virus carriers are still in progress.

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Additions and Corrections

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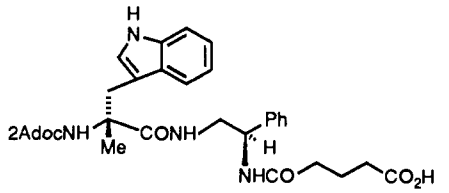
David C. Horwell,* John Hughes, John C. Hunter, Martyn C. Pritchard, Reginald S. Richardson, Edward Roberts, and Geoffrey N. Woodruff: Rationally Designed "Dipeptoid" Analogues of CCK. α-Methyltryptophan Derivatives as Highly Selective and Orally Active Gastrin and CCK-B Antagonists with Potent Anxiolytic Properties.

Page 404. In Table I, the data for **10c** was inadvertently omitted. The line should read: $C_{23}H_{28}N_2O_4$ (molecular formula), 202–210 °C (mp), and C,H,N (anal.).

Page 408. The last sentence in the right-hand column should read: CCK is known to coexist with GABA in some cortical interneurons,[8] and agents that modify GABA may have utility as anxiolytic agents.[9]

Page 408. In Scheme IV the correct structure for **28** is as shown below:



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Page 409. In ref 13, the correct name of the publication is *Proc. Natl. Acad. Sci.*