Review J. Trad. Med. 22 (Suppl.1), 116－128, 2005

Developing of anti-HIV agents from natural resources

Meselhy Ragab MESELHY,[a] Sahar EL-MEKKAWY,[b] Chaomei MA,[c] Norio NAKAMURA,[d] Supinya TEWTRAKUL,[e] and Masao HATTORI*[d]

Faculty of Pharmacy, [a]Cairo University, Cairo, Kasr El-Aini Street, Cairo 11562, Egypt. Division of Pharmaceutical Industry, [b]National Research Center, El-Tahrir Street, Dokki, Cairo, Egypt. School of Pharmacy, [c]Memorial University of Newfoundland, St. John's, NL, Canada AIB 3V6. Institute of Natural Medicine, [d]Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan. Faculty of Pharmaceutical Sciences, [e]Prince of Songkla University, Thailand. *(Accepted April 13, 2005.)*

Most drugs clinically used to suppress replication of HIV, the virus that causes AIDS, are nucleosides and their analogs, but their use is limited due to their severe toxicity, emerging drug resistance and cost. These challenges prompt the search for new anti-HIV-1 agents that are affordable for resource-poor countries and have fewer side effects. As an approach, traditional medicines from different places of the world were evaluated with a panel of *in vitro* bioassays [designed to monitor inhibition of HIV-1-induced cytopathic effect (CPE), and HIV essential enzymes; reverse transcriptase (RT), protease (PR), and integrase (IN)], and active leads were then isolated and identified. In this review, structurally diverse compounds that have potent anti-HIV activity were classified to CPE inhibitors (phorbol diterpenes), RT inhibitors (caffeic acid derivatives and polyphenols), PR-inhibitors (derivatives of oleanane, ursane and lupane type triterpenes). Other compounds included are IN inhibitors. Structural modification of the active compounds and conjugation with synthesized anti-HIV agents were carried out, which, hopefully, could help for developing novel and effective anti-HIV agents.

Key words anti-HIV agent, reverse transcriptase, protease, integrase, phorbol ester.

Introduction

It is worth mentioning that traditional medicines that have been used for thousands of years are employed worldwide in a variety of health care preparations. Hundreds of plants are cultivated for substances useful in medicine and pharmacy, and several lead and biologically active compounds have been isolated, and numerous drugs used in modern medicine are either chemical modifications of these compounds or natural products themselves.[1] Based on this information, it is likely that there has been a resurgence in interest in natural products from plants and other types of organisms for the identification of novel active compounds as new chemotypes for drug development. Our group has performed phytochemical work aimed at the discovery of potential anti-human immunodeficiency virus (anti-HIV) agents. HIV is the causative agent of the acquired immunodeficiency syndrome (AIDS). Since it was discovered, the virus has invaded nearly every branch of medical science along with the bloodstream of more than 36 million and killed over 22 million individuals, in which most of them are from Africa. The Joint United Nations Program on HIV/AIDS (UNAIDS) reported that HIV has swept through the continent leaving behind a cumulative of 17 million dead, and 12 million orphans. In addition, an estimation of more than 25 million people are now HIV-infected, 3.8 million new cases appeared in 2000, and an estimated 2.4 million deaths occurred during the year. Because of the pandemic nature of AIDS, therapeutic measures should also be aggressively set into motion for the prevention of this devastating disease. Scientists have made much progress in clarifying many of the steps by which the virus, HIV, attacks T cells. It became clear that a spike-like glycoprotein gp 120 on the viral envelope interacts with CD4 and CXCR4 receptors on T lymphocytes, and this interaction leads to membrane fusion. After uncoating, and by the help of its specific enzymes, the virus starts its replication inside T lymphocyte. Replication of HIV-1 in T lymphocytes leads to the dysfunction and depletion of CD4-positive T cells causing a profound immunodeficiency, which hampers the body's ability to fight not only HIV infection, but other infections as well. Therefore, direct inhibition of the viral replication as well as different steps in its life cycle, e.g. adsorption, fusion, uncoating, reverse transcription, integration, DNA replication, transcription, translation, maturation and budding (assembly/release) are considered promising approaches for developing anti-HIV drugs. Until now, scientists were able to develop two major classes of drugs, reverse transcriptase (RT) inhibitors and protease (PR) inhibitors. These drugs used alone or in combinations, have helped patients live much longer than ever before. However, they are apparently expensive and require a long term-therapy, which is almost accompanied with viral resistance. Problems with drug resistance and cost have focused efforts on the development of alternative approaches to find effective, cheap and less toxic anti-HIV agents that will be useful adjuncts or replacements for the currently used drugs.

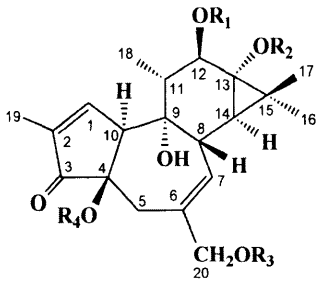
As an approach, traditional medicines from different places of the world were evaluated with a panel of *in vitro* bioassays [designed to monitor inhibition of HIV-1-induced cytopathic effect (CPE), and HIV essential enzymes; reverse

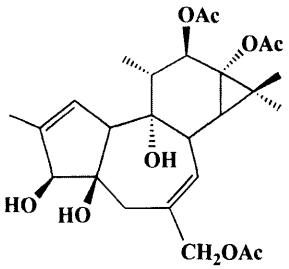
*To whom correspondence should be addressed. e-mail : saibo421@ms.toyama-mpu.ac.jp

transcriptase (RT), protease (PR) and integrase (IN)]. Examples highlighted in this review include lead compounds isolated and identified through bioassay-guided fractionation of crude extracts, and those derived from pure natural products. Compounds are classified according to their mode of action, for example: 1) CPE inhibitors (tigliane-type diterpenes and lanostane-type triterpenes), 2) RT inhibitors (caffeic acid derivatives and polyphenols), 3) PR-inhibitors (derivatives of lanostane, oleanane, ursane and lupane triterpenes), and 4) IN inhibitors (flavonoid and caffeic acid derivatives). Structural modification of the active compounds and conjugation with synthetic anti-HIV agents were carried out, which hopefully, could help for developing effective and less toxic anti-HIV agents.

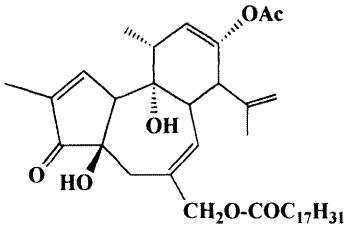
1. Inhibition of HIV-1-induced cytopathic effect (CPE)

In a primary screening, the effects of plant extracts were evaluated as the concentration that prevents HIV-induced cytopathic effect (CPE), observed with an optical microscope. Then plant extracts showing significant anti-HIV activity were submitted to a more accurate test using MT-4 cells, a human T4-positive cell line carrying HTLV-1. When MT4-cells are infected with HIV-1, the virus rapidly proliferates and the MT-4 cells are destroyed within 5 to 6 days.[2] From this test, the dose that reduces the viability of uninfected cells by 50% (CC_{50}) and that required to inhibit HIV-1 replication by 50% (IC_{50}) are obtained and the ratio of these values (CC_{50}/IC_{50}) is given as the selectivity index (SI) in order to assess whether the observed anti-HIV-1 activity is a specific or a general toxic effect. When extracts of several medicinal plants used in Egypt were screened some years ago, the MeOH extract of the seeds of *Croton tiglium* L. (Fam. Euphorbaceae) showed especially strong anti-HIV-1 activity (IC_{50} of 0.025 μg/ml) with a high selectivity index (SI value of 34.4).[3] Moreover, this extract suppressed giant cell formation in co-cultures of HIV-infected and -uninfected MOLT-4 cells, suggesting that this extract can inhibit virus adsorption or fusion, as giant cell formation depends on the interaction of HIV envelop protein with virus receptors on the cell surface.[4-6] On the basis of these findings, it was decided to pursue the isolation and identification of the active components in the MeOH extract of these seeds. The seeds of *C. tiglium* are known to contain tigliane-type phorbol esters. These compounds have been shown to be responsible





17



38

	R_1	R_2	R_3	R_4
1	H	Ac	$C_{18}H_{31}O$	H
2	H	Tig	$C_{18}H_{31}O$	H
3	Ac	Tig	H	H
4	$C_{10}H_{19}O$	2-Me butyryl	H	H
5	Tig	2-Me butyryl	H	H
6	Ac	$C_{10}H_{19}O$	H	H
7	2-Me butyryl	$C_{12}H_{23}O$	H	H
8	$C_{14}H_{27}O$	Ac	H	H
9	H	H	H	H
12	Ac	Ac	Ac	H
13	Bz	Bz	Bz	H
14	Ac	H	H	H
15	Ac	Ac	H	H
16	Ac	Ac	Ac	Me
18	Ac	Ac	Ac	Ac
37	Ac	Ac	$C_{18}H_{31}O$	H
39	$C_{10}H_{19}O$	2-Me butyryl	Ac	H
40	Ac	$C_{10}H_{19}O$	Ac	H
41	$C_{14}H_{27}O$	Ac	Ac	H
42	Ac	$C_{10}H_{19}O$	Ac	Me
43	$C_{14}H_{27}O$	Ac	Ac	Me
44	Ac	$C_{14}H_{27}O$	H	H
45	Ac	$C_6H_{11}O$	H	H
46	Ac	$C_6H_{11}O$	$C_6H_{11}O$	H
47	Ac	$C_9H_{17}O$	H	H
48	Ac	$C_9H_{17}O$	$C_9H_{17}O$	H
49	Ac	$C_{12}H_{23}O$	H	H
50	Ac	$C_{12}H_{23}O$	$C_{12}H_{23}O$	H
51	H	Ac	H	H
52	H	$C_{14}H_{27}O$	$C_{14}H_{27}O$	H

Chart 1

for eliciting a remarkable range of biochemical effects on a variety of biological systems.[7] The ability of these compounds to promote tumors presents one potential limitation to their utility. It has been reported that many phorbol esters have a tumor promoting effect, which is associated with activation of protein kinase C (PKC).[8] However, prostratin, 12-deoxyphorbol 13-acetate, from a Samoan *Homalanthus nutans* does not exhibit a tumor promoting effect, but showed potent anti-HIV activity.[9] This finding suggested that tumor promoting and anti-HIV-1 effects of phorbol esters could be dissociated from each other, and that particular structural features were necessary for the esters to provoke these effects.

In an attempt to isolate a selective anti-HIV substance that does not promote tumor, a bioactivity-guided approach to fractionation and isolation was pursued and activation of PKC, taken as a marker for tumor promotion, was also investigated. This approach led to the isolation of 8 compounds (**1-8**) (Chart 1)[10,11] from the methanol extract of the seeds of *C. tiglium*.

These compounds were phorbol diesters and their structures were determined by chemical degradation and spectroscopic methods. The acyl groups and the sites of acylation were determined by GC/MS after selective hydrolysis. These compounds were tested for their ability to inhibit the HIV-1-induced CPE on MT-4 cells and to activate PKC under the conditions where PKC from mouse brain was activated by calcium and phosphatedylserine. The increase in the activity of PKC by phorbol esters was investigated at a standard concentration of 10 ng/ml. PKC activation was assayed by measuring the incorporation of ^{32}P radioactivity from [γ-^{32}P]ATP into peptide, Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-OH. Activation of PKC was calculated relative to that shown by TPA.

From the results compiled in Table 1, the most potent anti-HIV-1 compounds, 12-*O*-tetradecanoylphorbol 13-acetate (**8**) and 12-*O*-acetylphorbol 13-decanoate (**6**), had complete inhibition of HIV-1-induced CPE at concentrations (IC_{100}) of 0.48 and 7.6 ng/ml, with minimum cytotoxic concentrations (CC_0) of 31.3 and 62.5 μg/ml, respectively. Compound **8** showed the strongest activation of PKC at 10 ng/ml (similar results were previously reported by Chowdhury *et al.*[12]). Of most interest was the fact that **6** showed specific anti-HIV activity without activating PKC (no activation was seen at concentrations of 10-100 ng/ml). This finding suggested that **6**, which demonstrated preference to HIV inhibition than to PKC activation, was a potential lead aimed at the development of therapeutically effective anti-HIV agents.

Based on the results obtained above, two approaches were followed: a) preparation of larger quantity of **6** for further advanced testing, and b) preparation of a series of phorbol derivatives to establish their anti-HIV-1 activity on structural basis. Preparation of a large quantity of **6** was followed through digestion of croton oil, which is commercially available, with $Ba(OH)_2$/MeOH to obtain the tetracyclic diterpenes, phorbol (**9**), 4α-phorbol (**10**, isophorbol) and 4-deoxy-4α-phorbol (**11**). These compounds were identified by comparison of their spectral data with reported values. Selective acetylation/transestrification gave **6** in a good yield. Forty-one derivatives based on phorbol and isophorbol structures were also synthesized and evaluated for their anti-HIV-1 activity (Charts 1 and 2).[13]

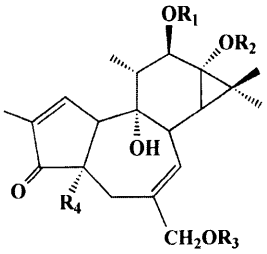
Phorbol derivatives **12** and **13** were synthesized from **9**, while **14-18** were from 12 (Chart 1), and the isophorbol derivatives **19-23** and **24-30** were from **10** and **20**, respectively, while **31** was obtained from **21** after irradiation with UV light at 254 nm (Chart 2). Compounds **1**, **4**, **6** and **8**, having appreciable inhibition of CPE were further modified. Acetylation of **1** gave **37**, while reaction of **1** with mesyl chloride in pyridine at room temp. afforded 38. In addition, acetylation of **4**, **6**, and **8** gave the respective 20-*O*-acetyl derivatives **39**, **40** and **41**, respectively. Methylation of **6** and **8** with methyl iodide and Ag_2O afforded 4-*O*-methyl derivatives **42** and **43**, respectively. Compound **44** (a positional isomer of **8**) was obtained by acylation of 12-*O*-acetylphorbol (**14**). Various 12-*O*-acetylphorbol 13-acylates

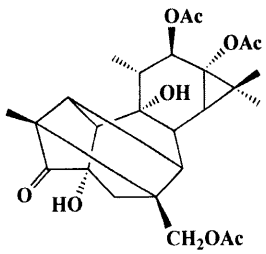
Table 1 Inhibition of HIV-1-induced CPE* and activation of PKC# by **1-8**

Compound	Anti-HIV-1(μg/ml)		% Activation of PKC at 10 ng/ml
	IC_{100}	CC_0	
13-*O*-Acetylphorbol 20-linoleate (**1**)	15.60	62.5	0
13-*O*-Tigloylphorbol 20-linoleate (**2**)	7.81	62.5	14
12-*O*-Acetylphorbol 13-tigliate (**3**)	125	500	16
12-*O*-Decanoylphorbol 13-(2-methylbutyrate) (**4**)	7.81	31.3	0
12-*O*-Tigloylphorbol 13-(2-methylbutyrate) (**5**)	31.30	62.5	10
12-*O*-Acetylphorbol 13-decanoate (**6**)**	0.0076	62.5	0
12-*O*-(2-Methylbutyroyl)phorbol 13-dodecanoate (**7**)	15.60	62.5	16
12-*O*-Tetradecanoylphorbol 13-acetate (**8**)	0.00048	31.3	100
DS 8000	3.90	>1000	

*Measured by the method of ref. 2 using HLTV-I-carrying cell line MT-4. IC_{100}: the minimum concn. for complete inhibition of HIV-1-induced CPE in MT-4 cells, determined by microscope observation; CC_0: the minimum concn. for appearance of MT-4 cell toxicity, determined by microscopic observation. DS, dextran sulfate.

#Assayed by measuring the incorporation of ^{32}P radioactivity from [γ-^{32}P]ATP into peptide, Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-OH, using a Biotrak PKC enzyme assay system code RPN 77 kit except that the TPA in the kit was replaced by **1-8** (10 ng/ml) in DMSO, final concentration of 0.02%. **Activation of PKC was not observed at 100 ng/ml. #Relative to that shown by TPA.





31

	R_1	R_2	R_3	R_4
10	H	H	H	OH
11	H	H	H	H
19	H	Ac	H	OH
20	H	Ac	Ac	OH
21	Ac	Ac	Ac	OH
22	Bu	Bu	Bu	OH
23	Ac	Ac	Ac	OAc
24	$C_8H_{15}O$	Ac	Ac	OH
25	$C_{10}H_{19}O$	Ac	Ac	OH
26	10-Undecenoyl	Ac	Ac	OH
27	$C_{12}H_{23}O$	Ac	Ac	OH
28	$C_{14}H_{27}O$	Ac	Ac	OH
29	$C_{17}H_{33}O$	Ac	Ac	OH
30	1-Adamantanoyl	Ac	Ac	OH
32	H	Ac	H	H
33	H	Ac	Ac	H
34	Ac	Ac	Ac	H
35	$C_{10}H_{19}O$	Ac	Ac	H
36	$C_{14}H_{27}O$	Ac	Ac	H

Chart 2

45-50 were prepared from **1** *via* **14**. Other derivatives of phorbol, **51** and **52**, were also prepared.

Phorbol alcohols (**9-11**) were inactive for inhibition of CPE. While addition of acetyl group(s) to the parent alcohols produced compounds with variable activities. Several compounds of isophorbol and 4-deoxy-4α-isophorbol derivatives (**19**, **20**, **24-36**) were inactive and others have reduced activity. Of the isophorbol derivatives, a 12-*O*-octanoyl 13,20-diacetate derivative (**24**) as well as mono-, and di-acetyl derivatives of **24** (**32** and **33**) were moderately active (IC_{100} values of 31.25 and 62.5 μg/ml, respectively, and the activity of **33** was remarkably enhanced by further acetylation (as in **34**, IC_{100} value of 7.81 μg/ml). Decanoyl and tetradecanoyl derivatives of **11** (**35** and **36**) were moderately active (IC_{100} values of 31.25 and 62.5 μg/ml, respectively).

As for the phorbol derivatives, 12-*O*-acetyl derivative (**14**) was inactive, while the 13-*O*-acetyl counter part (**51**) was a weak inhibitor of HIV-1. Although phorbol 12,13-diacetate (**15**) and pentaacetate (**18**) were inactive, the triacetate derivative (**12**) was moderately active (IC_{100} value of 62.5 μg/ml). Methylation of **12** enhanced the anti-HIV activity (as in **16**, IC_{100} value of 31.3 μg/ml), while reduction of a carbonyl group at C-3 abruptly reduced the inhibition of CPE as observed in 3β-hydroxyphorbol 12,13,20-triacetate (**17**) (IC_{100}=500 μg/ml) *vs* phorbol 12,13,20-triacetate (**12**) (IC_{100}=62.5 μg/ml). At a concentration of 10 ng/ml, selective activation of PKC was demonstrated by phorbol 12,13,20-tribenzoate (**13**, 100% activation). Acetylation of a hydroxy group at C-20 in phorbol derivatives significantly reduced inhibition of the CPE as observed in **12**, 20-*O*-diacetylphorbol 13-decanoate (**40**) (IC_{100}=15.6 μg/ml) *vs* 12-*O*-acetylphorbol 13-decanoate (**6**) (IC_{100}=0.0076 μg/ml), and 12-*O*-tetradecanoylphorbol 13,20-diacetate (**41**) (IC_{100}=15.6 μg/ml) *vs* 12-*O*-tetradecanoylphorbol 13-acetate (**8**) (IC_{100}=0.00048 μg/ml) except for 12-*O*-decanoylphorbol 13-*O*-(2-methylbutyrate) (**4**) and phorbol 12,13-diacetate (**15**).

Two-fold increase in the anti-HIV activity of **1** was demonstrated by **38** (IC_{100} value of 7.81 μg/ml). The similar enhancement in anti-HIV-1 activity was also observed after introducing an acetyl group at C-12 as in **37**. Selective enhancement of inhibition of CPE of **4** was observed by introducing an acetyl group at C-20 (as in **39**, IC_{100} value of 3.9 g/ml), while acetylation of **6** (as in **40**) and 4-*O*-methylation (as in **42**) significantly reduced the anti-HIV-1 activity.

Although TPA (**8**) was found to be equipotent to the inhibition of HIV-1 induced CPE and the activation of PKC, both activities were dramatically decreased by introducing an acetyl group at C-20 (as in **41**) and by methylation of a free hydroxyl group at C-4 (as in **43**). On the other hand, its positional isomer (**44**) was almost inactive. Removal of the long chain acyl group from **8** resulted in a substantial loss of both activities, as in **51** (IC_{100} value of 125 μg/ml). This finding together with the fact that **6** (with C_2 and C_{10} at C-12 and C-13, respectively) and **4** (with C_{10} and C_5 at C-12 and C-13, respectively), were potent inhibitors of the HIV-1-induced CPE, and no activation of PKC was observed, suggested that the difference in chain lengths of acyl groups and its relative positions significantly influenced both activities. Therefore, similar phorbol derivatives having acyl residues with different chain length (C6:0, C9:0, C12:0 and C14:0) were synthesized from **14**, and their activities were investigated. Compounds **47** and **48**, were weak inhibitors, while other derivatives **45**, **46**, **49** and **50** did not show a significant anti-HIV activity. It was obvious that a chain length of C_{14} at C-12 (with C_2 at C-13) is an essential requirement for a maximal anti-CPE activity as well as for PKC activation (as in **8**). However, acyl groups with a chain length of C_{10} at C-12 (with C_5 at C-13, as in **4**), at C-13 (with C_2 at C-12, as in **6**) were found essential for selective anti-HIV activity of these compounds. It could be concluded that activities were influenced by configuration of the diterpene ester in that all active phorbol derivatives were of the A/B *trans* configuration. The A/B *cis* analogs (isophorbol-type) had no remarkable inhibition on the HIV-1 induced CPE. Chain lengths of the acyl groups and their positions significantly influenced both activities. Diesters containing two short acyl groups were weak inhibitors of HIV-1. Acyl groups with a chain length of C_{10} at C-12, or at C-13 were found to be essential for selective anti-HIV activity of these compounds.

Having of these compounds identified **6** as a selective anti-HIV agent that showed very low cytotoxicity, the present study provided more evidence that phorbol esters should serve as useful lead structures in the development of new classes of anti-HIV agents.

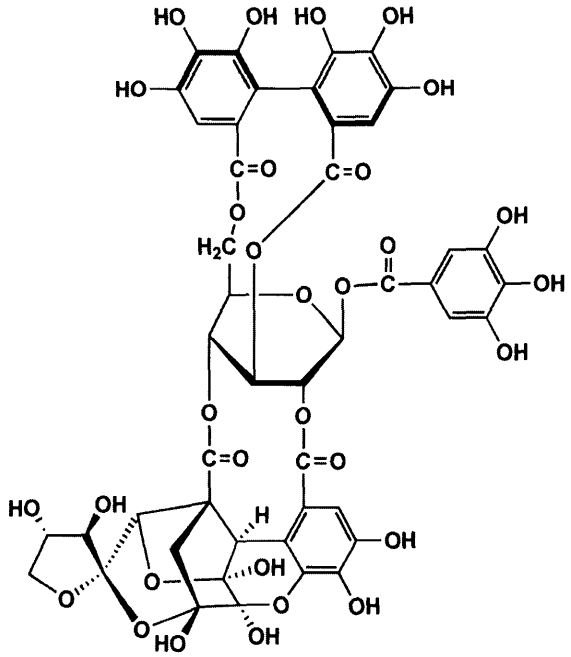
2. Inhibition of HIV-1 reverse transcriptase (RT)

In the early stages of the HIV-1 life cycle, RT is required for conversion of single-stranded genomic RNA to double-stranded proviral DNA.[14] The enzyme possesses not only RT (RNA-dependent DNA polymerase, RDDP) but also DNA-dependent DNA polymerase (DDDP) and ribonuclease H (RNase H) activities. The single-stranded RNA genome is reverse-transcribed by RDDP activity into the minus DNA strand to form RNA-DNA hybrid. Then, the RNase H domain catalyses hydrolysis of the RNA component of this hybrid, leaving small RNA primers for a subsequent synthesis of complementary plus DNA strand by DDDP activity.[15,16] Potential lead inhibitions of each catalytic function of RT were isolated and their modes of action were determined.

a) Inhibition of RT (RNA-dependent DNA polymerase) activity:

In an *in vitro* assay system designed to monitor the incorporation of [^{3}H]-dTTP into a polymer fraction by HIV-1 RT in the presence of $(rA)_n$-$(dT)_{12-18}$ as a template primer, a MeOH extract of the fruit of *Phyllanthus emblica* L. (Fam. Euphorbaceae),[17] and a water extract of the leaves of *Cordia spinescens* L. (Boraginaceae)[18] showed significant inhibitory effects with IC_{50} values of 2-49 μg/ml. In Unani system of medicine, the fresh fruit of *P. emblica* (sananir) are used for the treatment of jaundice, hepatitis and blood heat, while dried fruits are useful in diarrhea and dysentery, and are also used as stomachic.[19] While infusions of the roots and leaves of *C. spinescens* are used by Indians of northwestern Venezuela to relieve fever and headache,[20] also the powdered stem bark is used externally for wound healing.[21] Through a bioactivity-guided fractionation of the MeOH extract of *P. embilica,* the inhibitory effects were found in both the ethanol-soluble and -insoluble fractions of the methanol extract. The ethanol-soluble fraction was selected for further studies (the ethanol-insoluble fraction was found to be a mixture of inorganic salts and sugars and was not further investigated). Repeated chromatography of the active fraction led to the isolation of 6 compounds. Their structures were determined by spectroscopic and chemical means.[17] Of these compounds, putranjivain A (**53**, Chart 3) was found to demonstrate the most potent inhibitory activity against HIV-1 RT with an IC_{50} value of 3.9 μM.

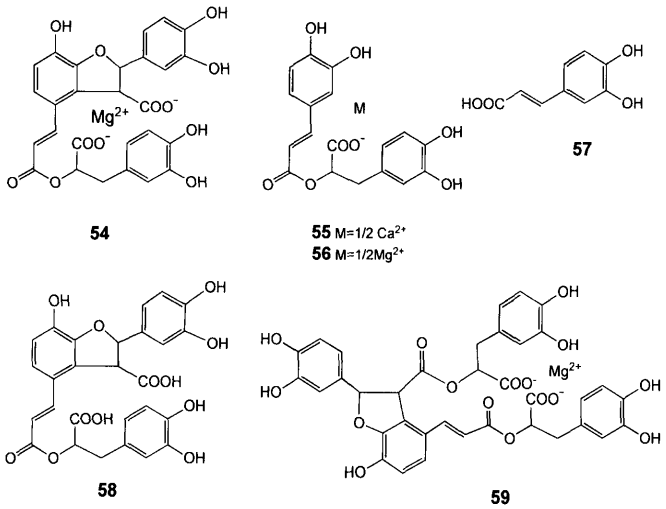
The Lineweaver-Burk plots for **53** showed that the inhibitory mode of action was non-competitive with respect to the substrate, but competitive with respect to the template-primer, suggesting that its action may be due to conformational changes of the enzyme, rather than of binding to the substrate-binding site. The respective inhibitory constants (*Ki*) were 0.89 μM for the substrate and 0.25 μM for the template-primer. When **53** was added to a reaction mixture 5 min after the initiation of DNA synthesis, the reaction was significantly suppressed depending on its concentration, suggesting that **53** inhibits not only the initiation of the polymerization, but also the chain elongation.



53

Chart 3

After fractionation of the aqueous extract of the leaves of *C. spinescens* collected in Panama, using an ion exchange resin column of IRA-400, the HIV-RT inhibitory activity was enriched in the neutral fraction (93.0%) followed by the basic fraction (69%), while the acidic fraction showed a very weak inhibitory activity (15%). Further chromatography of the neutral fraction over Sephadex LH-20 yielded magnesium lithospermate (**54**), calcium rosmarinate (**55**),



54 **55** M=1/2 Ca^{2+} **56** M=1/2Mg^{2+} **57** **58** **59**

Chart 4

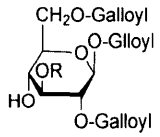
and magnesium rosmarinate (**56**) as potent inhibitory substances (Chart 4).[18]

Since these compounds are derivatives of caffeic acid (**57**), related compounds were synthesized and their structural activity relationship was investigated. Caffeic acid (**57**) was found inactive (IC_{50} value > 1000 μM), and lithospermic acid (**58**), a trimer of caffeic acid, was a weak inhibitor of HIV-RT (IC_{50} value of 34 μM), while its magnesium salt (**54**) was the most potent RT inhibitor (IC_{50} value of 0.8 μM). Next in potency were the salts of the caffeic acid dimers, **55** and **56** (IC_{50} values of 5.8 and 3.1 μM, respectively), while the magnesium salt of the tetramer, lithospermate B (**59**), was a very weak inhibitor (IC_{50} value of 68 μM).

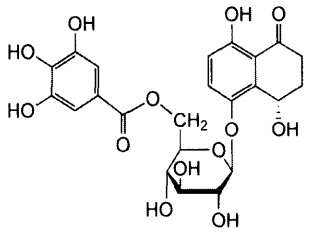
The inhibitory mode of action was kinetically analyzed and the mode of RT inhibition by magnesium lithospermate (**54**), calcium and magnesium rosmarenate (**55** and **56**) was found non-competitive with respect to dTTP. The *Ki* values were 0.8, 5 and 3 μM, respectively. Moreover, on the study of their specificity for inhibition of viral RT, a test was conducted using DNA polymerase I, a different DNA polymerase. These compounds inhibited DNA polymerase I in a lesser extent than RT, showing their specificity to inhibit RNA-dependent-DNA polymerase or RT than for DNA-dependent DNA polymerase.

Potent inhibition of RT was also demonstrated by 1,2,6-trigalloylglucose (**60**) and 1,2,3,6-tetragalloylglucose (**61**) (IC_{50} values of 0.067 and 0.040 μM, respectively) isolated from the stem-bark of *Juglans mandshurica* (Chart 5).[22] While 4α,5,8-trihydroxy-α-tetralone 5-*O*-β-D-[6'-*O*-(3",4",5"-trihydroxybenzoyl)]glucose (**62**) showed inhibitory activity with an IC_{50} value of 5.8 μM.

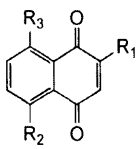
J. mandshurica has been used in Korea as a folk medicine to treat cancer. Several naphthoquinones, naphthalenyl glucosides and other phenolic compounds[23-25] have been isolated from this plant and have been shown to display cytotoxicity to human colon and lung carcinoma.[25]



60 R=H
61 R=Galloyl



62

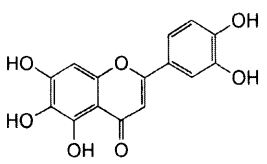


63 R_1=R_3=H, R_2=OH
64 R_1=H, R_2=R_3=OH
72 R_1=R_2=R_3=H
73 R_1=CH_3, R_2=R_3=H

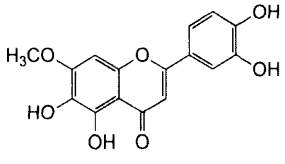
Chart 5

Of the naphthoquinones tested for their inhibitory effects on RNA-dependent DNA polymerase, juglone (**63**) and naphthazarin (**64**) were found to be moderate inhibitors of RDDP activity with IC_{50} values of 8 and 10 μM, respectively.[26]

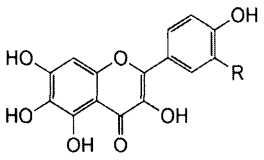
A comparative assay with HIV- and AMV-RT demonstrated that the inhibitory effects of the two enzymes occurred at a similar extent.[27] Of 190 flavonoids examined, 46 had over 50% inhibition at a concentration of 1 mM. 6-Hydroxyluteolin (**65**) (IC_{50}= 7 μM), pedalitin (**66**) (IC_{50}= 10 μM), 6-hydroxy kaempferol (**67**) (IC_{50}= 8 μM) and quercetagetin (**68**) IC_{50}= 8 μM) were the most potent inhibitory substances against AMV-RT (Chart 6).



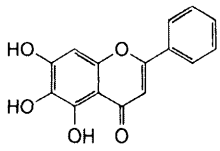
6-Hydroxyluteolin (**65**)



Pedalitin (**66**)



6-Hydroxykaempferol (**67**, R=H)
Quercetagetin (**68**, R=OH)



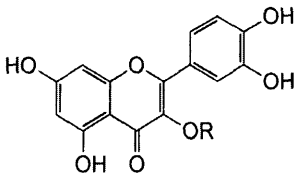
Baicalein (**69**)

Chart 6

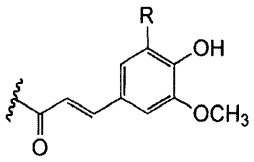
Of the flavonoids having potent inhibitory effects, the number of hydroxyl groups and their positions were found to be related to the potency of inhibition. As to the number of hydroxyl groups, all hexahydroxyl flavonoids showed potent inhibition but those having less than three hydroxyl groups were not effective, except for baicalein (**69**). When hydroxyl groups are arranged at the neighboring positions, such as 5,6,7 and 3', 4', and 5', the flavonoids showed extremely strong inhibition of RT. When these flavonoids were tested for DNA polymerase I, contrary to the results with RT, their inhibitory effects were not strong against this enzyme. This finding suggests that, at least in part, the inhibition by these compounds is more specific to RNA-dependent DNA polymerase (RT) than to DNA-dependent DNA polymerase (DDDP).

b) Inhibition of DNA-dependent DNA polymerase (DDDP) activity

Inhibition of DDDP activity is also considered as one of the approaches for possible inhibition of RT enzyme. Screening of Thai medicinal plants against HIV-1 revealed that the EtOH extract of *Thevetia peruviana* SCHUM. demonstrated high anti-HIV-1 activity (IC_{100}= 1.56 μg/ml). Therefore, this extract was subjected to bioactivity-guided fractionation to give compounds with moderate HIV-1 DDDP inhibitory activity, namely, quercetin 3-[(6-*O*-



70 R=gal^2-gl^6-sinapoyl
71 R=gal^2-gl^6-feruloyl



Feruloyl (R=H)
Sinapoyl (R=OCH_3)

Chart 7

sinapoyl)-*O*-β-D-glucopyranosyl (1→2)-β-D-galactopyranoside] (**70**, IC_{50} value of 69 μM) and quercetin 3-[(6-*O*-feruloyl)-*O*-β-D-glucopyranosyl (1→2)-β-D-galactopyranoside] (**71**, IC_{50} value of 42 μM).[28]

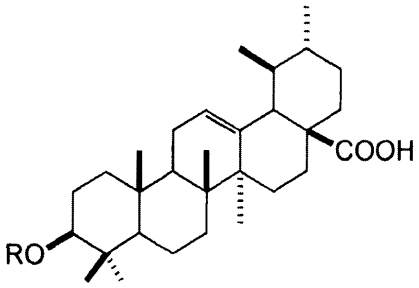
c) Inhibition of ribonuclease H (RNase H) activity

Compared with the relatively large volume of research on the inhibition of RT activity of HIV-1, there are only a few reports on the selective inhibition of RNase H activity. Therefore, our interest was focused on natural products to explore potent inhibitors of HIV-1 RT-associated RNase H. RNase H inhibitory activity was measured as the inhibition of the degradation of RNA in a DNA-RNA hybrid in the presence of a test sample. Illumaquinone was used as a positive control, which inhibited RNase H activity with an IC_{50} of 50 μM under the experimental conditions used.[22] During our screening program, we found that an EtOAc-soluble fraction of the MeOH extract of *J. mandshurica* (stem-bark) appreciably inhibited both HIV-RT and RNase H activities with IC_{50} values of 0.047 and 22 μg/ml, respectively. Of 14 compounds isolated from the EtOAc-soluble fraction, **61** was comparable in inhibitory potency to illimaquinone with an IC_{50} of 50 μM, while **60** and **62** showed moderate inhibition against RNase H with IC_{50} values of 310 and 330 μM, respectively (Chart 5).

Out of 30 quinone derivatives isolated from natural resources or obtained as intestinal bacterial metabolites from natural products,[29] naphthoquinone (**72**) was found to demonstrate potent inhibitory activity against RNase H (IC_{50} of 9.5 μM),[26] and vitamin K_3 (**73**) was moderately active (IC_{50} value of 75 μM).

3. Inhibition of HIV-1 protease (PR)

During the replication of HIV, the viral polyprotein must be cleaved by viral protease (PR) to generate essential viral enzymes, such as RT, IN and PR itself, as well as the viral structural proteins. HIV-1 PR is an aspartic protease composed of two identical monomers, which assemble by non-covalent interactions to form the composite active site. This structural peculiarity has provided the possibility of a special inhibitory mechanism, i.e. the dimerization inhibition mechanism. A dimerization inhibitor of HIV-1 PR could dissociate the enzyme into the inactive monomer form and thus inhibit the enzyme activity. In a preliminary screening of Chinese and Mongolian herbal drugs for inhibitory activity against HIV-1 PR, an extract of the stems of *Cynomorium songaricum* RUPR. was found to be active.



74 R=H
75 R=$COCH_2COOH$

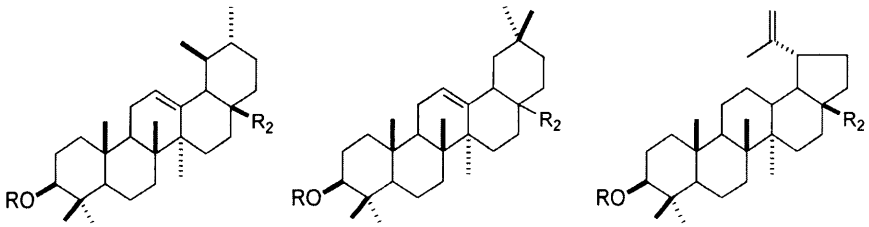
Chart 8

C. songaricum is a parasitic plant mainly growing in the Inner Mongolia region of China, where the stems are reputed to have medicinal use as a tonic.[30] From CH_2Cl_2 and MeOH extracts of the stems of *C. songaricum,* ursolic acid (**74**) and its hydrogen malonate (**75**) were isolated as inhibitors of HIV-1 PR (IC_{50} values of 8 and 6 μM, respectively) (Chart 8).[31] This finding encouraged us to prepare a series of dicarboxylic acid hemiesters of ursolic acid and related triterpenes. In ursolic acid (**74**), oleanolic acid (**82**) and betulinic acid (**90**), introduction of carboxyl groups linked at C-3 by an ester bond tended to increase the inhibitory activity in the order of oxalyl, malonyl, succinyl and glutaryl hemiesters [the most potent inhibition was observed for the glutaryl hemiesters (80, 88 and 95, IC_{50} of 4 μM, about half the values of the original triterpenes)] (Table 2).

Oleanolic acid (**82**), which is abundant in nature, was used as an example to investigate the effects of triterpene derivatives. The lengths of the acidic chains were optimized to 6 and 8 carbons with an IC_{50} value of 3.0 μM for both compounds (97 and 98) (Table 3). Further extension of the acyl chain by two additional methylene units (**99**) led to a slight decrease in the inhibitory potency.

Changing a 3-hydroxyl of **82** to an oxo or a hydroxyimino group retained their inhibitory activity against HIV-1 PR (**105** and **106**) (Table 4).[32] Replacing a 3-hydroxyl of 28-methyl oleanolate to an oxo (**111**) or amino group (**113**) did not ameliorate the poor activity. However, 3-hydroxyimino-olean-12-en-28-oic acid methyl ester (**112**) exhibited two-fold increased activity as compared to methyl oleanolate (**83**).

These findings indicated that introducing an additional acidic chain to the oleanane skeleton might be potential to the HIV-1 PR inhibitory activity of these classes of compounds. Acylation of sophoradiol (**121**, an aglycone of kaikasaponin III),[33] which has two hydroxy groups at C-3 and C-22 with a distance of 12.321 Å between the two groups [quite similar to that between 3-OH and the 17-COOH in the structure of **82** (12.409 Å)] with adipoyl chloride yielded 3, 22-di-*O*-adipoylsophoradiol (**122**) which showed more than 8-fold activity than the parent triterpene (IC_{50} value of 2.3 μM *vs.* 18.8 μM for **121**) (Table 5). A similar finding was obtained when a second carboxyl group was introduced at C-28 of oleanolic acid (**123**, IC_{50} value of



Ursene-type Oleanene-type Lupane-type

Table 2 Inhibitory effects of triterpenes on HIV-protease enzyme

R_1	R_2	Ursene-type Cmpd	IC_{50} (μM)	Oleanene-type Cmpd	IC_{50} (μM)	Lupane-type Cmpd	IC_{50} (μM)
H	CH_3	α-amyrin	80	β-amyrin	>100		
H	COOH	urolic acid (**74**)	8	oleanolic acod (**82**)	8	betulinic acid (**90**)	9
H	$COOCH_3$	**76**	14	**83**	20	**91**	>25
$COCH_3$	COOH	**77**	13	**84**	9		
COCOOH	COOH	**78**	7	**85**	20	**92**	7
$COCH_2COOH$	COOH	**75**	6	**86**	8	**93**	6
$CO(CH_2)_2COOH$	COOH	**79**	6	**87**	4	**94**	6
$CO(CH_2)_3COOH$	COOH	**80**	4	**88**	4	**95**	4
$CO(CH_2)_2COOCH_3$	$COOCH_3$					**96**	40
$CO(CH_2)_3COOCH_3$	$COOCH_3$	**81**	>50	**89**	>50		

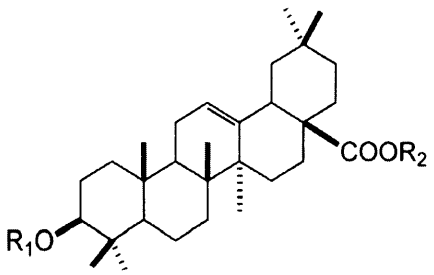


Table 3 Anti-HIV-1 PR activity of triterpene derivatives with different lengths of a 3-acyl chain

R_1	R_2=H		R_2=CH_3	
	Cmpd	IC_{50} (μM)	Cmpd	IC_{50} (μM)
H	**82**	8.0	**83**	20.0
COCOOH	**85**	20		
$COCH_2COOH$	**86**	8.0		
$CO(CH_2)_2COOH$	**87**	4.0		
$CO(CH_2)_3COOH$	**88**	4.0		
$CO(CH_2)_4COOH$	**97**	3.0	**103**	7.5
$CO(CH_2)_6COOH$	**98**	3.0		
$CO(CH_2)_8COOH$	**99**	4.0		
$COCH_2C(CH_3)_2CH_2COOH$	**100**	3.8		
$CO(CH_2)COOCH_3$	**101**	5.6	**104**	>20
$CO(CH_2)_3CH_3$	**102**	>20		

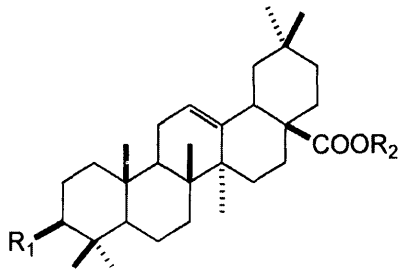


Table 4 Anti-HIV-1 PR activity of triterpene derivatives with different bond and chain nature at position 3

R_1	R_2=H		R_2=CH_3	
	Cmpd	IC_{50} (μM)	Cmpd	IC_{50} (μM)
β-OH	**82**	8.0	**83**	20
=O	**105**	5.5	**111**	20
=NOH	**106**	5.5	**112**	9.5
α-NH			**113**	>20
=NOCO$(CH_2)_4$COOH	**107**	5.5	**114**	>20
β-NHCO$(CH_2)_4$COOH	**108**	3.0	**115**	4.0
α-NHCO$(CH_2)_4$COOH	**109**	2.1	**116**	3.0
β-NHCO$(CH_2)_4$CONH$(CH_2)_3$COOH			**117**	3.5
β-NHCO$(CH_2)_4$CONH$(CH_2)_3$COOCH$_3$			**118**	6.0
β-NHCO$(CH_2)_4$CONH-β-oleanolic acid 28-R_2	**110**	3.3	**119**	>20
			120	>20

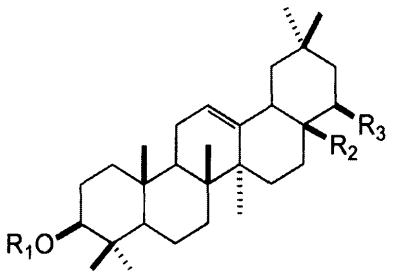


Table 5 Anti-HIV-1 PR activity of oleanene type triterpenes with hydroxyls or acidic chains at C-3 and C-22 (or) C-17.

Cmpd	R_1	R_2	R_3	IC_{50} (μM)
121	-H	-CH_3	-OH	18.8
122	-CO$(CH_2)_4$COOH	-CH_3	-OCO$(CH_2)_4$COOH	2.3
82	-H	-COOH	-H	8.0
97	-CO$(CH_2)_4$COOH	-COOH	-H	3.0
123	-CO$(CH_2)_4$COOH	-CONH$(CH_2)_5$COOH	-H	1.7
124	-H	-CONH$(CH_2)_5$COOH	-H	1.7

1.7 μM, though a derivative with only one acidic chain linked at C-28 (**124**) also showed the same activity against HIV-1 protease, and 4 times more than the inhibitory activity of oleanolic acid (**82**). The ester bond, which links the acidic chain and the triterpene skeleton, was found stable to lipase enzyme even after incubation for 18 hr.

The dissociation of HIV-1 PR by these compounds was monitored directly by size exclusion chromatography. Two main proteins at retention times of 29.4 and 39.0 min were assigned to HIV-1 PR dimer and monomer, respectively, by interpolation of a standard protein curve. After incubation with **97**, the peak was dominant and that of the dimer disappeared completely. On the other hand, after treatment with an active site inhibitor, acetyl pepstatin, the dimer was dominant and the monomer disappeared. This finding indicated that the triterpene compound could dissociate the dimeric polypeptides of HIV-1 PR into a monomeric one, i.e. it inhibited the activity of HIV-1 PR through the mechanism of dimerization inhibition. These compounds showed no inhibitory effects on other aspartic protease, pepsin, suggesting that it may not be interacting with the enzyme active sites. The scaffold of triterpene compounds matches in its volume that of the backbone of a cyclic hexapeptide, and a computer docking study has revealed that some triterpenes could fit well into the hydrophobic interface site of the relaxed HIV-1 PR monomers. The potency of most of the derivatives prepared was comparable to that of peptide compounds reported recently based on the same inhibition mechanism.[34,35] Therefore, it is not surprising that structural modification of triterpene compounds could lead to HIV-1 PR inhibitors with the same inhibitory mechanism and similar inhibitory potency as some peptide compounds. Though these compounds are potent inhibitors of HIV-1 PR, they did not show inhibitory effects on viral replication. When oleanolic acid derivatives were conjugated with known anti-HIV drugs like AZT (Chart 9), potent anti-HIV-1 activity was observed and PR inhibitory activity was also maintained (**133** *vs.* **124**), though AZT itself showed no PR inhibitory activity (Table 6).[36] Because the triterpene skeleton is more rigid and stable than peptide, it is expected that triterpene derivatives might be more specific to HIV-1 PR and have better pharmacokinetic properties.

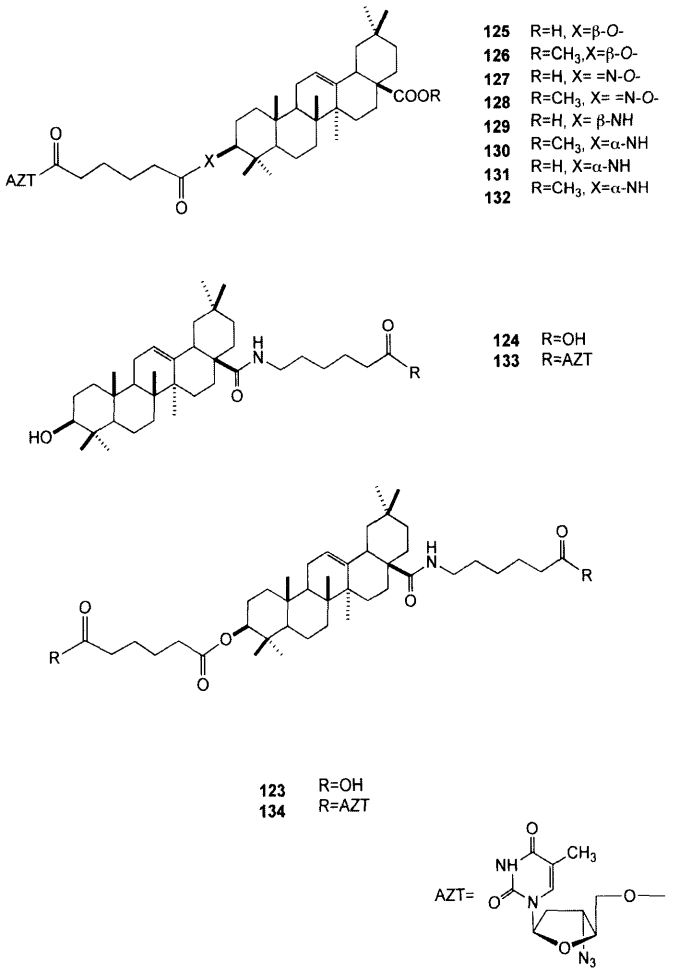


Chart 9

Table 6 Anti-HIV protease and anti-HIV-1 activity of triterpene-AZT conjugates

Cmpd No.	v.s. PR	v.s. HIV-1	
	IC_{50} (μM)	IC_{100} (mM)	CC_0 (mM)
125	3.2	3.78	15.0
126	4.0	18.40	148.0
127	1.9	1.84	29.6
128	16.0	4.53	145.0
129	1.2	0.589	120.0
130	20.0	0.370	73.9
131	1.9	0.469	120.0
132	8.0	7.39	>118.0
133	2.4	1.90	122.0
134	4.4	836	>836.0
123	1.7	NE	179.0
124	1.7	NE	110.0

4. Inhibition of HIV-1 integrase (IN)

Viral integrase (IN) is an enzyme that integrates the viral transcribed DNA into host-cell DNA. During viral infection, IN catalyzes the excision of the last two nucleotides from the linear viral DNA, leaving the terminal dinucleotide CA-3'-OH at the recessed 3'-end (3'-processing). After transport to the nucleus as a nucleoprotein complex, IN catalyzes a DNA strand transfer reaction involving the nucleophilic attack at the ends on the host DNA, which is called strand transfer or joining.[37-39] Clinically useful antiviral drugs targeting this enzyme still have not yet been developed. Accordingly, our strategy was directed towards natural resources in the hope that we can find HIV-1 IN inhibitory substance. There have been many reports on various HIV-IN inhibitory assay systems using isotope-labeled substrates and denaturing gel separation of reaction products.[40-45] These *in vitro* methods are referred to as standard integration assays and give clear results. However, they are inconvenient and time consuming, especially when screening inhibitors from many samples. Recently, an assay for

HIV-1 IN activity using DNA-coated plates has been described.[46-48] It is a non-radioisotopic method, which screens for both 3'-processing and 3'-strand transfer. This method was used for screening 50 medicinal plants used in Thailand for their activity against HIV-1 IN, and suramin was used as a positive control, which inhibits HIV-1 IN *in vitro* with an IC_{50} value of 2.4 μM. Among them, *Coleus parvifolius* (water and EtOH extracts) and *Thevetia peruviana* SCHUM. (EtOH extract) showed potent anti-HIV-1 IN activity (IC_{50} values of 2.9, 9.2 and 12 μg/ml, respectively).[49] Herbal preparations of *Coleus* species have long been used in Hindu and Ayurvedic traditional medicine, particularly for the treatment of heart diseases, abdominal pain and convulsion.[50] From the water extract of *C. parvifolius*, rosmarinic acid (**135**), luteolin (**137**) and luteolin 7-*O*-methyl ether (**138**) were isolated as potent inhibitors of HIV-IN (IC_{50} values of 5.0, 11.0 and 11.0 μM, respectively). Rosmarinic acid methyl ester (**136**) was obtained from an EtOAc-soluble fraction of the EtOH extract of *C. parvifolius* and was found to be the most potent constituent isolated from this plant (IC_{50} = 3.1 μM) (Chart 10). Rosmarinic acid derivatives (dimers, trimers, tetramers and their metal-binding derivatives) were also tested for their inhibitory effects on HIV-1 IN. Magnesium lithospermate (**54**), a magnesium salt of a trimer of caffeic acid, showed the highest activity, followed by calcium rosmarinate (**55**), magnesium rosmarinate (**56**), lithospermic acid B (**139**), lithospermic acid (**58**), rosmarinic acid methyl ester (**136**) and rosmarinic acid (**135**) with IC_{50} values of 0.7, 0.8, 1.0, 1.0, 1.4, 3.1 and 5.0 μM, respectively.[49]

From the EtOH extract of the leaves of *T. peruviana*, flavonoid glycosides **70** and **71** were isolated as potent IN inhibitors with IC_{50} values of 5 and 7 μM, respectively.[28] Since various flavonoids are known to demonstrate inhibitory action against various enzymes, it is of interest to investigate their inhibitory activity against HIV-1 IN. Of 183 flavonoids tested, 6-hydroxyluteolin (**65**), scutellarein (**140**), scutellarin (**141**), pedalitin (**66**), biacalein dimer (**142**), hypolaetin (**143**), 7-*O*-benzyl-6-hydroxyluteolin (**144**) and biacalein (**69**) showed appreciable inhibition with IC_{50} values of 0.4, 0.6, 1.7, 1.3, 2.0, 2.1, 3.0 and 3.6 mM, respectively.[51] The potent inhibition was observed with flavonoids having at least one pair of vicinal hydroxyl groups and the activity was highly dependent on the number of vicinal hydroxyl groups. On the other hand, the inhibitory activity tended to be decreased by replacing a hydroxyl group with one of methoxyl, acetoxyl, isopropoxyl, isopentenyl, benzyloxyl, glucuronyl and glucosyl groups. Flavanones, flavanonols and chalcones examined in this experiment did not show significant inhibitory activity.

Acknowledgments

The authors would like to dedicate this review to the late emeritus professor Tsuneo Namba, who died on July 24, 2004, at the age of 72.

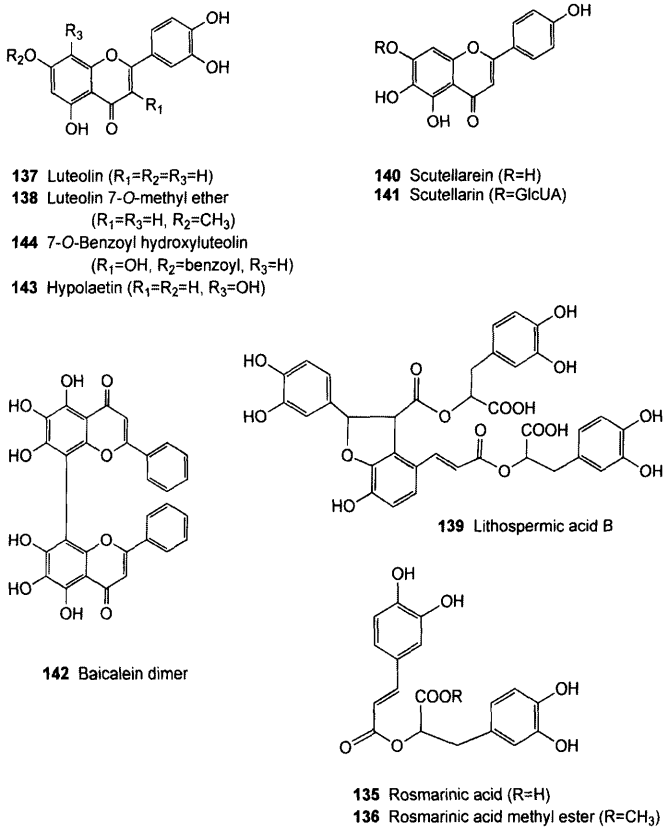


Chart 10

References

1) Kinghorn, A. D., Balandrin, M. F. (eds.): Human Medicinal Agents from Plants (ACS Symposium Series No. 534). A. C. S., Wash. D. C., p. 356, 1993.
2) Harada, S., Koyanagi, Y. and Yamamoto, N.: Infection of HTLV-III/LAV-I-carrying cells MT-2 and MT-4 and application in a plaque assay. *Science,* **229**, 563-566, 1985.
3) Kawahata, T., Otake, T., Mori, H., Morimoto, M., Ueba, N., Kusumoto, I. T., El-Mekkawy, S., Hattori, M. and Namba, T.: Screening of Egyptian folk medicinal plant extracts for anti-human immunodeficiency virus type-1 (HIV-1) activity. *J. Trad. Med.,* **13**, 59-65, 1996.
4) Otake, T., Miyano, K., Mori, H., Morimoto, M., Ueda, N., Kunita, N., Nakashima, H. and Kurimura, T.: Anti-HIV-1 activity of sulfated amphotericin B *in vitro*. *Antiviral Res.,* **16**, 243-255, 1991.
5) Otake, T., Mori, H., Morimoto, M., Ueda, N., Sutardjio, S., Kusumoto, I. T., Hattori, M. and Namba, T.: Screening of Indonesian plant extracts for anti-human immunodeficiency virus-type 1 (HIV-1) activity. *Phytother. Res.,* **9**, 6-10, 1995.
6) Mitsuya, H., Looney, D. J., Kuno, S., Ueno, R., Wong-Staal, F. and Broder, S.: Dextran sulfate suppression of viruses in the HIV family: Inhibition of virion binding to $CD4^+$ cells. *Science* **240**, 646-649, 1988.
7) Evans, F. J. and Taylor, S. E.: Pro-inflammatory tumor-promoting and anti-tumor diterpenes of the plant families Euphorbiaceae and Thymeleaceae. In "Progress in The Organic Chemistry of Natural Products, vol. 44 (Eds. by Herz, W., Grisebach, H. and Kirby, G. W.), Springer-Verlag, New York, pp. 1-99, 1983.
8) Nishizuka, Y. : The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature,* **334**, 661-665, 1988.
9) Gustafson, K. R., Cardellina II, J. H., McMahon, J. B., Gulakowski, R.

J., Ishitoya, J., Szallasi, Z., Lewin, N. E., Blumberg, P. M., Weislow, O. S., Beutler, J. A., Buckheit Jr., R. W., Cragg, G. M., Cox, P. A., Bader, J. P. and Boyd, M. R.: A nonpromoting phorbol from the Samoan medicinal plant *Homalanthus nutans* inhibits cell killing by HIV-1. *J. Med. Chem.,* **35**, 1978-1986, 1992.

10) El-Mekkawy, S., Meselhy, M. R., Nakamura, N., Hattori, M., Kawahata, T. and Otake, T.: 12-*O*-Acetylphorbol-13-decanoate potently inhibits cytopathic effects of human immunodeficiency virus type 1 (HIV-1), without activation of protein kinase C. *Chem. Pharm. Bull.,* **47**, 1346-1347, 1999.
11) El-Mekkawy, S., Meselhy, M. R., Nakamura, N., Hattori, M., Kawahata, T. and Otake, T.: Anti-HIV-1 phorbol esters from *Croton tiglium. Phytochemistry,* **53**, 457-464, 2000.
12) Chowdhury, M. I. H., Koyanagi, Y., Kobayshi, S., Hamamoto, Y., Yoshiyama, H., Yoshida, T. and Yamamoto, N.: The phorbol ester TPA strongly inhibit HIV-1-induced syncytia formation but enhances virus production: Possible involvement of protein kinase C pathway. *Virology,* **176**, 126-132, 1990.
13) El-Mekkawy, S., Meselhy, M. R., Abdel-Hafez, A. A. A., Nakamura, N., Hattori, M., Kawahata, T. and Otake, T.: Inhibition of cytopathic effects of human immunodeficiency virus type-1 by various phorbol derivatives. *Chem. Pharm. Bull.,* **50**, 523-529, 2002.
14) Palaniappan, C., Fay, P. J. and Bambara, R. A.: Nevirapine alters the cleavage specificity of ribonuclease H of human immunodeficiency virus 1 reverse transcriptase. *J. Biol. Chem.,* **270**, 4861-4869, 1995.
15) Loya, S. and Hizi, A.: The interaction of illimaquinone, a selective inhibitor of the RNase H activity, with the reverse transcriptase of human immunodeficiency and murine leukemia retroviruses. *J. Biol. Chem.,* **268**, 9323-9328, 1993.
16) Starnes, M. C. and Cheng, Y. C.: Human immunodeficiency virus reverse transcriptase-associated RNase H activity. *J. Biol. Chem.,* **264**, 7073-7077, 1989.
17) El-Mekkawy, S., Meselhy, M. R., Kusumoto, I., Kadota, S., Hattori, M. and Namba, T.: Inhibitory effects of Egyptian folk medicines on human immunodeficiency virus (HIV) reverse transcriptase. *Chem. Pharm. Bull.,* **43**, 641-648, 1995.
18) Lim, Y. A., Kojima, S., Nakamura, N., Miyashiro, H., Fushimi, H., Komatsu, K., Hattori, M., Shimotohno, K., Gupta, M. and Correa, M.: Inhibitory effects of Cordia spinescens extracts and their constituents on reverse transcriptase and protease from human immunodeficiency virus. *Phytother. Res.,* **11**, 490-495, 1997.
19) Usmanghani, K., Saeed, A. and Alam, M. T.: Indusyunic Medicine. Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, Karachi-75270, Pakistan, 1997.
20) Morton, J. F.: Atlas of Medicinal Plants of Middle America. Charles C. Thomas, Spingfield, p. 428, 1981.
21) Joly, L. G., Guerra, S., Septimo, R., Solis, P. N., Correa, M. D., Gupta, M. P., Levy, S., Sandberg, F. and Perera, P.: Ethnobotanical inventory of medicinal plants used by the Guaymi Indians in Western Panama. Part II. *J. Ethonopharmacology,* **28**, 191-206, 1990.
22) Min, B.-S., Nakamura, N., Miyashiro, H., Kim, Y.-H. and Hattori, M.: Inhibition of human immunodeficiency virus type 1 reverse transcriptase and ribonuclease H activities by constituents of *Juglans mandshurica. Chem. Pharm. Bull.,* **48**, 194-200, 2000.
23) Son, J. K.: Isolation and structure determination of a new tetralone glucoside from the roots of *Juglans mandshurica. Arch. Pharm. Res.,* **18**, 203-205, 1995.
24) Joe, Y. K., Son, J. K., Park, S. H., Lee, I. J. and Moon, D. C.: New naphthalenyl glucosides from the roots of *Juglans mandshurica. J. Nat. Prod.,* **59**, 159-160, 1996.
25) Agrawal, P. K., Agrawal, S. K., Rastogi, R. P. and Osterdahal, B. G.: Dihydroflavanonols from *Cedrus deodara,* a ^{13}C NMR study. *Planta Med.,* **43**, 82-85, 1981.
26) Min, B.-S., Miyashiro, H. and Hattori, M.: Inhibitory effects of quinones on RNase H activity associated with HIV-1 reverse transcriptase. *Phytother. Res.,* **16**, S57-S62, 2002.
27) Kusumoto, I. T., Hattori, M., Miyaichi, Y., Tomimori, T., Hanaoka, M. and Namba, T.: Effects of flavonoids and alkaloids on reverse transcriptase. *Shoyakugaku Zasshi,* **45**, 240-254, 1991.
28) Tewtrakul, S., Nakamura, N., Hattori, M., Fujiwara, T. and Supavita, T.: Flavanone and flavonol glycosides from the leaves of *Thevetia peruviana* and their HIV-1 reverse transcriptase and HIV-1 integrase inhibitory activities. *Chem. Pharm. Bull.,* **50**, 630-635, 2002.
29) Meselhy, M. R., Kadota, S., Tsubono, K., Hattori, M. and Namba, T.: Biotransformation of shikonin by human intestinal bacteria. *Tetrahedron,* **50**, 3081-3098, 1994.
30) New Medical College of Jangsu (ed.): Dictionary of Chinese Materia Medica. Shanghai Scientific and Technological Publishing Co., Shanghai, p. 2395, 1977.
31) Ma, C., Nakamura, N., Miyashiro, H., Hattori, M. and Shimotohno, K.: Inhibitory effects of constituents from *Cynomorium songaricum* and related triterpenes derivatives on HIV-1 protease. *Chem. Pharm. Bull.,* **47**, 141-145, 1999.
32) Ma, C., Nakamura, N. and Hattori, M.: Chemical modification of oleanene type triterpenes and their inhibitory activity against HIV-1 protease dimerization. *Chem. Pharm. Bull.,* **48**, 1681-1688, 2000.
33) Ma, C., Nakamura, N. and Hattori, M.: Saponins and *C*-glycosyl flavones from the seed of *Abrus precatorius. Chem. Pharm. Bull.,* **46**, 982-987, 1998.
34) Zutshi, R., Franciskovich, J., Shultz, M., Schweitzer, B., Bishop, P., Wilson, M. and Chmielewski, J.: Targeting the dimerization interface of HIV-1 protease: Inhibition with cross-linked interfacial peptides. *J. Am. Chem. Soc.,* **119**, 4841-4845, 1997.
35) Bouras, A., Boggetto, N., Benatalah, Z., Rosny, E. D., Sicsic, S. and Reboud-Ravaux, M.: Design, synthesis, and evaluation of conformationally constrained tongs, new inhibitors of HIV-1 protease dimerization. *J. Med. Chem.,* **42**, 957-962, 1999.
36) Ma, C., Nakamura, N., Hattori, M., Kawahata, T. and Otake, T.: Inhibitory effects of triterpene-azidothymidine conjugates on proliferation on human immunodeficiency virus type 1 and its protease. *Chem. Pharm. Bull.,* **50**, 877-880, 2002.
37) Fujiwara, T. and Mizuuchi, K.: Retroviral DNA integration: Structure of an integrase intermediate. *Cell,* **54**, 497-504, 1988.
38) Vink, C. and Plasterk, R. H. A.: The human immunodeficiency virus integrase protein. *Trends Genet.,* **9**, 433-437, 1993.
39) Katz, R. A. and Skalka, A. M.: The retroviral enzymes. *Annu. Rev. Biochem.,* **63**, 133-173, 1994.
40) Fesen, M. R., Pommier, Y., Leteurtre, F., Hiroguchi, S., Yung, J. and Kohn, K. W.: Inhibition of HIV-1 integrase by flavones, caffeic acid phenethyl ester (cape) and related compounds. *Biochem. Pharmacol.,* **48**, 595-608, 1994.
41) Burke, C. J., Sanyal, G., Bruner, M. W., Ryan, J. A., LaFemina, R. L., Robbins, H. L., Zeft, A. S., Middaugh, C. R. and Cordingley, M. G.: Structural implications of spectroscopic characterization of a putative zinc finger peptide from HIV-1 integrase. *J. Biol. Chem.,* **267**, 9639-9644, 1992.
42) Mazumder, A., Wang, S., Neamati, N., Nicklaus, M., Sunder, S., Chen, J., Milne, G. W. A., Rice, W. G., Burke Jr., T. R. and Pommier, Y.: Antiviral agents as inhibitors of both human immunodeficiency virus type 1 integrase and protease. *J. Med. Chem.,* **39**, 2472-2481, 1996.
43) Mazumder, A., Neamati, N., Sunder, S., Schulz, J., Pertz, H., Eich, E. and Pommier, Y.: Curcumin analogs with altered potencies against HIV-1 integrase as probes for biochemical mechanisms of drug action. *J. Med. Chem.,* **40**, 3057-3063, 1997.
44) Neamati, N., Mazumder, A., Zhao, H., Sunder, S., Burke Jr., T. R., Schultz, R. J. and Pommier, Y.: Diarylsulfones, a novel class of human immunodeficiency virus type 1 integrase inhibitors. *Antimicrob. Agents Chemother.,* **41**, 385-393, 1997.
45) Reddy, M. V. R., Rao, M. R., Rhodes, D., Hansen, M. S. T., Rubins,

K., Bushman, F. D., Venkateswarlu, Y. and Faulkner, D. J.: Lamellarin 20-sulfate, an inhibitor of HIV-1 integrase active against HIV-1 virus in cell culture. *J. Med. Chem.,* **42**, 1901-1907, 1999.

46) Hazuda, D. J., Hastings, J. C., Wolfe, A. C. and Emini, E. A.: A novel assay for the DNA strand-transfer reaction of HIV-1 integrase. *Nucleic Acids Res.,* **22**, 1121-1122, 1994.

47) Vink, C., Banks, M., Bethell, R. and Plasterk, R. H. A.: A high-throughput non-radioactive plate assay for activity of human immunodeficiency virus integrase protein. *Nucleic Acids Res.,* **22**, 2176-2177, 1994.

48) Chang, Y. C., Ching T. T. and Syn, W.: Assaying the activity of HIV-1 integrase with DNA-coated plates. *J. Viol. Methods,* **59**, 135-140, 1996.

49) Tewtrakul, S., Miyashiro, H., Nakamura, N., Hattori, M., Kawahata, T., Otake, T., Yoshinaga, T., Fujiwara, T., Supavita, T., Yuenyongsawad, S., Rattanasuwon, P. and Dej-Adisai, S.: HIV-1 integrase inhibitory substances from *Coleus parvifolius. Phytother. Res.,* **17**, 232-239, 2003.

50) Evans, W. C.: Trease and Evans' Pharmacology, 13th edition. Bailliere Tindall, London, 1989.

51) Tewtrakul, S., Miyashiro, H., Hattori, M., Yoshinaga, T., Fujiwara, T., Tomimori, T., Kizu, H. and Miyaichi Y.: Inhibitory effects of flavonoids on human immunodeficiency virus type-1 integrase. *J. Trad. Med.,* **18**, 229-238, 2001.