Specific Inhibition of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Mediated by Soulattrolide, a Coumarin Isolated from the Latex of *Calophyllum teysmannii*

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Soulattrolide (**1**), a coumarin isolated from *Calophyllum teysmannii* latex, was found to be a potent inhibitor of HIV-1 reverse transcriptase (RT) with an IC_{50} of 0.34 μM. Inhibition was remarkably specific, with no appreciable activity being observed toward HIV-2 RT, AMV RT, RNA polymerase, or DNA polymerases α or β.

Human immunodeficiency virus (HIV) is generally considered responsible for acquired immunodeficiency syndrome (AIDS). As with other retroviruses, the virus-coded enzyme reverse transcriptase (RT) is critical in the early stages of the viral life cycle, during which viral DNA is synthesized from the viral RNA template. Thus far, RT has served as a major target in chemotherapeutic regimens employed for the treatment of HIV infection.[1] Accordingly, the nucleoside analogue AZT was the first agent approved for clinical use against HIV, followed by ddC, ddI, and d4T.[2−4] These compounds inhibit viral replication by acting as DNA chain terminators and thereby have provided certain benefits in delaying disease progression and improving the survival of persons with AIDS, but long-term monotherapy using nucleoside analogues has been limited by toxicity and by the emergence of drug-resistant viral strains.[5−9] Use of combination therapy and protease inhibitors that have recently been approved by the U.S. Food and Drug Administration may help to alleviate this situation.

An additional class of compounds of interest for development as clinical agents is non-nucleoside inhibitors of RT. Examples include nevirapine, tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-one and -thione (TIBO) derivatives, pyridinone, bis(heteroaryl)-piperazines (BHAP), spiro[2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)pyrimidine-3′,5″-4″-amino-1″,2″-oxathiole 2″,2″-dioxide] (TSAO), 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT), α-anilinophenylacetamide (α-APA), psychotrine, and diarylsulfones.[10−18] Unlike nucleoside analogues that can inhibit a number of human and animal retroviruses,[19,20] non-nucleoside inhibitors are highly specific for HIV-1 RT. However, mutant HIV-1 strains that are resistant to a number of non-nucleoside inhibitors have also been identified,[21,22] and problems of this type further emphasize the critical need to identify new anti-HIV agents for use in monotherapy or in combination with other anti-retroviral agents.

Coumarins such as calanolide A[23] and inophyllums[24] have been established as non-nucleoside inhibitors of HIV-1 RT. Calanolide A, isolated from a tropical rainforest tree *Calophyllum lanigerum* Miq. var. *austrocoriaceum* (T. C. Whitmore) P. F. Stevens (Guttiferae) collected in Sarawak, Malaysia, provided complete protection against the in vitro replication and cytopathicity of HIV-1 in lymphoblastic cells (CEM-SS).[23] Unfortunately, subsequent field studies failed to identify a reliable natural source of calanolide A. Rather, another potent anti-HIV coumarin, costatolide (**2**),[25] was identified as the major active constituent from a tropical rainforest tree *Calophyllum teysmannii* Miq. var. *inophylloide* (King) Stevens. Unlike calanolide A, costatolide (**2**) is abundant (ca. 48% of latex extractables) in the latex of *C. teysmannii* var. *inophylloide*.[26] A related coumarin, soulattrolide (**1**),[27] is also a major component (ca. 29% of latex extractables) in the latex.[26] In comparative anti-HIV tests using CEM-SS cells and the XTT tetrazolium assay, soulattrolide (**1**) was found to mediate a response similar to calanolide B, but it was less potent than costatolide.[28] Nonetheless, soulattrolide (**1**) bears a close structural resemblence to costatolide (**2**), and substantial quantities of **1** are obtained during the process of extracting costatolide.[26] Due to these factors, we have evaluated inhibition profiles mediated by soulattrolide (**1**) with HIV-1 RT and a variety of other nucleic acid polymerases.

Results and Discussion

As summarized in Table 1, compound **1** specifically inhibited HIV-1 RT and was not active (<30% inhibition at 200 μg/mL) against HIV-2 or avian myeloblastosis virus (AMV) RTs. Therefore, compound **1** functions as

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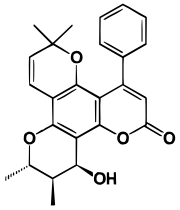
Table 1. Inhibitory Effects of Soulattrolide (**1**) on Nucleic Acid Polymerases and Ribonuclease H

enzyme	% inhibition at 200 μg/mL (495 μM)	IC_{50} (μM)
HIV-1 RT[a] (DDDP activity)	87.2	0.73[b]
HIV-1 RT[a] (RDDP activity)	96.2	0.34[b]
E. coli RNase H	86.5	69.5[b]
HIV-2 RT	0.7	inactive
TIBO-resistant (Tyr181) HIV-1 RT	31.0	inactive
AMV RT[c]	20.2	inactive
DNA polymerase α	28.1	inactive
DNA polymerase β	49.6	>495
RNA polymerase	29.7	inactive

[a] HIV-1 RT was assessed for DNA-dependent DNA polymerase (DDDP) and RNA-dependent DNA polymerase (RDDP) activities. The IC_{50} value for 3′-azido-3′-deoxythymidine triphosphate (AZT-TP) for the RDDP activity of HIV-1 RT was 0.051 μM. [b] IC_{50} values observed with costatolide (**2**) were 1.4 μM and 0.29 μM, respectively, when the DDDP and RDDP activities of HIV-1 RT were assessed. Costatolide (**2**) had no inhibitory activity against *E. coli* RNase H. [c] AMV RT, avian myeloblastosis virus reverse transcriptase.



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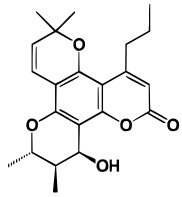

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a non-nucleoside HIV-1-specific RT inhibitor. The compound also effectively inhibits the DNA-dependent DNA polymerase (IC_{50} = 0.73 μM) and RNA-dependent DNA polymerase (IC_{50} = 0.34 μM) activities of HIV-1 RT. Therefore, soulattrolide (**1**) may be considered a potent inhibitor of the polymerase activity of HIV-1 RT, inasmuch as activity is only about 6-fold less than that observed with 3′-azido-3′-deoxythymidine triphosphate (AZT-TP, IC_{50} = 0.051 μM),[29] and similar to that observed with (+)-calanolide A (IC_{50} = 0.32 μM), with respect to inhibition of the RNA-dependent DNA polymerase activity of HIV-1 RT.[30] In addition, ribonuclease H activity was inhibited by compound **1** (IC_{50} = 69.5 μM), even though this activity was not affected by compound **2** (unpublished data).

Another unique characteristic of coumarins (e.g., calanolide A, costatolide) is their ability to inhibit certain mutant HIV-1 RTs.[23,24] Calanolide A, for instance, was active against AZT-resistant G9106 and pyridinone-resistant A17 strains of HIV-1, which are cross-resistant to TIBO and nevirapine.[23,30] Therefore, the ability of compound **1** to inhibit mutant RT was investigated. As shown in Table 1, compound **1** had no significant inhibitory activity against a mutant (TIBO-resistant, Tyr181) HIV-1 RT (31.5% inhibition at 495 μM), possibly suggesting that the binding sites for soulattrolide (**1**) and TIBO compounds overlap.

A common problem associated with HIV RT inhibitors is toxicity. Several studies have reported that cellular DNA polymerase α, the enzyme primarily involved in cell replication, is resistant to nucleoside analogues, but both polymerases β and γ are sensitive (e.g., IC_{50} values for AZT-TP with polymerases β and γ are 11 and 16 μM, respectively).[31] The toxicity of AZT could be due to inhibition of these enzymes, and this interference with cellular polymerases could seriously limit clinical usefulness.[5,32,33] As shown in Table 1, among the cellular polymerases tested, DNA polymerase β seems to be most sensitive to compound **1**, but activity was weak and only observed at high concentrations (i.e., 49.2% inhibition at concentration of 495 μM). This inhibitory response was weaker than that observed with (+)-calanolide A (IC_{50} = 205 μM),[30] and neither DNA polymerase α nor RNA polymerase were inhibited significantly in the presence of high concentrations of compound **1** (Table 1).

In summary, comparison of the IC_{50} values obtained with HIV-1 RT (0.2−1.5 μM) with the IC_{50} values obtained with cellular polymerases (>500 μM) indicates the potential efficacy of soulattrolide (**1**). Also, as noted above, soulattrolide (**1**) represents about 29% of *C. teysmannii* latex extractables,[26] thus a sustainable source of natural product drug, nondestructive to the forest ecosystem, is readily available.

Experimental Section

Test Compounds. Soulattrolide (**1**)[27] was isolated from *C. teysmannii* latex as described previously.[26] Final purification was accomplished by preparative silica TLC using 30% EtOAc in hexane. Purity was confirmed by silica TLC using the same solvent system, with visulization by UV and iodine staining.

Reagents and Templates. [*Methyl*-^{3}H]TTP (15 Ci/mmol) and [*methyl*-^{3}H]UTP (6.5 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, CA); [^{3}H]-poly(rA)$_n$ from Amersham (Arlington Heights, IL); DNA polymerases α and β from Molecular Biology Resources, Inc. (Milwaukee, WI); RNA polymerase (*Escherichia coli*) and poly(dT)$_n$ from Pharmacia (Piscataway, NJ); and DEAE-cellulose filter disks (Whatman DE 81) from VWR Scientific (Batavia, IL). Activated calf thymus DNA, native DNA, herring sperm DNA, TTP, ATP, CTP, GTP, dATP, dCTP, dGTP, poly (rA), oligo (dT)$_{12-18}$, dithiothreitol, glutathione, gelatin, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Reverse Transcriptases (RT). Dimeric HIV-1 (p66/p51) RT was purified by modification of the procedures of Clark *et al.*[34] HIV-2 (p68/p54) RT is a recombinant enzyme consisting of two polypeptide subunits. It was synthesized in an *E. coli* expression system using a genetically engineered plasmid.[35] The enzyme possesses DNA-dependent DNA polymerase (DDDP), RNA-dependent DNA polymerase (RDDP), and ribonuclease H (RNase H) activities typical of retroviral RTs. RNase H (*E. coli*) was purchased from Promega (Madison, WI), and avian myeloblastosis virus RT (AMV RT) was purchased from Sigma Chemical (St. Louis, MO).

RT Assay. HIV-2, mutant (TIBO-resistant; Tyr181) HIV-1, and AMV RTs were assessed for RDDP activities, and HIV-1 RT was assessed for DDDP, RDDP, and RNase H activities. All RT assays were performed as previously described.[36] For RDDP assays, the reaction mixture contained the following: 50 mM Tris·HCl buffer (pH 8.0), 150 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.3 mM glutathione, 0.5 mM ethylene glycobis(β-aminoethyl ether) *N*,*N*′-tetraacetic acid (EGTA), 250 μg/mL bovine serum albumin, 41 μM poly (rA) [ϵ_{260} (mM) = 7.8], 9.5 μM oligo (dT)$_{12-18}$ [ϵ_{260} (mM) = 5.6], 20 μM TTP, and 0.5 μCi [^{3}H]TTP. The required amount of each

test compound in DMSO (10 μL) was added to the reaction mixture (80 μL) in 96-well microtiter plates (in duplicate). The reaction was started by the addition of 10 μL (0.08 μg) of RT, followed by incubation at 37 °C for 1 h. The reaction was terminated by heating the mixture to 80 °C (5 min) and then chilling on ice (15 min). Aliquots of each reaction mixture (90 μL) were spotted uniformly onto circular 2.5-cm DE-81 (Whatman) filters and kept at ambient temperature for 15 min before washing four times with 5% aqueous $Na_2HPO_4 \cdot 7H_2O$. This was followed by two more washings with distilled H_2O. Finally, the filters were thoroughly dried and subjected to scintillation counting in a nonaqueous scintillation fluid.

Each test compound was prescreened at 200 μg/mL. If inhibition at 200 μg/mL was greater than 50%, 50% inhibition concentration (IC_{50}) values were determined by performing assays with at least 10 different concentrations (in duplicate) prepared by half-log serial dilutions. For soulattrolide (**1**), the assay concentrations ranged from 5.0×10^{-4} to 1.0×10^{-9} M. AZT-TP was analyzed in a similar manner.[29] Fagaronine chloride ($IC_{50} = 13$ μM) and DMSO (10 μL) were used as positive and negative controls, respectively. Percentage inhibition was calculated as (1 − test/negative control) × 100, and IC_{50} values were estimated from dose−response curves.

DDDP assays with HIV-1 RT were performed using similar procedures, except activated calf thymus DNA (final concentration, 20 μg/mL), dGTP, dCTP, and dATP (each at a final concentration of 50 μM) and 5 μCi [^{3}H]-TTP were used. RNase H assays were performed by measuring the release of radiolabeled molecules from the synthetic substrate [^{3}H]poly(rA)$_n$·poly(dT)$_n$ into the trichloroacetic acid (TCA)-soluble fraction.[37] The reaction mixture (total volume 100 μL) contained 50 mM Tris·HCl (pH 8.0), 50 mM KCl, 8 mM $MgCl_2$, 2.5 mM DTT, and approximately 110 pmol (total nucleotides) of [^{3}H]poly(rA)$_n$·poly(dT)$_n$. The reaction was initiated by the addition of 0.5 unit of *E. coli* RNase H [1 unit of *E. coli* RNase is the amount of enzyme required to produce 1 nmol of TCA-soluble ribonucleotides from [^{3}H]-poly(rA)$_n$·poly(dT)$_n$ in 20 min at 37 °C]. The reaction mixtures were incubated at 37 °C for 30 min, terminated by transferring onto ice, followed by addition of 250 μg of herring sperm carrier DNA and 0.6 mL (10% w/v) of cold TCA. After 15 min on ice, the turbid solutions were centrifuged at 10 000 g for 10 min and the supernatants were transferred to a counting vial containing 4 mL of a scintillant and counted in a scintillation counter. The substrate [^{3}H]poly(rA)$_n$·poly(dT)$_n$ was prepared following a standard procedure:[37] 0.625 A_{260} units of poly(dT)$_n$ were annealed to 17.4 nmol of [^{3}H]poly(rA)$_n$ (specific activity 1200 dpm/pmol) in 0.3 mL of 25 mM Tris·HCl (pH 7.5) containing 0.1 M NaCl and 1 mM EDTA. The mixture was heated to 70 °C for 10 min, allowed to cool gradually to 37 °C, incubated at 37 °C for 10 min, removed to room temperature for 30 min, and finally stored on ice.

DNA Polymerase Assay. The procedure was the same as that performed for the RT assay. The reaction mixture contained the following: 80 mM Tris·HCl buffer (pH 7.5); 5 mM KCl; 10 mM $MgCl_2$; 1.5 mM dithiothreitol; 25 μg/mL bovine serum albumin; 12% (v/v) glycerol; 41 μM activated calf thymus DNA; 80 μM each of dATP, dCTP, dGTP, and ATP, and 2.0 μCi [*methyl*-^{3}H]TTP. The required amount of each test compound in DMSO (10 μL) was added to 80 μL of the reaction mixture, and the reaction was initiated by adding 0.5 units of either DNA polymerase α or DNA polymerase β. One unit is the amount of enzyme required to incorporate 1 nmol of total nucleotide into an acid-insoluble form in 60 min at 37 °C.

RNA Polymerase Assay. The procedure was the same as that performed for the RT assay. The assay mixture contained the following: 40 mM Tris·HCl buffer (pH 7.9); 150 mM KCl; 10 mM $MgCl_2$; 0.1 mM dithiothreitol, 25 μg/mL bovine serum albumin, 41 μM native calf thymus DNA; 80 μM each of ATP, CTP, and GTP; and 1.5 μCi [*methyl*-^{3}H]UTP. The required amount of each test compound in DMSO (10 μL) was added to 80 μL of the assay mixture, and the reaction was initiated by adding 0.5 units of RNA polymerase. One unit is the amount of enzyme required to incorporate 1 nmol of AMP into acid-insoluble product using poly(dA)·poly-(dT) as template-primer in 10 min at 37 °C.

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