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3',4'-DI-*O*-(–)-CAMPHANOYL-(+)-*CIS*-KHELLACTONE AND RELATED COMPOUNDS: A NEW CLASS OF POTENT ANTI-HIV AGENTS[1]

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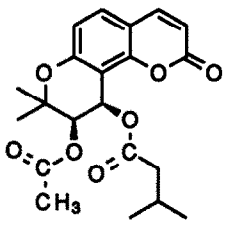
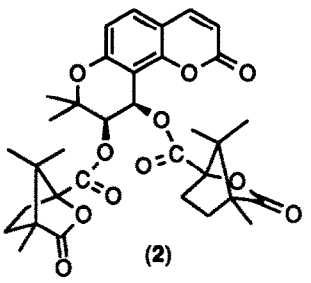
Abstract : Dihydroseselin type pyranocumarin derivatives were synthesized based on the discovery that suksdorfin (**1**) was isolated from *Lomatium saksdorfii* as an anti-HIV agent. Compound **2** demonstrated extremely potent inhibitory activity against HIV-1 replication in H9 lymphocyte cells with an EC_{50} value of 0.00041 μM and therapeutic index range of > 78,049 but < 390,244.

Recently, much effort has been focused on the search for compounds effective in the inhibition of HIV, the etiologic agent of AIDS. The result has been the identification of numerous inhibitors of HIV reverse transcriptase (RT) and protease. These include nucleoside analogs and peptide mimics, respectively. Although the RT inhibitors, such as AZT, ddI, and ddC, are available as anti-AIDS drugs, their clinical effectiveness is limited by their toxicity as well as the development of drug resistant virus. The discovery and development of a new class of anti-HIV agents with structures and mechanisms of action different from those of nucleoside analogs mentioned above are of current interest.

In the course of our continuing search for novel anti-HIV agents from natural products, suksdorfin (**1**) was isolated as an active principle from the fruits of *Lomatium suksdorfii* (Umbelliferae).[2] Compound **1** exhibited inhibitory activity against HIV-1 replication in acutely infected H9 lymphocytes with an EC_{50} value of 1.3 μM and a therapeutic index of > 40. Moreover, compound **1** was found to demonstrate a synergistic effect against HIV replication when it was co-administered with either AZT, ddI, or ddC (data not shown).

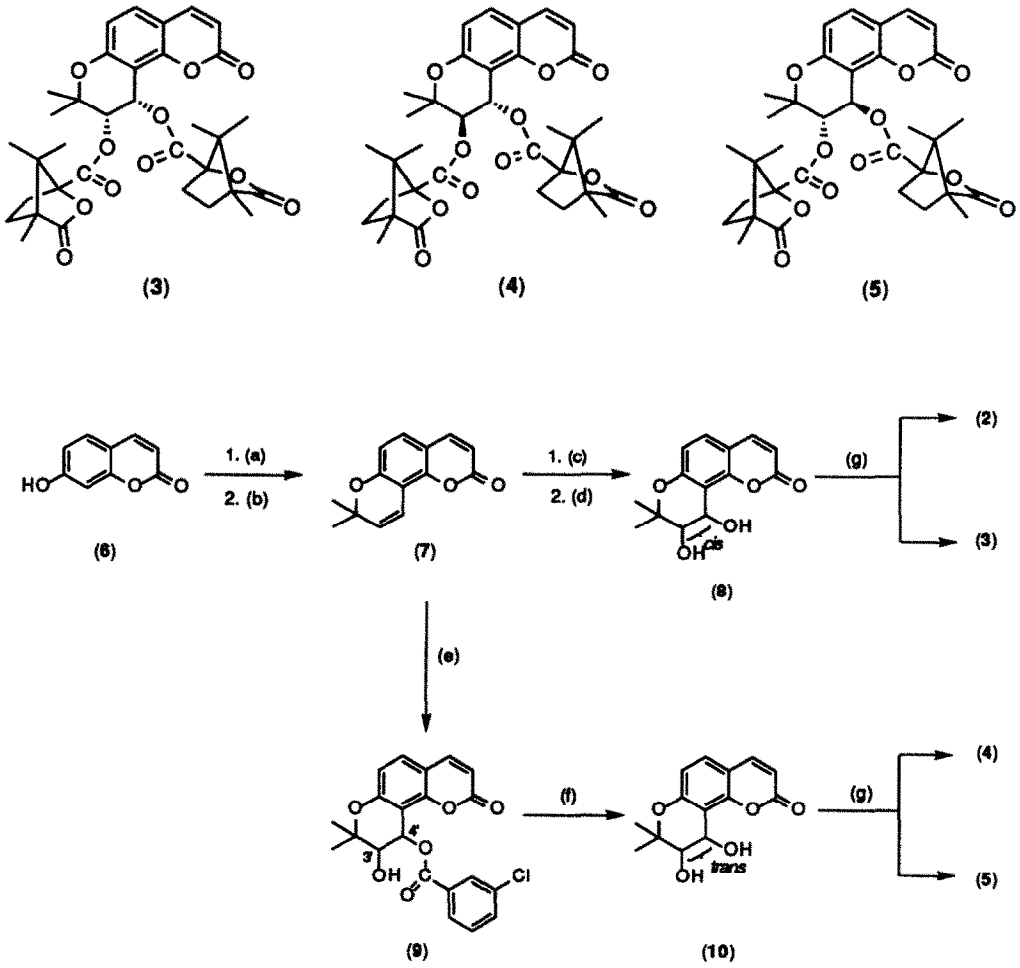


(1)



(2)

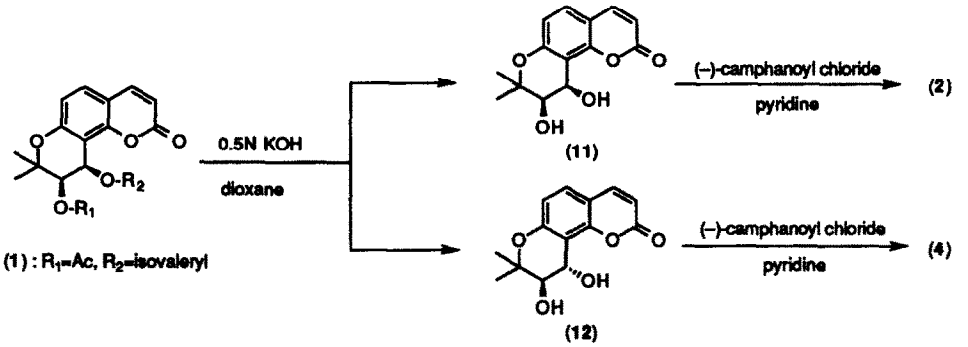
This discovery has prompted our synthesis of the dihydroseselin type pyranocoumarin derivatives (2 – 5) as a new class of anti-HIV agents.



Scheme 1. Synthesis of 3',4'-Di-*O*-Camphanoylkhellactones (2 – 5)
(a) 3-Chloro-3-methylbut-1-yne, KI, K_2CO_3 in acetone (b) diethylaniline, reflux
(c) OsO_4, dioxane (d) $NaHSO_3$ (e) *m*-chloroperbenzoic acid, $CHCl_3$ (f) 0.5 N KOH-dioxane
(g) (–)-camphanoyl chloride, pyridine

The synthesis of **2** – **5** is shown in Scheme 1. Seselin (**7**) was prepared from the commercially available 7-hydroxycoumarin (**6**) according to a procedure reported in the literature.[3] Subsequent oxidation[4] of **7** with OsO_4 gave the racemic *cis*-khellactone (**8**). Alternatively, compound **7** was treated with *m*-chloroperbenzoic acid[5] to furnish 4'-*O*-*m*-chlorobenzoyl-(±)-*trans*-khellactone (**9**), which was then hydrolyzed to produce the racemic *trans*-khellactone (**10**). Treatment of **8** and **10** with (–)-camphanoyl chloride[6] afforded diastereoisomers in each case. The diastereoisomers were separated by repeated column chromatography to yield four isomers of di-*O*-(–)-campanoylkhellactone (**2** – **5**).[7]

The stereochemistries of **2** – **5** were assigned as follows: the naturally occurring di-*O*-acyl-(+)-*cis*-khellactone (e.g., **1**) was hydrolyzed with base to give (+)-*cis*- (**11**) as well as (+)-*trans*- (**12**) khellactones.[8] Treatment of **11** and **12** with (–)-camphanoyl chloride afforded their corresponding diesters, which were found to be identical with **2** and **4**, respectively, by direct spectral comparison (Scheme 2).



Scheme 2.

As shown in Table I, compound **2** demonstrated extremely potent inhibitory activity against HIV-1 replication in acutely infected H9 lymphocytes with an EC_{50} value of 0.00041 μM. The IC_{50} range against uninfected H9 cell growth was >32 but < 160 μM, which was less toxic than the active principle (compound **1**). The therapeutic index for **2** was > 78,049 but < 390,244. Since the EC_{50} value and the therapeutic index of AZT in this assay system are 0.15 μM and 12,500, respectively, compound **2** is more potent than AZT as an anti-HIV agent.

Compound **3**, the diastereoisomer of **2**, as well as the *trans*-khellactone derivatives with same acyl groups (**4** and **5**) showed much less anti-HIV activity than **2**. Since only **1** and **2** show potent anti-HIV activity and both contain the same configuration at C-3' and C-4', the (+)-*cis*-khellactone skeleton may be required for the enhanced anti-HIV activity.

Table 1. HIV Inhibition[9] by Di-*O*-(–)-Camphanoylkhellactones (**2** – **5**), Suksdorfin (**1**), and AZT

Compounds	IC_{50} (μM)	EC_{50} (μM)	Therapeutic index
2	>32 but <160	0.00041	> 78,049 but < 390,244
3	1,700	51	>33.3
4	> 6.4 but < 32	>6.4 but < 32	>1
5	> 32	32	> 1
Suksdorfin (**1**)	>52	1.3	>40
AZT	1875	0.15	12,500

In order to determine whether the anti-HIV activity of **2** was limited to acute HIV-1 infections of the T cell line, H9, both PHA-stimulated peripheral blood mononuclear cells (PBMCs) and the promonocytic cell line, U937, were separately infected with HIV-1. The results showed that there was suppression detected no matter which type of target cell was used. This indicates that compound **2** was an effective inhibitior of virus replication regardless of cell lines tested. The EC_{50} value and the therapeutic index against PBMCs were 0.029 μM and >222 but < 1,111, while those against U937 were 0.0021 μM and >3,125 but < 15,625.

In conclusion, compound **2** and its related compounds, such as **1**, represent a new class of potent anti-HIV agents, which are structurally unique compared with other known anti-AIDS drugs. Calanolide A,[10,11] a dipyranocoumarin isolated recently from *Calophyllum lanigerum*, possesses the same pyranocoumarin subunit as **2**. However, its anti-HIV activity appears to be less potent.

Studies on the mechanism of action for **1**, **2** and other related compounds are in progress.

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7. **3',4'-Di-*O*-(–)-Camphanoyl-(+)-*cis*-Khellactone (2)** : Colorless needles (from EtOH); mp 200 – 202 °C; $[\alpha]_D^{20}$ +31.1 ° (*c*=0.5, $CHCl_3$); Positive FAB MS *m/z* 623 $(M+H)^+$, 425 (M – camphanic acid)$^+$, 227 (M – 2×camphanic acid)$^+$; IR (KBr) 1790, 1745 (COO), 1605 (C=C); ^1H NMR (300 MHz, $CDCl_3$) δ 7.62 (1H, d, *J*=9.5 Hz, H-4), 7.41 (1H, d, *J*=8.5 Hz, H-5), 6.82 (1H, d, *J*=8.5 Hz, H-6), 6.66 (1H, d, *J*=5 Hz, H-4'), 6.24 (1H, d, *J*=9.5 Hz, H-3), 5.39 (1H, d, *J*=5 Hz, H-3'), 2.50, 2.23, 1.94, 1.70 (each 2H, m, camphanoyl CH_2), 1.50, 1.45 (each 3H, s, 2'-CH_3), 1.12, 1.11, 1.10, 1.08, 1.01, 0.98 (each 3H, s, camphanoyl CH_3). Anal. Calcd for $C_{34}H_{38}O_{11}$: C, 65.58; H, 6.15. Found: C, 65.41; H, 6.21.

3',4'-Di-*O*-(–)-Camphanoyl-(–)-*cis*-Khellactone (3) : Colorless needles (from EtOH); mp 242 – 244 °C; $[\alpha]_D^{20}$ –67.7 ° (*c*=0.5, $CHCl_3$); Positive FAB MS *m/z* 623 $(M+H)^+$, 425 (M – camphanic acid)$^+$, 227 (M – 2×camphanic acid)$^+$; IR (KBr) 1780, 1750 (COO), 1605 (C=C); ^1H NMR (300 MHz, $CDCl_3$) δ 7.61 (1H, d, *J*=9.5 Hz, H-4), 7.40 (1H, d, *J*=8.5 Hz, H-5), 6.82 (1H, d, *J*=8.5 Hz, H-6), 6.74 (1H, d, *J*=4.5 Hz, H-4'), 6.22 (1H, d, *J*=9.5 Hz, H-3), 5.47 (1H, d, *J*=4.5 Hz, H-3'), 2.55, 2.34, 2.10, 1.93, 1.70 (8H in total, each m, camphanoyl CH_2), 1.56, 1.45 (each 3H, s, 2'-CH_3), 1.13, 1.12, 1.06, 1.04, 0.94 (18H in total, each s, camphanoyl CH_3). Anal. Calcd for $C_{34}H_{38}O_{11}$: C, 65.58; H, 6.15. Found: C, 65.46; H, 6.12.

3',4'-Di-*O*-(–)-Camphanoyl-(+)-*trans*-Khellactone (4) : Colorless needles (from EtOH); mp 249 – 251 °C; $[\alpha]_D^{20}$ +18.4 ° (*c*=0.5, $CHCl_3$); Positive FAB MS *m/z* 623 $(M+H)^+$, 425 (M – camphanic acid)$^+$, 227 (M – 2×camphanic acid)$^+$; IR (KBr) 1790, 1770, 1750 (COO), 1610 (C=C); ^1H NMR (300 MHz, $CDCl_3$) δ 7.63 (1H, d, *J*=9.5 Hz, H-4), 7.42 (1H, d, *J*=8.5 Hz, H-5), 6.86 (1H, d, *J*=8.5 Hz, H-6), 6.30 (1H, d, *J*=3.5 Hz, H-4'), 6.24 (1H, d, *J*=9.5 Hz, H-3), 5.39 (1H, d, *J*=3.5 Hz, H-3'), 2.50, 2.46, 2.07, 1.93, 1.66 (8H in total, each m, camphanoyl CH_2), 1.50, 1.41 (each 3H, s, 2'-CH_3), 1.12, 1.09, 1.08, 1.00, 0.98, 0.97 (each 3H, s, camphanoyl CH_3). Anal. Calcd for $C_{34}H_{38}O_{11}$: C, 65.58; H, 6.15. Found: C, 65.60; H, 6.17.

3',4'-Di-*O*-(–)-Camphanoyl-(–)-*trans*-Khellactone (5) : Colorless needles (from EtOH); mp 253 – 254 °C; $[\alpha]_D^{20}$ –42.0 ° (*c*=0.5, $CHCl_3$); Positive FAB MS *m/z* 623 $(M+H)^+$, 425 (M – camphanic acid)$^+$, 227 (M – 2×camphanic acid)$^+$; IR (KBr) 1800, 1750, 1735 (COO), 1605 (C=C); ^1H NMR (300 MHz, $CDCl_3$) δ 7.64 (1H, d, *J*=9.5 Hz, H-4), 7.41 (1H, d, *J*=8.5 Hz, H-5), 6.84 (1H, d, *J*=8.5 Hz, H-6), 6.29 (1H, d, *J*=3.5 Hz, H-4'), 6.26 (1H, d, *J*=9.5 Hz, H-3), 5.40 (1H, d, *J*=3.5 Hz, H-3'), 2.49, 2.12, 1.92, 1.68 (each 2H, m, camphanoyl CH_2), 1.50, 1.41 (each 3H, s, 2'-CH_3), 1.10, 1.09, 1.07, 1.06, 0.99 (18H in total, each s, camphanoyl CH_3). Anal. Calcd for $C_{34}H_{38}O_{11}$: C, 65.58; H, 6.15. Found: C, 65.66; H, 6.19.

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9. HIV Growth Inhibition Assay: The T cell line, H9, and the promonocytic cell line, U937, were maintained separately in continuous culture with complete medium (RPMI 1640 and 10% fetal calf serum [FCS]) at 5% CO_2 and 37° C. Cell lines were used in experiments only when in log phase of growth;

whereas, uninfected peripheral blood mononuclear cells (PBMCs) were first stimulated with PHA (1 μg/ml) for 3 days. All cell targets were incubated with HIV-1 (IIIB isolate, $TCID_{50}$ 10^4 IU/ml, at a multiplicity of infection of 0.1-0.01 IU/cell) for 1 hour at 37° C and 5% CO_2. The cell lines and PBMCs were washed thoroughly to remove unadsorbed virions and resuspended at 4 X 10^5 cells/ml in complete medium or complete medium with 10% v/v interleukin 2, IL-2, respectively. Aliquots (1 ml) were placed in wells of 24-well culture plates containing an equal volume of test compound (diluted in the appropriate culture medium). After incubation for 4 days at 37° C, cell density of uninfected cultures was determined by counting cells in a Coulter counter to assess toxicity of the test compound. A p24 antigen ELISA assay was used to determine the level of virus released in the medium of the HIV-infected cultures. The p24 antigen assay uses a HIV-1 anti-p24 specific monoclonal antibody as the capture antibody coated-on 96-well plates. Following a sample incubation period, rabbit serum containing antibodies for HIV-1 p24 is used to tag any p24 "captured" onto the microtiter well surface. Peroxidase conjugated goat anti-rabbit serum is then used to tag HIV-1 p24 specific rabbit antibodies which have complexed with captured p24. The presence of p24 in test samples is then revealed by addition of substrate. The cut-off for the p24 ELISA assay is 12.5 pg/ml. P24 in the culture medium was quantitated against a standard curve containing known amounts of p24. The effective (EC_{50}) and inhibitory (IC_{50}) concentrations for anti-HIV activity and cytotoxicity, respectively) were determined graphically. Both the EC_{50} and IC_{50} values were calculated by plotting drug concentration versus percent inhibition, and then identifying a 50% inhibition value from the graph.

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