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ANTI-AIDS AGENTS 33.[1] SYNTHESIS AND ANTI-HIV ACTIVITY OF MONO-METHYL SUBSTITUTED 3',4'-DI-*O*-(-)-CAMPHANOYL-(+)-*CIS*-KHELLACTONE (DCK) ANALOGUES

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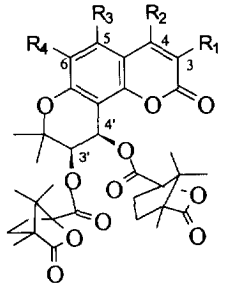
Abstract: Four isomeric methyl substituted DCK analogues (**2-5**) were asymmetrically synthesized from different starting materials. 3-Methyl, 4-methyl, and 5-methyl-3',4'-di-*O*-(-)-camphanoyl-(+)-*cis*-khellactone (**2**–**4**) all were extremely potent against HIV-1 replication in H9 lymphocyte cells with EC_{50} and therapeutic index values of $< 4.23 \times 10^{-7}$ μM and $>3.72 \times 10^{8}$, respectively, which are much better than those of DCK and AZT in this assay.

In previous research,[2,3] we found that 3',4'-di-*O*-*S*-(-)-camphanoyl-*3'R,4'R*-(+)-*cis*-khellactone (DCK) (**1**) exhibited potent inhibitory activity against HIV-1 replication in H9 lymphocyte cells with EC_{50} and therapeutic index values of 0.000256 μM and 136,719, and was more potent than AZT as an anti-HIV agent in the same assay. The three stereoisomers of DCK were much less active; thus, the anti-HIV activity of DCK was stereoselectivity for its target. In addition, the presence of the (-)-*S*-camphanoyl group greatly enhanced the anti-HIV activity of DCK. These results suggested that (+)-*cis*-*3'R,4'R*-khellactone derivatives should be a novel class of anti-HIV compounds and prompted us to further explore the structure-activity relationships for this compound type.



DCK (1)	R_1= R_2= R_3= R_4= H
2	R_1= CH_3, R_2= R_3= R_4= H
3	R_2= CH_3, R_1= R_3= R_4= H
4	R_3= CH_3, R_1= R_2= R_4= H
5	R_4= CH_3, R_1= R_2= R_3= H

In the structure of DCK, substituents can be introduced at four positions on the coumarin ring. Recently, we reported the synthesis of four isomeric mono-methoxy substituted DCK analogues,[4] in which the methoxy group was at the 3-, 4-, 5-, or 6-position of the coumarin ring. Their anti-HIV assay results indicated that the 3-ethoxy, 4-methoxy, and 5-methoxy substituted DCK analogues all were more potent than AZT. The best

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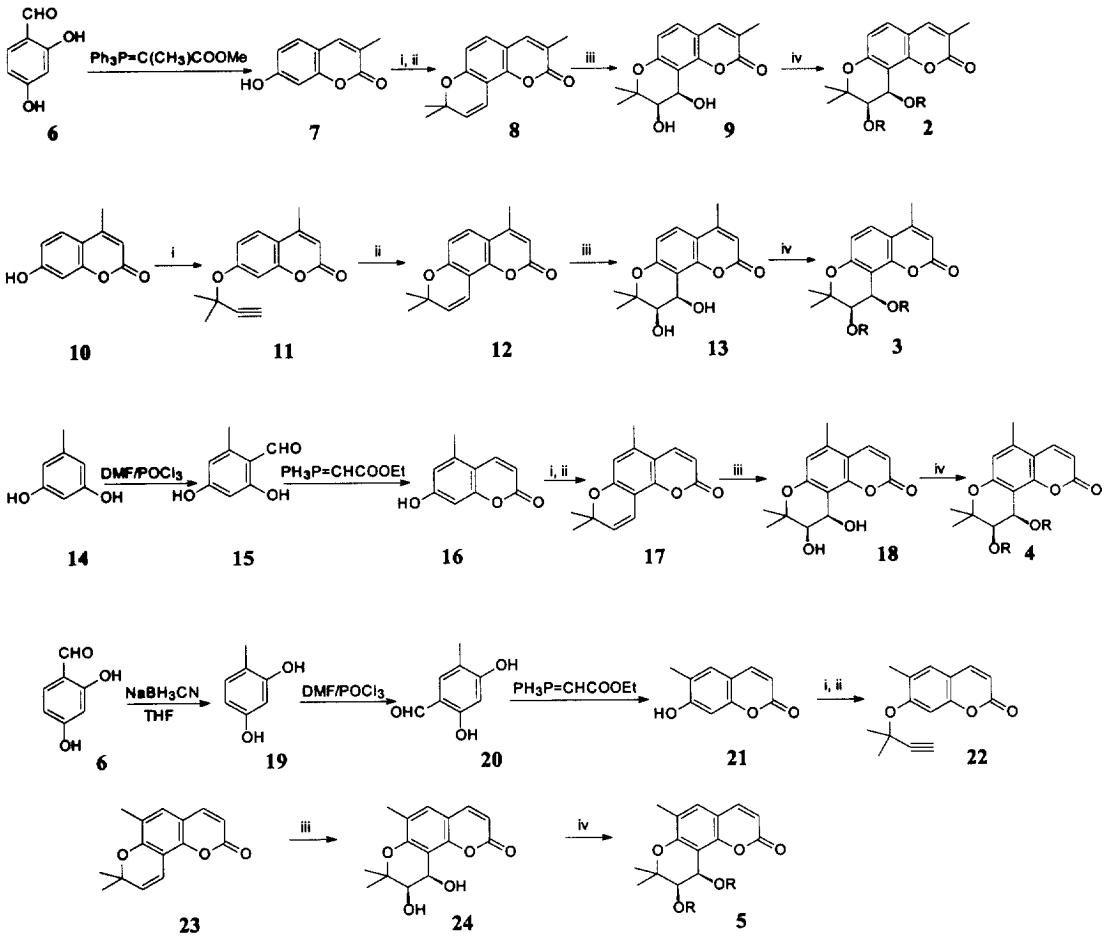
compound was 5-methoxy DCK with EC_{50} and TI values of 0.00013 μM and 867,555, respectively, which were better than those of DCK. In order to explore the effect of different substituents at these four positions, we asymmetrically synthesized four isomeric methyl substituted DCK analogues, 3-methyl, 4-methyl, 5-methyl, and 6-methyl-di-*O*-*S*-(-)-camphanoyl-*3'R,4'R*-(+)-khellactone (**2–5**). In this paper, we report their synthesis and anti-HIV activity in the H9 cell line.

The four isomeric compounds **2–5** were synthesized from different starting materials by corresponding synthetic routes, as shown in Scheme 1. First, the substituted 7-hydroxy coumarins (**7, 16,** and **21**) were synthesized. 3-Methyl-7-hydroxy-coumarin (**7**) was prepared by the Wittig reaction of 2,4-dihydroxybenylaldehyde (**6**) with $Ph_3PC{=}C(CH_3)COOMe$. 3,5-Dihydroxy-toluene (**14**) reacted with DMF/$POCl_3$ complex to produce 2,4-dihydroxy-5-methyl-benzlaldehyde (**15**). Compound **15** then underwent a Wittig reaction with $Ph_3PC{=}CHCOOEt$ to afford 5-methyl-7-hydroxy-coumarin (**16**). 2,4-Dihydroxy-toluene (**19**) was prepared in a 98% yield by reducing **6** with $NaBH_3CN$.[5] Using the same methods as in the preparation of **16**, 6-methyl-7-hydroxy-coumarin (**21**) was synthesized from **19**. 4-Methyl-7-hydroxy-coumarin (**10**) was commercially available.

The methyl substituted seselins (**8, 12, 17,** and **23**) were separately synthesized in two steps — a nucleophilic substitution reaction with 3-chloro-3-methylbut-1-yne and a Claisen rearrangement — from the correspondingly substituted 7-hydroxy-coumarins **7, 10, 16,** and **21**. The yields ranged from 30–60%. Next, these four isomeric methyl substituted seselins (**8, 12, 17,** and **23**) separately underwent the Sharpless' asymmetric dihydroxylation reaction[6] with $(DHQ)_2$-PYR as a chiral catalyst[7] to selectively produce the methyl substituted *3'R,4'R*-(+)- *cis*-khellactones (**9, 13, 18,** and **24**).[8] Their percent enantiomeric excess (% e.e.) ranged from 81–90%.[9] Finally, compounds **9, 13, 18,** and **24** were esterified with (-)-*S*-camphanoyl chloride at rt for 48 h to afford four isomeric methyl substituted DCK analogues (**2, 3, 4,** and **5**, respectively).[10] The ^1H NMR data of **2–5** are listed in Table 1.

The methyl substituted DCK analogues **2–5** were tested against HIV-1 replication in the H9 cell line. Their anti-HIV activities are shown in Table 2. The 3-methyl and 4-methyl DCK compounds (**2** and **3**) exhibited significant anti-HIV activity in this assay. Both had the same EC_{50} (1.57×10^{-7} μM) and TI (1×10^9) values. The 5-methyl DCK compound (**4**) also showed potent anti-HIV activity with an EC_{50} value of 4.23×10^{-7} μM and TI of 3.72×10^8, and was only slightly less active than compounds **2** and **3**. However, all of these values are much better than those of DCK. These results indicated that modification at the 3-, 4-, and 5-positions on the coumarin nuclei enhanced the anti-HIV activity of DCK, whereas substitution at the 6-position (compound **5**) significantly decreased the anti-HIV activity. These results were consistent with those found with the methoxy DCK analogues. However, the methyl substitution was obviously preferable to the methoxy substitution. Thus, we suggest that the presence of a methyl group on the coumarin nucleus might be important to the anti-HIV activity for this compound type.



Scheme 1. Synthesis of mono-methyl substituted 3',4'-di-*O*-(-)-camphanoyl-(+)-*cis*-khellactones **(2–5)**

i. 3-Chloro-3-methylbut-1-yne, KI, K_2CO_3 in DMF
ii. N,N-Diethylaniline, reflux
iii. $K_2OsO_2(OH)_4$, K_2CO_3, $K_3Fe(CN)_6$, $(DHQ)_2$-PYR in t-BuOH/H_2O, 0°C
iv. (-)-Camphanoyl chloride, pyridine in CH_2Cl_2

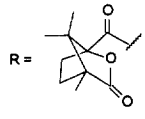


Table 1. ^{1}H NMR Data of Methyl Substituted-DCK Analogues **(2–5)**

Proton	Compound 2 δ ppm (*J*)	Compound 3 δ ppm (*J*)	Compound 4 δ ppm (*J*)	Compound 5 δ ppm (*J*)
H-3	2.16 (s, CH_3)	6.13 (s)	6.23 (d, 9.8)	6.22 (d, 9.8)
H-4	7.43 (s)	2.41 (s, CH_3)	7.80 (d, 9.8)	7.58 (d, 9.8)
H-5	7.35 (d, 8.8)	7.54 (d, 8.8)	2.43 (s, CH_3)	7.26 (s)
H-6	6.80 (d, 8.8)	6.85 (d, 8.8)	6.67 (s)	2.23 (s, CH_3)
H-3'	5.40 (d, 4.8)	5.40 (d, 4.8)	5.36 (d, 4.8)	5.39 (d, 4.8)
H-4'	6.66 (d, 4.8)	6.67 (d, 4.8)	6.62 (d, 4.8)	6.66 (d, 4.8)
CH_2 (x 4)	2.50–2.47 (m)	2.52–2.41 (m)	2.53–2.43 (m)	2.53–2.43 (m)
	2.28–2.21 (m)	2.25–2.16 (m)	2.26–2.17 (m)	2.28–2.18 (m)
	1.96–1.89 (m)	2.20–1.88 (m)	1.95–1.87 (m)	1.98–1.84 (m)
	1.76–1.62 (m)	1.76–1.66 (m)	1.75–1.65 (m)	1.75–1.56 (m)
CH_3 (x8)	2.19 (s, 3H)	2.41 (s, 3H)	2.05 (s, 3H)	2.05 (s, 3H)
	1.49 (s, 3H)	1.69 (s, 3H)	1.48 (s, 3H)	1.49 (s, 3H)
	1.45 (s, 3H)	1.50 (s, 3H)	1.44 (s, 3H)	1.47 (s, 3H)
	0.96–1.15 (m.s.)	1.27 (s, 3H)	1.24 (s, 3H)	0.92–1.12 (m.s.)
		0.98–1.14 (m.s.)	0.97–1.13 (m.s.)	

Table 2. Anti-HIV Activity of DCK and Its Analogues **(2–5)** in Acutely Infected H9 Lymphocytes[11]

Compound	IC_{50} (μM) [a]	EC_{50} (μM) [b]	Therapeutic index [c]
2	> 157[d]	0.000000157	> 1,000,000,000
3	> 157[d]	0.000000157	> 1,000,000,000
4	> 157[d]	0.000000423	> 372,000,000
5	33	0.15	220
DCK (1)	35	0.000256	136,719
AZT	1875	0.045	41,667

[a] concentration that inhibits uninfected H9 cell growth by 50%.
[b] concentration that inhibits viral replication by 50%.
[c] TI = IC_{50}/EC_{50} .
[d] Maximum IC_{50} value possible for this assay due to the presence of DMSO which is used to solubilize the agents tested.

Based on our research results, we therefore considered that modification of the coumarin moiety in DCK is significant for enhancing anti-HIV activity. In principle, the molecular skeleton and stereochemistry determine the three-dimensional orientation of a molecule. On the other hand, various substituents located on the boundary of this molecular skeleton may directly influence molecular bioactivity because they can change the molecular size, shape and some pharmacological properties, such as affinity with receptor/enzyme, solubility, and stability. The synthesis and anti-HIV evaluation of methyl substituted DCK analogs, as well as methoxy substituted DCK analogs reported previously, indicate that modification can take place at the 3-, 4-, and 5-positions of coumarin nuclei. Introduced methyl and methoxy groups all increased the molecular size, which may be profitable for binding between the molecule and its target. However, the effect of different substituents at these positions for anti-HIV activity should be more extensively investigated. Further modification of DCK is in progress.

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7. $(DHQ)_2$-PYR: Hydroquinine 2,5-diphenyl-4,6-pyrimidinediyl diether.
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9. The percent enantiomeric excess was determined by ^1H NMR analysis of the bis-(-)-camphanic esters.
10. **3-Methyl-3',4'-di-*O*-*S*-camphanoyl-(+)-*cis*-khellactone (2)** (% d.e. >90)**:** mp 143–5 °C; $[\alpha]_D$ +21.08° (*c* 0.37, $CHCl_3$); EA for $C_{35}H_{40}O_{11} \cdot H_2O$: Theory: C, 64.21; H, 6.47; Found: C, 64.56; H, 6.49.
4-Methyl-3',4'-di-*O*-(-)-camphanoyl-(+)-*cis*-khellactone (3) (% d.e. 84)**:** mp 264–7 °C; $[\alpha]_D$ +8.4° (*c* 0.50, $CHCl_3$); EA for $C_{35}H_{40}O_{11} \cdot 2½\ H_2O$: Theory: C, 61.76; H, 6.52. Found: C, 61.94; H, 6.23.

5-Methyl-3',4'-di-*O*-(-)-camphanoyl-(+)-*cis*-khellactone (4) (% d.e. 81): mp 163–4°C; $[\alpha]_D$ +18.92° (*c* 0.37, $CHCl_3$); EA for $C_{35}H_{40}O_{11}\cdot 1\frac{1}{2}\ H_2O$: Theory: C, 63.34; H, 6.53. Found: C, 63.74; H, 6.35.

6-Methyl-3',4'-di-*O*-(-)-camphanoyl-(+)-*cis*-khellactone (5) (% d.e. 87): mp 206–7°C; $[\alpha]_D$ +8.45° (*c* 0.97, $CHCl_3$); EA for $C_{35}H_{40}O_{11}\cdot H_2O$: Theory: C, 64.21; H, 6.47. Found: C, 64.47; H, 6.39.

11. HIV Growth Inhibition Assay. The T cell line, H9, was maintained in continuous culture with complete medium (RPMI 1640 with 10% fetal calf serum [FCS] supplemented with L-glutamine at 5% CO_2 and 37 °C. Aliquots of this cell line were only used in experiments when in log-phase of growth. Test samples were first dissolved in dimethyl sulfoxide (DMSO). The following were the final drug concentrations routinely used for screening: 100, 20, 4 and 0.8 μg/mL, but for active agents additional dilutions were prepared for subsequent testing so that an accurate EC_{50} value could be achieved. As the test samples were being prepared, an aliquot of the T cell line, H9, was infected with HIV-1 (IIIB isolate) while another aliquot was mock-infected with complete medium. The mock-infected aliquot was used for toxicity determinations (IC_{50}). The stock virus used for these studies typically had a $TCID_{50}$ value of 10^4 Infectious Units/mL. The appropriate amount of virus for a multiplicity of infection (moi.) between 0.1 and 0.01 Infectious Units/cell was added to the first aliquot of H9 cells. The other aliquot of H9 cells only received culture medium and then was incubated under identical conditions as the HIV-infected H9 cells. After a 4 h incubation at 37 °C and 5% CO_2, both cell populations were washed 3 times with fresh medium and then added to the appropriate wells of a 24 well-plate containing the various concentrations of the test drug or culture medium (positive infected control/negative drug control). In addition, AZT was also assayed during each experiment as a positive drug control. The plates were incubated at 37 °C and 5% CO_2 for 4 days. Cell-free supernatants were collected on Day 4 for use in our in-house p24 antigen ELISA assay. P24 antigen is a core protein of HIV and therefore is an indirect measure of virus present in the supernatants. Toxicity was determined by performing cell counts by a Coulter Counter on the mock-infected H9 cells that had either received culture medium (no toxicity) or test sample or AZT.