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Equisetin and a Novel Opposite Stereochemical Homolog Phomasetin, Two Fungal Metabolites as Inhibitors of HIV-1 Integrase

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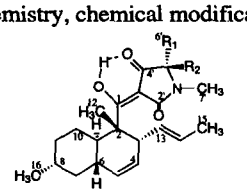
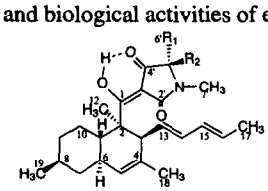
Abstract: Integration is an essential step in HIV replication and is catalyzed by an enzyme called integrase. We have isolated equisetin (**1a**), and a novel opposite stereochemical homolog, phomasetin (**2a**), from *Fusarium heterosporum* and a *Phoma* sp. respectively. They inhibit the *invitro* recombinant integrase enzyme with IC_{50} values of 7-20 μM. Unlike known inhibitors, these compounds also inhibit the integration reactions catalyzed by preintegration complexes isolated from HIV-1 infected cells.

Integration is an essential and defining step in the replication of retroviruses[1] in general and HIV in particular and is carried out by an enzyme appropriately called integrase. This enzyme is the single known enzyme that catalyzes all of the steps that ultimately lead to insertion of viral DNA into the genome of the host cell. The catalysis includes 3'-end processing cleavage of viral DNA, non-specific nicking of host DNA and strand transfer of processed viral DNA to the host DNA.[2] This enzyme appears to be very specific to HIV and seems to be absent in the host, and therefore, is a potential target for the development of highly selective anti-HIV chemotherapeutic agents.

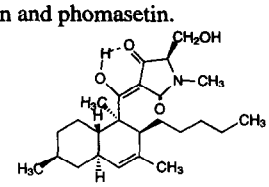
Screening of microbial extracts for natural product inhibitors of integrase using recombinant enzyme[3] resulted in identification of a number of active broths. Bioassay guided separation[4] of the extracts of one the active broth, identified as *Fusarium heterosporum*, resulted in the isolation of equisetin (**1a**). Extract of another organism, identified as *Phoma* sp., led to the isolation of a novel enantiomeric homolog, phomasetin (**2a**). Both equisetin and phomasetin possess two distinct structural halves: the upper tetramic acid type hydrophilic half and the lower hydrophobic bicyclic half. In this paper, we wish to describe the isolation, structure elucidation, stereochemistry, chemical modification and biological activities of equisetin and phomasetin.



1a: R_1=CH_2OH, R_2=H
1b: R_1=H, R_2=CH_2OH



2a: R_1=H, R_2=CH_2OH
2b: R_1=CH_2OH, R_2=H



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Size exclusion chromatography (Sephadex LH-20) of methyl ethyl ketone extracts of the fermentation broth of *F. heterosporum* (MF6069, ATCC 74349) and *Phoma* sp. (MF6070, ATCC 74348) followed by reverse phase (C-8) HPLC afforded equisetin, **1a** (340 mg/L) and phomasetin, **2a** (90 mg/L) respectively as colorless powders.

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STRUCTURE ELUCIDATION

***Equisetin* (1a).** Electron impact (EI) mass spectral analysis of **1a** indicated a molecular ion at *m/z* 373 (48%) and subsequent high resolution measurement indicated the molecular formula $C_{22}H_{31}NO_4$. The ^1H NMR spectrum in $CDCl_3$ gave broad signals, but the ^{13}C NMR spectrum revealed 22 carbons, consistent with the molecular formula. C-13 similarity search in our local SIMSER[5] database indicated that this newly isolated compound was a perfect match for equisetin, a toxin originally isolated from *F. equiseti* in 1974.[6b,c] In 1989, it was reisolated by Phillips et al,[6c] the structure elucidated and subsequently confirmed by total synthesis.[7] Physical and spectroscopic data including NMR, UV, IR, mass spectral fragmentations, specific rotation[8] and CD spectrum of **1a** were identical to those of equisetin. The identity of equisetin was confirmed by comparison (HPLC and LC-MS) with an authentic sample.[7] Equisetin is reported to be a potent inhibitor (IC_{50} 8 nM) of DNP-stimulated ATPase activity of liver mitochondria and mitoplast.[9]

***Phomasetin* (2a).** EI mass spectral analysis of phomasetin gave a molecular ion at *m/z* 413 (100%). High resolution measurements of the molecular ion suggested the molecular formula $C_{25}H_{35}NO_4$which was supported by the ^{13}C NMR spectrum. This formula indicated that phomasetin has an additional C_3H_4 unit compared to equisetin. Except for these three additional carbons and their effect on the respective chemical shifts, ^{13}C NMR spectra (Table 1) of phomasetin and equisetin were virtually identical. The UV spectrum of **2a** was also similar to that of **1a** except for the expected difference in the extinction coefficient.[10] The infrared spectrum showed absorption bands for hydroxy (3389 cm^{-1}), and conjugated ketones (1690, 1664 cm^{-1}). As in the case of equisetin, the ^1H NMR spectrum of phomasetin in $CDCl_3$ was extremely broad, but the spectrum sharpened considerably when recorded in CD_3CN. Careful analysis of the ^1H NMR spectrum indicated that phomasetin contained an additional olefin and an additional olefinic methyl group both in the hydrophobic half of the molecule. This result was corroborated by the presence of two additional olefinic carbons in the ^{13}C NMR spectrum of **2a**. Like equisetin, phomasetin shows a broad singlet for the chelated exchangeable hydroxy group at δ 17.42 ppm. The ^1H NMR spectrum (Table 1) was completely assigned by application of ^1H-^1H COSY and TOCSY experiments. The ^{13}C NMR spectrum (Table 1) of **2a** was assigned with the help of HMQC and HMBC experiments.

The COSY and TOCSY correlations of H-3 to H-13 and subsequent correlations to the respective olefinic protons, ultimately extending to the H-17 methyl group, indicated that the extra olefin was a part of the extended conjugation in the side chain at C3. This was further substantiated by the HMBC correlations (Table 1). Lack of a coupling partner for H-5 of the $\Delta^{4,5}$ endocyclic double bond indicated substitution at C4 by the remaining methyl group, and this was confirmed by the HMBC correlations of H_3-18 to C-3, C-4 and C-5. The H-5 proton did not show any measurable coupling to H-6 and appeared as a broad singlet. This could be explained by a ~90° dihedral angle between these two protons. The ^{13}C NMR spectrum was unambiguously assigned by careful examination of the HMBC correlations and these assignments are presented in Table 1. Mass spectral fragmentation of phomasetin gave major fragment ions at *m/z* 170 ($C_7H_8NO_4$) and 243 ($C_{18}H_{27}$) due to α-cleavage between C1 and C2 and supported the structural assignment of **2a**. Hydrogenation with 5% Pd/C at ambient conditions gave tetrahydro derivative **3** (*m/z* 417) selectively.

RELATIVE AND ABSOLUTE STEREOCHEMISTRY

The relative stereochemistry of phomasetin was determined by a NOESY experiment (400 MHz, CD_3CN). Strong 1,3-diaxial correlations between protons at H-6, 8 and H-10; H-7, 9, and H-11; and H-6 to H_3-12 indicated a 6,11-*trans* ring fusion between the cyclohexyl chair and cyclohexenyl boat rings. The NOESY correlations between H_3-12 and H-3 indicated their *syn* relationship. The NOESY derived stereochemistry and conformation of phomasetin is depicted in Figure 1. The optical rotation (+93.9°) and the CD spectrum ($+5.2_{290}$)[10] of phomasetin (**2a**) were opposite in sign to those of equisetin ($[\alpha]_D$ -273° and CD -8.9_{290})[8] (**1a**) suggesting opposite stereochemical relationship. Epimerization of **1a** and **2a** in pyridine gave a ~3:2 epimeric mixtures of **1a/1b** and **2a/2b** respectively. HPLC purification of the respective mixtures gave 5'-epiequisetin (**1b**) and 5'-epiphomasetin (**2b**). The CD spectrum of **1b** was qualitatively similar to the spectrum of **1a**; likewise the CD spectrum of **2b** was similar to that of **2a**. However, the stereochemistry

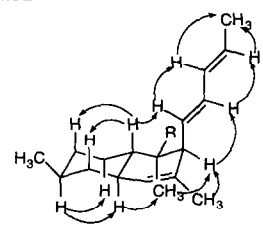

Figure 1: NOESY

at C-5' for **2a** was determined to be opposite to that of equisetin based on the comparison of the ^{1}H NMR shifts of H-5' and H-6' in CD_3CN at -10 °C. Each α and β H-6' prochiral protons of all four compounds showed a broad and a sharp doublet of doublets in between 3-4 ppm. The stereochemical insight for C-5' came from the careful examination of these resonances. In **1a** and **2a** pair, the broad doublet of doublets was shifted downfield when compared to the sharp doublet of doublets whereas it was shifted upfield in **1b** and **2b** pair. The similarites and dissimilarities of the ^{1}H NMR spectrum of these protons would indicate that these pairs would have similar relative stereochemistries. Therefore, the absolute stereochemistry of the labile center at C-5' may also be opposite to that of equisetin and thus the stereochemistry of equisetin and phomasetin at all stereocenters are opposite, a rather rare occurence in nature. As expected, the CD spectrum[11] of the synthetic C-5'-equilibrated *ent*-equisetin (**4**) showed positive Cotton effects ($+12.5_{291}$), and was almost identical to the CD spectrum of phomasetin, and was a mirror image of the CD spectrum of equisetin.

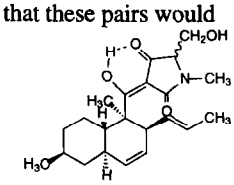

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Table 1: NMR assignments of Phomasetin in CD_3CN at 25°C

position	Equisetin (1a) δC[6c] ($CDCl_3$) -20°C	Phomasetin (2a) δC CD_3CN	mult (APT)	δH CD_3CN	HMBC (C ⇨ H)
1	190.6	197.52	C°	—	H-12
2	48.4	49.90	C°	—	H-3, H-12
3	44.6	50.22	CH	3.17, brd, 9	H-5, H-12, H-14, H-18
4	127.1*	132.45	C°	—	H-3, H-6, H-18
5	130.4**	127.08	CH	5.22, brs	H-3, H-6, H-18
6	38.4	40.07	CH	1.84, m	H-5
7	41.9	43.14	CH_2	*e*: 1.78, m	H-5, H-6, H-19
				a: 0.85, appq, 12	
8	33.3	34.28	CH	1.50, m	H-6, H-7, H-19
9	35.5	36.59	CH_2	*a*: 1.74, m	H-19
				e: 1.04, m	
10	28.1	28.97	CH_2	*e*: 1.96, m	H-6
				a: 1.05, m	
11	39.6	40.55	CH	1.62, m	H-3, H-5, H-6, H-7, H-12
12	13.7	14.23	CH_3	1.38, brs	H-3,
13	129.8**	131.59	CH	5.25, dd, 12, 10	H-3, H-15
14	126.2*	133.43	CH	5.78, dd, 15, 11	H-3, H-15, H-16
15	18.2	132.16	CH	5.91, appt, 13	H-13, H-14, H-17
16	22.5	129.24	CH	5.55, dq, 13, 7	H-14, H-17
17	---	18.15	CH_3	1.66, d, 7	H-15, H-16
18	---	22.53	CH_3	1.55, appt, 1.5	H-3, H-5
19	---	22.75	CH_3	0.90, d, 6.5	H-7
2'	176.7	178.02	C°	—	H-5', H-7'
3'	99.8	101.60	C°	—	---
4'	198.9	191.54	C°	—	H-5', H-6'
5'	66.4	68.79	CH	3.60, t, 2.5	H-6', H-7'
6'	60.0	59.62	CH_2	3.87, brdd, 12, 3	H-5'
				3.80, dd, 12, 3	
7'	27.2	27.45	CH_3	2.97, brs	—
C1-OH		–	–	17.42, brs	—

* and ** may be interchanged[6c]

BIOGENESIS

These compounds are probably biosynthesized from a suitably substituted olefinic polyketide through a biological intramolecular Diels-Alder-type reaction. The stereochemistry of cyclization is dictated by that of the methyl group at C-8 with a predisposed stereochemistry. In order to avoid steric hindrance the methyl group must occupy an equatorial orientation in both cases hence resulting in opposite stereochemistries during cyclization.

BIOLOGICAL ACTIVITY

Equisetin (**1a**) and phomasetin (**2a**) and derivatives **1b**, **2b** and **3** inhibited the 3' end-processing reaction with IC_{50} values between 7 to 15 μM and the strand transfer reaction IC_{50} values between 15 and 20 μM respectively.[4] Despite being enantiomeric, the compounds are almost equally active in these assays. Unlike previously described inhibitors, these compounds also inhibited the integration reactions catalyzed by preintegration complexes isolated from HIV-1 infected cells. To the best of our knowledge, these are the first natural product inhibitors of integrase.

ACKNOWLEDGMENTS

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8. **1a:** $[\alpha]^{22}_{D}$ = -278° (c, 0.77, $CHCl_3$), was higher than that of synthetic equisetin -253° (c, 0.038, $CHCl_3$)[7] and almost twice that of previously isolated natural equisetin (-146°). This discrepancy may be due to higher degree of purity of the current sample, and better accuracy of the measurement due to a higher concentration used in the current measurement. CD (CH_3OH) $\Delta\varepsilon$ (λ_{max}): 0 (330), -8.9 (290), -3 (260), -7.5 (235), -5.5 (227). ^1H NMR (CD_3CN at -10 °C): only important signals shown, 3.57 (1H, brt, J = 2.5 Hz, H-5'), 3.77 (1H, dd, J = 2.5, 12 Hz, H-6'), 3.85 (1H, brdd, J = 2.5, 12 Hz, H-6'); **1b**: CD spectrum identical to **1a**, ^1H NMR (CD_3CN at -10 °C): only important signals shown, 3.62 (1H, t, J = 2.5 Hz, H-5'), 3.81 (1H, brdd, J = 2.5, 12 Hz, H-6'), 3.86 (1H, dd, J = 2.5, 12 Hz, H-6').
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10. Phomasetin (**2a**): Crystallized from hot benzene as powder, mp.140-43 °C, $[\alpha]^{22}_{D}$ = +93.9° (c, 1.05, $CHCl_3$), UV (CH_3OH) λ_{max}: 290 (ε 5422), 235 (7541), CD (CH_3OH) $\Delta\varepsilon$ (λ_{max}): 0 (330), + 5.2 (290), +1.0 (260), +4.4 (232), +3.2 (225), IR (ZnSe) ν_{max} 3389, 2924, 1690, 1664 (fused with 1690), 1568, 1455, 1379, 1266, 1169, 1059, 976 cm^{-1}, ^1H NMR (CD_3CN at -10 °C): only important signals shown, 3.60 (1H, brt, J = 2.5 Hz, H-5'), 3.78 (1H, dd, J = 2.5, 12 Hz, H-6'), 3.86 (1H, brdd, J = 2.5, 12 Hz, H-6'); HREIMS (*m/z*): 413.2565 (M^+, 100% calcd. for $C_{25}H_{35}NO_4$: 413.2566), 243.2044 (50% calcd. for $C_{18}H_{27}$: 243.2113), 170.0472 (60% calcd. for $C_7H_8NO_4$: 170.0453), **2b**: CD spectrum identical to **2a**, ^1H NMR (CD_3CN at -10 °C): only important signals shown, 3.61 (1H, brt, J = 2.5 Hz, H-5'), 3.84 (1H, brdd, J = 2.5, 12 Hz, H-6'), 3.87 (1H, dd, J = 2.5, 12 Hz, H-6').
11. CD spectrum of C-5' equilibrated *ent*-equisetin (**4**): (CH_3OH) $\Delta\varepsilon$ (λ_{max}): 0 (330), +12.5 (291), +4.0 (250), +6 (235).