

KAMPANOLS: NOVEL RAS FARNESYL-PROTEIN TRANSFERASE INHIBITORS FROM *STACHYBOTRYS KAMPALENSIS*

Sheo B. Singh,^{*} Deborah L. Zink, Marie Williams, Jon D. Polishook,
Manuel Sanchez,[†] Keith C. Silverman, and Russell B. Lingham

Natural Products Drug Discovery, Merck Research Laboratories, P.O. Box 2000, Rahway, NJ 07065, U.S.A.

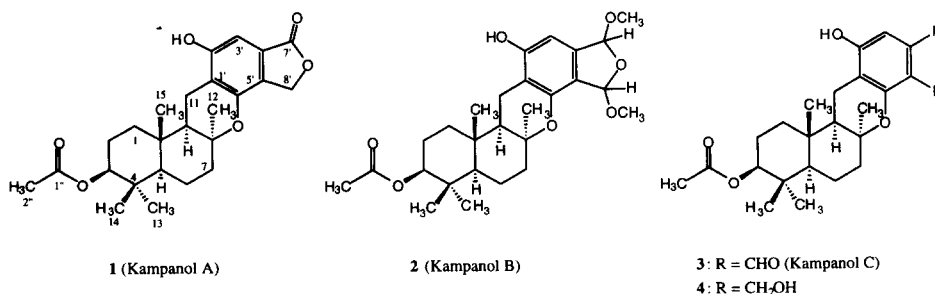
[†]Centro de Investigación Básica, Merck Sharp & Dohme de España S.A.,
Josefa Valcárcel 38, 28027, Madrid, Spain

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Abstract: Farnesyl-protein transferase (FPTase) is a critical enzyme that participates in the post-translational modification of the Ras protein. Inhibitors of this enzyme have the potential of being novel anticancer agents for tumors in which the *ras* oncogene is found mutated and contributes to cell transformation. Continued screening of natural product extracts led to the isolation of kampanols, which are novel and specific inhibitors of FPTase. The most active kampanols exhibited IC₅₀ values between 7 to 13 μ M against human recombinant FPTase. The isolation, structure determination, and biological activity of these compounds are described. © 1998 Elsevier Science Ltd. All rights reserved.

The *ras* oncogene, is mutated in a large number of different types of cancer, and codes for a protein known as Ras (p21). The carboxy terminal CaaX box of Ras undergoes several post-translational modifications that are essential for cell transforming activity.¹ The first and critical step of post-translational modification is farnesylation of the cysteine of the CaaX box by FPTase. It has now been demonstrated that inhibitors of FPTase have the potential to become anticancer agents for tumors in which the *ras* gene is mutated and contributes to cell transformation.²

Recently, we and several other groups have reported the discovery of a number of inhibitors of FPTase. These inhibitors are either rationally designed CaaX mimetics (L-731,734,³ benzodiazepines⁴) or derived from random screening of natural product extracts or chemical collections. Examples of natural product inhibitors are chaetomelic acids,⁵ actinoplanic acids,⁶ oreganic acid,⁷ cylindrols,⁸ fusidienols,⁹ gliotoxin,¹⁰ CP-225,917 and CP-263,114,¹¹ and peptidinnamins.¹² Examples of leads derived from the screening of chemical collections are Sch44342 and analogs.¹³ Our continued interest to find non peptide inhibitors of FPTase led to the discovery of kampanols A–C (1–3), which are novel, from a fungal culture *Stachybotrys kampalensis* Hansf., isolated from leaf litter, collected from Bagaces, Parque Nacional Palo Verde, Province de Guanacaste, Costa Rica.



The bioassay guided isolation, structural elucidation, relative stereochemistry and FPTase activity of these tetra and pentacyclic aromatic sesquiterpenoids named herein as kampanols A–C (1–3) and the sodium borohydride reduction product (4) are described.

Isolation

A 20 mL solid state fermentation of fungus *S. kampalensis* (MF 6199, ATCC74357) was extracted with methyl ethyl ketone and the extract was partitioned between aqueous methanol, hexane, methylene chloride and ethyl acetate. All of the FPTase activity was concentrated in the latter two extracts. Size exclusion chromatography of the combined active extracts on Sephadex LH-20 resulted the FPTase activity in a broad zone. Reverse-phase (Zorbax RX C-8) chromatography of these fractions gave kampanol A (1, 2.6 mg, 130 mg/L)¹³ and kampanol B (2, 4.4 mg, 220 mg/L)¹⁴ as amorphous powders. Kampanol C (3),¹⁴ an unstable compound, was isolated from a separate larger batch in a non bioassay directed isolation method.

Structure Elucidation

Kampanol A (1): Electron impact (EI) and NH_4Cl induced chemical ionization (CI) mass spectral analysis of kampanol A (1) gave highest ions at m/z 428 (M^+) and 429 ($\text{M}+\text{H}^+$), respectively. High-resolution mass measurement of the molecular ion in the EI spectrum led to a molecular formula of $\text{C}_{25}\text{H}_{32}\text{O}_6$. This formula suggests that kampanol A has ten degrees of unsaturation. The infra red spectrum of 1 showed absorption bands for a hydroxy group (3369 cm^{-1}), lactone/ester groups ($1745, 1735\text{ cm}^{-1}$) and an aromatic ring (1620 cm^{-1}). The UV spectrum exhibited a major absorption band at 223 ($\epsilon = 13330$) nm and two minor bands at 262 ($\epsilon = 5118$) and 308 ($\epsilon = 2408$) nm.

As expected from the molecular formula of kampanol A, the ^{13}C NMR spectrum of 1 in CD_2Cl_2 displayed 25 carbon resonances (Table 1). The DEPT and APT spectrum of kampanol A indicated the presence of five methyls, six methylenes, and four methines (an aromatic, an oxymethine and two aliphatic), and ten quaternary carbons. The distribution of the quaternary carbon resonances was as follows: two were ester/lactone carbonyls, two phenolic, three aromatic/olefinic, two aliphatic and an oxygen containing aliphatic.

The 400 MHz ^1H NMR spectrum of kampanol A exhibited resonances for four methyl singlets in the upfield region of the spectrum (δ 0.70, 0.86, 0.91, and 1.16) and one in a lower field at δ 2.01. Apart from a number of resonances in the upfield region of the spectrum, the ^1H NMR spectrum displayed downfield signals readily assignable to an oxymethine at δ 4.47 (dd, $J = 11.8, 4.8\text{ Hz}$), an oxymethylene at δ 5.14 (ABq, $J = 16\text{ Hz}$) and an aromatic methine singlet at δ 6.80 (Table 1).

The ^1H - ^1H connectivities of kampanol A were assigned by ^1H - ^1H COSY and TOCSY experiments and ^1H - ^{13}C connectivities were assigned by an HMQC experiment. The COSY and TOCSY experiments produced only three fragments C1-C3, C5-C7, and C9-C11 (Figure 1). The connectivities of these fragments with the remaining independent pieces of kampanol A were established by an HMBC experiment. Selected HMBC correlations are summarized in Figure 1.

The angular methyl group (H_3 -15, δ 0.70) gave two and three bond HMBC correlations to C-1 (δ 38.1), C-5 (δ 54.4), C-9 (δ 48.7) and C-10 (δ 38.4). The correlations of this angular methyl group turned out to critical

as it helped in connecting the three COSY derived fragments to each other. Aside from giving HMBC correlations to each other and establishing their geminal nature, the methyl groups (H₃-13 and H₃-14) showed two and three bond correlations to the oxymethine carbon C-3 (δ 80.8), C-4 (δ 38.0), and C-5. Similarly, the remaining angular methyl group H₃-12 gave correlations to the quaternary oxymethine C-8 (δ 76.8); C-7 (δ 40.4) and C-9. These HMBC correlations helped to unambiguously establish the decalin ring system. The HMBC correlations from both H α -11 (δ 2.73) and H β -11 (δ 2.84) to C-8, C-9, C-10 of the decalin ring and C-1' (δ 117.1), C-2' (δ 155.3) and C-6' (δ 150.6) of the aromatic ring ascertained the linkage of the decalin ring to the aromatic ring.

Table 1. NMR Assignments of Kampanols (1–4) in CD₂Cl₂ and acetone-*d*₆.

Position	1 (δ C) CD ₂ Cl ₂	1 (δ H) CD ₂ Cl ₂	2 (δ C) CD ₂ Cl ₂	2 (δ H) CD ₂ Cl ₂	3 (δ H) acetone- <i>d</i> ₆	4 (δ C) acetone- <i>d</i> ₆	4 (δ H) acetone- <i>d</i> ₆
1	38.1	α : 1.19, m β : 1.90, m	37.8	α : 1.10, m β : 1.90, m	1.10, m 1.90, m	39.01	1.18, m 1.90, m
2	23.7	β : 1.56, m α : 1.66, m	23.7	β : 1.53, m α : 1.53, m	1.62, m 1.62, m	27.74	1.60, m 1.60, m
3	80.8	4.47, dd, 11.8, 4.8	80.8	4.47, dd, 11.2, 4.8	4.48, dd, 9.0, 7.2	81.48	4.48, dd, 9.2, 7.2
4	38.0	---	37.9	---	---	38.86	---
5	54.4	1.05, m	54.6	1.05, m	1.05, m	55.36	1.15, m
6	18.1	β : 1.50, m α : 1.60, m	18.2	β : 1.50, m α : 1.65, m	1.75, m 1.75, m	19.09	1.50, m 1.60, m
7	40.4	β : 1.62, m α : 2.2, brd, 11.6	40.6	β : 1.53, m α : 2.19, brd, 13.6	1.53, m 2.25, m	41.67	1.60, m 2.20, m
8	76.8	---	76.0	---	---	76.23	---
9	48.7	1.49, brd, 7.6	48.5	1.45, brd, 7.4	1.60, m	49.45	1.50, brd, 8
10	38.4	---	38.3	---	---	39.25	---
11	18.5	α : 2.73, dd, 19.6, 7.6 β : 2.84, d, 20	17.9	α : 2.65, dd, 18, 7.2 β : 2.72, d, 18	α : 2.70, dd, 18, 8.4 β : 2.80, d, 18	19.13	α : 2.76, dd, 18, 8 β : 2.80, d, 18
12	27.1	1.19, s	26.7	1.17, s	1.28, s	27.65	1.17, s
13	28.5	0.91, s	28.4	0.90, s	0.92, s	29.22	0.91, s
14	16.9	0.86, s	16.8	0.86, s	0.88, s	17.71	0.87, s
15	14.3	0.70, s	14.1	0.70, s	0.79, s	13.20	0.75, s
1'	117.1	---	111.2	---	---	110.10	---
2'	155.3	---	155.6	---	---	155.22	---
3'	101.4	6.80, s	100.2	6.31, s	6.79, s	107.94	6.47, s
4'	124.9	---	139.0	---	---	141.60	---
5'	127.7	---	118.1	---	---	120.44	---
6'	150.6	---	151.6	---	---	154.76	---
7'	171.7	---	105.6	5.77, s	10.41, s	64.26	4.65, brd, 4.4
8'	68.4	5.12, 51.5, d, 16	104.6	6.05, s	10.41, s	56.00	4.57, brd, 3.0
1''-CO	171.1	---	171.1	---	---	171.17	---
2''-CH ₃	21.4	2.01, s	21.3	2.01, s	2.05, s	21.55	2.00, s
7'-OCH ₃	---	---	55.2	3.47, s	--	--	8.16, brs
8'-OCH ₃	---	---	52.5	3.2, s	--	--	3.66, brt, 4.0
OH	--	---	---	--	--	---	4.21, brs

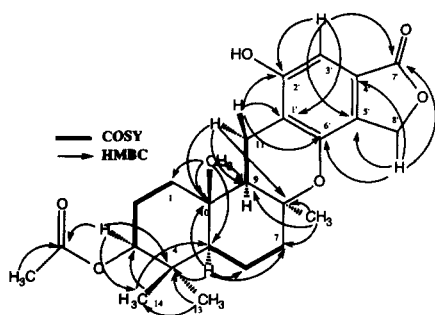


Figure 1. Selected COSY and HMBC ($J_{\text{CH}} = 7 \text{ Hz}$) correlations of kampanol A.

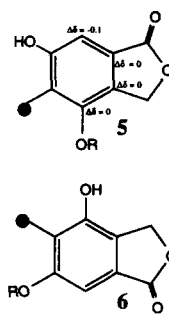


Figure 2. Alternative structures showing aromatic ring substitution.

For the most part the substitution pattern of the aromatic ring was determined by the HMBC correlations of the $\text{H}_2\text{-8}'$ (δ 5.14) and $\text{H-3}'$ as shown in Figure 1. However, it was difficult to distinguish between the alternative structures **5** and **6** based on the HMBC correlations alone. The two structures were distinguished by the application¹⁴ of deuterium induced shifts of ^{13}C resonances from the deuterium exchange of the active phenolic hydrogen. The ^{13}C NMR spectra of kampanol A was recorded separately in CD_2Cl_2 , $\text{CD}_2\text{Cl}_2+\text{D}_2\text{O}$, and $\text{CD}_2\text{Cl}_2+\text{H}_2\text{O}$ and chemical shifts of all the carbons were assigned. The differences in the chemical shifts ($\Delta\delta = \delta(\text{CD}_2\text{Cl}_2+\text{D}_2\text{O}) - \delta(\text{CD}_2\text{Cl}_2 + \text{H}_2\text{O})$) are shown on the partial structure **5**. The ^{13}C resonances for $\text{C-1}'$ and $\text{C-2}'$ experienced $+0.05$ and $+0.16$ ppm downfield shifts in $\text{CD}_2\text{Cl}_2 + \text{D}_2\text{O}$ spectrum. However, the resonances for these two carbons were not visible when the spectrum was recorded in $\text{CD}_2\text{Cl}_2+\text{H}_2\text{O}$ which was attributed to exchange broadening of the resonances in the latter solvent mixture. The most pronounced shift was observed for $\text{C-3}'$ ($\Delta\delta = -0.1$ ppm) thereby indicating that the free phenolic group should be located *ortho* to $\text{C-3}'$. Since one of the two possible *ortho* positions to $\text{C-3}'$ is already occupied by the lactone carbonyl, as implied from the strong three bond HMBC correlation from $\text{H-3}'$ (δ 6.80) to the lactone carbonyl (δ 171.7), the free phenolic group must be located at $\text{C-2}'$. This observation indicated that the aromatic ring substitution in kampanol A (**1**) should be the same as illustrated in the partial structure **5**.

The $\text{C-1}''$ carbonyl (δ 171.1) experienced the HMBC correlations from H-3 (δ 4.47) and the acetate methyl group $\text{H}_3\text{-2}''$ (δ 2.01) and thus confirming the placement of the acetate group at C3 .

Relative stereochemistry: The relative stereochemistry of kampanol A (**1**) was deduced from NOEDS experiments (400 MHz) and the measurements of the scalar coupling constants. Irradiation of the angular methyl group ($\text{H}_3\text{-15}$) gave strong enhancements to $\text{H}_3\text{-14}$, and $\text{H}_\beta\text{-6}$ indicating 1,3-diaxial relationships. Similarly strong NOE's were observed between H-3 , H-5 and $\text{H}\alpha\text{-1}$ due to their 1,3-diaxial relationships. NOE's were also observed between H-5 and H-9 ; $\text{H}_3\text{-13}$ and $\text{H}_\alpha\text{-6}$. These and other observed NOE enhancements (see Figure 3) could be explained from a *trans* ring fusion and a

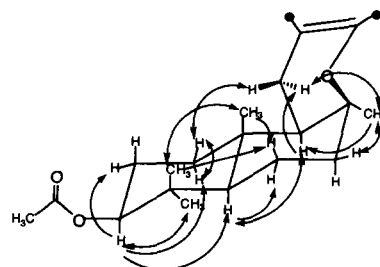


Figure 3. Kampanol A showing NOE's.

chair–chair conformation of decalin rings and a *cis* ring fusion and a chair-boat conformation of B ring of decalin unit and dihydropyran ring. The stereochemistry and conformation of kampanol A is shown in Figure 3.

Kampanol B (2): The mass spectral analysis of kampanol B (2) furnished a molecular weight of 474 and the molecular formula of $C_{27}H_{38}O_7$. This formula has an additional C_2H_6O compared to the formula of kampanol A. The 1H and ^{13}C NMR spectra (Table 1) of the two compounds were virtually identical except for the absence of C-7' lactone carbonyl and C-8' methylene group, and the presence of two methoxy and two additional methine groups. These new groups occupy the position where the lactone is present in kampanol A. The structure **2** was assigned to kampanol B on the basis of the NMR spectral comparison with kampanol A. The structural assignment was firmly supported by the HMBC experiment. This type of hemiacetal compound has been reported to be an artifact of isolation from dialdehydes (e.g., stachybotridial).¹⁶ Extensive use of methanol during the bioassay guided purification of this compound would indicate that this compound is an artifact of the isolation conditions. Aside from these two compounds there were no other compounds present in the mixture that showed any FPTase activity.

Kampanol C (3): Kampanol C could not be isolated during the initial bioassay directed isolation. However, the presence of this compound in the broth extract was predicted due to the isolation of kampanol B. A different isolation method, that did not involve use of methanol was designed for the isolation of this compound. The methyl ethyl ketone extract was chromatographed on a preparative HPLC on a Dynamax C-18 column. The column was eluted with a 30 to 80% gradient of acetonitrile in water without addition of any pH modifier. The fractions containing kampanol C (3) were immediately lyophilized to give a buff color powder. Kampanol C was extremely unstable in CD_2Cl_2 with a half life of less than 1 h but showed reasonable stability in acetone. The 1H NMR spectral data of kampanol C was recorded in acetone- d_6 and summarized in Table 1. The chemical shifts were assigned based on a COSY spectrum.

A small portion of the broth extract was reduced with $NaBH_4$ in a mixture of THF and methanol. The resulting diol (4) was purified by silica gel followed by a similar reverse phase HPLC chromatography. The complete assignment of the 1H and ^{13}C NMR spectra of **4** is summarized in Table 1.

The HPLC analysis of the methyl ethyl ketone extract of the fungal broth indicated the presence of kampanol A but did not show the presence of kampanol B. Methanol treatment of the broth extract showed a significant reduction of the aldehyde peak but the formation of kampanol B was proportionally negligible. However, when the extract was treated with methanol and Sephadex LH-20 in the presence of trace of TFA a significant formation of kampanol B (2) was observed.

Biological Activity

Kampanol A and B inhibited Ras rHFPTase^{17,18} with IC_{50} values of 13 and 7 μM , respectively. Kampanol C (3) and diol (4) were inactive and displayed IC_{50} values of 560 and 460 μM , respectively. The lack of inhibitory activities of dialdehyde (3) and diol (4) is surprising and indicates that the additional ring in **1** and **2** is important for activity. Obviously, the structural integrity of kampanol C in the assay mix is questionable given its inherent instability. All these compounds were inactive in the GGTase I assay ($IC_{50} \gg 100 \mu M$).

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- Physical data (1): crystallized from acetone-hexane as granules, mp 260 °C (dec); UV: λ_{max} (CH₃OH): 223 (ϵ = 13330), 262 (ϵ = 5118), 308 (ϵ = 2408); IR: ν_{max} (ZnSe): 3369, 2948, 1745, 1735, 1680, 1620, 1470, 1343, 1245, 1168, 1136, 1080, 1015, 936, 915, 773, 737 cm⁻¹; $[\alpha]_{\text{D}}^{25}$ -11.54° (c, 0.26, CH₃OH); HREIMS (m/z) 428.2206 (calcd for C₂₅H₂₅O₆: 428.2199). (2): IR: ν_{max} (ZnSe): 3346, 2948, 1733, 1690, 1615, 1456, 1370, 1246, 1067, 1033, 1005, 897, 733 cm⁻¹; $[\alpha]_{\text{D}}^{25}$ -25° (c, 0.008, CH₃OH); HREIMS (m/z) 474.2601 (calcd for C₂₇H₃₈O₇: 474.2617); (3): UV: λ_{max} (CH₃CN): 208 (ϵ = 23369), 230 (sh), 282 (ϵ = 7480), 314 (ϵ = 3360); IR: ν_{max} (ZnSe): 3317, 2948, 1733, 1651, 1594, 1427, 1368, 1316, 1246, 1167, 1137, 1085, 1026 cm⁻¹; HREIMS (m/z) 428.2224 (calcd for C₂₅H₂₅O₆: 428.2199); (4): crystallized from acetone-hexane as granules; mp >290 °C (dec); UV: λ_{max} (CH₃CN): 214 (ϵ = 24495), 230 (sh), 285 (ϵ = 1565); IR: ν_{max} (ZnSe): 3400, 3285, 2943, 1733, 1598, 1443, 1352, 1259, 1166, 1136, 1088, 1036, 1017, 999, 973 cm⁻¹; $[\alpha]_{\text{D}}^{25}$ -66.6° (c, 0.45, CH₃CN); HREIMS (m/z) 432.2558 (calcd for C₂₅H₃₆O₆: 432.2528).
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