Pergamon

Tetrahedron Letters 42 (2001) 8587–8591

TETRAHEDRON LETTERS

Natural anti-HIV agents. Part 2: Litseaverticillol A, a prototypic litseane sesquiterpene from *Litsea verticillata*

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Received 25 July 2001; revised 28 September 2001; accepted 2 October 2001

Abstract—We report herein the first isolation of a novel structural type sesquiterpene designated as 'litseane' from the twigs and leaves of *Litsea verticillata* Hance (Lauraceae). The isolate (litseaverticillol A, **1**) was obtained as a racemate through bioassay-guided fractionation and found to inhibit the replication of human immunodeficiency virus (HIV) type 1 with an IC_{50} value of 5.0 μg/mL (21.4 μM) and a selectivity index of 2.6. Spectroscopic data and a potential biosynthetic pathway are given.

Litsea verticillata Hance (Lauraceae), a perennial shrub or arbor, was collected in the Cuc Phuong National Park (Nho Quan District, Ninh Binh Province, Vietnam) as part of our International Cooperative Biodiversity Group (ICBG) project.[1] The goal of the ICBG is to address the related issues of biodiversity conservation, economic growth and promotion of human health through the discovery of anti-HIV, anti-malarial, anticancer and anti-tuberculosis natural products.[1,2] During an initial screen for anti-HIV activity, the chloroform extract of the leaves and twigs of *L. verticillata* inhibited HIV-1 replication by 50% at a concentration of 20 μg/mL with minimal toxicity (90% cell viability). Bioactivity-guided fractionation of the re-collected material was initiated in an attempt to isolate and identify the active constituent(s).

As described previously,[3] the dried leaves and twigs of *L. verticillata* (4.5 kg) was milled and extracted with MeOH. The extract was then defatted with hexane and partitioned with $CHCl_3$ to afford an active $CHCl_3$ extract (93 g). Bioassay-directed fractionation of the

Table 1. ^1H and ^{13}C NMR data for litseaverticillol A (**1**)[a]

Position	δ_H	δ_C	Position	δ_H	δ_C
1	4.51, m	76.21 d[c]	9	2.04, m	26.44 t
2	7.09, m	155.57 d	10	5.04, brt, 6.7	123.84 d
3		142.25 s	11		131.51 s
4		206.87 s	12	1.62, s	25.54 q
5	3.10, dd, 9.0, 2.4[b]	55.97 d	13	1.73, t, 1.6	10.07 q
6	4.95, brd, 9.0	118.81 d	14	1.69, s	16.97 q
7		141.62 s	15	1.54, s	17.56 q
8	2.00, m	39.51 t			

[a] Recorded on Bruker DRX-500 MHz spectrometer at 24°C in $CDCl_3$ (Sigma).
[b] Coupling constant in Hz.
[c] Multiplicity was determined by DEPT data.

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0040-4039/01/$ - see front matter
PII: S0040-4039(01)01852-4

$CHCl_3$ extract by repeated flash column chromatography on Si gel and RP-18 Si gel, followed by preparative HPLC afforded an active isolate. This isolate, assigned the trivial name of litseaverticillol A (**1**), was found to be a new sesquiterpenoid racemate with a unique skeleton and. The present paper describes the structure elucidation of **1**, its biological evaluation as an anti-HIV agent, and a possible biosynthetic pathway.

Litseaverticillol A (**1**), a colorless gum, was purified from an anti-HIV fraction by separation on a preparative HPLC column (GROM-Suphir 110 C18, 120 Å, 12 μm, 300×40 mm; MeCN/H_2O 50:50, 20 mL/min).[4] The molecular formula ($C_{15}H_{22}O_2$) of **1** was established by analysis of the ^{13}C NMR and DEPT spectra, and confirmed by HRTOFMS ($[M+H]^+$ m/z 235.1703, calcd. 235.1698). The 15 carbons were characterized by DEPT-135 and DEPT-90 spectra as four non-oxymethyl carbons (δ chemical shifts between 10 and 20 ppm), two non-oxymethylene carbons (δ 39.51, 26.44), a non-oxymethine carbon (δ 55.97), an oxymethine carbon (δ 76.21), three olefinic methane carbons (δ chemical shifts between 110 and 160 ppm), three olefinic quaternary carbons (δ chemical shifts between 130 and 145 ppm), and a quaternary carbonyl carbon (δ 206.87) (Table 1). Three double bonds were present. One was deduced to form an α,β-conjugated keto group with the carbonyl carbon (δ 206.87) due to the downfield shift of the olefinic methine carbon (δ 155.57) and the upfield shift of the carbonyl carbon in the ^{13}C NMR spectra. More conclusive structural information was obtained by applying ^1H–^1H COSY, HMQC, and HMBC techniques. ^1H–^1H COSY spectra normally reveal direct proton–proton coupling,[5,6] while HMQC spectra uncover direct proton–carbon coupling.[7,8] In addition, the more powerful HMBC spectra suppress direct proton–carbon coupling, but reveal two- or three-bond couplings between protons and carbons.[9] The four non-oxymethyl groups (δ_H 1.73, 1.69, 1.62, 1.54) were used as a starting point for deducing three substructures in **1** (Fig. 1). The methyl protons at δ_H 1.73 were shown to have three long-range correlations to δ_C 206.87 (s), 142.25 (s) and 155.57 (d) in the HMBC spectrum (Fig. 2), thus establishing a sub-structural unit of Me-C(-C=O)=CH- (unit A). The presence of HMBC correlations between δ_H 1.69 and δ_C 141.62 (s), δ_C 118.81 (d), or δ_C 39.51 (t), respectively, suggested a second sub-structural unit of Me-C(CH_2)=CH- (unit B). Lastly, the protons in the two methyl groups (δ_H 1.62, 1.54) were shown to be long-range-coupled with δ_C 131.51 (s), δ_C 123.84 (d) and δ_C 26.44 (t), respectively, suggesting the presence of a third potential sub-structural unit of Me_2C=CH- (unit C) (Fig. 1). One of the proton signals (δ_H 5.04) in unit C was observed to have correlations with the methylene protons (δ_H 2.04) in the ^1H–^1H COSY spectrum. This observation further defines the third substructural unit as Me_2C=CH-CH_2-. This last unit was observed to be connected to another methylene group based on the presence of the ^1H–^1H COSY correlation between the two methylenes. This indicates a connection between units C and B to afford yet another sub-structural unit of Me_2C=CH-CH_2-CH_2-C(Me)=CH- (unit D). Further analysis of the ^1H–^1H COSY spectrum revealed the proton signal in unit D at δ_H 4.95 to be coupled with the proton signal at δ_H 3.10,

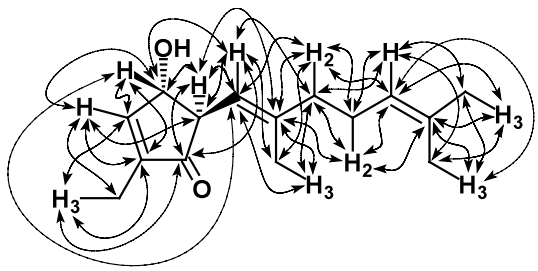

Figure 2. HMBC correlation for litseaverticillol A (**1**) ($CDCl_3$).

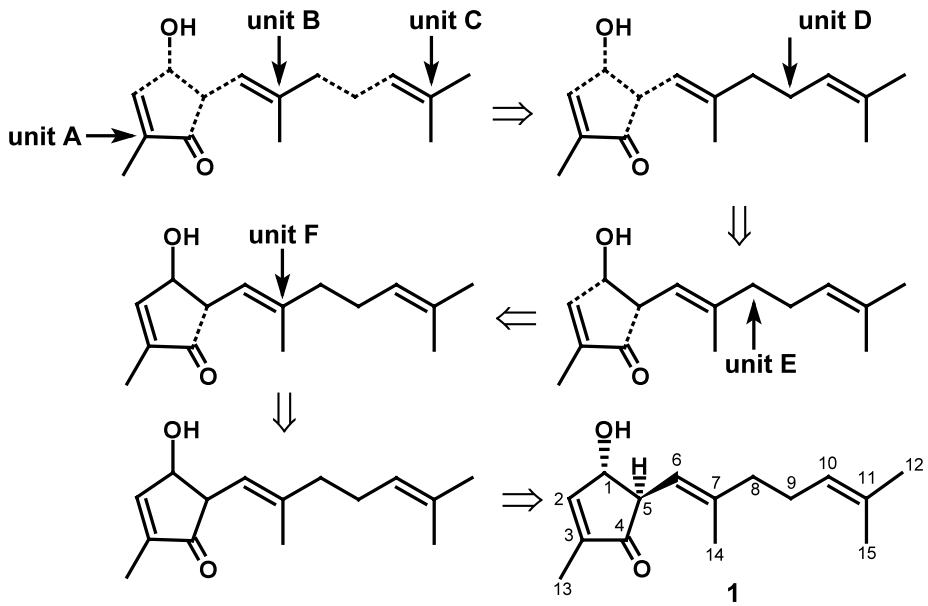

Figure 1. Structure deduction of litseaverticillol A (**1**).

which was in turn coupled with the proton signal at δ_H 4.51. Taken together, this suggested that **1** contains a Me_2C=CH-CH_2-CH_2-C(Me)=CH-CH-CH(OH)- group (unit E). The fact that the proton signal in unit A at δ_H 7.09 was coupled with the proton signal in unit E at δ_H 4.51 in the ^1H–^1H COSY spectrum linked the two units (A and E) as Me_2C=CH-CH_2-CH_2-C(Me)=CH-CH-CH(OH)-CH=C(-C=O)-Me (unit F). Five double-bond equivalents were calculated from the molecular formula ($C_{15}H_{22}O_2$) of **1**. Four of these were accounted for by the presence of the three carbon–carbon double bonds and a carbonyl double bond, with the remaining unsaturated bond equivalent being unassigned. Conceivably, this unassigned double bond equivalent could be due to the presence of a ring structure in **1**. This assumption was confirmed by the HMBC correlations between the carbonyl carbon (δ_C 206.87) and the proton signals at δ_H 3.10 and δ_H 4.95, which demonstrated that a five-member ring was formed by connecting the carbonyl carbon and the non-oxymethine carbon in unit F. The final planar structure was thus elucidated for **1** (Fig. 1). This represents a unique sesquiterpene structural skeleton that has not been reported previously from nature. We have designated this type structure as 'litseane'. In addition, compound **1** represents a new anti-HIV chemotype.

The relative stereochemistry of **1** was obtained through an analysis of coupling constants and a ROESY experiment[10] (Fig. 3). The protons on C-1 and C-5 in compound **1** were determined to be of β- and α-orientation, respectively, based on the small coupling constant (J=2.4 Hz) between H-1 and H-5, which was caused by the proximate 90° dihedral angle between the two protons. The geometric isomerism at C-6 and C-7 was assigned the *E*-configuration through the observation of an ROE correlation between H-5 and H-14. This observation, when taken together with the ROE correlation between H-1 and H-6, confirmed the hydroxy group of C-1 as being in an α-orientation and the side chain of C-5 to be in a β-orientation.

The optical rotation of **1**, $[\alpha]_D$=0°, suggested that **1** may be a racemate. To determine the optical purity of **1**, a Mosher ester reaction was performed.[11,12] Theoretically, the Mosher reaction of an optically pure compound will result in a single Mosher ester derivative being formed. However, treatment of **1** with (*R*)- or (*S*)-α-methoxy-α-trifluoromethylphenylacetyl chlorides (MTPA-Cl) afforded two mono-ester derivatives.[13] These esters appeared to exist in a 1:1 ratio based on the ^1H and ^{13}C NMR spectra, in which the signals either overlapped or existed in pairs, with the pairing signals exhibiting almost identical areas of integration. Thus, **1** was determined to be an equimolar racemic mixture.

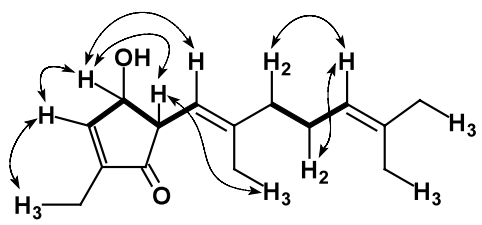

Figure 3. ^1H–^1H COSY (shown as bold bonds) and ROESY correlations for litseaverticillol A (**1**) ($CDCl_3$).

Accordingly, **1** was established to be (±)-1α-hydroxy-(*E*)-litse-2,6,10-trien-4-one,[14] and given the trivial name of litseaverticillol A.

By virtue of its novelty, the biosynthetic pathway of **1** has not been previously established. However, given its proposed litseane structure, it is most probable that **1** is formed through the mevalonate pathway characteristic of sesquiterpenes. In fact, the side chain represents a geranyl unit. Thus, it may be postulated that **1** is formed by the condensation of an isopentenyl diphosphate with a geranyl diphosphate to give farnesyl diphosphate. Cyclization and oxidation of the latter leads to **1**, as proposed in Fig. 4.

The isolate **1** was tested for in vitro inhibitory effects against HIV-1 replication in HOG.R5, a reporter cell line constructed for quantitating HIV-1 replication.[15] This microtiter assay is based on the transactivation of a stably integrated HIV-1 LTR-green fluorescent protein (GFP) transcription unit by the viral Tat protein. The system was validated and adapted in our laboratory as a moderately high-throughput procedure for screening natural products for anti-HIV activity. We recently reported the isolation of two lignans from *L. verticillata* that possess anti-HIV activity.[3] The present litseane, **1**, inhibited the replication of HIV-1 with an IC_{50} value of 5.0 μg/mL (21.4 μM). It also demonstrated toxicity to HOG.R5 cells, with a CC_{50} value of 13.2 μg/mL (56.4 μM). This yields an unfavorable selectivity index value (CC_{50}/IC_{50}) of 2.6 that excludes **1** from consideration for more advanced studies with in vivo models of HIV infection. However, this prototypic molecule may warrant a more detailed in vitro evaluation as a lead compound for the development of novel anti-HIV agents.

Acknowledgements

All work involving plant sample collection, taxonomic identification, bioassay-guided chemical isolation, and structure elucidation in connection with this paper were carried out under a grant administered by the Fogarty International Center, NIH (Grant 1 UO1-TW01015-01), as part of an International Cooperative Biodiversity Group (ICBG) program. The authors are grateful to the Research Resources Center, University of Illinois at Chicago for access to the Bruker DRX 500 MHz instrument, and to the Center Research Group of the College of Pharmacy, University of Illinois at Chicago for the acquisition of MS data.

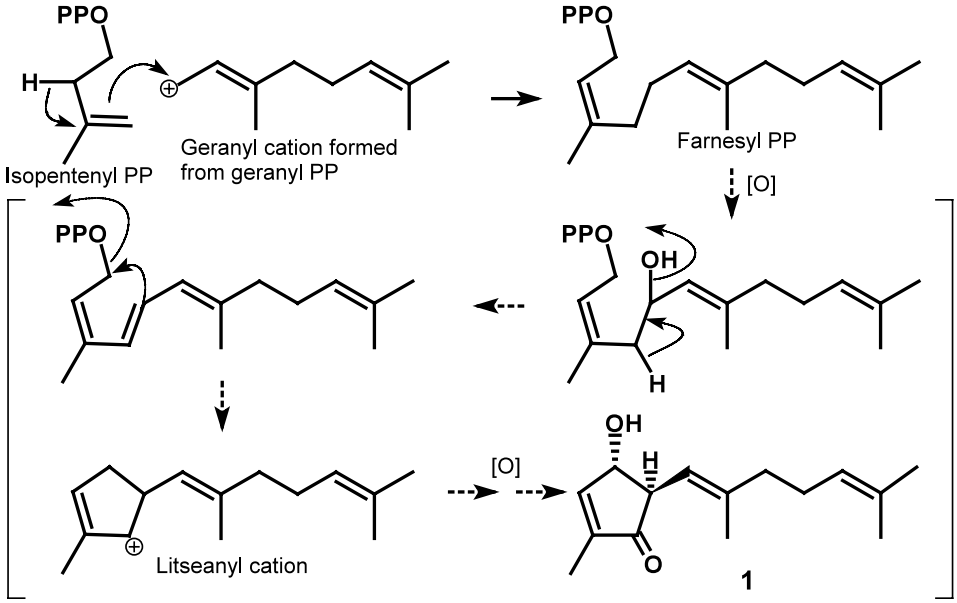

Figure 4. A proposed biosynthetic pathway for litseaverticillol A (**1**).

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4. Litseaverticillol A (**1**): colorless gum, $[\alpha]_D$ 0° (*c* 2.7, MeOH); UV (MeOH) λ_{max} (log ε) 232 (4.56), 320 (3.14) nm; IR (film) ν_{max} 3380.6 (br), 2956.3, 2928.4, 2866.7, 1701.9, 1608.3, 1509.0, 1458.9, 1423.2, 1363.4, 1259.3, 1203.4, 1165.8, 1129.1, 1097.3, 1041.4, 960.4, 886.1, 813.8 cm^{-1}; TOFMS/MS *m*/*z* (10 eV, from 235) 217, 163, 123; HRTOFMS *m*/*z* 235.1703 $[M+1]^+$ (calcd for $C_{15}H_{23}O_2$, 235.1698, Δ +0.5 mmu).
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13. Treatment of **1** (5.0 mg) with 4-(dimethylamino)pyridine (1.8 mg) and (*S*)-(+)-α-methoxy-α-trifluoromethylphenylacetic chloride (20 μL, MTPACl) at room temperature afforded two (*R*)-MTPA esters (3.2 mg) of **1**: ^{1}H NMR (Bruker DRX 500 MHz, $CDCl_3$, *J* in Hz) δ 7.50–7.45 (4H, m, aromatic), 7.42–7.35 (6H, m, aromatic), 7.16 (1H, m, H-2), 7.11 (1H, m, H-2), 5.72 (2H, m, H-1), 5.06 (2H, m, H-10), 5.01 (1H, br d, J=9.4, H-6), 4.99 (1H, br d, J=9.1, H-6), 3.54 (3H, s, OMe), 3.52 (3H, s, OMe), 3.35 (1H, dd, J=9.4, 2.6, H-5), 3.24 (1H, dd, J=9.1, 2.4, H-5), 2.15–2.00 (8H, m, H-8/H-9), 1.83 (3H, t, J=1.6, Me-13), 1.81 (3H, t, J=1.6, Me-13), 1.66 (6H, brs, Me-14), 1.63 (3H, d, J=1.3, Me-12), 1.58 (6H, s, Me-13), 1.49 (3H, d, J=1.3, Me-12); ^{13}C NMR (Bruker DRX 500 MHz, $CDCl_3$) δ 204.48 (1C, s, C-4), 204.27 (1C, s, C-4), 166.36 (2C, s, MTPA), 149.50 (1C, d, C-2), 149.45 (1C, d, C-2), 145.83 (1C, s, C-3), 145.67 (1C, s, C-3), 142.64 (1C, s, C-7), 142.54 (1C, s, C-7), 131.81 (2C, s, C-11), 129.79 (1C, d, MTPA), 129.74 (1C, d, MTPA), 128.53 (2C, d, MTPA), 128.50 (3C, d/s, MTPA), 127.24 (3C, d/s, MTPA), 127.11 (2C, d, MTPS), 124.30 (2C, s, MTPA), 123.70 (1C, d, C-10), 123.66 (1C, d, C-10), 122.02 (2C, s, MTPA), 117.69 (2C, d, C-6), 79.54 (1C, d, C-1), 79.52 (1C, d, C-1), 55.52 (1C, q, MTPA), 55.36 (1C, q, MTPA), 51.72 (1C, d, C-5), 51.65 (1C, d, C-5), 39.52 (1C, t, C-8), 29.49 (1C, t, C-8), 26.38 (1C, t, C-9), 26.35 (1C, t, C-9), 26.65 (2C, q, Me-12), 17.70 (2C, q,

Me-15), 16.75 (1C, q, Me-14), 16.61 (1C, q, Me-14), 10.46 (1C, q, Me-13), 10.44 (1C, q, Me-13). Treatment of **1** with (*R*)-(−)-MTPA-Cl as described above yielded a colorless gum that contained the two (*S*)-MTPA esters in a 1:1 ratio; the ^{1}H NMR spectrum (Bruker DRX 500 MHz, $CDCl_3$, *J* in Hz) was identical to that of the (*R*)-MTPA esters of **1**.

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