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Isolation of an anti-HIV diprenylated bibenzyl from *Glycyrrhiza lepidota*

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Abstract

The organic soluble extract from the leaves of *Glycyrrhiza lepidota* showed moderate activity in the US National Cancer Institute in vitro anti-HIV-1 bioassay. Chromatographic separation of this extract resulted in the identification of a new diprenylated bibenzyl as the compound responsible for the observed anti-viral activity. Extensive spectroscopic experiments provide the complete ^{1}H NMR and ^{13}C NMR spectral assignments to support the proposed structure. Known compounds glepidotin B and glepidotin A were also isolated from the extract and shown to be inactive in the anti-viral assay.

Keywords: *Glycyrrhiza lepidota*; Fabaceae; Prenylated; Glepidotin; HIV-1

1. Introduction

Over the past several years, this laboratory has been investigating the chemistry of medicinal native plants of the North American prairie. Plant collection was guided by historical accounts of plant medicinal uses by early European settlers traveling across the plains and by indigenous North Americans (Dilwyn, 1980). The ethnobotanical literature cites 203 plant species used for medicinal purposes by the Indian tribes of the Prairie Bioregion of North America (Kindscher, 1992). With the ethnobotanical uses of plants for medicinal purposes clearly documented, a study of native prairie plants as sources of new pharmaceuticals was begun. The initial investigation examined the anti-HIV activity of 22 species of medicinal plants using the US National Cancer Institute's (US NCI) in vitro AIDS anti-viral assay (Weislow et al., 1989). The assay results identified three organic extracts as "active" in the anti-HIV screen (Kindscher et al., 1998). An active extract was defined as a crude extract that showed at least 50% growth inhibition in HIV-infected white blood cells compared to the non-infected control at a maximum test concentration of 250 μg/ml. One of the three active species was *Glycyrrhiza lepidota* (wild licorice). This communication describes the isolation and structure elucidation of the diprenylated bibenzyl **1** responsible for the observed anti-HIV activity.

The genus *Glycyrrhiza* ("sweet root") is a member of the Fabaceae family and contains 18 species world-wide (Mabberly, 1997). *Glycyrrhiza lepidota* (common names: wild licorice, American licorice, and desert root) is the only member of the genus native to North America and is widely distributed throughout the western United States and Canada. It is a perennial herb that grows to 0.3–1.1 m in height. Its use by native North Americans has been well documented (Gilmore, 1977; Camazine and Bye, 1980; Munson, 1981). The antimicrobial agents from *G. lepidota* have been previously studied (Mitscher et al., 1983; Gollapudi et al., 1989). In the work by Mitscher et al. (1983) antimicrobial bioassay-guided fractionization afforded the flavanol glepidotin A (**2**), the flavanol glepidotin B (**3**), and the prenylated bibenzyl glepidotin C (**4**). Additionally, a number of other flavanoids and bibenzyls were isolated and identified that had been previously isolated from *G. glabra* (Saitoh et al., 1976; Mitscher et al., 1980), *Helichrysum umbraculigerum* (Bohlmann and Hoffmann, 1979), and *Radula camplanata* (Asakawa et al., 1978).

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2. Results and discussion

The dried leaves of *G. lepidota* were extracted with both water and CH_2Cl_2:MeOH (1:1) . Both the aqueous and organic extracts were submitted for in vitro screening against HIV-1 as previously described by Wieslow et al. (1989). The water extract was inactive in the assay while the organic extract gave an EC_{50} of 30 μg / ml in duplicate testing. Subsequent bioassay-guided fractionation (LH-20) yielded one active fraction which was then subjected to normal phase HPLC purification to yield three pure compounds.

The first eluting compound from HPLC was identified as glepidotin B (**2**) based on its high resolution accurate mass, ^{13}C, and ^{1}H NMR spectral characteristics. The coupling constant of 13.4 Hz and the lack of a NOE between H-2 and H-3 suggested a *trans* stereochemistry. The second eluting compound was identified as glepidotin A (**3**). Subsequent in vitro assay for anti-HIV activity showed that neither of these two compounds were responsible for the observed anti-viral activity of the parent fraction.

The third compound isolated proved to be somewhat difficult to work with. After its initial purification by HPLC and removal of solvent, spectral data (^{1}H and ^{13}C NMR, IR and UV) could be obtained if the experiments were performed immediately after solvent removal. However, if the solvent was removed and the sample stored overnight, only about 50% would re-dissolve in the NMR solvent ($CDCl_3$ or CD_3OD) the next day. The insoluble material appeared as a white film on the flask. Attempts to dissolve this material in any common solvent (e.g. DMSO, THF, H_2O) failed. It was determined that if the sample was stored dry for any extended period of time (i.e. over approximately 6 h), substantial decomposition occurred and reisolation by HPLC was required with a concomitant loss of material. Eventually it was determined that the material could be stored for long periods of time in solution without decomposition. Therefore, once isolated, **1** would be quickly weighed and stored in solution ($CDCl_3$) for subsequent experiments.

The ^{1}H NMR spectrum of **1** showed a four-proton multiplet at δ 2.82. A subsequent ^{1}H–^{1}H COSY analysis showed no coupling outside the multiplet which suggested an isolated pair of non-equivalent methylene protons. This observation initially led us to believe that the compound was the earlier isolated bibenzyl **4**. However, high resolution mass spectrometry indicated a molecular formula of $C_{24}H_{30}O_3$. This molecular formula suggested a diprenylated bibenzyl. Two prenyl groups could be identified from two methyl groups at δ 1.76 and δ 1.78 and an overlapping six-proton methyl singlet at δ 1.73 ppm thus accounting for the expected four methyl groups. The one-proton signals for the prenyl olefin triplets were at δ 5.25 and δ 5.45. There was a substantial difference between the chemical shifts of the prenyl methylene groups, δ 3.39 and δ 4.45 ppm, which suggested that one of the prenyl groups was bonded to an oxygen (Williams and Flemming, 1989). Analysis of the aromatic region of the ^{1}H NMR 1D and ^{1}H–^{1}H COSY spectra identified an isolated *meta* coupled spin system at δ 6.29 and δ 6.34. This suggested that one of the aromatic rings was 1,2,3,5-tetra-substituted. Four other aromatic protons appeared as a triplet at δ 7.15 ($J = 7.65$), a *meta* coupled doublet of triplets at δ 6.78 ($J = 7.65$, 1.02), and a pair of overlapping signals centered at δ 6.66 and δ 6.65. The signal at δ 6.66 was a poorly resolved, finely coupled triplet while the one centered at δ 6.65 appeared as an unresolved mulitplet. Careful examination of the ^{1}H–^{1}H COSY identified the proton signal centered at δ 6.65 as being coupled to the triplet at δ 7.15. This implied that the proton at δ 6.66 showed only *meta* coupling. These observations suggested that this ring was *meta* disubstituted with the resonance centered at δ 6.66 corresponding to the isolated proton.

In order to establish the ring substitution patterns, a HMBC experiment was performed. Shown in Fig. 1 are the key long range correlations. Essential to establishing the structure was the correlation from the non-oxygenated prenyl methylene protons at δ 3.39 to C-1 and C-3. The oxygen-bonded prenyl methylene protons at δ 4.45 ppm also correlated to C-3, thus establishing the ether oxygen carbon at δ 157.0. This indicated that the tetra-substituted ring had the two prenyl groups *ortho* to each other and a hydroxy group *ortho* to the non-oxygenated prenyl group. These HMBC data, in combination with the 1D proton data, established the substitution pattern for the tetra-substituted ring as shown in Fig. 1. This is also consistent with the substitution previously established for glepidotin C (**4**) (Gollapudi et al., 1989).

The *meta* substitution pattern on the other aromatic ring had been established from its ^{1}H–^{1}H COSY spectrum. The results of the HMBC experiment confirmed the assignment of the signal at δ 6.66 to be assigned to H-2″ based on its long range correlation to the hydroxyl bearing carbon at δ 155.3 (C-3″) and the bibenzyl carbon at δ 37.7 (C-1″a). The assignment of the H-6″ signal at δ 6.78 was established based on its long range correlation to the same bibenzyl carbon as H-2″ (C-1″a). Given in Table 1 are the complete ^{1}H and ^{13}C NMR spectra assignments for **1** based on the acquired HMBC, ^{13}C–^{1}H HETCOR, and ^{1}H–^{1}H COSY experiments.

Compound **1** is, therefore, 2-(3-methyl-2-butenyl)-3-*O*-(3-methyl-2-butenyl)-5-[2-(3-hydroxyphenyl)ethyl]-1, 3-benzenediol. However, Mitscher et al. (1980, 1983) have established the common name ''glepidotins'' for bibenzyls and flavanols from *G. lepidota*. Therefore, we refer to this compound as glepidotin D.

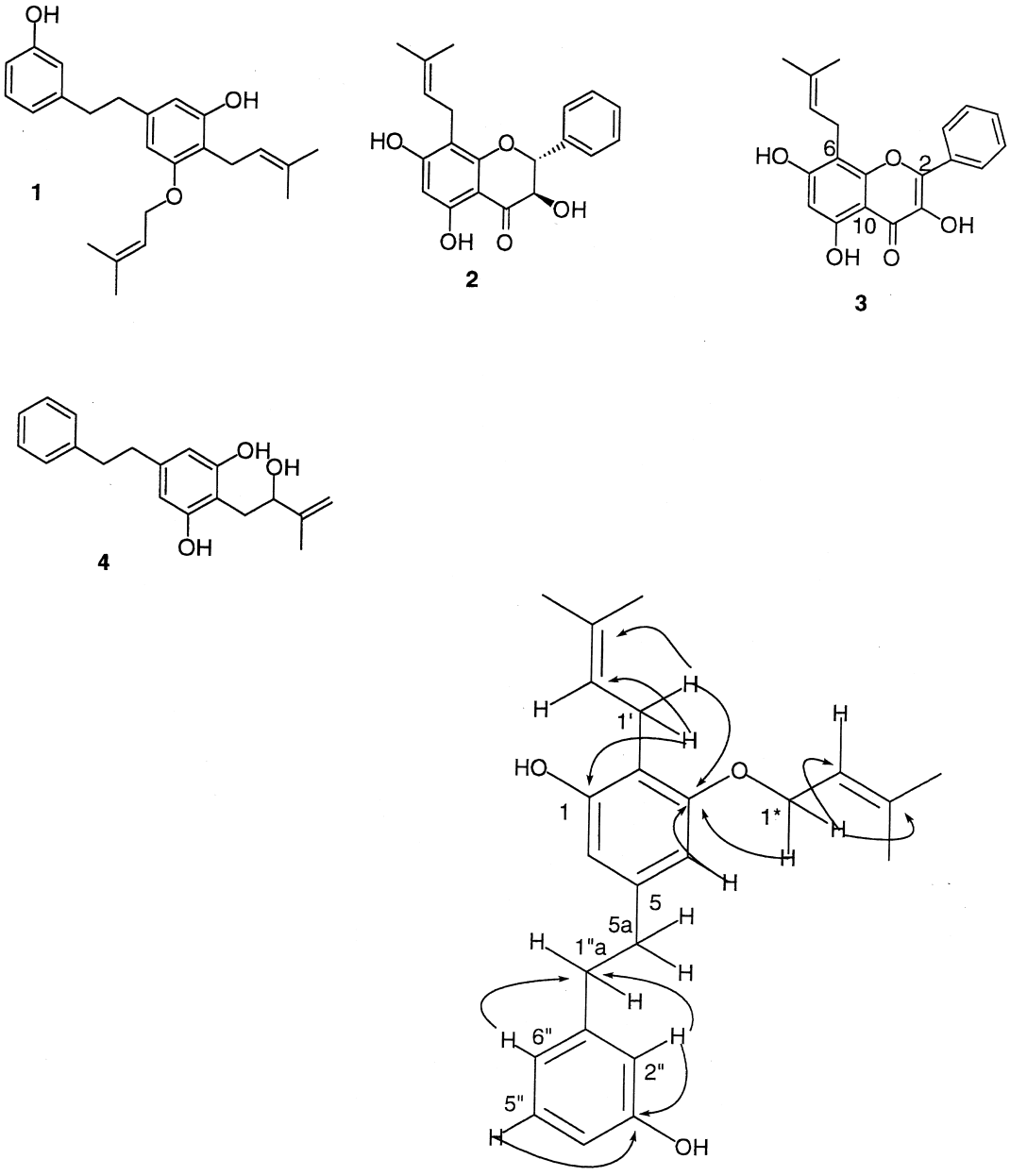

Fig. 1. Key long range HMBC correlations for **1**.

3. Biological activity

In conjuction with the Laboratory of Drug Discovery, Research and Development (LDDRD) at the US NCI, an in vitro assay against HIV-1 was performed. Compound **1** achieved an EC_{50} of 2.0 μg/ml and an IC_{50} of 5.0 μg/ml. The activity of **1** is a full order of magnitude greater than that of the crude extract and supports the hypothesis that the observed anti-HIV activity may solely be due to **1**. We were unable to isolate any glepidotin C (**4**) from the plant to determine if this related compound possessed anti-viral properties. The activity associated with this compound may be suspect because of its instability. However, we have determined that it is stable for prolonged periods of time in organic solvents, but are unsure of its stabilty under the assay conditions. Because of the instability of compound **1** and its small therapuetic index (TI: EC_{50}/IC_{50}) no further testing in the anti-viral assays is planned.

4. Experimental

4.1. General

NMR experiments were acquired on a Tecmag Aquerius (GE QE 300) at 300 or 75 MHz. The inverse-detected HMBC experiment was acquired on a Varian

Table 1
^1H and ^{13}C spectral assignments for **1** ($CDCl_3$)

Carbon	^1H (ppm, *m*, *J*)	^{13}C (ppm)	Long range (HMBC)
1		155.3	
2		113.1	
3		157.0	
4	6.29 (*d*, 1.02)	105.1	157.0, 113.1, 108.7, 37.4
5		141.0	
6	6.34 (*d*, 1.02)	108.7	155.3, 113.1, 105.1, 37.4
1″		143.8	
2″[a]	6.66 (*dt*)	112.8	155.5, 115.4, 37.7
3″		155.5	
4″[a]	6.65 (*t*)	115.4	155.5, 112.8
5″	7.15 (*t*, 7.65)	129.5	155.5, 143.8
6″	6.78 (*dt*, 7.65,1.1)	120.9	115.4, 112.8, 37.7
1″a, 5a[b]	2.82 (*m*)	37.4, 37.7	37.7, 37.4, 143.8, 141.0, 108.7, 112.8, 120.9
1′	3.39 (*d*, 7.14)	22.3	157.0, 155.3, 134.1
2′	5.25 (*t*, 7.14)	122.3	17.7, 25.8
3′		134.1	
4′, 5′	1.73, 1.78 (*s*)	25.8, 17.8	134.1, 137.2, 120.2, 122.3
1*	4.45 (*t*, 6.63)	65.4	157.0, 137.2, 120.2
2*	5.45 (*t*, 6.63)	120.2	18.7, 25.8
3*		137.2	
4*, 5*	1.76, 1.73 (*s*)	18.2, 25.8	134.1, 137.2, 20.2, 122.3
OH	4.42 (*s*, *br*)		
OH	4.70 (*s*, *br*)		

[a] Because these signals overlapped, the coupling constants could not be determined from the 1D ^1H NMR spectrum at 300 MHz.

[b] These carbons and hydrogens could not be resolved. The long range correlations must be assigned based on the proposed structure.

VXR 500 at LDDRD at the US NCI in Frederick, Maryland. All chemical shifts are reported in ppm relative to TMS. Mass spectral data were obtained on a JOEL SX 102 mass spectrometer operating at 10 kV (NIDDK, US NIH, Bethesda, Maryland). Samples for FABMS were desorbed from a nitrobenzyl alcohol matrix using 6 keV xenon atoms. Accurate mass FAB was performed at a resolution of 10,000. IR data were acquired on a Galaxy series 2020 FT–IR spectrometer. HPLC chromatography was performed on an ISCO gradient HPLC with a ISCO V-4 UV detector. All solvents were purchased from Fisher Scientific and were HPLC grade. HPLC columns were either Altech (Econosil) or Rainin (Varian) Dynamax.

4.2. Biological testing

The anti-HIV assay was carried out at LDDRD at the US NCI in Frederick, Maryland as previously described by Wieslow et al. (1989). The assay involves the killing of T4 (CD4+) lymphoid cells (CEM-SS line) by HIV-1 (RF strain). The EC_{50} is the concentration at which a compound or fraction inhibits the killing of the lymphoid cells by 50% relative to the HIV infected, non-treated lymphoid cells. The IC_{50} is a measure of the compound's or fraction's toxicity to uninfected lymphoid cells. The growing lymphoid cells were pelleted from the growth medium, infected at a multiplicity of infection (MOI) of 0.05 at room temperature for 45 min with constant shaking. The infected cells were then diluted in the growth medium to yield a desired concentration of infected cells of 5000 cells/well after inoculation into individual wells of a 96-well microtiter plate. The extracts, fractions and compounds were dissolved in H_2O–DMSO and incubated for 6 days at 37°C. (Typically, fractions and extracts were tested at an initial concentration of 250 μg /ml and serially diluted. Pure compounds were tested at an initial concentration of 100 μg/ml.) After incubation, cell viability and growth were measured using the XTT-tetrazolium method as described. *G. lepidota* extract and subsequent fractions were tested through the high-flux assay lab by SAIC Frederick, PO Box B, Frederick, MD. Pure compounds were tested by Dr. Robert Gulowkoski at LDDRD. To avoid decomposition associated with **1** when stored neat, all samples were sent to LDDRD in $CDCl_3$ solutions in sealed tubes.

4.3. Plant material

G. lepidota was collected and identified by Hillary Loring at a location 1 km west and 0.5 km north of Brookville, KS. Plant identification follows the *Flora of the Great Plains* (Great Plains Flora Association, 1986). A voucher specimen is archived at the R.L. McGregor Herbarium at the University of Kansas.

4.4. Extraction and isolation

The air-dried leaves and stems of *G. lepidota* (500 g) were chopped into small pieces and covered with CH_2Cl_2:MeOH (1:1) for 24 h. The solvent was then removed and the plant material covered with MeOH. After another 24 h, this MeOH extract was added to the CH_2Cl_2:MeOH extract and the solvent removed at reduced pressure. The plant material was then extracted with H_2O and freeze-dried. Small samples of the organic and aqueous extract were submitted for HIV testing. All activity was associated with the organic phase (EC_{50} = 30 μg/ml). The crude organic extract (5.4 g) was dissolved in MeOH (100 ml) and H_2O (10 ml). This suspension was then extracted with three aliquots (50 ml each) of hexane, which was removed at reduced pressure to give a residue (1.0 g). Water was then added to the aqueous layer to bring the total H_2O content to 20% and then extracted with CCl_4 which was removed at reduced pressure to give extract (1.8 g). (CCl_4 extraction has now been eliminated in our lab due to environmental concerns.) Water was then added to the aqueous layer to bring the total concentration to 30%, and extracted with $CHCl_3$ to give a residue (0.90 g). The methanol was then removed from the aqueous layer under reduced pressure and the subsequent water layer

extracted with EtoAc to give a residue (0.28 g). The aqueous layer was then freeze dried to give a residue (1.3 g). Subsequent anti-HIV assay indicated that all of the activity was associated with the CCl_4 fraction. The CCl_4 fraction was then applied to a Sephadex LH-20 column (Pharmacia-Upjohn; 100×2.5 cm i.d.; 1:1 CH_2Cl_2:MeOH). Collected fractions were analyzed by TLC using a (H_2SO_4–EtOH–H_2O–vanillin) spray reagent and UV for visualization. Like fractions were combined and assayed (8 total).

4.5. Glepidotin B (2)

Compound **2** (32 mg) was isolated using HPLC purification on a silica HPLC column and a solvent gradient of hexane : 2-propanol (6% 2-propanol increasing to 15%). HRMS (FAB) calc. for $C_{20}H_{21}O_5$ (M + H) 341.1389, obs. 341.1379. ^{13}C NMR spectral data (75 Mhz, $CDCl_3$): δ 196.0 (*s*, C-4), 164.6, 161.2, 161.0 (*s*, C-5, C-7, C-9), 136.1 (*d*, C-2″), 136.0 (*s*, C-1′), 129.3 (*d*, C-4′) 128.7, 127.5 (4C, *d*, C-2′, 3′, 5′,6′), 121.0 (*d*, C-2″) 107.5 (*s*, C-6), 100.8 (*s*, C-10) 96.0 (*d*, C-8), 83.3 (*d*, C-3), 72.5 (*d*, C-2), 25.8 (*t*, C-1″), 21.0, 18.0 (*q*, C-4″, C-5″). Its ^1H NMR and IR spectral data were identical to those reported by Mitscher et al. (1983).

4.6. Glepidotin A (3)

Compound **3** (56 mg) was isolated using the same solvent gradient as above, with **3** eluting 0.5 min after **2**. HRMS (FAB) calcd. for $C_{20}H_{19}O_5$ $(M-H)^+$ 339.1227, obs. 339.1234. All spectral data were consistent with that previously reported by Mitscher et al. (1983).

4.7. Glepidotin D (1)

Compound **1** (18 mg) was isolated using the same HPLC solvent system as for **2** and **3**, and eluted approximately 0.1 min after **3**. Attempts to eliminate traces of **3** were initially thwarted by the apparent decomposition of **1** when it was stored neat for any length of time (>6 h). An insoluble white material formed on the walls of the flask which could not be dissolved in any common solvent. To circumvent this problem, repurification of **1** was accomplished by keeping the compound in a small amount of hexane-isopropanol HPLC solvent. Once purified, **1** could be stored in solution without appreciable decomposition as measured by periodic ^1H NMR spectral comparisons. The ^1H and ^{13}C NMR spectral data are given in Table 1. IR spectral data (neat, thin film, NaCl, ν_{max} cm^{-1}): 3382, 1588, 1489, 1453, 737, 697. EIMS (probe) 70 eV, *m*/*z* (rel. int): 366 $[M]^+$ (18), 298 (33), 69 (100). HRMS (FAB) calc. for $C_{24}H_{31}O_3$ $(M-H)^+$ 367.2273, obs. 367.2260.

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