Pergamon 0031-9422(95)00478-5 Phytochemistry, Vol. 40, No. 6, pp. 1659–1665, 1995

0031-9422/95 $9.50 + 0.00

BIOSYNTHETIC STUDIES OF LACTUCIN DERIVATIVES IN HAIRY ROOT CULTURES OF *LACTUCA FLORIDANA*

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(*Received* 4 *May* 1995)

IN HONOUR OF PROFESSOR ANTONIO G. GONZALEZ

Key Word Index—*Lactuca floridana*; Lactuceae; Asteraceae; hairy root cultures; sesquiterpene lactones; guaianolides; ^{13}CNMR; biosynthesis.

Abstract—Biosynthetic studies of the guaianolide-type sesquiterpene lactones 11βH,13-dihydrolactucin-8-*O*-acetate and 8-desoxylactucin were performed in *Agrobacterium rhizogenes*—transformed hairy root cultures of blue-flowered lettuce, *Lactuca floridana*. The ^{13}CNMR spectra of the two guaianolides labelled by incorporation of [1-^{13}C], [2-^{13}C], [1,2-$^{13}C_2$]acetate and [2-^{13}C]mevalolactone showed patterns of enrichment consistent with a previously proposed biogenetic pathway for guaianolide-type sesquiterpene lactones via the acetate–mevalonate–germacradiene route.

INTRODUCTION

Sesquiterpene lactones, which have been isolated from fungi, liverworts and members of angiosperm families, mainly the Asteraceae, represent one of the largest classes of plant products with over 4000 reported naturally occurring compounds [1, 2]. Biogenetic proposals for the various skeletal types of sesquiterpene lactones suggest that they are derived from germacradiene precursors which, in turn, are formed via the mevalonic acid–farnesyl/nerolidyl pyrophosphate pathway (Scheme 1) [1]. A wealth of information on the structural aspects and biological activities of sesquiterpene lactones is contrasted by the lack of data on the biosynthesis of these compounds [3, 4]. Possible reasons for the absence of biosynthetic data on sesquiterpene lactones obtained in plants or cell cultures might be due to the fact that earlier biosynthetic attempts were performed with plants which poorly incorporate precursors into the terpenoid skeleton. Also, undifferentiated plant cell cultures frequently alter the biosynthetic pathways, leading to changes in the compositions and structures of the secondary metabolites [5, 6]. In contrast, *Agrobacterium rhizogenes*-induced transformed roots, popularly known as hairy roots, represent differentiated cells and provide a convenient tissue source for the production of root-derived secondary metabolites at high and stable production levels [7]. Previous biosynthetic studies in our laboratory of different structural types of polyacetylenes in hairy root cultures of *Tagetes patula* [8] and *Ambrosia artemisiifolia* [9] have demonstrated that hairy roots are very well suited for biosynthetic studies and give high incorporations of the ^{13}C-labelled precursors into the secondary plant metabolites.

In continuation of our biosynthetic investigations, we have studied the biosynthesis of sesquiterpene lactones in hairy root cultures of blue-flowered lettuce, *Lactuca floridana*, tribe Lactuceae (Asteraceae). We isolated the known guaianolides 11βH,13-dihydrolactucin-8-*O*-acetate (**1**) [10], lactucin-8-*O*-acetate (**2**) [10] and 8-desoxylactucin (**3**) [11] from natural roots as well as hairy roots of *L. floridana*. Below we describe the results of ^{13}C-labelling experiments on hairy root cultures of *L. floridana*, which provided detailed information on the biosynthesis of the guaianolide skeleton of lactones **1** and **3**.

RESULTS AND DISCUSSION

Previous chemical investigations of *L. floridana* roots of unknown origin reported the isolation of the triterpenes lupeyl acetate, its $\Delta^{12,13}$-isomer and stigmasterol [10]. In our present study, dried roots of natural *L. floridana* were extracted with dichloromethane and the crude extract was chromatographed using vacuum liquid chromatography (VLC) with mixtures of hexane and ethyl acetate of increasing polarity followed by preparative reverse phase HPLC separations. The two known guaianolides, (**1**) and (**2**), which had been previously isolated from aerial parts of *L. floridana* [10], were the major constituents, and trace amounts of **3** [11] were detected by GC–mass spectrometric analysis. In addition, the two known eudesmanolides, reynosin (**4**)

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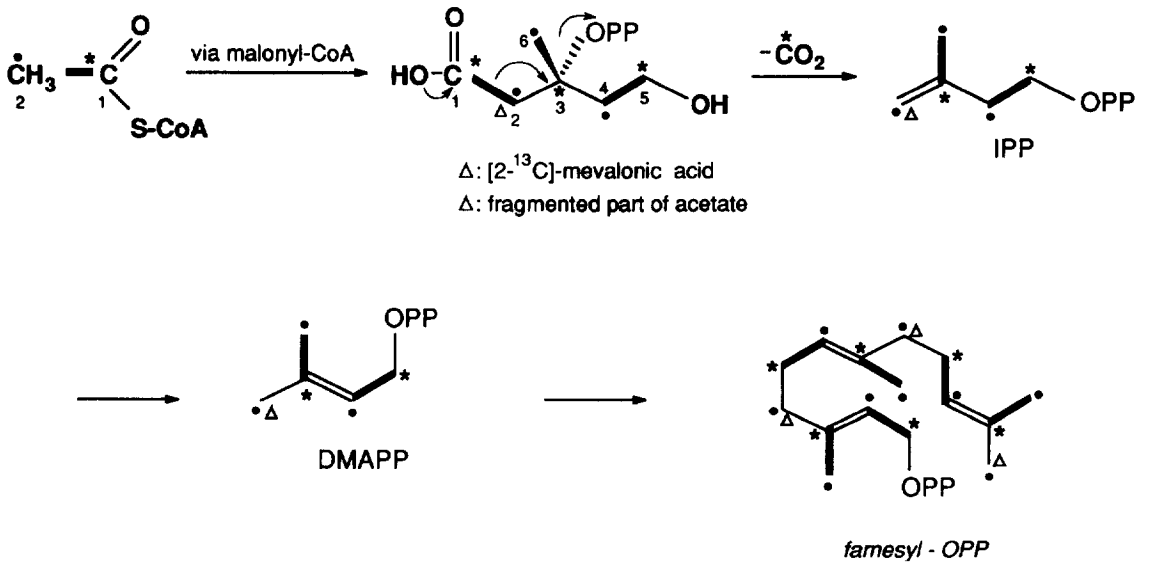
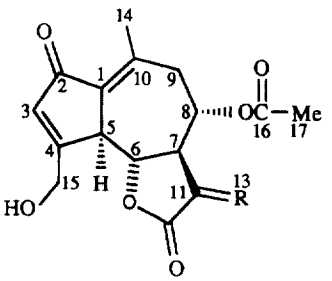
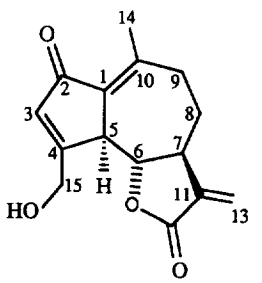


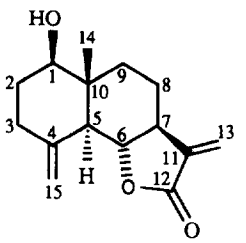
Scheme 1. Biosynthetic pathway of farnesyl pyrophosphate.



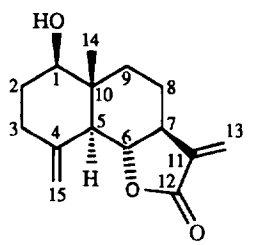
R
1 βH, α-Me
2 CH_2



3 8-Desoxylactucin



4 Reynosin



5 Santamarine

[12, 13] and santamarine (**5**) [12, 14], were found as minor products. The latter lactones might represent artefacts, which could have been formed from costunolide-1,10-epoxide during chromatographic procedures on silica gel [12]. Since only the NMR spectra of a derivative of **1** were previously reported [10], ^{1}H NMR assignments of lactone **1** were carried out by 2D COSY and 2D HETCOR experiments, and detailed assignments of its ^{13}C NMR spectrum (Table 1) were achieved by the use of DEPT 135° and DEPT 90° experiments, ^{1}H–^{13}C correlations [15] as well as spectral comparison with reported values for **2** [10] and the acetate of **3** [11]. The ^{1}H NMR spectral data for **2** was essentially identical with reported values [10] and its ^{13}C NMR absorptions are listed in Experimental.

Hairy root clones of *L. floridana* were established by the root-inducing plasmid of soil bacterium *A. rhizogenes* into the genome of *L. floridana* [16]. These clones were grown in bioreactors [8, 9] and the labelled precursors were added aseptically to the reactors two weeks after

Table 1. ^{13}C NMR spectral data and percent of ^{13}C enrichments in [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$]acetate-derived 11βH,13-dihydrolactucin-8-*O*-acetate (**1**)

Carbon	δ(ppm)*	Percentage enrichment			$^2J(^{13}C–^{13}C)$ (Hz)
		[1-^{13}C]acetate	[2-^{13}C]acetate	[1,2-^{13}C]acetate	[1,2-^{13}C]acetate
1	132.37 *s*	—	0.34	0.92	52.9
2	194.40 *s*	2.99	—	1.39	53.0
3	133.55 *d*	—	0.99	0.86	32.2
4	171.28 *s*	5.40	—	1.41	46.2
5	48.50 *d*	—	2.09	1.44	39.4
6	80.54 *d*	1.30	—	1.91	39.3
7	58.76 *d*	—	1.96	1.48	42.2
8	70.10 *d*	0.78	—	1.90	42.2
9	44.77 *t*	—	1.96	0.70	37.0
10	146.40 *s*	3.03	—	1.92	39.8
11	40.86 *d*	1.45	0.28	1.20	37.1
12	176.38 *s*	0.59	6.37	0.71	48.6
13	14.90 *q*	—	1.92	2.13	37.4
14	21.49 *q*	—	3.22	3.15	39.9
15	62.49 *t*	—	1.23	2.48	46.1
16	169.68 *s*	4.97	—	1.98	59.2
17	21.10 *q*	—	1.77	2.18	59.7

*Recorded at 100 MHz in $CDCl_3$; inverse gated ^{13}C NMR data were obtained for singly labelled precursors and broad band decoupled spectra were recorded for the doubly labelled precursor.

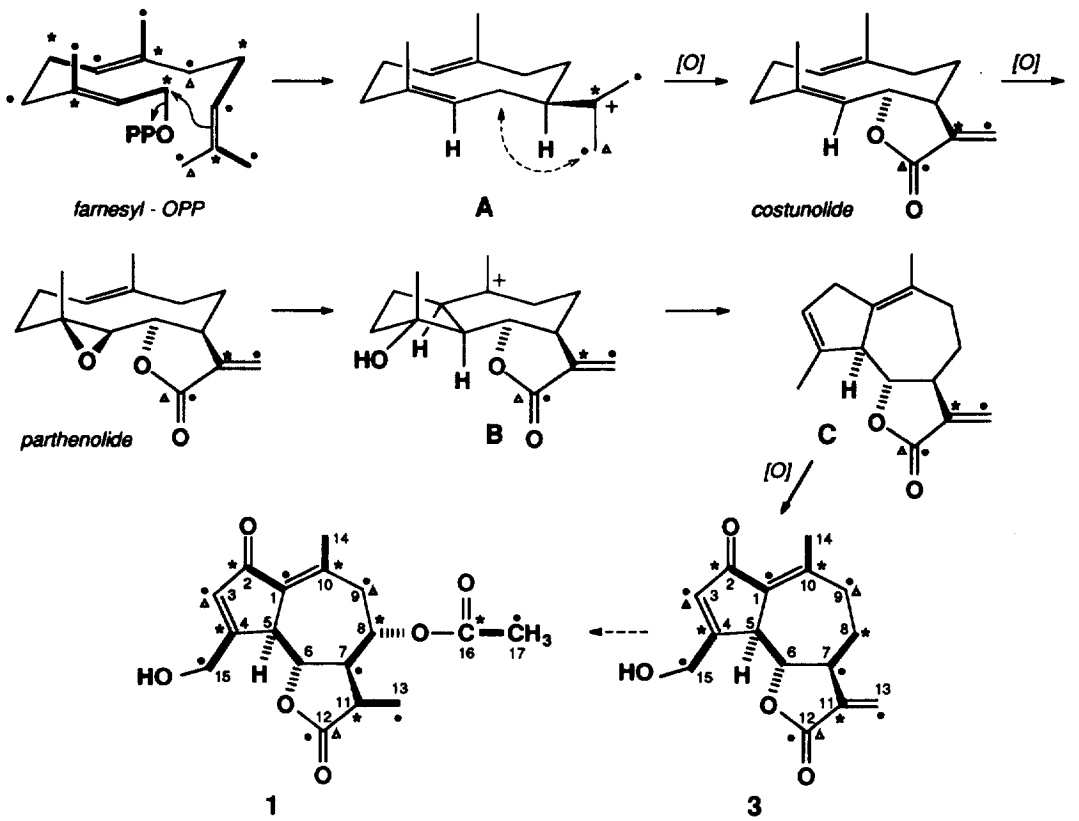
inoculation. After harvest, the hairy roots were extracted with dichloromethane, and the crude extract was chromatographed as outlined above for natural roots. Unlabelled and labelled **1** and **3** were isolated in pure form, and their chemical structures were verified by spectroscopic methods [15] and by spectral comparison with reported data [10, 11]. In several instances, mixtures of unlabelled and labelled lactones **1** and **2** were submitted to $NaBH_4$ reduction in methanol to obtain pure **1**, which was used for further spectroscopic studies.

Biogenetic hypothesis proposes that the guaianolide skeleton is formed via the acetate–mevalonate pathway to give farnesyl (nerolidyl) pyrophosphate (Scheme 1). As proposed in Scheme 2, cyclization of farnesyl pyrophosphate provides the germacradiene skeleton **A** [1, 2]. Enzymic oxidation at C-6 and C-12 of **A** is most likely followed by lactonization to give the germacranolides costunolide, and upon bio-epoxidation, parthenolide. Trans-annular cyclization of the hypothetical intermediate parthenolide would provide the guaianolide skeleton **C** via cation **B**. Further oxidative bio-modifications of **C** would lead to lactones **3** and **1** (Scheme 2). The most probable initial lactone would be **3**, which must be further bio-modified to give lactone **1**.

The ^{13}C NMR data for unlabelled **1**, [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$] acetate-enriched **1** are given in Table 1. The ^{13}C NMR spectra of the unlabelled and the singly labelled experiments were recorded on the same scale (absolute intensity) to allow for easy comparison of relative peak intensities, thus deriving the magnitude and the distribution of ^{13}C incorporations. The ^{13}C enrichments of single-labelled precursors were calculated using a previously reported method [17, 18]. As summarized in Table 1, [1-^{13}C]acetate-enriched **1** exhibited significantly enhanced signals for carbons 2, 4, 6, 8, 10, 11 and 16, and [2-^{13}C]acetate-enriched **1** showed significant enhancements of carbon signals 1, 3, 5, 7, 9, 12, 13, 14, 15 and 17. Results similar to those for **1** were obtained for **3** enriched with singly labelled acetates (Table 2). These findings are in full agreement with the proposed biogenesis of guaianolides [2], as outlined in Scheme 2 for **3** and **1**.

In both compounds **1** and **3**, it was observed that the lactonic carbonyl carbons (C-12) and the C-13 methyl group in **1** as well as the exocylic methylene carbon (C-13) in **3** represent non-equivalent carbons. This indicated that the two prochiral methyl groups at the cationic centre C-11 in intermediate cation **A** remain non-equivalent throughout the biosynthetic steps towards lactones **1** and **3**. This suggests that the cationic carbon centre C-11 must be associated with the enzyme surface not allowing free rotation of the C-7/C-11 bond in intermediate **A** or its biosynthetic equivalent.

In the ^{13}C NMR spectrum of [1,2-$^{13}C_2$]acetate-enriched **1**, most carbons exhibited the characteristic 'triplets' which result when doubly labelled acetate is incorporated into the molecule as an intact unit. The centre peak of a 'triplet' is due to the natural abundance signal, and the two satellite signals represent doublets caused by ^{13}C–^{13}C spin–spin coupling from incorporations of intact acetate units [17]. Of significance in doubly labelled **1** were the vicinal coupling constants ($^2J_{c,c}$) observed between C-1/C-2 (53.0 Hz), C-4/C-15 (46.2 Hz), C-5/C-6 (39.4 Hz), C-7/C-8 (42.2 Hz), C-10/C-14 (39.9 Hz), C-11/C-13 (37.3 Hz) and C-16/C-17



Scheme 2. Proposed biosynthesis of 8-desoxylactucin (3) and 11βH,13-dihydrolactucin-8-*O*-acetate (1).

Table 2. ^{13}C NMR spectral data and percentage of ^{13}C enrichments in [2-^{13}C]mevalolactone-derived and [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$] acetate-derived 8-desoxylactucin (3)

Carbon	δ(ppm)*	Percentage enrichment				$^2J(^{13}C–^{13}C)$ (Hz)
		[1-^{13}C]acetate	[2-^{13}C]acetate	MVA-[2-^{13}C]	[1,2-^{13}C]acetate	[1,2-^{13}C]acetate
1	131.13 *s*	—	0.32	—	0.92	53.0
2	195.16 *s*	1.74	—	0.30	0.63	53.3
3	133.26 *d*	—	1.59	1.31	0.74	31.0
4	171.53 *s*	1.68	—	—	0.74	46.7
5	49.83 *d*	—	1.76	0.26	1.16	38.7
6	83.87 *d*	3.80	0.14	0.17	1.32	38.7
7	52.74 *d*	—	2.47	0.09	1.05	36.6
8	24.25 *t*	4.20	0.83	—	1.19	36.6
9	37.31 *t*	—	2.62	1.26	0.59	34.7
10	153.34 *s*	1.42	—	—	1.22	39.3
11	138.37 *s*	1.99	—	—	0.71	75.3
12	168.87 *s*	—	0.82	0.54	0.59	63.8
13	119.31 *t*	—	1.68	0.19	1.34	75.6
14	21.95 *q*	—	1.78	0.19	1.39	39.5
15	62.48 *t*	0.17	2.86	0.17	1.54	46.1

*Recorded at 100 MHz in $CDCl_3$; inverse gated ^{13}C NMR data were obtained for singly labelled precursors and broad band decoupled spectra were recorded for the doubly labelled precursor.

(59.4 Hz). This indicated that the above carbon pairs (shown in bold in structures **1** and **3** of Scheme 2) were derived from intact acetate units, which were incorporated in accord with the proposed pathway outlined in Schemes 1 and 2. Carbons 3, 9 and 12 of **1** appeared as multiplets with J values of 32.2 Hz for C-3, 37.0 Hz for C-9 and 48.6 Hz for C-12. These couplings are known to occur by substantial $^{13}C–^{13}C$ couplings between adjacent carbons not derived from the same acetate moiety, which exhibit couplings due to high overall ^{13}C

enrichment of the doubly labelled molecule [8, 18]. The absence of strongly enhanced specific ^2J values for C-3, C-9 and C-12 in **1** indicated that during the biosynthesis these three carbons must be derived from the acetate units which lost the carboxyl carbon during the fragmentation step, leading from mevalonic acid to isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Scheme 1). The above incorporations of [1,2-$^{13}C_2$]acetate into the guaianolide skeleton of **1** are highly specific and are in full accord with the labelling patterns shown in Scheme 2. The incorporation of doubly labelled [1,2-$^{13}C_2$]acetate into **2** (not shown) and **3** gave similar results (Table 2). The data obtained from acetate-[1,2-$^{13}C_2$]-enriched **1** and **3** are consistent with the values obtained with singly labelled [1-^{13}C] and [2-^{13}C]acetate experiments and support our biogenetic proposal for guaianolide-type sesquiterpene lactones via the acetate–mevalonate–germacradiene route, leading to the guaianolide skeleton, as outlined in Scheme 2.

Additional support for the above proposed biogenetic pathway was obtained by biosynthetic studies of lactone **3** by feeding hairy root cultures of of *L. floridana* with [2-^{13}C]mevalolactone. As outlined in Scheme 2, incorporation of [2-^{13}C]mevalonic acid into **3** was expected to show ^{13}C enrichments at positions C-3, C-9 and C-12, as marked by symbol △ in **1** and **3**. The ^{13}C NMR spectra of ^{13}C-enriched **3**, obtained by incorporation of [2-^{13}C]mevalolactone, showed specific enrichments at C-3, C-9 and C-12 (Table 2). These results provided further evidence for the specific transformation of mevalonic acid into the guaianolide skeleton of **3**, as outlined in Schemes 1 and 2.

In conclusion, all ^{13}C-incorporation data obtained in the experiments described above confirmed the previously proposed biogenesis for guaianolide-type sesquiterpene lactones [2] via the acetate–mevalonate–germacradiene pathway. The question remains open at which stages of the biosynthetic sequence hydroxylation/lactonization of the sesquiterpenoid skeleton takes place.

EXPERIMENTAL

^{1}H and ^{13}C NMR spectra were recorded on Bruker AC 200 and AM 400 spectrometers. IR spectra were obtained as a film on KBr platex. Mass spectra were determined on Hewlett-Packard 5971A GC and TSQ70 FAB mass spectrometers. Semi-prep. HPLC sepns were performed on a 10 μm C18 reverse phase column (250 × 10 mm, Alltech) coupled with a LDC/Milton Roy CM 4000 multi-solvent delivery system and an ISCO UV detector. Analyt. HPLC was carried out on a 10 μm C8 or C18 reverse phase column (250 × 4.6 mm, Alltech) coupled with a Hewlett-Packard 1090 HPLC system with diode array detection. VLC [19] sepns were run on silica gel (MN Kieselgel G) and TLC was performed on precoated MN Sil-G 25 UV254 plates (0.25 and 1.0 mm).

Materials. Roots of *L. floridana* (L.) Gaertn. were collected on 15 November 1989 in East Baton Rouge Parish, LA, U.S.A.; the voucher specimen (N. H. Fischer No. 409) is deposited at the Louisiana State University Herbarium.

Hairy root cultures. The hair root clones T1, L1-3 and L4-6 of *L. floridana* were established by infection with *A. rhizogenes* strain TR 105 as described before [8, 9]. The hairy roots were transferred and subcultured in 50 ml of phytohormone free, modified Murashige and Skoog's medium [20] in 125 ml flasks on a rotary shaker in the dark at 150 rpm (at 25°) every 3–4 weeks. The modified MS medium, in which the MS vitamins and Fe were replaced by B5 vitamins and Fe, was supplemented with 30 g l^{-1} sucrose, and the pH was adjusted to 5.7–5.8, prior to autoclaving of the medium, with 0.1 N KOH/HCl.

Incorporation experiments. Hairy roots (20–35 g per bioreactor) from 3-week-old liquid cultures were inoculated into a bioreactor at the same medium conditions described above for subcultures. The bioreactor used for culturing the hairy roots was an inverted 10 l polycarbonate carboy (Nalgene) with a fritted glass air sparger placed in the cap opening. The top of the reactor was fitted with an air outlet and inoculation port. The ^{13}C-labelled precursor soln ([1-^{13}C], [2-^{13}C], [1,2-$^{13}C_2$] NaOAc, and [2-^{13}C]mevalolactone) was added aseptically to each bioreactor (0.4 or 0.2 g 9 l^{-1}) through a sterilized filter 2 weeks after inoculation. All experiments were conducted in aseptic conditions. The hairy roots were harvested after a further 4 weeks, rinsed with H_2O, blotted dry and weighed immediately to give the fr. weight.

Extractions and sepns of constituents from natural and hairy roots. Air-dried natural roots of *L. floridana* were exhaustively extracted at room temp. with CH_2Cl_2, yielding *ca* 2% of extract. The crude extract (0.88 g) was subjected to VLC using mixts of hexane and EtOAc of increasing polarity to yield 36 frs of 20 ml each. The combined frs 25–27 were purified by HPLC with 30% CH_3CN–H_2O, affording 10 mg **1** and 5 mg **2**. Frs 18–24, after prep. TLC with 1% MeOH–$CHCl_3$ (× 4) yielded 7 mg **4** and 3 mg **5**.

Fresh hairy roots of *L. floridana* (1068 g) fed with non-labelled acetate were extracted under the same conditions described above. After removal of CH_2Cl_2, the crude extract (2.5 g) was subjected to VLC using mixts of hexane and EtOAc of increasing polarity to yield 49 frs of 20 ml each. Frs 30–35 were combined and further purified by HPLC with 30% CH_3CN–H_2O producing 18 mg of a mixt. of **1** and **2**, and 19.0 mg **3**. The mixt. of **1** and **2** was converted into pure **1** by reduction with $NaBH_4$ as described below.

Fresh hairy roots of *L. floridana* (194 g) fed with 0.4 g [1-^{13}C]acetate were extracted under conditions described above. VLC of the crude extract (105 mg) using mixts of hexane and EtOAc of increasing polarity yielded 38 frs of 20 ml each. Frs 25–28 were combined and further purified by HPLC with 30% CH_3CN–H_2O to afford 10.8 mg **1**, 4.6 mg **2** and 9.7 mg **3**.

Fresh hairy roots of *L. floridana* (348 g) fed with 0.4 g [2-^{13}C]acetate were extracted as described above. The crude extract (511 mg) was subjected to VLC using mixts s of hexane and EtOAc of increasing polarity to give 48 frs of 20 ml each. Further purification of frs 34–37 by HPLC under the above conditions gave 6.2 mg **1**, 3.4 mg **2** and 10.3 mg **3**.

Fresh hairy roots of *L. floridana* (880 g) fed with 0.4 g [1,2-^{13}C]acetate were extracted as described above. The crude extract (1.0 g) was subjected to VLC using mixts of hexane and EtOAc of increasing polarity providing 31 frs of 20 ml each. The combined frs 24–26 were further purified by HPLC with 30% CH_3CN–H_2O affording 5.8 mg **1**, 6.1 mg **2** and 20.5 mg **3**.

Fresh hairy roots of *L. floridana* (1261 g) fed with 0.25 g [2-^{13}C]mevalolactone were extracted as described above. After removal of solvent, the crude extract (3.70 g) was subjected to VLC using mixts of hexane and EtOAc of increasing polarity to yield 52 frs of 20 ml each. Frs 34–39 were combined and further purified by HPLC with 30% CH_3CN–H_2O providing 19.5 mg of a mixt. of **1** and **2**, and 26.3 mg **3**. The mixt. of **1** and **2** was converted into pure **1** by reduction with $NaBH_4$ in MeOH (see above).

11βH,13-*Dihydrolactucin*-8-*O*-*acetate* (**1**). $C_{17}H_{20}O_6$, Needles; mp 107–109° (dec.); FABMS *m/z* (rel. int.): 320 $[M]^+$ (2), 260 $[M - Ac]^+$ (13), 231 (58), 187 (100); ^{1}H NMR (400 MHz, $CDCl_3$): δ1.33 (3H, *d*, *J* = 6.8 Hz, H-13), 2.11 (3H, *s*, Ac), 2.33 (1H, *m*, H-7), 2.39 (1H, *dd*, *J* = 11, 13 Hz, H-9α), 2.45 (3H, *br s*, H-14), 2.57 (1H, *m*, H-11), 2.73 (1H, *dd*, *J* = 13, 1.8 Hz, H-9β), 3.71 (1H, *dd*, *J* = 10, 1.5 Hz, H-5), 3.75, (1H, *t*, *J* = 10 Hz, H-6), 4.53 (1H, *br d*, *J* = 17.5 Hz, H-15a), 4.84 (1H, *ddd*, *J* = 10, 10, 1.8 Hz, H-8), 4.86 (1H, *br d*, *J* = 17.5 Hz, H-15b), 6.44 (1H, *d*, *J* = 1.0 Hz, H-3). ^{13}C NMR data: Table 1.

Lactucin-8-*O*-*acetate* (**2**). $C_{17}H_{18}O_6$, gum; EIMS *m/z* (rel. int.): 318 $[M]^+$ (0.9), 276 $[M - CO_2]^+$ (1), 258 $[M - Ac]^+$ (10), 229 (40), 43 $[Ac]^+$ (100). ^{13}C NMR (100 MHz, $CDCl_3$): δ132.70 *s* (C-1), 194.22 *s* (C-2), 133.34 *d* (C-3), 171.03 *s* (C-4), 48.43 *d* (C-5), 80.87 *d* (C-6), 54.74 *d* (C-7), 69.10 *d* (C-8), 44.46 *t* (C-9), 146.01 *s* (C-10), 135.87 *s* (C-11), 167.99 *s* (C-12), 122.30 *t* (C-13), 21.42 *q* (C-14), 62.49 *t* (C-15), 169.64 *s* (CH_3–$\underline{C}O$–), 21.01 *q* ($\underline{C}H_3$–CO–).

8-*Desoxylactucin* (**3**). $C_{15}H_{16}O_4$, gum; EIMS *m/z* (rel. int.): 260 $[M]^+$ (100), 242 $[M - H_2O]^+$ (4), 231 (10), 214 (32). ^{13}C NMR data: Table 2.

Reductive conversion of lactucin-8-*O*-*acetate* (**2**) *into* **1**. A *ca* 1:1 mixt. of **1** and **2** (30 mg) in 6 ml dry MeOH was treated with $NaBH_4$ (6.6 mg). The soln was allowed to react for 1 hr at 0°, then quenched with 5% aq. HCl. The solvent was removed *in vacuo*, the residue taken up in 20 ml H_2O, and extracted with CH_2Cl_2 and dried over $MgSO_4$. Removal of CH_2Cl_2 under red. pres. yielded crude **1**, which was further purified by HPLC as described above to provide pure **1** used for spectral analysis.

^{13}C NMR spectroscopy. For precise calculation of incorporation yields, inverse gated ^{13}C NMR experiments were performed in order to exclude nuclear Overhauser effects [21]. Integration of ^{13}C satellite peaks served as an alternative method for calculation of incorporation yields. The distribution of the label was ascertained by comparing the ^{13}C NMR spectra of the labelled and natural abundance spectra recorded under identical conditions. Relative ^{13}C enrichments were expressed as the ratio of the intensity of each peak in the labelled compound divided by the intensity of the same peak in the natural abundance spectrum, normalized to give a ratio of 1.0 relative to selected non-enriched peaks.

Acknowledgements—This research was supported by the National Science Foundation Biotechnology Program (Project No. EET-8713078). We thank the LSU Office of Research and Economic Development for a Biomedical Research Sub-Grant.

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