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QUINOLIZIDINE ALKALOIDS AND THE ENZYMATIC SYNTHESES OF THEIR CINNAMIC AND HYDROXYCINNAMIC ACID ESTERS IN *LUPINUS ANGUSTIFOLIUS* AND *L. LUTEUS*

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Key Word Index—*Lupinus angustifolius*; *L. luteus*; Fabaceae; quinolizidine alkaloids; hydroxycinnamic acid esters; acyltransferase; hydroxycinnamoyl-coenzyme A; biosynthesis.

Abstract—The accumulation pattern of quinolizidine alkaloids during development of seedlings and young plants of *Lupinus angustifolius* and *L. luteus* are described. The enzymes catalysing the syntheses of the cinnamic acid *O*-ester of 13-hydroxylupanine and hydroxycinnamic acid *O*-esters of lupinine (4-coumaroyl- and feruloyllupinine) are characterized and classified as cinnamoyl-CoA:13-hydroxylupanine *O*-cinnamoyltransferase (EC 2.3.1.-) and hydroxycinnamoyl-CoA:lupinine *O*-hydroxycinnamoyltransferase (EC 2.3.1.-).

INTRODUCTION

In continuation of our work on the enzymatic formation of hydroxycinnamic acid esters occurring in a wide array of plants' secondary structures [e.g. 1, 2], we have now investigated two lupine species, known to form 13-*O*-cinnamoyllupanine (Cin-lupanine) (*Lupinus angustifolius*) [3] and 4-coumaroyl- and feruloyllupinine (Coum-, Fer-lupinine) (*L. luteus*) [4, 5]. Evidence has been presented that Coum-lupinine in *L. luteus* is formed through the Coum-coenzyme A ester (Coum-CoA) [6] and recently the enzymatic synthesis of another 13-hydroxylupanine ester, i.e. 13-*O*-tigloyllupanine, in *L. albus* has been shown to proceed via tigloyl-CoA as the activated donor molecule [7]. These CoA-dependent acyltransferase activities are widespread [8, 9]. Alternative mechanisms, so far described, are the 1-*O*-acylglucose- [9] and *O*-acylquinic acid-dependent reactions [10–12], and it is quite possible that other transacylation mechanisms might also exist.

We describe (i) the accumulation patterns of the quinolizidine alkaloids focusing on the cinnamic acid ester in *L. angustifolius* and the 4-coumaric and ferulic acid esters in *L. luteus* and (ii) some properties of the CoA-dependent acyltransferases involved in the formation of Cin-lupanine, Coum-lupinine and Fer-lupinine.

RESULTS AND DISCUSSION

Accumulation pattern of alkaloids

With the aid of GC-MS analyses 16 alkaloids, including one new structure, i.e. 13-*O*-caproyllupanine (**7**), have been identified from *L. angustifolius* and *L. luteus* (see structure scheme in Fig. 1). These compounds, extracted from dry and germinating seeds and the different organs of developing seedlings and young plants, have been quantified by GC. Traces of GC analyses from the cotyledons and primary leaves are shown in Fig. 2. *Lupinus angustifolius* showed tetrahydrorhombifoline (**1**) [13, 14], angustifoline (**2**) [15, 16], isolupanine (**3**) [17, 18], lupanine (**4**) [19, 20], 13-hydroxylupanine (**5**) [18, 21], 13-*O*-tigloyllupanine (**6**) [3, 13, 22], the new 13-*O*-caproyllupanine (**7**), 13-*O*-benzoyllupanine (**8**) [3, 22], 13-*O*-*Z*-cinnamoyllupanine (**9**) [3, 22], and 13-*O*-*E*-cinnamoyllupanine (**10**) [3, 22]. *Lupinus luteus* gave lupinine (**11**) [23, 24], gramine (**12**) [25, 26], sparteine (**13**) [24, 27], ammodendrine (**14**) [28–30], *E*-4-coumaroyllupinine (**15**) [4, 31], and *E*-feruloyllupinine (**16**) [5, 31, 32].

The retention index of the new 13-*O*-caproyllupanine (**7**) is in accordance with the value calculated by extrapolating the retention indices of homologous esters with shorter chain length [33]. The mass spectral data (GC-MS) showed the characteristic fragmentation pattern of esters of 13-hydroxylupanine with *m/z* 246 as base peak formed by elimination of the carboxylic acid moiety [22] and other intense ions at *m/z* 112, 134, and 148. The molecular ion at *m/z* 362 substantiates the proposed structure of **7**. This structure was confirmed by comparison with a chemically synthesized sample.

Some of the quantitative data are summarized in Table 1. Both *Lupinus* species showed high amounts of alkaloids in the cotyledons in the dry seeds, i.e. **2**, **4** and **5** in *L. angustifolius* and predominantly **11** in *L. luteus*. The amounts of these compounds in the cotyledons declined rapidly during seedling development, whereas they appeared in the hypocotyl, primary leaves and roots. It seems likely that the newly accumulating alkaloids in the developing organs of the young plants of *L. angustifolius* are derived from the storage pools of the dry seeds, which is indicated by calculating the total quinolizidine alkaloid balance. On the other hand, the alkaloids (mainly **11** as quantitatively relevant component) in *L. luteus* seem to be partly newly synthesized in the roots and overall disappeared (more than 80%) during development of the

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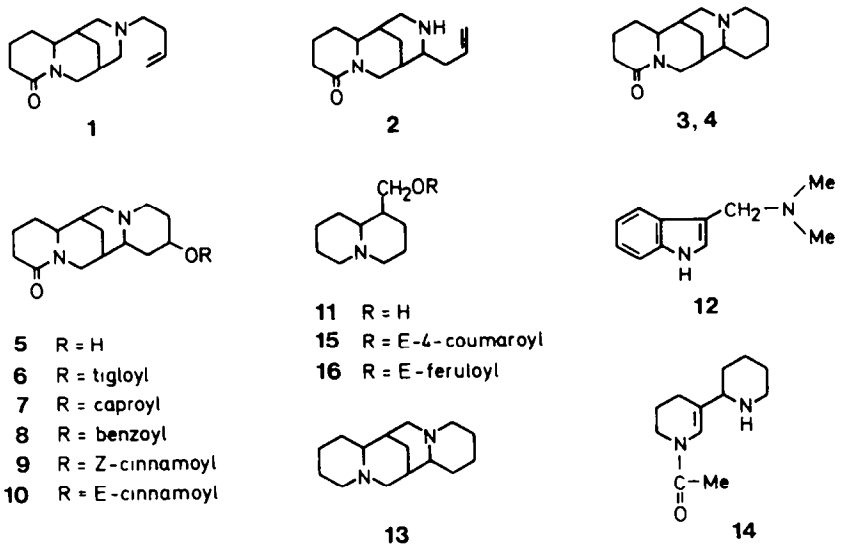

Fig. 1. Structures of the alkaloids of *L. angustifolius* (**1**–**10**) and *L. luteus* (**11**–**16**).

young plant (degradation or conjugation reactions?). The quantitative pattern of the alkaloids from *L. luteus* is in agreement with results of Wink and Witte [34] on lupin seedlings, who observed significant decreases of the amounts of quinolizidine alkaloids during germination and seedling development. The fate of these alkaloids is unknown.

Appreciable amounts of these compounds accumulate in their conjugated forms (*O*-esters), i.e. in *L. angustifolius* the tigloyl (**6**), caproyl (**7**), benzoyl (**8**) and cinnamoyl (**9**, **10**) esters and in *L. luteus* the 4-coumaroyl (**15**) and the feruloyl (**16**) esters. In the cotyledons and primary leaves of *L. angustifolius* the *O*-esters reached 80 and 50% (day 30), respectively, of the total alkaloids; in the cotyledons of *L. luteus* the HCA-*O*-esters reached between 15 and 20% (days 6–8). With the exception of **6** the *O*-esters are exclusively present in the aerial plant organs.

Enzymatic syntheses of Cin-lupanine and Coum- and Fer-lupinine

To investigate the enzymatic syntheses of the HCA-acylated alkaloids, $(NH_4)_2SO_4$-precipitated (30–70% saturation) protein from freshly prepared acetone powders of six-day-old *L. angustifolius* (accumulation of **9**, **10**) and four-day-old *L. luteus* plants (accumulation of **15**, **16**), respectively, were used. It was found that these plants contain alkaloid-specific CoA-dependent HCA-*O*-transferases, the cinnamoyl-CoA : 13-hydroxylupanine *O*-cinnamoyltransferase (Cin-transferase; EC 2.3.1.-) from *L. angustifolius* and the hydroxycinnamoyl (4-coumaroyl, feruloyl)-CoA : lupinine *O*-hydroxycinnamoyltransferase (HCA-transferase; EC 2.3.1.-) from *L. luteus* in their aerial parts (hypocotyl, cotyledons, primary leaves; not documented). This conclusion is supported by the general criteria such as reaction linearity with time and amount of protein as well as substrate dependence and enzyme kinetics. The enzyme reactions showed hyperbolic saturation curves with increasing substrate concentrations. Lineweaver–Burk plots were linear from which apparent K_m values at fixed concentrations (suboptimal) of the respective second substrate were determined. Both enzyme activities were stable for at least six months when stored as acetone powder at −20°. However, frozen (−20°) intact plants lost more than 90% of enzyme activities within three months. There were no activities as determined by GC when protein was extracted with buffer from freshly harvested plants, avoiding preparation of the acetone powder.

Some properties of the two transferase reactions, catalysed by the 13-hydroxylupanine Cin-transferase from *L. angustifolius* and the lupinine HCA-transferase from *L. luteus*, are summarized in Table 2. The formation of the products can be formulated as shown in Fig. 3. The reactions showed quite different properties. They are acceptor- and donor-specific and are different with respect to their cofactor requirements as well as optimal buffer concentration, pH optimum and temperature maximum. Interestingly only the maximal activity of the 13-hydroxylupanine Cin-transferase is dependent on the presence of EDTA and a SH-group agent (e.g. DTT) which is in accordance with the previously described 13-hydroxylupanine tigloyltransferase from *L. albus* [7]. In addition, the pH optimum and the temperature maximum correspond to those of the tigloyl transferase reaction. The strict acceptor specificity of the Cin-transferase among the hydroxycinnamic acids tested is a good indication that there are separate, highly specific transferases responsible for the formation of the other 13-hydroxylupanine *O*-esters in *L. angustifolius*. This should be proved by purification of the individual enzymes.

The identification of the lupinine HCA-transferase from *L. luteus* substantiates the results of Murakoshi and coworkers [6] who demonstrated with *L. luteus* seedlings the formation of **15** in the presence of adenosine triphosphate (ATP) and free coenzyme A. In summary, the results of the present paper support the central role of the HCA-CoAs in HCA conjugation reactions along with the 1-*O*-acylglucoside-dependent ones [9].

EXPERIMENTAL

Plant material, growing conditions and harvest. Seeds of *Lupinus angustifolius* and *L. luteus* were purchased from 'Sämerei

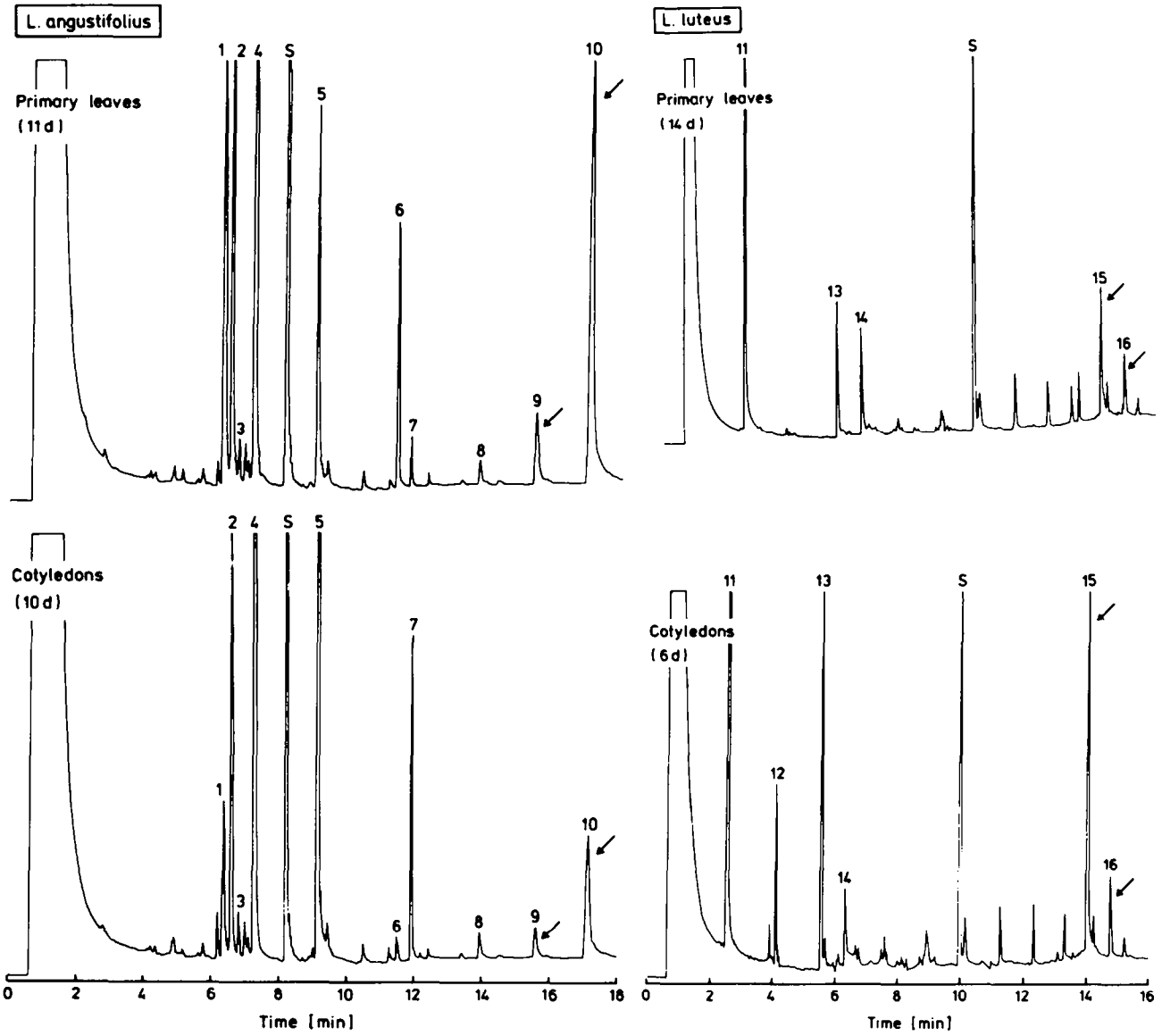

Fig. 2. GC analysis traces of alkaloid extracts from cotyledons and primary leaves of *L. angustifolius* and *L. luteus*. For peak identification see numbering system in Fig. 1; S = scopolamine as standard. The arrows indicate the peaks corresponding to 13-*O*-*Z*- (**9**) and 13-*O*-*E*-cinnamoyllupanine (**10**) as well as *E*-4-coumaroyl- (**15**) and *E*-feruloyllupinine (**16**).

Schmitz und Laux' (Hilden, F.R.G.) and 'Landhandel Schridde' (Peine, F.R.G.), respectively. Seedlings and young plants were grown in a greenhouse under a 16-hr-day at 20–25° in a defined soil (standard soil Type T, Einheitserdewerk, Hameln, F.R.G.). Plants were harvested at the same time of day (4–5 p.m.), dissected into their different organs which were immediately processed for protein preparation or treated with liq. N_2 and kept at −20° until further processing.

Substrates. Cinnamic acid and HCAs were obtained from Fluka (Buchs, Switzerland) and lupinine–HCl from Roth (Karlsruhe, F.R.G.). 13-Hydroxylupanine was kindly supplied by M. Wink (Heidelberg, F.R.G.). Hydroxycinnamoyl-CoA esters were chemically synthesized by the ester-exchange reaction via the acyl *N*-hydroxysuccinimide esters [35] and purified on polyamide (CC 6; Macherey-Nagel, Düren, F.R.G.) columns [36]. The filtrate of the reaction mixt. in question containing the succinimide ester and crystalline dicyclohexylurea was dried under red. pres. The ester was redissolved in Me_2CO and carefully poured dropwise into an aq. $NaHCO_3$-containing solution of CoA (Serva, Heidelberg, F.R.G.). After a reaction time of 12 hr in a stoppered tube under N_2, the mixt. was concd under red. pres. to remove the organic solvent. The aq. residue was filtered through glass wool onto a H_2O-equilibrated polyamide column (24 cm × 2 cm i.d.) and fractionated using 300–350 ml each of H_2O, MeOH, and 0.15, 0.44, 0.73, 1.5, 4.4 and 7.3% aq. NH_3 in MeOH. Whereas the 0.15–0.44% NH_3 and 4.4–7.3% NH_3 frs contained the free HCAs and free CoA, respectively; the HCA-CoAs were eluted with the 0.73–1.5% NH_3 eluants. Purities and yields (30–40%) were estimated UV-spectroscopically [8, 35] and by HPLC (on prepacked Nucleosil C_{18} [Macherey-Nagel] gradient elution with solvents A [1.5% H_3PO_4 in H_2O] and B [1.5% H_3PO_4, 20% HOAc and 25%

Table 1. Amounts of quinolizidine alkaloids* in the dry and germinating seeds as well as the different organs of *L. angustifolius* and *L. luteus* during development of the seedlings and young plants

	Alkaloids† from *L. angustifolius*							Alkaloids† from *L. luteus*			
Day‡	**2**	**4**	**5**	**6**	**7**	**8**	**9+10**	**11**	**13**	**15**	**16**
Cotyledons (μmol × 10^2 per pair of cotyledons)§											
0	111	672	341	‖	—	—	—	200	32		
1	109	552	290	—	—			191	24		—
2	111	684	356			—	—	292	26		
3	78	484	334	2	33	15	1	265	30	3	2
4	74	457	295	1	197	27	12	213	19	14	3
6	39	284	184	1	287	42	16	121	23	27	3
8	26	177	116	1	219	54	19	131	25	23	4
12	24	131	73	1	280	57	18	90	23	13	3
16	11	72	33	1	220	59	14	64	19	11	3
20	13	77	60	1	144	59	13	46	20	6	2
30	6	21	23	3	105	67	13	17	12	4	3
Hypocotyl (μmol × 10^2 per hypocotyl)											
3	7	59	6	2	—	—	1	¶			
4	25	140	15	4	7	10	3	45	2	5	—
6	37	240	41	7	30	20	8	31	3		
8	33	210	54	14	10	38	11	52	4	4	—
12	36	180	64	13	12	35	9	41	5	4	
16	22	150	52	14	9	34	6	37	4		
20	16	99	63	17	7	32	6	29	5	—	—
30	11	45	29	7		42	3	10	3	—	
Primary leaves (μmol × 10^2 per pair of primary leaves)											
6	11	57	6	4	31	13	15				
8	22	62	15	6	14	30	33	21	2	10	—
12	45	110	41	15	27	59	60	59	6	10	2
16	27	88	26	18	21	52	47	78	8	9	5
20	29	99	29	23	21	58	48	81	4	7	4
30	20	72	22	18	18	79	21	21	4	4	4
Roots (μmol × 10^2 g^{-1} fr. wt)											
3	64	401	56	31		—	—	285	—		—
4	80	338	55	30	—	—	—	275			—
6	70	470	27	52	—	—	—	157			—
8	76	336	34	63			—	182			—
12	107	459	56	75	—	—		134		—	—
16	104	503	76	32	—	—		86	—		
20	94	388	139	46	—	—		78	—	—	
30	72	280	85	28	—		—	7			—

*Mean of two separate GC analyses from two separate groups of plants ($n = 20$).

†Numbering as in the structure scheme.

‡Age of growing plants: day 0 = dry seed; days 1, 2 (*L. angustifolius*) and days 1–3 (*L. luteus*) = seed germination; day 5 (*L. angustifolius*) and day 7 (*L. luteus*) onwards = developing young plant.

§Actual data multiplied by 10^2.

‖ Not detected.

¶Blank if organ not yet developed.

MeCN in H_2O]). The frs from the polyamide column containing the HCA–CoAs were carefully taken to dryness under red. pres. at room temp., redissolved in an appropriate amount of H_2O to give concns of *ca* 2–5 mM based on an average log ε of 4.3 at λ_{max} [35]. The polyamide used for this purification was freed from fine particles and thoroughly washed with 1 M aq. NH_3 in MeOH, followed by H_2O, 1 M aq. HCl and finally H_2O.

TLC. On microcrystalline cellulose ('Avicel'; Macherey-Nagel): S1, *n*-BuOH–HOAc–H_2O (3:1:1); S2, *n*-BuOH–HOAc–H_2O (6:1:2); detection with a Dragendorff spray reagent. The cinnamoyl- and HCA-alkaloids were detected under UV light with and without NH_3 vapour: cinnamoyl absorbing at 254 nm, 4-coumaroyl absorbing at 350 nm changing to dark blue fluorescence with NH_3 vapour, feruloyl blue fluorescence at 350 nm changing to greenish blue with NH_3 vapour. The following alkaloids (R_f values in S1 and S2) were detected: **4** (0.60 and 0.48), **5** (0.48 and 0.41), **9** and **10** (0.80 and 0.72), **11** (0.87 and 0.59), **13** (0.81 and 0.67), **15** (0.66 and 0.85), **16** (0.71 and 0.82).

GC. Separation of the alkaloids (1 and 2 μl injection vol. from alkaloid extracts and enzyme assays, respectively) was achieved on a 15 m WCOT DB1-15N silica glass capillary column (0.23 mm i.d.) on a Dani 6800 chromatograph with a temp.

Table 2. Properties of the 13-hydroxylupanine Cin-transferase reaction from *L. angustifolius* and the lupinine HCA-transferase reaction from *L. luteus*

Property	Cin-transferase	HCA-transferase
pH optimum	7.8	7.0
K-Pi optimum	22 mM (50% at 55 mM)	30–140 mM
Temperature maximum	30°	37°
EDTA + DTT	Maximal activity (100%)	—*
EDTA	37% of maximal activity	—
DTT	39% of maximal activity	—
EDTA and DTT omitted	55% of maximal activity	—
V Cin-lupanine	1.0 pkat/mg protein	0†
V Coum-lupinine	0	1.1 pkat mg^{-1} protein
V Fer-lupinine	0	0.6 pkat mg^{-1} protein
K_m 13-hydroxylupanine	0.38 mM with Cin-CoA	0
K_m lupinine	0	0.12 mM with Coum-CoA 0.16 mM with Fer-CoA
K_m Cin-CoA	1.26 mM with 13-hydroxylupanine	0
K_m Coum-CoA	0	0.55 mM with lupinine
K_m Fer-CoA	0	0.71 mM with lupinine
K_m Caf-CoA	0	0
K_m Sin-CoA	0	0

*No effect.
†No activity.
Key: Cin, cinnamoyl; Coum, coumaroyl; Fer, feruloyl; Caf, caffeoyl; Sin, sinapoyl.

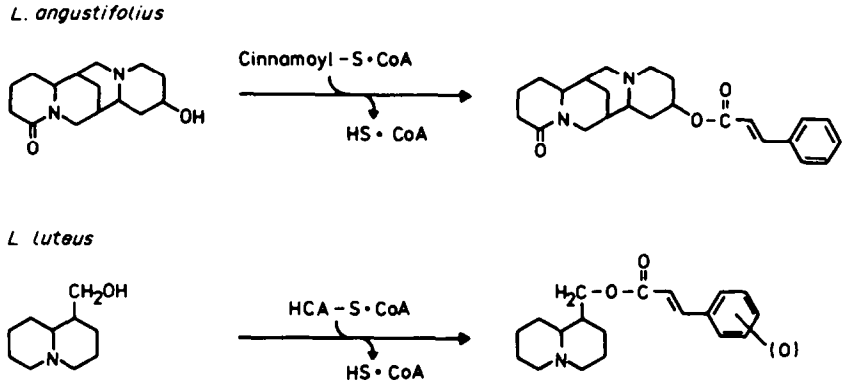

Fig. 3. Reaction scheme for the formation of 13-*O*-cinnamoyllupanine (EC 2.3.1.-) in *L. angustifolius* and hydroxycinnamoyllupinine (EC 2.3.1.-) in *L. luteus*. The ring-attached oxygen represents different stages of oxidation, here 4-hydroxycinnamic acid (4-coumaric acid) and 4-hydroxy-3-methoxycinnamic acid (ferulic acid).

program from 150–300° with 12° min^{-1} for *L. angustifolius* and 120–300° with 12° min^{-1} for *L. luteus*, followed by 7 min at 300°; carrier gas He (0.7 bar); split ratio 1:20; FID detection; quantification (Shimadzu C-R1B Chromatopac) through internal standardization with scopolamine; recorder at 2 mV. Kovat's retention indices were obtained by co-chromatography with a homologous series of hydrocarbons according to ref. [37]. The following values were obtained: **1**, 2050; **2**, 2085; **3**, 2105; **4**, 2170; **5**, 2400; **6**, 2750; **7**, 2825; **8**, 3090; **9**, 3280; **10**, 3405; **11**, 1395; **12**, 1620; **13**, 1780; **14**, 1865; **15**, 2850; **16**, 2965; scopolamine (internal standard), 2295.

GC-MS. GC on a 30 m WCOT DB1-30W silica glass capillary column (0.32 mm i.d.), with the same conditions as described above at 0.55 bar He and a split ratio of 1:30, was coupled with a quadrupole-mass spectrometer (Finnigan MAT 4515). Mass spectra were recorded at 45 eV every second with an Incos Data System. *m/z* (rel. int. %) of caproyllupanine (**7**): 362 ($[M]^+$, 3), 263 (4), 247 (48), 246 (100), 231 (10), 148 (27), 134 (60), 112 (37), 55 (28). The same MS data were obtained from a chemically synthesized sample of **7** with a seed extract from *L. angustifolius* and caproate-Cl (Merck-Schuchardt, Hohenbrunn, F.R.G.) according to a described method [22].

Extraction and quantification of alkaloids. The organs from the seedlings and young plants of the lupines were cut into small pieces which were treated for 4–5 min with an Ultra Turrax homogenizer in 20 ml 50% aq. MeOH containing 1 M HCl and 100 µl of a standard aq. soln (100 mM) of scopolamine (Roth, Karlsruhe, F.R.G.). The homogenates were allowed to stand for 30 min with continuous stirring and then centrifuged at 26000 *g* for 10 min. Aliquots (5 ml) of the clear supernatants were each mixed with 2 ml 25% aq. NH_4OH which were applied to Extrelut columns (10 g hydromatrix; Merck, Darmstadt,

F.R.G.). The alkaloids were eluted with 100 ml CH_2Cl_2 which was evapd to dryness under red. pres. and the residues redissolved in 2 ml 50% aq. MeOH. Quantifications were achieved by capillary GC using scopolamine as int. standard.

Preparation and activity determination of acyltransferases. Enzymatic activities were prepared by Ultra Turrax homogenization of the plant material in cold (−20°) Me_2CO. The resulting powder ('acetone powder') was washed with Me_2CO and Et_2O (× 3 each) and finally dried in a desiccator and kept at −20° until required. Standard extraction of protein was performed by a modified method according to ref. [7]. *Lupinus angustifolius*: the acetone powder was mixed with 200 mM K-Pi buffer (pH 7.8; 1 ml per 35 mg powder) and allowed to stand for 30 min at 4° with continuous stirring. Then polysterene resin XAD was added (0.4 g per ml buffer) and allowed to stand for another 5 min. The homogenate was passed through Miracloth and the filtrate centrifuged for 30 min at 43000 *g*. The enzymic activity from the supernatant was precipitated by $(NH_4)_2SO_4$ (30–70% saturation) and redissolved in a minimal vol. of 22 mM K-Pi buffer (pH 7.8). Excess $(NH_4)_2SO_4$ was removed by filtration through Sephadex G-25. *Lupinus luteus*: preparation of the enzyme was essentially performed as described for *L. angustifolius* except that both buffers (105 mM, pH 7.0), that for protein extraction and that for the enzyme assay, contained 1 mM EDTA and 10 mM DTT. Quantification of the enzymatically formed products was achieved by GC (see above) and internal standardization (addition of 5 μl of 25 mM aq. solution of scopolamine). Incubations and activity determinations were carried out as described elsewhere [7]. The assays (300 μl) were run at 30° at their optimal pHs and buffer concns, were terminated within reaction time linearity after 15–20 min by the addition of 200 μl 25% NH_4OH, applied to Extrelut columns and the products quantified by GC. Some properties of the enzymatic activities, listed in Table 2, were investigated as previously described (standard procedures) with another enzyme [12].

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