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ALKALOIDS OF YOUNG PONDEROSA PINE SEEDLINGS AND LATE STEPS IN THE BIOSYNTHESIS OF PINIDINE*

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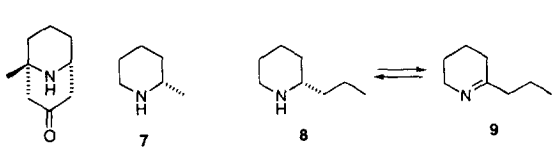
Key Word Index—*Pinus ponderosa*: Pinaceae; piperidine alkaloids; pinidine; biosynthesis.

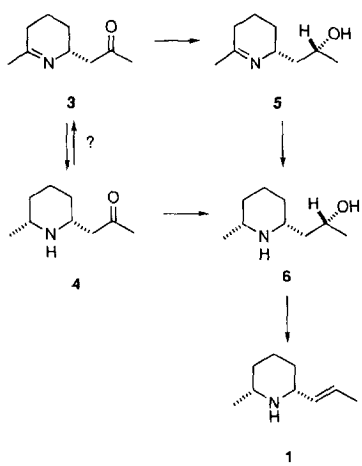
Abstract—The pattern of ponderosa pine (*Pinus ponderosa*) piperidine alkaloid accumulation from seeds to 28-day-old seedlings was assessed. Seeds were devoid of alkaloids but eight-day-old seedlings contained several alkaloids whose concentrations remained low with continued growth, while the concentration of pinidine [*cis*-2-methyl-6-(2-propenyl)-piperidine] steadily increased. ^{13}C-Labelled *cis*-pinidinone [*cis*-2-methyl-6-(2-oxopropyl)-piperidine] was taken up through seedling roots and was incorporated into pinidine.

INTRODUCTION

Ponderosa pine (*Pinus ponderosa*) was recently found [1, 2] to contain several novel *cis*-2,6-disubstituted piperidine alkaloids, along with pinidine, **1**, a long-known similar alkaloid [3]. Early biosynthetic studies on pinidine in Jeffrey pine showed it to be acetate-derived, but no more proximate precursors could be established [4–6]. The new alkaloids we discovered compose a structurally-related sequence which suggested a reasonable series of late biosynthetic steps for the formation of **1** (Scheme 1 [1].) This also provided for formation of 1-methyl-9-*nor*-3-granatanone, **2**, now found to be common among pine and spruce alkaloids [2]. This alkaloid was originally isolated from *Euphorbia atoto* (Euphorbiaceae) [7], later from ladybird beetles (*Cryptolaemus montrouzieri*) [8] and then from the Mexican bean beetle, *Epilachna varivestis* [9], where it was named euphococcinine. More recently, additional 2,6-disubstituted piperidines similar to those of pine and spruce have been identified from *E. varivestis* [10, 11].

We report here the alkaloid composition of *P. ponderosa* as a function of early growth from seed and the results of feeding studies with a labelled precursor alkaloid.





Scheme 1. Possible pathways in pinidine biosynthesis as suggested by structural relationships of isolates.

RESULTS AND DISCUSSION

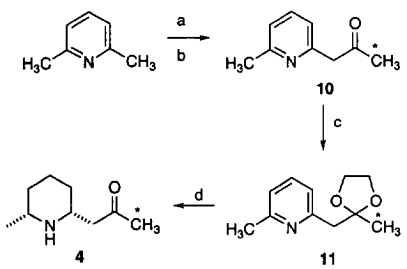
Pinus ponderosa seeds contained no detectable alkaloids. The time-course and pattern of alkaloid production was then assessed in seedlings from germination to 28 days growth (Table 1). Whole seedlings were analysed. Pinidine content increased continually until it levelled out at *ca* 24 days; it appears to be the final product of alkaloid biosynthesis in the seedlings. The levelling-off of pinidine production is most likely a consequence of the nature of the experiment, because no nutrients were added. In addition to pinidine, seedlings produced the previously described [1–3] alkaloids **2**–**7**. Unknown alkaloids of M_r 153 (isomeric, but not identical with **2** and **5**),

*Part 5 in the series 'Conifer Alkaloids'. For Part 4, see ref. [2].

and 169 were also detected by GC-mass spectrometric analysis. An unknown with *m/z* 137 (which may not be the M_r) was also present. The data are consistent with the hypothesis of Scheme 1, since the concentrations of the supposed early precursors (e.g. **3** and **5**) are relatively high in young seedlings (6–10 days) when compared with **1** at this time.

One group of 22-day-old seedlings was divided into hypocotyls, cotyledons and roots for separate analyses. Seedlings of this age had not yet developed true stems and juvenile leaves (needles). Total alkaloid content was in the order cotyledons > hypocotyls > roots. Only one significant difference was observed among the three groups in alkaloid pattern. The concentration of the iminoketone **3** was especially high (130 $\mu g\, g^{-1}$ of tissue) in the roots, where it equalled that of **1**.

In order to probe the biosynthesis of **1**, synthesis of ^{13}C-labelled precursor alkaloids was undertaken. Alkaloid **3** was prepared but was unstable in aqueous solution under the conditions we wished to use for the incorporation experiment. In one branch of Scheme 1, ketone **4** is postulated as a pinidine precursor. We therefore prepared ^{13}C-labelled (±)-**4** (Scheme 2) and fed it to growing 12-day-old *P. ponderosa* seedlings through the roots. Seedlings were sampled after nine, 15 and 25 days of further growth. A total alkaloid mixture was obtained and, because of volatility problems, the alkaloids were converted to the HCl salts for chromatographic purification. Per cent incorporations into pinidine of 1, 5 and 7.5% for the nine, 15 and 25 day growth periods, respectively, were observed with the label exclusively in C-10 (Fig. 1). The ^{13}C NMR spectrum of the crude mixture of alkaloid HCl salts before chromatography indicated the presence of two additional alkaloids, each with a single ^{13}C resonance enhancement, but they did not survive the purification process. Only traces of **2** and **3** were detected by GC-mass spectrometry of the total alkaloid mixture and the possible incorporation of label could not be



a) BuLi, Et_2O. b) $^{13}CH_3CON(OMe)Me$ from $^{13}CH_3COCl$.
c) $HOCH_2CH_2OH$, p-TsOH. d) PtO_2; aq. HCl work-up.

Scheme 2. Synthesis of ^{13}C-labelled *cis*-pinidinone, **4**.

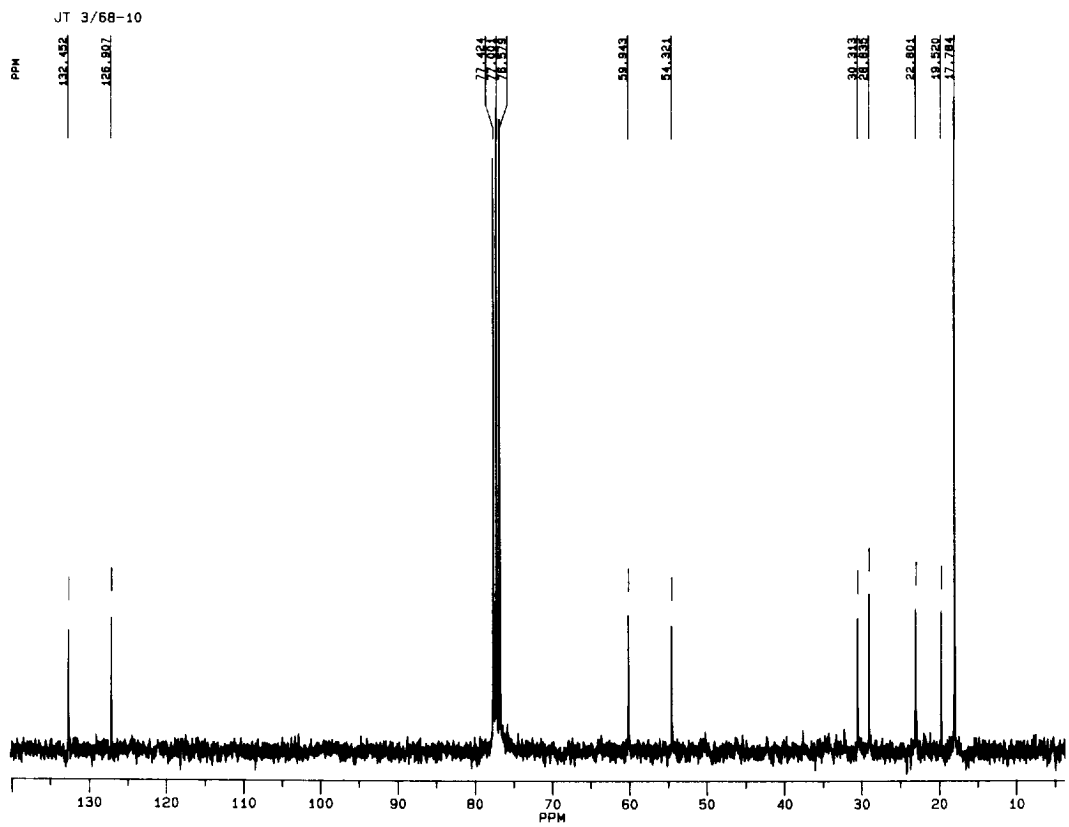


Fig. 1. ^{13}C NMR spectrum of biosynthesized pinidine hydrochloride from pine seedlings fed labelled *cis*-pinidinone, **4**.

Table 1. Alkaloid concentrations in *Pinus ponderosa* seedlings ($\mu g\ g^{-1}$ fresh plant)

Age (days)	1	2	3	4	5	6	7
5	trace	trace	trace	trace	trace	trace	trace
6	0.67 (0.28)*	4.1 (2.3)	5.2 (2.1)	—	6.1 (4.0)	1.3 (0.9)	0.36 (0.05)
8	11 (3)	15 (2)	27 (7)	2.0 (0.01)	14 (7)	3.7 (2.4)	1.4 (0.4)
10	39 (4)	21 (1)	42 (5)	4.2 (0.9)	19 (6)	8.6 (2.4)	2.4 (1.3)
12	80 (22)	23 (2)	40 (10)	7.1 (2.4)	10 (3)	12 (4)	3.7 (0.8)
14	140 (49)	27 (4)	30 (17)	8.3 (2.0)	trace	17 (5)	6.0 (1.7)
16	240 (38)	24 (1)	34 (11)	11 (1)	—	19 (4)	9.4 (1.6)
18	290 (48)	21 (3)	25 (6)	11 (1)	—	trace	8.0 (3.7)
20	380 (52)	24 (5)	31 (8)	12 (3)	—	21 (3)	9.4 (4.8)
22	440 (55)	25 (5)	20 (14)	15 (2)	—	trace	7.1 (1.1)
24	490 (38)	21 (8)	16 (1)	18 (3)	—	20 (6)	6.8 (2.3)
26	470 (58)	17 (1)	—	14 (1)	—	—	5 7 (0.7)
28	470 (58)	—	—	trace	—	—	5.1 (1.4)

*Standard deviation.

calculated. The concentration of **6** was below the level of detection.

In the hope that the presence of excess pinidine might exert feedback control on the biosynthesis and allow accumulation of precursors, a second feeding experiment was performed in which three groups of seedlings were fed with (i) labelled **4** and unlabelled **1**, (ii) unlabelled **1** and (iii) no added alkaloids (control). A moderate accumulation (after nine days growth) of **2** and **3** from $< 5\ \mu g\ g^{-1}$, in the control (iii) to 97 and $42\ \mu g\ g^{-1}$, respectively, in experiment (i) and 78 and $49\ \mu g\ g^{-1}$, respectively, in experiment (ii) was observed.

The good incorporation of **4** shows that it serves as an important precursor of pinidine but does not clarify its relationship to **3**. Coniine, **8**, and γ-coniceine, **9**, are known to be in equilibrium in *Conium maculatum* [12]; there might be a similar equilibrium between **3** and **4** in *P. ponderosa*. Although **3** would be a direct product of cyclization from an acyclic aminoketone precursor, the presence of **3** (and other imines) in conifers could also be a result of dehydrogenation–reduction processes similar to those which occur in *C. maculatum* [12]. Intermediacy of **6** between **4** and **1** is presumed. Failure to find **6** suggests that it is very rapidly converted to **1** in the seedlings. This and other biosynthetic hypotheses will be tested in future studies.

EXPERIMENTAL

General instrumental methods, including GC and GC-MS analysis and isolation of alkaloids were as previously described [1, 2].

Seedling growth and feeding studies. Seeds of *P. ponderosa* (Dougl. ex Laws) were purchased from the Dean Swift Seed Co., Jaroso, Colorado. They were treated briefly with 5% Chlorox soln, rinsed with distilled H_2O, and germinated in the dark in plastic containers on wet paper towels. The containers were placed under a grow lamp (15 hr light cycle) 11 days after germination and the paper towels were moistened with H_2O. Alkaloid analyses were conducted on the seeds and every 2 days thereafter by GC and/or GC-MS. One additional analysis was performed at day 5. For each analysis date, replicates were performed by separate analysis of four groups of seedlings to obtain the standard deviations of Table 1. No alkaloids were detected in seeds or in the 2nd and 4th day analyses, but trace levels (unquantifiable) were detected on the 5th day. Complete data, including information on unknown alkaloids, are available in ref. [13].

For the feeding of ^{13}C-labelled (±)-**4**, 9-day-old seedlings were transferred in 3 groups to small beakers containing wet polymer beads (Agrosoak, Bath Nursery, Inc., Fort Collins). Preliminary studies showed this method of seedling growth to be preferable to immersion of the roots in water without beads. Labelled **4** was added to the beakers on alternate days beginning at day 12 (3.8 mg per feeding). Seedlings were removed and groups of 46–59 seedlings were combined for alkaloid analyses at 9, 15 and 25 days after the beginning of feeding. A differential pH extraction [1] yielded a $CHCl_3$ soln of alkaloids which could be analysed by GC-MS. A few drops of aq. HCl were then added to the soln and evapd. The residue was chromatographed (Me_2CO) on a column of basic alumina. As each fr. was eluted, one drop of 2M aq. HCl was added. Frs were combined according to ^1H NMR spectra and pure pinidine HCl (Fig. 1) was obtained after a final differential pH purification to remove traces of a nonalkaloidal contaminant. Per cent incorporation was determined by the method of ref. [15] based upon normalization of ^{13}C NMR peak intensities of all the carbons in the spectrum of the HCl salt of **1**.

A second expt was carried out similarly, except that 3 groups of seedlings were placed in beakers with polymer beads as described above. To one were added equal amounts of **1** and labelled **4**, to a second group was added only **1**, while the third group was given only distilled H_2O. Each soln was adjusted to pH 7–8 with dilute

base. Feeding commenced when the seedlings were 8 days old and terminated 9 days later. Whole seedlings were analysed for alkaloids at that time.

10-^{13}C-*Methyl*-2-(2-*oxopropyl*)-*piperidine* (±)-**4**. Prepd by a sequence of reactions through intermediates which were not purified, but carried through to the final product which was then purified by chromatography. Butyllithium (1.6 M in hexane, 6 ml) was slowly added to a stirred soln of 2,6-lutidine (1.23 g, 1.33 ml, 0.0115 mol) in Et_2O (20 ml) at 0° and the reaction mixt. stirred for 40 min at room temp. The stirred mixt. was cooled to − 65° and to it was slowly added 5 ml of an Et_2O soln containing 0.84 g (0.0096 mol) of ^{13}C-labelled *N*-methoxy-*N*-methylacetamide [^{13}MeCON(OMe)Me]. The acetamide had been prepd from ^{13}MeCOCl and *N*,*O*-dimethylhydroxylamine [14]. The reaction mixt. was left stirring at room temp. for 10 hr, after which it was acidified and extracted with 20% aq. HCl (10 ml × 4). The acidic extracts were combined, made basic and extracted with Et_2O to yield the crude ketone **10** (1.67 g, 76%). To the ketone, was added 1.41 ml (0.026 mol) of ethylene glycol, 50 ml of benzene and 2.9 g (0.015 mol) of *p*-toluenesulphonic acid. The reaction mixt. was refluxed with a Dean-Stark trap for 3 hr, washed with satd aq. $NaHCO_3$ soln, dried and evapd to leave 3.64 g (87%) of crude **11**. This was dissolved in 50 ml MeOH and 2.67 g of benzoic acid and 0.4 g of Adams catalyst added. The mixt. was hydrogenated (H_2, 60 psi) for 48 hr, was filtered, evapd, the residue dissolved in $CHCl_3$ and the soln extracted with 10% aq. HCl (10 ml × 4). The acidic aq. soln was made basic, extracted with $CHCl_3$ and the $CHCl_3$ evapd to leave 1.4 g (80%) of crude **4**, which was contaminated with lutidine and minor piperidine products. Final purification was achieved by VLC (silica gel, MeOH–$CHCl_3$, 1:9) to yield pure ^{13}C-labelled (±)-**4**, identical with previously isolated and semi-synthetically prepd (−)-**4** by ^{1}H and ^{13}C NMR and GC-MS. The ^{13}C NMR spectrum showed an intense resonance at δ 30.6 for the labelled C-10 carbon, while the natural abundance peaks corresponded to those reported for (−)-**4** [1], but were barely visible above the base line. The ^{1}H NMR spectrum showed a δ 2.12 doublet (J = 127.1 Hz) for the labelled methyl. The $[M]^+$ in the GC-MS was at m/z 156 instead of m/z 155. The presence of an m/z 141 fragment (loss of 15 mu) showed that the C-7 methyl, and not the C-10 methyl, was cleaved from the $[M]^+$.

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REFERENCES

1. Tawara, J. N., Blokhin, A., Foderaro, T. A. and Stermitz, F. R. (1993) *J. Org. Chem.* **58**, 4813.
2. Stermitz, F. R., Tawara, J. N., Boeckl, M., Pomeroy, M., Foderaro, T. A. and Todd, F. G. (1994) *Phytochemistry* **35**, 951.
3. Tallent, W. H., Stromberg, V. L. and Horning, E. C. (1955) *J. Am. Chem. Soc.* **77**, 6361.
4. Leete, E. and Juneau, K. N. (1969) *J. Am. Chem. Soc.* **91**, 5614.
5. Leete, E., Lechleiter, J. C. and Carver, R. A. (1975) *Tetrahedron Letters* **44**, 3779.
6. Leete, E. and Carver, R. A. (1975) *J. Org. Chem.* **40**, 2151.
7. Hart, N. K., Johns, S. R. and Lamberton, J. A. (1967) *Aust. J. Chem.* **20**, 561.
8. Brown, W. V. and Moore, B. P. (1982) *Aust. J. Chem.* **35**, 1255.
9. Eisner, T., Goetz, M., Aneshansley, D., Ferstandig-Arnold, G. and Meinwald, J. (1986) *Experientia* **42**, 204.
10. Proksch, P., Witte, L., Wray, V. and Hartmann, T. (1993) *Entomol. Gener.* **18**, 1.
11. Attygalle, A. B., Xu, S.-C., McCormick, K. D. and Meinwald, J. (1993) *Tetrahedron* **49**, 9333.
12. Fairbairn, J. W. and Suwal, P. N. (1961) *Phytochemistry* **1**, 38.
13. Tawara, J. N. (1994) PhD Dissertation, Colorado State University.
14. Oster, T. A. and Harris, R. M. (1983) *Tetrahedron Letters* **24**, 1851.
15. Leete, E. and Bodem, G. B. (1976) *J. Am. Chem. Soc.* **98**, 6321.