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PIPERIDINE ALKALOID CONTENT OF *PICEA PUNGENS* (COLORADO BLUE SPRUCE)*

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Abstract—The content and pattern of piperidine alkaloid accumulation in *Picea pungens*, the Colorado blue spruce, was studied as a function of growth stage and plant part. Qualitative and quantitative differences were encountered between early seedlings, mature trees, needles, stems and other plant parts. A new natural piperidine imine alkaloid, 2-methyl-6-propyl-1,6-piperideine, was found and postulated to be an intermediate leading to epidihydropinidine, the major alkaloid found in spruce needles.

INTRODUCTION

An intriguing aspect of conifer alkaloid phytochemistry is that the *Pinus* (pine) species so far studied contain only *cis*-2,6-disubstituted piperidines, while *Picea* (spruce) species contain both *cis*- and *trans*-2,6-disubstituted piperidines [1, 2]. We recently investigated piperidine alkaloid patterns in young seedlings of *Pinus ponderosa* and established that *cis*-pinidinone, 2-methyl-6-(2-oxopropyl)piperidine, is converted to *cis*-pinidine, (2*R*,6*R*)-2-methyl-6-(*Z*-2-propenyl)piperidine, in such seedlings [3].

In order to explore the genesis of the *trans*-2,6-disubstituted piperidines and provide a spruce study complementary to that of the pine, *Picea pungens* Englem, the Colorado blue spruce, was chosen for detailed analysis. Blue spruce was previously reported [4] to contain epidihydropinidine, (2*R*,6*S*)-2-methyl-6-propylpiperidine, and pinidinol, (2*R*,2*R*)-2-methyl-6-[(2*R*)-2-hydroxypropyl] piperidine, as well as a variety of other piperidine alkaloids [2]. Alkaloid content and patterns were assessed in young seedlings grown from seed, in greenhouse raised young plants and in mature trees from several field locations. Within plant and plant part variations were also studied. Spruce are susceptible to numerous diseases and insect or other herbivore attack [5], so a knowledge of which of these potentially bioactive alkaloids might be encountered by different herbivores or disease organisms was deemed of importance.

*Part 6 in the series 'Conifer Alkaloids'. Part 5: Tawara, J. N., Stermitz, F. R. and Blokhin, A. V. (1995) *Phytochemistry* **39**, 705.

RESULTS

Picea pungens *seedling alkaloids*

Seeds contained 10 μg g^{-1} *cis*-pinidinol, **5**, and other alkaloids were detectable in small amounts about five days after germination. At various stages after germination, three to six groups of fresh seedlings were analysed for alkaloid content (Table 1). Because of their small size, each group was composed of 60–80 whole seedlings (total fr. wt 1.3 to 2.0 g). Thirteen different alkaloids were detected, of which seven (**1**–**5**, **7** and **8**) were previously known isolates. Alkaloid **6** (2-methyl-6-propyl-1,6-piperideine) is reported here for the first time as a natural isolate, but was a previously known synthetic compound [6]. Identification of the remaining five unknowns will require more extensive seedling growth and structure confirmation by synthesis or semi-synthesis, but mass spectral data are available for each [7].

Of particular note (Table 1) was an early and continual high accumulation of euphococcinine (**2**) and the iminoketone **3**. The seedling alkaloid content of pinidine (**1**), *cis*-pinidinol (**5**) and epidihydropinidine (**8**) started low and increased with growth so that they, along with **2**, became the dominant alkaloids. A gradual decrease in alkaloid content was observed in older seedlings. This could have been a reflection of the fact that nutrients were not applied during the growth period.

In order to examine older seedlings, 90- and 180-day-old plants which had been greenhouse grown were analysed. Needles, stems and roots could be examined individually for alkaloid content (Table 2). In the very new growth, needles are borne on a green stem, while needles from an earlier growth spurt are on a grey-brown stem clearly separated from the new growth. The

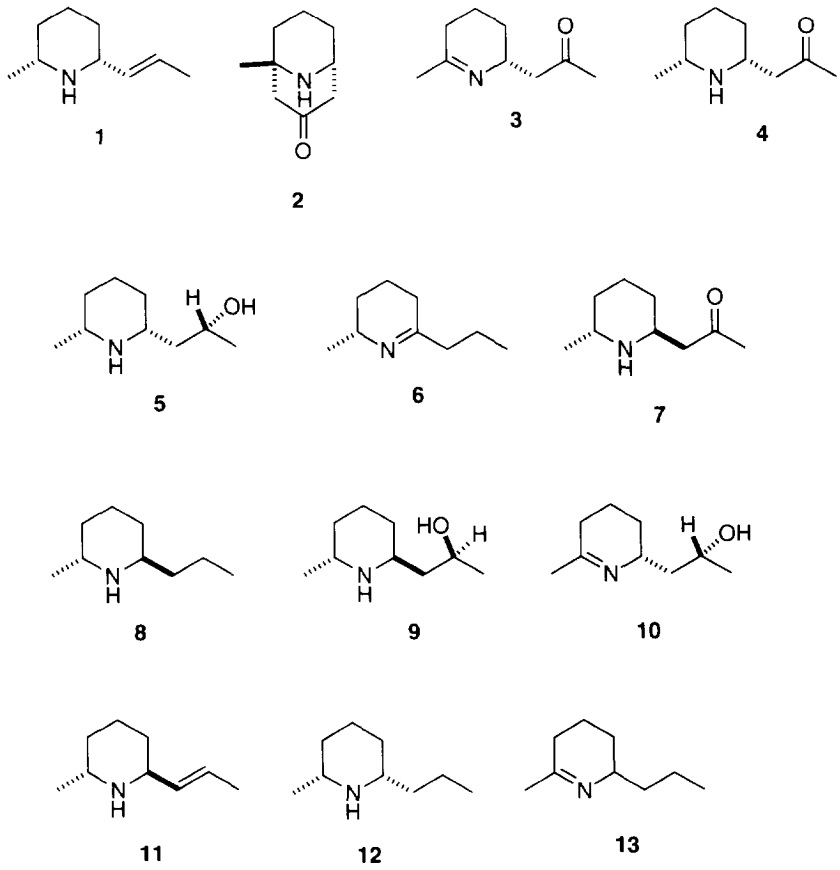


Table 1. Alkaloid content ($\mu g\ g^{-1}$ fr. wt; average of 3–6 samples) of *Picea pungens* seedlings

Age (days)	1	2	3	4	5	6	7	8
9	0(0)*	260(76)	31(9)	37(16)	36(2)	0(0)	60(22)	0(0)
13	0	660(128)	155(20)	66(4)	0(0)	20(5)	130(15)	0(0)
16	22(10)	550(56)	137(40)	45(4)	241(40)	58(11)	89(17)	0(0)
20	32(16)	511(34)	162(25)	32(6)	160(36)	63(14)	63(6)	5(9)
24	61(5)	481(105)	117(42)	29(3)	200(82)	59(10)	41(10)	74(12)
28	159(17)	659(117)	208(25)	40(5)	449(77)	128(12)	73(11)	185(20)
34	304(16)	762(61)	226(71)	51(2)	736(64)	135(12)	103(8)	525(54)
40	290(87)	602(174)	129(45)	51(11)	569(147)	70(16)	85(22)	507(115)
48	283(11)	469(34)	0(0)	55(1)	611(81)	11(7)	69(4)	430(31)
57	159(4)	360(70)	0(0)	38(2)	662(113)	0(0)	0(0)	286(54)

*Standard deviation in parentheses.

iminoketone **3** was not a major alkaloid so is not included in Table 2, nor are **4** and **7** which were also very low in concentration. A major change was the appearance of the 2,6-*trans*-piperidine alcohol (**9**), which was not detected in the younger seedlings (Table 1). The pinidinol **9** appeared in roots and stems, but not needles. The *cis*-alcohol **5** was found in new growth needles, but not older needles. Pinidine, **1**, was a major alkaloid at 90 days as it was in the young seedlings, but not in the 180-day-old plants. Epidihydropinidine, **8**, and the imine **6** were particularly high in concentration in new growth stems and new needles.

Alkaloids from mature Picea pungens

In contrast to very young seedlings, which contained a large array of different alkaloids, with several of somewhat comparable concentrations (Table 1), needles and stems from mature trees were heavily dominated by alkaloids **2**, **5** and **8**. The relative percentages of the six

major alkaloids found in two individual trees (A and B) growing close together with interwined branches are shown in Table 3, while the content of the three major alkaloids in three branches of a single tree was also examined (Table 4). These results show a dominance of **2** and **8** in needles, their low concentration or absence in stems and the reverse for concentration of **5**. This is particularly striking in Table 4, which displays results from a late winter sampling. There is a clear dichotomy in the alkaloid pattern of second year needles vs. stems, although this difference was less marked in first year needles vs. first year stems. Table 4 also provides a measure of variation in alkaloids between branches of an individual tree.

In late spring the new growth appears as small, tightly packed needle buds and these were analysed from trees A and B, as were buds from a third tree (C), from which male cones and the pistils within the cones were also examined (Table 5). Like the early seedlings (Tables 1 and 2), but unlike needles from more mature trees (Tables 3 and 4), alkaloids **3** and **6** were found and alkaloid **8** was absent. Of particular note was the high concentration of **6** in the new needle bundles and the remarkably high content of **5** in the male cone pistils. In two cases **10** was also present, a dominant alkaloid in *Pinus nigra* [1] and *Pinus ponderosa* [3], but not a common component of *Picea pungens*, other than in trace amounts.

At a local tree farm, aphids were found infesting a *Picea pungens* tree. They were clustered on branch twigs near the ends of many branches. Alkaloid content was determined for the aphids and for needles, branch bark and branch wood where aphids were feeding, along with analysis of bark from a branch that did not contain aphids (Table 6). The aphid content mirrored that of the branch bark, not that of the needles, as would be expected of aphids feeding on phloem [8, 9]. There was a slightly higher concentration of the major alkaloid **5** in the bark from a non-infested branch than in the aphid-infested branch, but these results from a single analysis need to be re-examined for a larger sample, particularly in view of some of the standard deviations seen in the seedling analyses (Table 1).

Table 2. The major alkaloids ($\mu g\ g^{-1}$ fr. wt) of 90- and 180-day-old *Picea pungens* seedlings

	1	**2**	**5**	**6**	**8**	**9**
90 day						
Roots	—	300	500	500	100	900
Stems*	700	2700	1200	3600	2300	600
Stems†	100	300	600	100	400	900
Needles*	900	1900	300	1100	3600	—
Needles†	400	1100			1400	—
180 day						
Roots	—		500	100	300	400
Needles*	—	300	700	100	800	
Needles†	—	800			300	—
Stems	—		900	—	200	700

*New growth.
†Older growth.

Identification of 6-methyl-2-propylpiperideine (**6**)

The imine **6** was found in several of the above plant analyses. It eluted just before epidihydropinidine (**8**) and just after pinidine (**1**) in the gas chromatograms. For example, the GC-mass spectral retention times under typical conditions were **1** (5.67 min), **6** (5.87 min) and **8** (6.05 min). Since the 2,6-*trans*-disubstituted piperidines always elute after the corresponding 2,6-*cis*-disubstituted piperidines [1], one possibility was that the unknown was **11**. Alkaloid **11** was prepared by dehydration of **9**, but it proved to have a 6.27 rather than a 5.87 min retention time under the same GC-mass spectral conditions [7]. The synthesis of **11** did allow us to use it as a standard and **11** was indeed identified as a trace component in spruce needles, but at level too low to quantify and include in the tables.

One needle sample which was available from a previous study on *Picea sitchensis* [2], contained a major amount of **6**, along with **8** and traces of **5**. This sample was reduced with $NaBH_4$ and submitted to GC-mass spectrometry. The peak for **6** had disappeared and was replaced by one at R_t 5.4 min, whose mass spectrum was essentially identical to that of **8** (R_t 6.05 min). The R_t 5.4 min alkaloid had a mass spectrum and retention

Table 3. Relative percentages of alkaloids in two mature *Picea pungens* trees (A and B). August sampling

	1	**2**	**5**	**8**	**9**	**10**	**12**
A Needles (1st year)	0.2	24	26	44	0.6	2.5	1.5
A Needles (2nd year)	0.4	42	3.5	45	1.1	4.8	1.3
B Needles (1st year)	—	57	6.5	36	—	—	—
B Needles (2nd year)	—	63	—	35	—	—	—
A Stems (1st year)		6.7	67	13	6.6	6.6	—
A Stems (2nd year)	—	3.4	73	5.0	13	5.4	—
B Stems (1st year)	0.7	2.9	75	7.6	9.6	3.5	—
B Stems (2nd year)	1.1	2.5	77	11	5.5	—	—

time identical to those for authentic **12**, which was prepared by catalytic reduction of **1** and by total synthesis, Scheme 1 [10, 11]. Only alkaloids **6** and **13** would yield **12** after reduction with $NaBH_4$. Alkaloid **12** was subjected to dehydrogenation with *t*-butyl hypochlorite and base [1] to yield a 5:2 mixture of two alkaloids (Scheme 1) of retention times 6.27 and 5.87 min. The mass spectral and retention time for the latter were identical to those for isolated **6**. The ^1H NMR spectrum of the mixture confirmed the GC analysis. In particular, the spectrum showed methyl doublets in a 5:2 ratio at δ1.90 (J = 1.9 Hz) and 1.21 (J = 6.8 Hz). The former peak corresponds to a methyl on the carbon of a C=N, as in **13** [1], while the latter would be typical of the C-2 methyl of **6**. Confirmation of the structure **6** was also evident from a comparison of the mass spectra of the two imines. Thus, **13** had a major peak at m/z 110 and a negligible one at m/z 111, while the reverse was true for **6**. The m/z 111 peak from **6** is readily attributable to a McLafferty rearrangement process (Scheme 2 [12, 13]) which is not available to **13**. The product ratio for the dehydrogenation is consistent with previous reactions, wherein **5** yielded exclusively **10**, while **9** gave no reaction [1].

Table 4. Major alkaloid ($\mu g\, g^{-1}$ fr. wt) of three different branches of *Picea pungens* trees. March sampling

	2	5	8
Branch 1 (needles, 1st year)	700	160	520
Branch 2 (needles, 1st year)	380	150	410
Branch 3 (needles, 1st year)	570	50	260
Branch 1 (needles, 2nd year)	1700	–	930
Branch 2 (needles, 2nd year)	1700	–	950
Branch 3 (needles, 2nd year)	730	–	660
Branch 1 (stems, 1st year)	–	1400	–
Branch 2 (stems, 1st year)	–	830	–
Branch 3 (stems, 1st year)	–	1360	–
Branch 1 (stems, 2nd year)	–	340	–
Branch 1 (stems, 2nd year)	–	380	–
Branch 1 (stems, 2nd year)	–	480	–

Table 5. Alkaloid content ($\mu g\, g^{-1}$ fr. wt) of new needles bundles, male cones and pistils of cones from *Picea pungens* mature trees

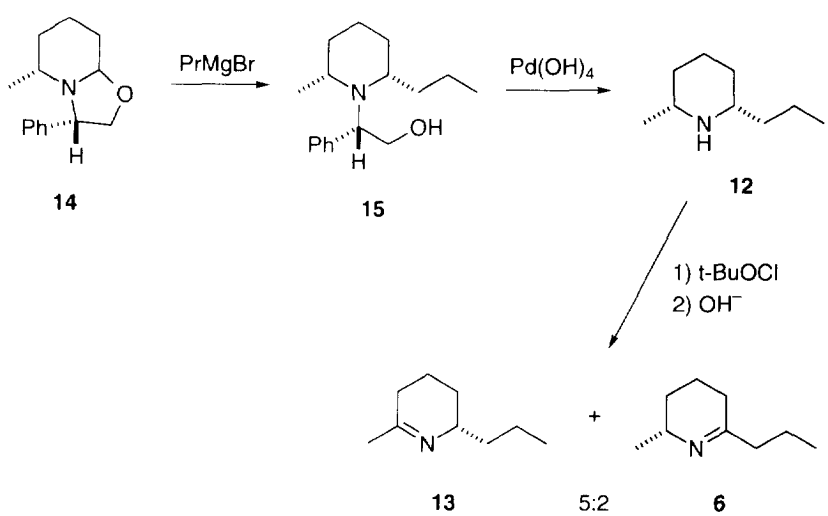
Tree	2	3	5	6	10
A Needles	1000	–	200	1000	100
B Needles	2200	–	–	520	–
C Needles	930	140	1300	2300	–
Male cones	310	50	410	810	–
Pistils	–	–	12800	–	2700

Table 6. Alkaloid content ($\mu g\, g^{-1}$ fr. wt) of aphids and infested and non-infested branches of *Picea pungens*

	2	5	8
Aphids	16	290	56
Infested branch bark	16	810	170
Infested branch bark needles	160	290	910
Infested branch bark wood	–	—	—
Non-infested branch bark	—	1100	190

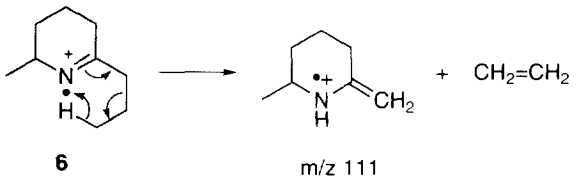
DISCUSSION

The results clearly demonstrate qualitative and quantitative differences in alkaloid content and patterns with



14 **15** **12** **13** **6**

Scheme 1. Synthesis of piperidine imines.



6 m/z 111

Scheme 2. McLafferty-type rearrangement of imine **6**.

growth stage, season and plant part. Insect herbivores will encounter different alkaloids depending on their phenological development and/or restricted feeding habits (e.g. budworms vs. bark beetles). Rational decisions on which alkaloids to examine for feeding deterrence (or attraction) against which insects, or insect life stages, can now be made. For example, early budworms will encounter mostly **2** and **6** (Table 5), while herbivores feeding on needles at a later growth stage will consume **2** and **8**. Phloem feeders are likely to encounter mostly the alcohol **5**. There is likely to be some variability within and among individual trees (Tables 5 and 6), but this aspect was not studied in a statistically valid sample.

Many of the alkaloids (e.g. **3**, **4**, **7**, **10**) appear never to be at particularly high concentration at any time or site and, based upon structural relationships, they seem to be either very early biosynthetic intermediates [3] or simple modifications of such intermediates. The final accumulated products of biosynthesis appear to be **2**, **5**, **8** and **9**. The position of pinidine, **1**, which is a major component of pines [2], is somewhat anomalous in blue spruce since it is a major alkaloid in seedlings (Tables 1 and 2), but sporadic in later growth occurrence (Table 3). Pinidine is thought to be dehydration product from **5** [3], which occurs in seedling or new growth needles, but **5** is apparently later shunted into stems and/or roots ([2] and Tables 3 and 5). This suggests that the dehydration of **5** to **1** may be occurring in needles.

The finding of **6** as a major alkaloid in early growth stages (seedlings, Table 1; new needle bundles, Table 5) is consistent with our hypothesis [2] that it is the precursor of **8** in spruce tree needles. This is also consistent with the fact that neither **6** nor **8** are found in pine tree needles. As has been pointed out [3, 14], there can be an equilibrium in the plant between a piperidine alkaloid and its corresponding imine (for example, between **3** and **4**, or **6** and **8**) so the relationship between **6** and **8** content can be a complex one. Nevertheless, the finding of a high concentration of **6** without appreciable **8** in new needle bundles (Table 5) suggests that **6** is the precursor. The relationship of **6** and/or **8** to the other *trans*-2,6-disubstituted piperidines is not clear. Since it was shown [3] that labelled **4** serves as an efficient precursor of **1** (presumably through **5**) in pines, **7** is a likely precursor for **9** in spruce. The finding that **7** is one of the major alkaloids in the 9- and 13-day-old seedlings (Table 1) is consistent with this idea. The rapid early growth accumulation of **6** and **8**, and absence of **9** and **11** at this time, suggests that the latter two alkaloids may not be directly involved in **6** and/or **8** synthesis. Additional feeding studies as well as continued identification of the structurally unknown alkaloids will be necessary to validate these or other hypotheses.

EXPERIMENTAL

Plant material was obtained from two mature (approximately 30-year-old) *Picea pungens* trees, vouchers FRS 416A and FRS 416B, growing in Fort Collins, Larimer Co., Colorado. They were identified by R. D. Moench, Colorado State Forest Service, Fort Collins, Colorado, who also provided seeds and the 90- and 180-day-old seedlings. Seeds originated from collections made in the Rio Grande National Forest by the Dean Swift Seed Co., Jaroso, Colorado. The aphid-infested *P. pungens* was at the T and M Tree Farm, north of Fort Collins. Alkaloid isolations and identifications, and GC-MS analysis methods, were generally as previously described [1]. For quantification, an HP58909A gas chromatograph with a J&W Scientific DB-1 capillary column (30 m × 0.32 mm; 0.1 mm film thickness), FID detector and an HP3392A integrator were used. Conditions were as follows: detector 300°, injector 250°, initial oven temp. 65° for 3 min; 5° min^{-1} to 75°; hold for 0.1 min, 3° min^{-1} to 87°, hold for 0.1 min then 40° min^{-1} to 290° and hold for 5.5 min (19.7 min total run time). GC response factors were obtained from the standard alkaloids **1**, **2** and **5** and the average response factor for these used for unknown peaks.

Preparation of piperideines **6** *and* **13**. The requisite precursor, **14**, was prepared according to the literature [10, 11] procedure. A soln of **14** (165 mg) in 5 ml ether was added slowly to a soln of PrMgBr in 4 ml ether at −60°. The temp. was raised to −10° and 5 ml of pH 6.2 phosphorous buffer was added. The ether layer was sepd and the aq. layer extracted with $CHCl_3$. The organic layers were combined, dried (Na_2SO_4) and evapd to yield 119 mg (60%) of **15**. A mixt. of 100 mg **15** and 10 mg $Pd(OH)_2$ on carbon in 20 ml MeOH was stirred for 12 hr at 20 p.s.i. H_2. The mixt. was filtered through Celite, 5 drops of 35% aq. HCl was added and the MeOH evapd. The residue was dissolved in 10 ml water and extracted with $CHCl_3$. The aq. layer was made basic (Na_2CO_3) to pH 10 and extracted with CH_2Cl_2. The organic layer was carefully evapd to leave 43 mg (80%) of dihydropinidine, **12**. *t*-Butyl hypochlorite (20 mg) was added to a stirred soln of 20 mg **12** in 5 ml ether and the soln stirred in the dark for 15 min. The ether was evapd, the residue was dissolved in 5 ml MeOH, 4 drops of a 20% aq. soln of Na_2CO_3 was added and the soln was stirred for 3 hr at 60°. The soln was extracted with CH_2Cl_2, the layers were sepd and the CH_2Cl_2 was very carefully evapd to yield a residue which was shown by GC-MS and NMR to be a 5:2 mixture of **13** and **6**.

GC-MS *m/z* (rel. intensity): **6**: 139 (16), 124 (31), 111 (42), 96 (100), 83 (12), 70 (35), 55 (15), 42 (37), 41 (44); **13**: 139 (26), 124 (7), 110 (96), 97 (89), 96 (100), 83 (32), 82 (64), 70 (18), 68 (25), 55 (51). ^{1}H NMR: δ1.21 (*d*, J = 6.8 Hz, H-7, **6**), 1.90 (*d*, J = 1.9 Hz, H-7, **13**), 3.25 (*m*, H-6, **13**), 3.40 (*m*, H-2, **6**). ^{13}C NMR: **6**: δ18.5, 20.1,

23.2, 28.2, 29.2, 29.5, 43.0, 52.8, 167.5; **13**: 14.0, 18.7, 19.2, 26.7, 27.2, 30.1, 39.8, 57.3, 166.7.

Preparation of **11**, *(2R,6S)-2-methyl-6-(Z-2-propenyl) piperidine, by dehydration of* **9**. Alkaloid **9**, isolated from *P. pungens*, was dehydrated by heating with fused potassium bisulphate according to the literature procedure [15]. After partial, careful evapn of a $CHCl_3$ extract of the total base fr., the soln was subjected to GC-MS. This showed, predominantly, a peak of R_t 6.27 min whose fragmentation pattern was essentially identical to that for **1** (R_t for **1** = 5.67 min). Complete careful evapn of solvent left a residue whose ^{13}C NMR spectrum showed δ132 and 125 (C=C) as well as δ52 and 47 (C-2 and C-6 for the *trans*-disubstituted piperidine, **11**. These resonances are at δ59.5 and 52.3 in the case of **1**, typical of a *cis*-disubstituted piperdine).

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