The leaf volatile terpenoids of *Pinus heldreichii* Christ from Bulgaria and comparisons with Greece and Montenegro-Serbia oils, and *P. leucodermis* oil, Italy.

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ABSTRACT

A detailed, complete analysis of volatile leaf oils of *Pinus heldreichii* from Bulgaria found the oil very high in limonene (46.5%), germacrene D (15.4%) and α-pinene (14.3%). The oil is similar to literature reports from Greece, Montenegro-Serbia and Bulgaria. Comparison with *P. h.* var. *leucodermis* (southern Italy) revealed many differences in concentrations of terpenoids. The differentiation in the oils of *Pinus heldreichii* and of *P. heldreichii* var. *leucodermis* (southern Italy) supports the recognition of var. *leucodermis* as a distinct taxon, but additional research is needed. While sampling, one tree (14734) was found that appeared to be a hybrid between *Pinus heldreichii* and *P. mugo*. Analysis of its volatile leaf oils revealed that 14734 has 10 compounds that are intermediate in concentration between *Pinus heldreichii* and *P. mugo*, 8 compounds are transgressive (shaded red in Table 2, i.e., larger than in either parent), 2 compounds are about the same concentration as in *P. heldreichii*, and 3 compounds are about the same concentration as in *P. mugo*. Taken together, these data strongly support that plant 14734 is a hybrid between *P. heldreichii var. heldreichii* and *P. mugo*.
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KEY WORDS: *Pinus heldreichii, P. mugo* hybrids, Bulgaria, volatile leaf oil, terpenes, composition, *P. leucodermis.*

Pinus heldreichii Christ was named after its discovered, von Heldreich in the mountains of northern Greece (Gymnosperm Database, 2019). It is rare and endangered in its range in the Balkans. A variety, *P. heldreichii* var. *leucodermis* (Antoine) Markgraf ex Fitschen, grows in the Calabrian region of southern Italy, the Balkans and Greece. It is often treated as a distinct species (*P. leucodermis* Antoine) in Italy (Pennacchini and Bonin, 1975).

There has been considerable research on the volatile leaf oils of *Pinus heldreichii* (see Mitic et al., 2017; Nikolic et al. 2011, for recent literature reviews). Petrakis, et al. (2001) examined the volatile oil of *P. heldreichii* growing on Mt. Katara, central Greece and reported the oil was dominated by limonene (34.3%), α-pinene (16.7%), germacrene D (12.8%), and (E)-caryophyllene (8.4%). This is similar to the report by Mitic et al. (2017) who analyzed plants from Montenegro and Serbia and noted the oils were dominated by limonene (25.8%), α-pinene (16.0%), germacrene D (15.3%), and (E)-caryophyllene (10.2%). Ioannou et al. (2014) reported similar composition from Mt. Pindos, Metsovo, Greece.

Nikolic et al. (2011) examined variation in the leaf oils of *P. heldreichii* in Montenegro and Serbia and reported the most distinguishing terpenes were germacrene D (13.5%), myrcene (2.2%), α-humulene (2.1%), α-muurolene (1.7%), α-muurolene (1.2%), γ-cadinene (1.0%), α-cadinene (0.5), and β-bourbonene (0.3%). They did not publish a complete analysis of *P. heldreichii* volatile leaf oil.

Naydenov et al. (2005) reported on chloroplast microsatellites and terpene analysis from *P. heldreichii* from Bulgaria. Thet found the volatile leaf oil was dominated by limonene (36.9 - 48.2%) and α-pinene (16.9 - 18.6%) and no significant correlation between cp microsatellite (cpSSR) and terpene variation patterns. They reported on the composition of only 10 terpenoids: α-pinene (16.9-18.6%), camphene (1.86 - 2.23%), β-pinene (5.07 - 6.49%), δ-3-carene (3.20 - 4.96%), limonene, 36.9 - 48.2%), terpinolene (0.85 - 1.01%), β-farnesene (4.73 - 7.64%), β-selinene (0.75 - 1.33%), γ-muurolene (14.85 - 22.87%), γ-cadinene (1.36 - 2.32%).

Searches of the literature revealed only one paper (Bonesi et al., 2010) reporting on the composition of *P. leucodermis* from southern Italy).

The purpose of this report, a continuation of research on leaf volatile oils of *Pinus* in Bulgaria (Adams and Tashev, 2019a,b), is to present a complete analysis of the volatile leaf of *Pinus heldreichii* from Bulgaria and compare its composition with reports from Montenegro-Serbia, and with the oil of *P. heldreichii* var. *leucodermis* (southern Italy). In the midst of this study, we found a tree with oil that was intermediate in composition between *P. heldreichii* and *P. mugo,* so the composition of this putative hybrid along with two nearby *P. mugo* tree's oils is also presented.

MATERIALS AND METHODS

Leaf samples of *P. heldreichii* collected:
BULGARIA: Pirin Mountain (North), National Park "Pirin". Between the huts "Banderitza" and "Vihren", in the valley of Banderishka river, together with *Pinus peuce, P. mugo, Juniperus communis*. 41º45'57.9" N, 23º25'26.1" E., 1815 m,18 May 2015. Coll. *Alexander & Nikolay Tashev* 2015 Sp.1-3 *PH1-PH5, Lab Acc. Robert P. Adams 14732-14736.* Population of *P. heldreichii, P. mugo* and a putative hybrid.
BULGARIA: North Pirin Mt., Bahderishka, glade location. 41˚ 45' 46" N to 41˚ 45' 57" N; 23˚ 25' 6" E, 23˚ 25' 22" E, 1807-1863 m. 12 Oct 2019, Coll. Alexander et. Nikolay Tashev Ph1-Ph10, Lab Acc. *Robert P Adams 15832-15841*. Population of only *P. heldreichii,* used to obtain a composite of oil *15832-15841* (10) to represent *P. heldreichii* oil.
Voucher specimens are deposited in the herbarium, University of Forestry, Dept. of Dendrology, 10, Kliment Ochridsky Blvd., 1797 Sofia, Bulgaria

Gently dried leaves (100g, 40 - 45˚C) were steam distilled for 2 h using a circulatory Clevenger-type apparatus (Adams, 1991). The oil samples were concentrated (ether trap removed) with nitrogen and the samples stored at -20ºC until analyzed. The extracted leaves were oven dried (100ºC, 48 h) for determination of oil yields.

The oils were analyzed on a HP5971 MSD mass spectrometer, scan time 1/ sec., directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column (see Adams, 2007 for operating details, Out of print, free pdf: www.juniperus.org). Identifications were made by library searches of the Adams volatile oil library (Adams, 2007), using the HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Limonene, sylvestrene and β-phellandrene co-elute as a single peak on DB-5, but their amounts can be quantitated by Single Ion Chromatography (SIC, Adams and Tashev 2019b). Quantitation was by FID on an HP 5890 gas chromatograph using a J & W DB-5, 0.26 mm x 30 m, 0.25 micron coating thickness, fused silica capillary column using the HP Chemstation software.

RESULTS AND DISCUSSION

The composition of the volatile leaf essentials oils of *P. heldreichii* from Bulgaria are shown in Table 1 in comparison with oils from Bulgaria (Naydenov et al. 2005), Greece (Petrakis et al. 2001), and Montenegro-Serbia (Metic, et al. 2017) and *P. heldreichii* var. *leucodermis,* Italy (Bonesi et al., 2010).

The oil composition of *P. heldreichii* var. *heldreichii* is very similar to the oil compositions reported from Bulgaria, Greece and Montenegro-Serbia (Table 1). It should be noted that the report of 18.4% γ-muurolene (Table 1, Naydenov, et al. 2005), is likely germacrene D, because the other reports (Table 1) list γ-muurolene as varying from 0.0 to 0.9%, and germacrene D ranging from 12.8% to 15.2% for *P. heldreichii*. The mass spectra of γ-muurolene and germacrene D are very similar, as are their retention times on DB5 (1478 and 1480) (Adams, 2007). It is easy to mis-identify these compounds.

It is of interest that the oil of *P. h.* var. *leucodermis* (southern Italy, Bonesi et al. 2010) are quite different from that of *P. heldreichii* from any location in numerous constituents (green, Table 1). The var. *leucodermis* (= *P. leucodermis* in many flora treatments) oil sample report (Bonesi et al. 2010)from southern Italy differed in many components from the oils of *P. heldreichii* (cpd, *leucodermis*, *heldreichii* range): α-pinene (24.2, 9.6-16.0%), limonene (7.8, 25.8-46.5%), (E)-β-ocimene (3.7, 0-0.2%), terpinolene (5.9, 0-0.6%), terpinen-4-ol (0.8, 0-0.2%), α-terpineol (1.7, 0-0.2%), linalool acetate (3.6, 0), bornyl acetate (2.7,0 - 0.7%), myrtenyl acetate (0.6%, 0%), α-cubebene (7.6; 0%), germacrene D (0.7, 12.8 - 15.4%), ethyl dodecanoate (0.3; 0-trace%), tetradecanal (0.5; 0-trace%) and manool oxide (0.6; 0-trace%). There are clearly differences in the volatile leaf oils between *P. heldreichii* var. *heldreichii*, var. *leucodermis* based on this study *and* Bonesi et al. 2010. Additional samples are needed of var. *leucodermis* to verify these differences. According to Farjon (1984) and Richardson and Rundel (1998), *P. h.* var. *leucodermis* grows in southern Italy, the Balkans and Greece, on sunny, dry slopes. Janković (1986) states that var. *leucodermis* grows in pure stands only on steep, dry rocky southern slopes. Boscherini et al. (1994) reported no variation in cpDNA amplifications and restriction patterns among all *P. leucodermis* individuals sampled from seven populations (two in Greece and five in Italy). This is remarkable, and indicates that whatever the taxonomic status of *P. leucodermis*, the same taxon grows in Italy and Greece. Unfortunately, they did not include any samples of *P. heldreichii* and so, were not able to determine if these taxa differ in the cpDNA characters.

A detailed examination of leaf oil variation among plants in a Bulgaria population revealed a tree with oil that was intermediate in composition between *P. heldreichii* and *P. mugo*, so the composition of this putative hybrid, along with two nearby *P. mugo* tree's oils were analyzed (analyses of other *P. mugo* trees are reported in Adams and Tashev (2019a). Although morphology is usually intermediate in hybrids, in two recent studies on the inheritance of terpenoids in *Cryptomeria japonica* (Adams and Tsumura, 2012) and *Pseudotsuga menziesii* (Adams and Stoehr, 2013) artificial hybrids revealed that many compounds are transgressive in the hybrids, (i.e., greater (or lower) concentration than found in either parent), the same concentration as one of the parents, or intermediate in concentration between the parents' values.

Examination of Table 2 reveals: 10 compounds are intermediate (shaded aqua): tert-butyl-benzene, sabinene, α-phellandrene, δ-3-carene, limonene, (E)-β-ocimene, terpinolene, m-cymen-8-ol, p-cymen-8-ol and bornyl acetate. Eight compounds are transgressive (shaded red in Table 2, i.e., larger than either parent in this case): myrcene, β-phellandrene, α-terpinyl acetate, β-elemene, bicyclogermacrene, γ-cadinene, δ-cadinene, and (E)-nerolidol. Two compounds are about the same concentration as in *P. heldreichii* (trace amount): α-thujene and cis-carveol, and 3 compounds are about the same concentration as in *P. mugo*: p-cymene, germacrene D, and α-muurolene (Table 2). Taken together, these data strongly support that plant 14734 is a hybrid between *P. heldreichii var. heldreichii* and *P. mugo*. It is very likely that other hybrids are in the area.

The differentiation reported in the oil of *P. h.* var. *leucodermis* (southern Italy) supports the recognition of this taxon as a distinct species, but additional research is needed to determine if its oil maintains its compositional profile throughout its range from Italy to Greece.

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Table 1. The leaf oil constituents of *Pinus heldreichii* from Bulgaria compared with other analyses on the volatile leaf oils. Compounds highlighted in yellow/green separate var. *heldreichii* and var. *leucodermis*, those with aqua highlight are unusual amounts. Compositional values less than 0.1% are denoted as traces (t). Unidentified components less than 0.5% are not reported. KI is the Kovat's Index using a linear calculation on DB-5 column.

KI	compound	Bulgaria comp oil 10 trees 15868	Bulgaria ex Naydenov[1]	Greece Mt. Pindos Ioannou[2]	Greece ex Petrakis[3]	Montenegro Serbia ex Metic[4]	*P. h.* var. *leucodermis* Italy Bonesi[4]
921	tricyclene	0.2	-	0.3	-	-	-
924	α-thujene	t	-	-	0.3	0.2	-
932	**α-pinene**	**14.3**	**17.4**	**0.1**	**13.8**	**16.0**	**24.2**
946	camphene	0.7	2.0	0.8	1.5	0.9	0.4
948	benzaldehyde	t	-	-	-	-	-
967	(tert-butyl benzene)	t	-	-	-	-	-
969	sabinene	t	-	0.6	0.8	0.1	0.1
974	**β-pinene**	**3.8**	**5.8**	**3.6**	**4.2**	**5.2**	**8.4**
988	myrcene	2.4	-	2.2	2.5	2.2	-
1002	α-phellandrene	t	-	-	-	0.0	-
1008	**δ-3-carene**	t	**4.3**	18.6	**2.8**	**2.1**	**0.2**
1014	α-terpinene	t	-	0.2	-	-	1.5
1020	p-cymene	t	-	t	-	-	0.2
1023	silvestrene	t	-	-	-	-	-
1024	limonene	46.5	42.0	23.7	34.3	25.8	7.8
1025	β-phellandrene	-	0.9	-	t	0.0	t
1044	(E)-β-ocimene	t	-	-	0.1	0.2	3.7
1054	γ-terpinene	t	-	0.3	0.1	-	1.0
1065	cis-sabinene hydrate	t	-	-	-	-	-
1082	m-cymenene	t	-	-	-	-	-
1086	terpinolene	0.2	-	2.1	0.6	0.5	5.9
1095	linalool	-	-	-	t	-	0.5
1118	trans-p-mentha-2,8-dien-1-ol	t	-	-	-	-	-
1118	cis-p-menth-2-en-1-ol	t	-	-	-	-	-
1122	α-campholenal	t	-	-	-	-	0.1
1126	cyclohexene <4-acetyl-1-me-1-	t	-	-	-	-	-
1135	trans-pinocarveol	t	-	-	-	-	0.2
1136	trans-sabinol	t	-	-	-	-	-
1133	cis-p-mentha-2,8-dien-1-ol	t	-	-	-	-	-
1141	camphor	t	-	-	-	-	-
1145	camphene hydrate	t	-	-	-	-	-
1160	pinocamphone	t	-	-	-	-	-
1165	borneol	t	-	-	t	-	-
1174	terpinen-4-ol	t	-	0.1	0.1	-	0.8
1176	m-cymen-8-ol	-	-	-	-	-	-
1179	p-cymen-8-ol	-	-	-	-	-	-
1186	α-terpineol	0.2	-	t	0.2	-	1.7
1195	myrtanol	t	-	-	-	-	-
1215	trans-carveol	t	-	-	-	-	-
1226	cis-carveol	t	-	-	-	-	-
1232	thymol, methyl ether	-	-	-	-	-	0.2
1239	carvone	t	-	-	-	-	-
1253	trans-sabinene hydrate acetate	-	-	-	-	-	-
1254	linalool acetate	-	-	-	-	-	3.6
1284	bornyl acetate	0.7	-	0.3	-	0.1	2.7
1293	2-undecanone	t	-	-	-	-	-
1309	p-vinyl guaiacol	t	-	-	-	-	-
1315	(2E,4E)-decadienal	t	-	-	-	-	-
1324	myrtenyl acetate	-	-	-	-	-	0.6
1345	α-terpinyl acetate	0.3	-	-	0.4	0.5	-
1348	α-cubebene	-	-	-	-	-	7.6
1350	citronellyl acetate	-	-	-	0.2	-	-
1374	α-copaene	t	-	t	-	-	0.4
1379	geranyl acetate	t	-	-	-	-	0.4
1387	β-bourbonene	t	-	t	-	-	0.5
1389	β-elemene	t	-	t	-	-	-

KI	compound	Bulgaria comp oil 10 trees 15868	Bulgaria ex Naydenov[1]	Greece Mt. Pindos Ioannou[2]	Greece ex Petrakis[2]	Montenegro Serbia ex Metic[3]	*P. h.* var. *leucodermis* Italy Bonesi[4]
1403	methyl eugenol	-	-	-	-	-	0.3
1400	β-longipinene	0.5	-	-	-	-	-
1409	α-gurjunene	-	t	-	0.2	-	-
1417	**(E)-caryophyllene**	**5.0**	**-**	**8.6**	**8.4**	**10.2**	**4.5**
1430	β-copaene	t	-	0.1	-	-	-
1431	β-gurjunene	-	-	-	-	1.1	-
1439	aromadendrene	t	-	t	-	0.7	-
1454	α-humulene	0.7	-	1.5	1.0	0.4	1.0
1454	**(E)-β-farnesene**	-	**6.1**	-	**-**	**-**	**0.9**
1465	(E)-ethyl cinnamate	t	-	-	-	-	-
1478	γ-muurolene	t	18.4(GRMD?	0.1	-	0.9	0.2
1480	**germacrene D**	**15.4**	**(18.4)?**	**21.3**	**12.8**	**15.2**	**0.7**
1484	aristolene	-	-	-	6.0	-	-
1489	β-selinene	-	1.1	-	-	-	-
1500	bicyclogermacrene	-	-	-	-	-	-
1500	α-muurolene	0.2	-	0.2	0.3	1.3	-
1508	germacrene A	-	-	-	-	-	-
1513	γ-cadinene	0.2	1.8	0.2	-	0.8	1.0
1522	δ-cadinene	0.4	-	0.6	0.6	1.4	1.8
1537	α-cadinene	t	-	t	-	-	t
1561	(E)-nerolidol	t	-	-	-	-	t
1565	dodecanoic acid	0.4	-	-	-	-	-
1574	germacrene-D-4-ol	t	-	t	-	0.3	t
1583	caryophyllene oxide	t	-	0.2	-	-	t
1594	ethyl dodecanoate (ethyl laurate	t	-	-	-	-	0.3
1608	humulene epoxide II	t	-	-	-	-	-
1611	tetradecanal	-	-	-	-	t	0.5
1638	epi-α-cadinol	t	-	0.1	-	-	-
1640	epi-α-muurolol	t	-	t	-	-	-
1644	α-muurolol	t	-	t	-	-	-
1652	α-cadinol	0.2	-	0.3	-	-	-
1685	germacra-4(15),5,10)14)-trien-1-al	-	-	-	-	-	-
1710	pentadecanal	-	-	-	-	-	-
1713	(2E,6Z)-farnesal	t	-	-	-	-	-
1722	(2Z,6E)-farnesal	1.0	-	-	-	-	-
1740	(2E,6E)-farnesal	t	-	-	-	-	-
1759	benzyl benzoate	-	-	-	-	-	-
1874	hexadecanol	-	-	-	-	-	-
1892	(7Z,10Z,13Z-hexadecatrienal)	t	-	-	-	-	-
1937	cembrene	t	-	t	-	-	-
1943	iso-cembrene	t	-	-	-	-	-
1959	hexadecanoic acid	0.6	-	-	-	-	-
1987	manool oxide	t	-	-	-	-	0.6
2048	thunbergol (isocembrol)	0.2	-	-	-	-	-
2055	abietatriene	t	-	-	-	-	-
2056	manool	0.2	-	-	-	-	-
2087	abietadiene	t	-	-	-	-	-
2116	phytol isomer	0.9	-	-	-	-	-
2220	isopimaral	t	-	-	-	-	-
2243	palustral (8,13-abietadien-18-al	0.2	-	-	-	-	-
2274	dehydro abietal	t	-	-	-	-	-
2310	isopimarol	t	-	-	-	1.3	-
2314	trans-totarol	t	-	-	-	-	-
2381	octadecanoic acid, butyl ester	t	-	-	-	-	-
2420	abietinol acetate (4-epi-dehydro	t	-	-	-	-	-

[1]Naydenov et al. 2005; [2]Ioannou et al. 2014, [3]Petrakis et al. 2001; [4]Mitic et al. 2017; [5]Bonesi et al. 2010.

Table 2. Putative hybridization between *P. heldreichii* and *P. mugo* in a population in Bulgaria. Compounds in yellow are typical of *P. heldreichii*; those in green - *P. mugo*; Compounds in aqua - intermediate; red - transgressive (larger than either parent). Compounds that were always less than 0.5 are not included to simplify the table.

		P. heldreichii			mugo x heldriechii	*P. mugo*		
KI	compound	tree *14732*	tree *14733*	comp oil *15868*(10)	tree *14734*	tree *14735*	tree *14736*	comp oil *15773*(5)
921	tricyclene	0.1	0.2	0.2	0.4	0.5	0.2	0.2
924	α-thujene	t	t	t	t	0.9	0.7	0.3
932	α-pinene	5.0	9.6	14.3	9.4	10.8	7.5	11.0
946	camphene	0.3	0.9	0.7	1.7	1.9	0.7	1.2
967	(tert-butyl benzene)	t	t	t	0.2	0.5	1.1	0.5
969	sabinene	t	0.1	t	0.6	1.4	1.1	1.2
974	β-pinene	1.4	2.3	3.8	3.8	3.3	5.9	2.7
988	myrcene	1.3	1.9	1.9	3.8	2.6	2.0	3.1
1002	α-phellandrene	t	t	t	0.9	0.6	0.4	0.5
1008	δ-3-carene	t	t	t	10.6	27.6	23.1	24.6
1020	p-cymene	t	t	t	0.6	0.7	0.8	0.2
1024	limonene	44.9	54.2	46.5	18.0	t	-	t
1025	β-phellandrene	-	-	-	17.8	14.4	13.1	16.7
1044	(E)-β-ocimene	t	0.1	t	0.5	0.9	0.8	0.6
1054	γ-terpinene	t	t	t	0.5	0.4	0.4	0.4
1086	terpinolene	0.2	0.2	0.2	2.3	3.4	2.9	3.9
1165	borneol	0.7	1.4	t	0.4	0.7	0.5	0.2
1174	terpinen-4-ol	0.2	0.2	t	0.3	0.5	0.6	0.4
1176	m-cymen-8-ol	t	t	-	0.1	0.3	0.7	0.2
1179	p-cymen-8-ol	t	t	-	0.2	0.5	0.7	0.3
1186	α-terpineol	0.7	1.1	0.2	0.5	0.1	0.2	t
1215	trans-carveol	0.3	0.6	t	t	0.2	0.3	t
1226	cis-carveol	0.1	0.3	t	t	0.6	1.2	-
1284	bornyl acetate	0.7	0.3	0.7	2.0	5.5	1.7	4.2
1345	α-terpinyl acetate	0.3	0.4	0.3	1.8	1.4	1.4	1.8
1389	β-elemene	t	t	t	0.6	t	0.2	0.2
1417	(E)-caryophyllene	3.5	4.7	5.0	4.0	5.2	4.9	5.3
1430	β-copaene	0.1	0.1	t	t	t	t	t
1454	α-humulene	0.6	0.8	0.7	0.6	0.9	0.8	0.8
1480	germacrene D	16.7	6.7	15.5	4.7	3.5	4.7	1.7
1500	bicyclogermacrene	0.2	0.1	-	1.8	t	t	1.1
1500	α-muurolene	0.2	0.2	0.2	0.6	0.4	1.2	0.3
1513	γ-cadinene	0.3	0.2	0.2	0.9	t	0.6	0.6
1522	δ-cadinene	0.8	0.3	0.4	1.4	0.2	0.9	1.2
1561	(E)-nerolidol	t	t	t	1.4	0.3	t	0.1
1574	germacrene-D-4-ol	0.2	1.9	t	0.2	0.9	1.8	2.3
1583	caryophyllene oxide	0.2	0.1	t	t	t	1.2	0.4
1652	α-cadinol	0.5	0.5	0.2	0.6	0.2	0.6	1.1
1710	pentadecanal	1.3	0.6	-	0.2	0.2	0.3	0.2
1722	(2Z,6E)-farnesol	0.5	0.2	1.0	t	t	t	t
1874	hexadecanol	2.4	0.3	-	t	0.2	t	t
1959	hexadecanoic acid	1.2	0.2	0.6	0.2	0.2	0.2	-
2116	phytol isomer	0.2	t	0.9	-	t	0.2	-
2243	palustral (8,13-abietadien-18-al	0.3	t	0.2	t	0.2	1.7	1.0