ANTINEOPLASTIC AGENTS, 88. *PIMELEA PROSTRATA*[1]

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Abstract.—The aerial portion of *Pimelea prostrata* (Thymelaeaceae) collected in New Zealand was evaluated as a source of substances that inhibit growth of the murine P-388 lymphocytic leukemia (PS). Simplexin (**1**) and Pimelea factor P_2 (**2**) were found to strongly inhibit growth (ED_{50} 5 x 10^{-3} and 8 x 10^{-4} µg/ml, respectively) of the PS *in vitro* cell line. The cyclic orthoester (**2**) was also found to inhibit growth (T/C 132 at 20 µg/kg) of the PS *in vivo* system. Detailed ^{1}H-nmr (at 400 MHz) and ^{13}C-nmr studies combined with fast atom bombardment mass spectral evidence were employed to confirm the structural assignments.

The Thymelaeaceae constitute a medium-sized plant family with up to 650 species distributed among some 50 genera. Six of the genera (e.g., *Daphne*) contain about 40 species (2) known to have found extensive (China and India to Europe) application in the primitive treatment of cancer. A substantial number of species are used in other primitive medical (such as epilepsy, malaria, snake-bite, and certain viral infections) treatment (3) and are known for pronounced toxic effects (4,5) when ingested by range animals, especially cattle (St. George disease) (6-9). Indeed, in the regions frequented by camels, only the Thymelaeaceae and one other plant family contain species poisonous to this animal (4).

Species (~80) of the genus *Pimelea* occur primarily in Australia and are usually unpalatable (6). But a few are responsible for St. George disease (weight loss, diarrhea, anemia, and circulatory failure). The toxin produced by *Pimelea simplex*, known as simplexin (**1**), was found to produce the characteristic symptoms of St. George disease (8). The Australian *Pimelea linifolia* and *ligustrina* (10) as well as the New Zealand shrub *Pimelea prostrata* (11) have been found to give extracts with antineoplastic activity. While the antitumor active constituents have not hitherto been identified, Hecker and colleagues (12,13) have isolated the cocarcinogenic (mouse skin) simplexin (**1**) and Pimelea factor P_2 (**2**) as well as three related irritant orthoesters from *P. prostrata*. Earlier (1972) this plant was uncovered in the U.S. National Cancer Institute's (NCI) exploratory botany programs[2] as a potential source of antineoplastic constituents. Typically, alcohol extracts were found to give a 40% (at a dose of 50 mg/kg) increase in life span against the murine P-388 lymphocytic leukemia (PS system).

A 1979 recollection (New Zealand) of *P. prostrata* (aerial portion, flowering stage) was investigated for antineoplastic components employing a variety of separation methods and the murine P-388 lymphocytic leukemia (PS system) for bioassay. The successful route has been outlined in figure 1. Rapid performance at each stage to reduce personnel contact[3] and decomposition of sensitive components was important. In this final procedure, the plant was extracted by our new methylene chloride-methanol technique, and the solution was diluted with water (14). Further separation of the methylene chloride phase was achieved using the solvent partitioning sequence (14) 9:1↦3:2 methanol-water with hexane↦methylene chloride. The PS active methylene chloride fraction was separated by an extensive series of column chromatographic steps

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[1]Consult reference (1) for part 87.

[2]In 1972 the joint NCI-USDA effort was directed by Drs. J.L. Hartwell and R.E. Perdue.

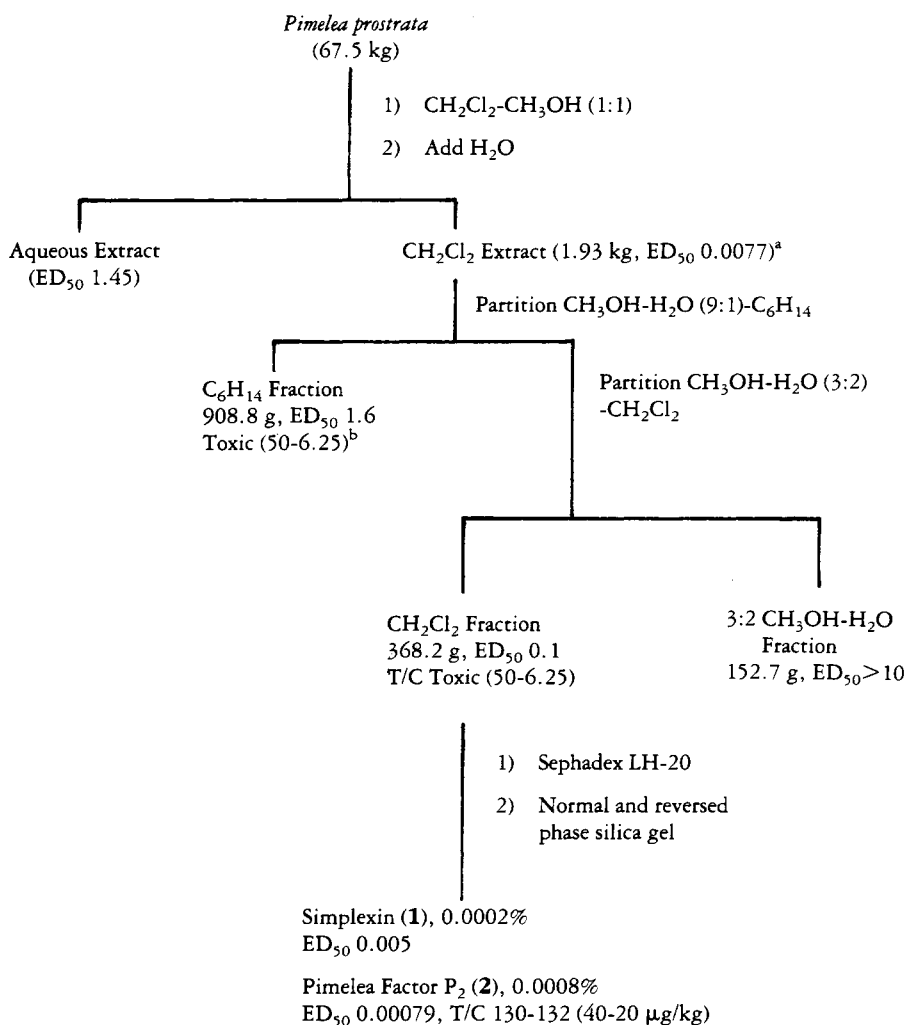[3]Two chemists experienced some facial swelling and other evidence of toxic effects.

Pimelea prostrata
(67.5 kg)

1) CH_2Cl_2-CH_3OH (1:1)

2) Add H_2O

Aqueous Extract
(ED_{50} 1.45)

CH_2Cl_2 Extract (1.93 kg, ED_{50} 0.0077)[a]

Partition CH_3OH-H_2O (9:1)-C_6H_{14}

C_6H_{14} Fraction
908.8 g, ED_{50} 1.6
Toxic (50-6.25)[b]

Partition CH_3OH-H_2O (3:2)
-CH_2Cl_2

CH_2Cl_2 Fraction
368.2 g, ED_{50} 0.1
T/C Toxic (50-6.25)

3:2 CH_3OH-H_2O
Fraction
152.7 g, ED_{50}>10

1) Sephadex LH-20

2) Normal and reversed
   phase silica gel

Simplexin (**1**), 0.0002%
ED_{50} 0.005

Pimelea Factor P_2 (**2**), 0.0008%
ED_{50} 0.00079, T/C 130-132 (40-20 µg/kg)

FIGURE 1.   Successful route of separation.

[a]µg/ml, PS *in vitro*.
[b]mg/kg, PS *in vivo*.

beginning with gel permeation (Sephadex LH-20), followed by partition (LH-20) and proceeding through normal and reverse-phase silica gels.

Although a number of PS active constituents were isolated and/or detected in trace amounts, efforts were concentrated on the two most abundant, namely the PS cell growth inhibitor orthoester **1** (ED_{50} 0.005 µg/ml, 0.0002% yield) and antineoplastic (PS) orthoester **2** (32% life extension at 20 µg/ml, ED_{50} 0.00079 µg/ml, 0.0008% yield). Initial characterization studies suggested that both inhibitors of the PS cell line were related to the daphnane-type diterpenes, and the crystal structure (15,16) of gnidimacrin (**3**) was used as a basis for interpreting spectral data. By this approach, the two diterpenes were found to resemble simplexin (**1**) and Pimelea factor P_2 (**2**). Detailed [1]H-nmr studies (table 1) at high field (400 MHz) became necessary to confirm assignment of orthoester structures **1** and **2**. The proton assignments presented in table 1 were substantiated, where possible, by decoupling experiments.

The strong coupling of the 10α-proton in Pimelea factor P_2 (**2**) with the 1β-proton

1

Simplexin

2

Pimelea Factor P$_2$

3

Gnidimacrin
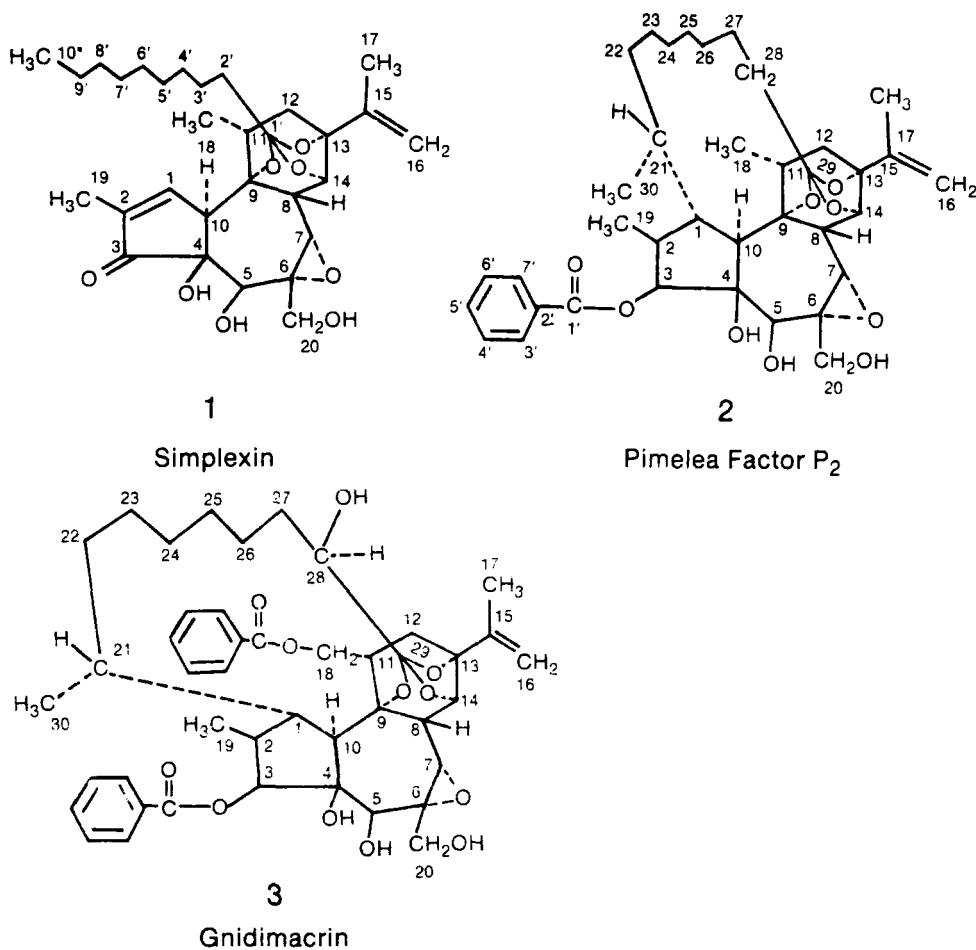
indicates a *trans*-diaxial arrangement. Hence the C-1 side chain has an α-orientation. The 1β-proton is also strongly coupled to the 2-proton, but is not coupled to the 21-proton at 2.547 ppm. Occurrence of the 21-proton at such low field (2.547 ppm) might possibly be due to a long-range deshielding effect of the orthoester moiety. Indeed, decoupling experiments indicate that the 21-proton is coupled only to 30-methyl group (δ 0.816) protons and to the 22-methylene protons in the region of 1.4 ppm. The absence of coupling between the 1β- and 21-protons indicates a dihedral angle of approximately 90°. Examination of molecular models suggests that a macrocyclic ring conformation with a 30α-methyl group is favored due to minimization of interactions between this methyl and those at C-2 (19-methyl) and C-11 (18-methyl).

Chemical shift values of the 18- and 30-methyl groups (1.424 and 0.816 ppm, respectively) in Pimelea factor P$_2$ (**2**) have been reversed from those reported earlier (13). The revision in assignments arises from the fact that irradiation of the signal at 2.633 ppm (11β-proton) results in decoupling of both the 18-methyl group doublet at 1.424 ppm and the double doublet at 2.116 ppm assigned to the 12β-proton. The latter is geminally coupled to the 12α-proton at 1.607 ppm. And this experiment thereby unequivocally established the position of the 18-methyl group protons at 1.424 ppm. Assignment of the multiplet at 1.994 ppm to one of the 28-protons was based on finding the analogous methylene protons of the model compound triethylorthopropionate at 1.79 ppm. Decoupling experiments indicated that the other 28-proton resonates at ap-

TABLE 1. Simplexin (**1**) and Pimelea factor P$_2$ (**2**) ^{1}H-nmr (400 MHz) assignments relative to tetramethylsilane in deuteriochloroform solution.

Structure 1 Assignment No.	Chemical Shift, ppm	Structure 2 Assignment No.	Chemical Shift, ppm
1	7.592 (1H, d, J=0.5 Hz)	3',7'	8.025 (2H, m)
16	5.00 (1H, s)	5'	7.609 (1H, m)
16	4.875 (1H, s)	4',6'	7.479 (2H, m)
14	4.345 (1H, d, J=2.4 Hz)	3	5.041 (1H. d, J=5 Hz)
5	4.233 (1H, s)	16	4.945 (1H, s)
20	3.864	16	4.833 (1H,s)
20	3.745 (2H, ABq, J=12.5 Hz)	14	4.261 (1H, d, J=2.2 Hz)
10	3.717 (1H, m)	5	4.089 (1H, s)
OH	3.550 (1H, br s)	20	3.855
7	3.414 (1H, s)	20	3.768 (2H, ABq, J=12 Hz)
8	2.880 (1H, d, J=2.4 Hz)	7	3.337 (1H, s)
11	2.424 (1H, m)	10	3.082 (1H, d, J=11 Hz)
12β	2.191 (1H, dd, J=14.4; 8.7 Hz)	8	2.869 (1H, d, J=2.2 Hz)
one of 2' hydrogens	1.925 (m)	OH	2.836 (1H, s)
19	1.781 (3H, s)	11	2.633 (1H, m)
17	1.754 (3H, s)	21	2.547 (1H, br s)
12α	1.622 (1H, d, J=14.4 Hz)	1	2.356 (1H, dd, J=11; 11 Hz)
one of 2' hydrogens	1.570 (m)	12β	2.116 (1H, dd, J=14; 8 Hz)
3'-9'	1.283 (br s)	28	1.994 (m)
18	1.139 (3H, d, J=7.1 Hz)	2	1.826 (m)
10'	0.854 (3H, t, J=6.7 Hz)	17	1.716 (3H, s)
		12α	1.607 (d, J=14 Hz)
		18	1.424 (3H, d, J=6.6 Hz)
			1.282 (m)
		19	1.038 (3H, d, J=6.7 Hz)
		30	0.816 (3H, d, J=6.6 Hz)

proximately 1.7 ppm. In the case of simplexin (**1**) the 2'-protons were accordingly assigned chemical shift values of 1.925 and 1.57 ppm.

The nmr spectral interpretations were supported by results of fast atom bombardment (FAB) mass spectral experiments. At this point, it was clear that the two most prominent cell-growth inhibitory constituents of *P. prostrata* must be the cocarcinogenic orthoesters **1** and **2**. An authentic specimen of Pimelea factor P$_2$ generously provided by Prof. E. Hecker was found to be identical with the substance assigned structure **2**. Structure **1** was confirmed by comparison with an authentic specimen of simplexin kindly provided by Prof. W. C. Taylor.

Interestingly, Hecker and co-workers (17) have found Pimelea factor P$_2$ (**2**) to be a new type of potent tumor promoter (cocarcinogen), whereas simplexin (**1**) exhibits only a moderate level of such activity (18). The presently reported discovery of Pimelea factor P$_2$ antineoplastic activity provides a striking illustration of a substance with superficially opposing biological properties. Such a possibility was correctly predicted by Hecker (17) for Pimelea factor P$_2$. Thus, orthoester **2**, as well as certain related diterpenes, may eventually prove very useful in better understanding the chemistry of cell-growth regulation.

EXPERIMENTAL

Each solvent was redistilled. Sephadex LH-20 (particle size, 25-100 μm) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ambient column chromatographic procedures employed silica gel (70-230 mesh), supplied by E. Merck, Darmstadt. Column chromatography under pressure was carried out using prepacked Lobar LichroPrep silica gel 60 (40-63 μm) and for reversed-phase using LichroPrep RP-8 (40-63 μm) size A and B columns (E. Merck). Fraction collection was partially automated, using a Gilson microfractionator. Thin layer chromatography was performed with silica gel GHLF and reverse phase silica gel RPS-F precoated (250 μ) plates from Analtech, Inc. The tlc plates were developed by uv light and/or ammonium metavanadate reagent.

All melting points are uncorrected and were observed by use of a Koefler-type melting point apparatus. Infrared spectra were recorded with a Nicolet MX-1 FT-IR spectrophotometer. Ultraviolet spectra were measured in methanol solution with a Hewlett-Packard model 8450A spectrophotometer. The ^{1}H-nmr spectra at 100 MHz were recorded by Dr. J. Witschel, Jr., using a Varian XL-100 instrument and the 400 MHz nmr spectra were measured (in deuteriochloroform solution) with a Brüker WH-400 nmr spectrometer by Dr. D. Gust. The ^{13}C-nmr spectra were determined (by Dr. Witschel) at 22.63 MHz with a Brüker WH-90 spectrophotometer. Tetramethylsilane was used as internal reference and δ values are reported. Mass spectra were obtained by Dr. P. Williams and Mr. D. M. Adams using a MAT 312 mass spectrometer equipped with a FAB inlet system.

PIMELEA PROSTRATA.—In 1979 about 110 kg of the stembark, stem, twigs, leaf, and flowers of this plant was collected in New Zealand. The collection was performed under auspices of the Economic Botany Laboratory, Agricultural Research Center-East, USDA, Beltsville,, MD, as part of a joint NCI-USDA program directed by Drs. M. I. Suffness and J. A. Duke.

PLANT EXTRACTION.—*P. prostrata* (67.5 kg dry weight) was ground and extracted with methylene chloride-methanol (1:1, 160 liters) at ambient temperature. Addition of 25% water caused separation into methylene chloride and aqueous phases. The methylene chloride fraction (1.93 kg, PS ED_{50} 7.7 x 10^{-3} μg/ml) was found to be very toxic (LD_{100} 50-6.25 mg/kg) in the PS *in vivo* system. The aqueous phase was less active (PS ED_{50} 1.45 μg/ml) and was not investigated further.

SOLVENT PARTITION SEQUENCE.—Successive partitioning of the methylene chloride fraction (1.93 kg) between 9:1 methanol-water with hexane and 3:2 methanol-water with methylene chloride, followed by removal of solvents from the hexane, methylene chloride, and methanol-water solutions, gave, respectively, 908.8-g (PS ED_{50} 1.6 μg/ml, *in vivo* toxic 50 to 6.25 mg/kg), 368.2-g (PS ED_{50} 0.1 μg/ml, *in vivo* toxic 50 to 6.25 mg/kg), and 152.7-g (PS ED_{50} 10 μg/ml, *in vivo* non-toxic, inactive at 50 to 6.25 mg/kg) fractions.

ISOLATION OF PIMELEA FACTOR P_2 (**2**) AND SIMPLEXIN (**1**).—A 120-g portion of the methylene chloride solvent partition fraction was subjected to gel permeation chromatography on Sephadex LH-20 (2 kg, 10.5 x 120 cm) using methanol. Elution volumes 5.16-10.42 liters gave an active fraction (42 g, PS ED_{50} 7.3 μg/ml). An aliquot (23.6 g) of the active fraction was subjected to rapid chromatography on silica gel (500 g, 8 x 18 cm). Gradient elution was performed using toluene followed by increasing amounts of ethyl acetate. The resulting active fraction (4.8 g, PS ED_{50} 0.1 μg/ml) was eluted with 40-50% ethyl acetate (2 liters each). A sample (3.5 g) was partitioned on a column of Sephadex LH-20 (600 g, 4.5 x 196 cm) using hexane-methylene chloride-methanol (10:10:1) as eluent. The active material (0.80 g, PS ED_{50} 7.3 x 10^{-3} μg/ml) was concentrated between volumes 1150-2750 ml. A 0.55-g amount was chromatographed on a silica gel (Lobar Size B) column using methylene chloride and increasing amounts of methanol. The 10% methanol eluate (0.42 g, PS ED_{50} 3.8 x 10^{-3} μg/ml) proved to be the most promising fraction. Rechromatography of an aliquot (0.24 g) on a silica gel (2 Lobar Size A) column using cyclohexane-ethyl acetate (3:2) as eluent afforded two fractions. Elution between volumes 70-80 ml gave a fraction (53.3 mg) enriched in Pimelea factor P_2 (**2**) and between volumes 94-130 ml another fraction (43 mg) enriched in simplexin (**1**).

The fraction (53.3 mg) containing Pimelea factor P_2 (**2**) was subjected to reverse-phase (RP-8) silica gel chromatography on a size A column using methanol-water (4:1) as eluent. Elution between volumes 144-204 ml afforded pure Pimelea factor P_2 (**2**, 29 mg, 0.0008%,[4] PS ED_{50} 7.9 x 10^{-4} μg/ml, T/C 130 and 132 at 40 and 20 μg/kg) as a colorless glass.

The fraction (43 mg) containing simplexin (**1**) was first chromatographed on silica gel (2 Lobar size A) using cyclohexane-ethyl acetate (6:5) as eluent. The portion (16.4 mg) eluted between volumes 114-150 ml was rechromatographed on reverse-phase (RP-8) silica gel (2 size A) using methanol-water (4:1). Elution volumes 455-585 ml gave pure simplexin (**1**, 13 mg, 0.0002%,[4] PS ED_{50} 5.1 x 10^{-3}, *in vivo* toxic 400 to 50 μg/kg) as a colorless glass.

CHARACTERIZATION OF PIMELEA FACTOR P_2 (**2**).—Pimelea factor P_2 (**2**) exhibited the following physical properties: mp 130-137°, FAB mass spectrum m/z 661 $C_{37}H_{50}O_9$ +Na); uv λ max (log ε) 202 (3.96), 229 (4.07) and 272 (2.95) nm; ir (KBr) ν max 3484, 3475, 3470, 3458, 3454, 3445, 2959, 2932, 1712, 1451, 1384, 1285, 1252, 1113, 1070, and 711 cm^{-1}; ^{1}H-nmr (see table 1); ^{13}C-nmr (22.63 MHz, $CDCl_3$) 167.71 (s, C-1'), 146.91 (s, C-2'), 133.49 (d, C-5'), 130.01 (s, C-15), 129.75 (d, C-3', C-7'), 128.71 (d, C-4', C-6'), 119.78 (s, C-29), 110.74 (t, C-16), 83.48 (s, C-13), 83.02 (d, C-3), 82.63 (d, C-14), 81.24 (s, C-9), 80.17 (s, C-4), 73.63 (d, C-5), 66.22 (t, C-20), 64.28 (d, C-7), 60.99 (s, C-6),

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[4]Percent yields are given on the basis of dry weight of plant material. The separation methods were not optimized and samples at each step were consumed in biological testing.

50.69 (C-10), 47.38 (d, C-8), 37.95, 36.85, 36.62, 36.33, 35.35, 33.60, 29.51, 27.95, 27.85, 26.74, 24.76, 21.38, 21.09, 18.85 (q, C-17), 14.46 (q, C-19), 12.71 (q, C-18).[5]

CHARACTERIZATION OF SIMPLEXIN (1).—Simplexin (1) showed mp 60-68° (Lit. 19, mp 68°), FAB mass spectrum m/z 555, $C_{30}H_{44}O_8$ +Na); uv λ max (log ϵ) 243 (3.92) nm; ir (KBr) ν max 3446, 2924, 1710 cm^{-1}; ^{1}H-nmr (see table 1); ^{13}C-nmr (22.63 MHz, CD_2Cl_2) 210.21 (s, C-3), 161.47 (d, C-1), 147.07 (s, C-2), 137.06 (s, C-15), 119.71 (s, C-1'), 110.97 (t, C-16), 84.65 (s, C-13), 82.15 (d, C-14), 79.19 (s, C-9), 72.72 (s, C-4), 72.33 (d, C-5), 65.58 (t, C-20), 64.57 (d, C-7), 60.96 (s, C-6), 48.61 (d, C-8), 37.11, 36.98 (d, C-10), 35.29 (d, C-11), 32.33 (t), 29.99, 29.73, 23.95, (t, C-12), 23.10 (t), 20.44 (c-19), 19.27 (C-17), 14.27 (q, C-10'), 10.01 (q, C-18).[5]

ACKNOWLEDGMENTS

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[5]The identity was confirmed by direct comparison (infrared spectrum in KBr) with authentic samples of Pimelea factor P_2 or simplexin provided, respectively, by Prof. E. Hecker and by Prof. W.C. Taylor.