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3,3′,5′-TRI-*O*-METHYLPICEATANNOL AND 4,3′,5′-TRI-*O*-METHYLPICEATANNOL: IMPROVEMENTS OVER PICEATANNOL IN BIOACTIVITY[1]

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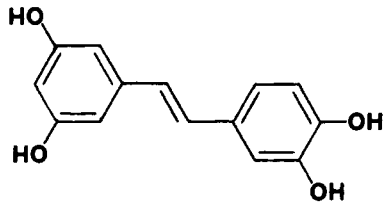
ABSTRACT.—Piceatannol (3,4,3′,5′-tetrahydroxy-*trans*-stilbene) [**1**] (NSC 365798) has recently been isolated and was subsequently synthesized for NCI tumor panel testing as a new antileukemic natural product from the seeds of *Euphorbia lagascae*. During the synthesis, a bioactive reaction mixture of several partially *O*-methylated piceatannol analogues was obtained. This mixture has now been maximized and subjected to bioactivity-directed fractionation, using brine shrimp lethality, to yield 3,3′,5′-tri-*O*-methylpiceatannol [**5**] (NSC 381281); this new compound has improved stability and better bioactivity in several systems than piceatannol itself. To confirm the structure, **5** was synthesized from vanillin [**2**]. In addition, the isovanillin [**3**] analogue, 4,3′,5′-tri-*O*-methylpiceatannol [**6**] (NSC 381864), another new compound, was sythesized and found to be bioactive.

Piceatannol (3,4,3′,5′-tetrahydroxy-*trans*-stilbene) [**1**] is an active 9PS and 3PS (P-388) murine antileukemic agent which has recently been isolated from the seeds of *Euphorbia lagascae* Spreng. (Euphorbiaceae) (1). This compound can be prepared in the laboratory due to its relatively simple structure, and a 10 g quantity has been synthesized (2) and submitted to the National Cancer Institute (NCI) for tumor panel evaluation (3). However, piceatannol [**1**], a polyphenolic, is readily subject to oxidation. This instability prompted our search for a more stable analogue which might retain or improve upon the antileukemic activity. In addition, such an analogue might enhance the recently reported antimicrobial, phytogrowth-inhibitory, and ichthyotoxic activities of piceatannol (4).



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The final step in the synthesis of piceatannol [**1**] (2) entailed the aryl methyl ether cleavage of tetra-*O*-methylpiceatannol (3,4,3′,5′-tetramethoxy-*trans*-stilbene). A controlled partial demethylation of the latter compound held a promise of yielding the desired, more stable, bioactive/analogue(s). A bioactivity-directed approach, using brine shrimp lethality (5) and involving fractionation of the reaction mixture, was clearly more expedient than the independent synthesis of all fourteen of the theoretically possible intermediates.

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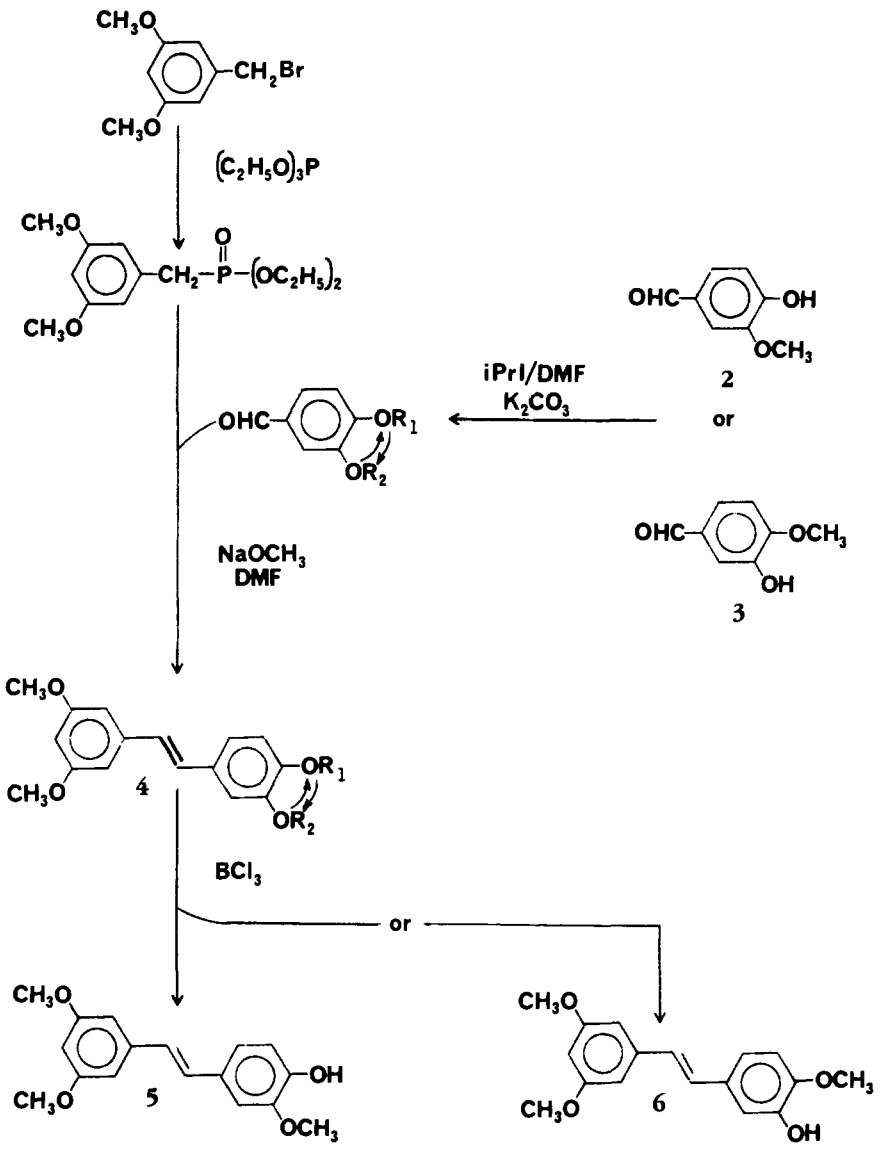
The research was initially directed at determining optimum reaction conditions which would yield the greatest number of partially demethylated analogues while maximizing the consumption of the starting material and minimizing the formation of piceatannol [**1**]. The method of demethylation utilizing pyridine HCl was selected; this reagent was known to result in a low yield (ca. 10%) of piceatannol (6). Optimal conditions for purposes of this research were determined and yielded six major fluorescing spots on tlc.

A scaled up reaction (2.07 g) yielded a brown syrup of the partially demethylated mixture that was chromatographed on a silica gel column. Pools of fractions were made on the basis of similarity in tlc patterns. Three pools proved to be toxic to brine shrimp (BS) (LC_{50}'s 31-147 ppm) (5) and were, surprisingly, more toxic than piceatannol [**1**] itself (LC_{50} 278 ppm) (1). All three active pools contained in common the starting material as well as an intermediate unknown compound [**5**] as evidenced by tlc patterns. It was surmised that the toxicity must reside with the unknown **5** because the fully methylated starting material was inactive (LC_{50}>1000 ppm) in the BS assay. Subsequent manipulations of these pools through crystallization and Chromatotron separation successfully isolated compound **5**. Brine shrimp toxicity testing (5) showed **5** to be quite toxic [LC_{50} 1.2 ppm, 95% confidence interval (CI): 0.7-1.9]. The total yield of the compound was only 4.53% of the demethylation mixture. Thus, the BS assay led to the isolation of a very active compound which was only a minor component of the total reaction mixture.

Eims analysis of active compound 5 showed an *m/z* of 286 (M^+). This observation readily indicated that a single methyl group (a mass loss of 14) had been cleaved from tetra-*O*-methylpiceatannol (M^+, *m/z* 300) to form **5**. Hrms confirmed via exact mass determination that the molecular formula was $C_{17}H_{18}O_4$. Theoretically, there are three possible tri-*O*-methyl analogues of **1**. To attempt to distinguish which methyl was missing, careful analyses of the ^{1}H-nmr spectra were made.

A comparison with the ^{1}H-nmr spectrum of tetra-*O*-methylpiceatannol showed that the six proton signal for the equivalent 3',5'-methoxyls was still intact. This placed the free hydroxyl at either the 3 or 4 position on the unknown structure [**5**]. A comparison with published ^{1}H-nmr spectra of vanillin (4-hydroxy-3-methoxybenzaldehyde [**2**]) and isovanillin (4-methoxy-3-hydroxybenzaldehyde [**3**]) suggested that the 4-methoxy, whose signal is more downfield, might have been lost (7). However, the close proximity of the 3 and 4 methoxy signals precluded the determination of unequivocal assignments. Furthermore, the vinyl proton signals, even with 470 MHz spectra, were superimposed on the aromatic proton signals and confused their assignments. Acetylation of **5** yielded a crystalline acetate, and the downfield shift of the apparent peaks for the aromatic proton at 2 suggested the vanillin [**2**] pattern of substitution. Obviously, sythesis of **5** through an unambiguous route was needed, both to confirm the structure and to obtain sufficient material for in vivo 3PS testing. Vanillin [**2**] was utilized as the starting aldehyde. After protection of its phenolic hydroxyl with an isopropyl group (8), a Wittig condensation (2) was performed (Scheme 1). The ^{1}H nmr of **4** confirmed its structure. Selective cleavage of the isopropyl ether of **4** was accomplished using boron trichloride (9) to yield **5**.

The tlc, mp, mmp, eims, ir, and ^{1}H-nmr data were all indicative of the same identity of these two substances and confirmed that the bioactive material in the demethylation mixture is 3,3',5'-tri-*O*-methylpiceatannol or 4-hydroxy-3,3',5'-trimethoxy-*trans*-stilbene [**5**]. This compound has not been previously reported. Compound **5** has significant cytotoxic activity (ED_{50} 0.25 μg/ml) in the 9KB in vitro system (10). In addition, **5** is quite active in the crown gall plant antitumor (potato disc) assay (11), showing 59%, 19%, and 65% inhibition of tumors in three independent runs; piceatannol



SCHEME 1. Synthesis of 3,3′,5′-trimethylpiceatannol [**5**] from vanillin [**2**] and 4,3′,5′-trimethylpiceatannol [**6**] from isovanillin [**3**]

[**1**] showed 20% and 32% inhibition in similar tests (1). A close correlation (p=0.000002) exists between the potato disc assay (1,11) and the 3PS in vivo murine antileukemic assay (10); thus, sufficient **5** (100 mg) was synthesized and submitted to NCI (NSC 381281) for 3PS testing. No 3PS activity was detected in doses up to 200 mg/kg. In 9PS in vitro testing, **4** indicated borderline activity at ED_{50} 10 μg/ml.

The isovanillin analogue 4,3′,5′,-tri-*O*-methylpiceatannol (3-hydroxy-4,3′,5′-trimethoxy-*trans*-stilbene) [**6**] was, in turn, sythesized by following the same route but starting with isovanillin [**3**] (Scheme 1). The structure of **6** was confirmed with ^{1}H-nmr, ir, and ms data. The isovanillin analogue **6** had identical tlc Rf values when compared to **5** in many different tlc systems and visualized with H_2SO_4-MeOH (1:1) spray reagent. Alternatively, a phenolic specific spray reagent, tetrazotized benzidine (TZB) (12), was employed with success, resulting in distinctive colorations; yellow and red for

5 and **6**, respectively, thus, permitting the identification of each compound in spite of their similar Rf values.

Compound **6** exhibited an LC_{50} of 2.8 ppm (CI: 1.8-4.3) in the BS bioassay and was also quite active in the potato disc assay (22%, 62% tumor inhibition) (11). Consequently, more of this compound was synthesized and submitted to NCI (NSC 381864) for 3PS testing; it had borderline activity (T/C 119% at 50 mg/kg) but failed to confirm in a repeat assay. Nevertheless, in the in vitro 9KB and 9PS tests, **6** was significantly cytotoxic (ED_{50}<0.01 μg/ml and 0.1 μg/ml, respectively) and was more active than either **1** or **5**. The acetates of both **5** and **6** were prepared and were inactive (LC_{50}>1000 ppm) in the BS bioassay.

EXPERIMENTAL

BIOLOGICAL TESTING.—3PS test results were obtained through the cooperation of the Natural Products Branch, Developmental Therapeutics Program, Division of Cancer Treatment, NCI (10). 9PS and 9KB cytotoxicities were determined at the Cell Culture Laboratory, Purdue Cancer Center. The potato disc (crown gall) plant antitumor inhibitions (11) and the brine shrimp (BS) lethalities (5) were determined in our laboratory.

PARTIAL *O*-DEMETHYLATION OF TETRA-*O*-METHYLPICEATANNOL.—2.07 g of 2,4,3′,5′-tetramethoxy-*trans*-stilbene (2) was heated with 31.887 g of pyridine HCl for 1 h at 150-160°. Upon cooling to room temperature, 100 ml of 2 N HCl was added, followed by 150 ml of Et_2O. The separated Et_2O layer was washed four times with 300-ml portions of H_2O, dried over anhydrous Na_2SO_4, and reduced in vacuo to brown syrup (1.975 g, 95.67% yield).

SEPARATION AND ISOLATION OF [**5**].—The total partial *O*-demethylation mixture was subjected to column chromatography over 50 g of Si gel using a gradient of hexane-C_6H_6 (1:1, 1,300 ml), C_6H_6 (100 ml), C_6H_6-MeOH (99:1, 100 ml; 98:2, 850 ml; 97:3, 200 ml; 96.5:3.5, 150 ml; 1:1, 100 ml), and $CHCl_3$-MeOH (1:1, 300 ml; 1:4, 200 ml). Column fractions of 50 ml were collected and then pooled on the basis of similarities upon tlc analysis (Si gel, hexane-$CHCl_3$-MeOH, 49:49:2). Three pools were significantly active in the BS bioassays (5): fractions 24-26, 27-41, and 42-47 (LC_{50}'s: 147, 31 and 98 ppm, respectively). The pool residue of fractions 27-41 was radially chromatographed (Chromatotron) on a 4 mm and then a 2 mm Si gel layer, using gradients of hexane/$CHCl_3$/MeOH and concentrating a flourescent unknown [**5**] which was common to the three active pools. Crystallization of **5** was effected with toluene and hexane. Two samples (the column pool of fractions 24-26 and pool 10-12 of the second Chromatotron run) were crystallized separately. The total yield of **5** was 89.5 mg (4.53% from tetra-*O*-methylpiceatannol).

IDENTIFICATION OF **5**.—Mp 85.5-86.5°; ^{1}H nmr ($CDCl_3$) 200 MHz δ 3.82 (s, 6H, 3′ and 5′-O*Me*), 3.94 (s, 3H, 3-O*Me*), 5.66 (s, 1H, 4-O*H*), 6.37 (t, *J*=2.2 Hz, 1H, 4′-H), 6.64 d, *J*=2.2 Hz, 2H, 2′ and 6′-H), 6.82-7.05 (complex overlapping, 5H, 3 aromatic, and 2 C*H*=C*H*); eims *m/z* 286 (M^+) hrms found, 286.1191; calculated for $C_{17}H_{18}O_4$: 286.1205; ir cm^{-1} 3490 (OH), 1580, 1500, 1445, 1420, 1340.

PREPARATION OF ACETATE OF **5**.—To a solution of **5** (10 mg) in pyridine (0.5 ml), Ac_2O (0.5 ml) was added and kept overnight at room temperature. The usual work-up provided white crystals of the acetate from $CHCl_3$ and MeOH: 9 mg, 78% yield, mp 120-122°; eims *m/z* 328 (M^+) and 286 (M^+- Ac); ^{1}H nmr ($CDCl_3$) 200 MHz δ 2.31 (s, 3H, acetate), 3.82 (s, 6H, 3′ and 5′-O*Me*), 3.88 (s, 3H, 3-O*Me*), 6.39 (t, *J*=2.2 Hz, 1H, 4″-H), 6.64 (d, *J*=2.2 Hz, 1H, 2′ and 6′-H), 6.90-7.09 (complex overlapping, 5H, 3 aromatic, 2 C*H*=C*H*); BS inactive (LC_{50}>1000 ppm).

SYNTHESIS OF **5** (Scheme 1).—To vanillin [**2**] (491 mg) in DMF (5 ml), anhydrous K_2CO_3 (0.653 g) and 2-iodopropane (2 ml) were added. The mixture was heated for 3 h at 90-100° under a N_2 atmosphere. After standing overnight at room temperature, the mixture was poured into H_2O and extracted with $CHCl_3$. The $CHCl_3$ layer was, in turn, extracted with three portions of H_2O to remove DMF, dried with $MgSO_4$, and then evaporated (residue weight, 0.6165 g).

The residue was placed on a column (15 g Si gel) and eluted with a solvent gradient of hexane and C_6H_6 to obtain 4-*O*-isopropylvanillin: 276 mg, 44% yield; ^{1}H nmr ($CDCl_3$) 80 MHz δ 1.42 [d, *J*=6.1 Hz, 6H, -CH(CH_3)$_2$], 3.92 (s, 3H, O*Me*), 4.69 [m, 1H, -C*H*(CH$_3$)$_2$], 6.97 (d, *J*=8.7 Hz, 1H, 5-H), 7.42 (d, *J*=1.8 Hz, 1H, 2-H), 7.44 (dd, *J*=8.7, 1.8 Hz, 1H, 6-H), 9.84 (s, 1H, -C*H*O).

Condensation between 102.5 mg of 3,5-dimethoxybenzylbromide and 1 ml of triethylphosphite yielded 3,5-dimethoxybenzyldiethylphosphonate. This was coupled through a Wittig condensation with

81.5 mg of 4-*O*-isopropylvanillin to obtain **4** (2). Compound **4** was isolated from the reaction mixture (0.115 g) by twice utilizing column chromatography (ca. 10 g Si gel each column) eluting with a C_6H_6/MeOH solvent gradient: mp 73.5-75°, 90 mg, 65.31% yield; ^{1}H nmr ($CDCl_3$) 80 MHz δ 1.36 [d, J=6.1, 6H, -CH(CH_3)$_2$], 3.82 (s, 6H, 3′ and 5′-O*Me*), 3.90 (s, 3H, 3-O*Me*), 4.54 [m, 1H, -C*H*(CH_3)$_2$], 6.37 (t, J=2.2 Hz, 1H, 4′-H), 6.64 (d, J=2.2 Hz, 2H, 2′ and 6′-H), 6.84-7.06 (complex overlapping, 5H, 3 aromatic H and 2 C*H*=C*H*), BS LC_{50} 467, CI: 118-1000$^+$.

Compound **4** (20 mg) was then dissolved in 2 ml of CH_2Cl_2 followed by the addition of 0.2 ml of BCl_3 and maintained for 15 min at −10° in a salted ice bath. H_2O was added slowly and the layers were separated, washing the CH_2Cl_2 three times with additional H_2O. The CH_2Cl_2 was dried over $MgSO_4$ and chromatographed over a microcolumn of Si gel developed with C_6H_6 (9). Compound **5** crystallized from toluene/hexane: 15 mg, 86% yield, mp 85-86°, mmp 85-86°; eims *m/z* 286 (M^+); ^{1}H-nmr and ir data indistinguishable from isolated **5**. A larger scale synthesis of **5** was made to obtain a sufficient quantity for 3PS testing.

SYNTHESIS OF **6** (Scheme 1).—The methods utilized to synthesize **5** were essentially repeated to synthesize **6**; 315.5 mg of 3-*O*-isopropylisovanillin: ^{1}H nmr ($CDCl_3$) 80 MHz δ 1.40 [d, J=3.7 Hz, 6H, -CH(CH_3)$_2$], 3.94 (s, 3H-O*Me*), 4.64 [m, 1H, -C*H*(CH_3)$_2$], 6.97 (d, J=8.8 Hz, 1H, 5-H), 7.42 (d, J=1.8 Hz, 2-H), 7.47 (dd, J=8.8, 1.8 Hz, 1H, 6-H), 9.84 (s, 1H, -C*H*O) was obtained from the reaction of 500 mg of isovanillin [**3**] with 2 ml of 2-iodopropane; **6** was isolated from the reaction mixture via a 14.5 g Si gel column eluted with C_6H_6 (yield 49%). The subsequent Wittig condensation reaction with 224 mg of 3-*O*-isopropylisovanillin and 3,5-dimethoxybenzyldiethylphosphonate, prepared from 277 mg of 3,5-dimethoxybenzylbromide and 1.2 ml triethylphosphite, yielded a brown syrup (326.5 mg); 220 mg of the syrup was dissolved in 2 ml of CH_2Cl_2 and treated with 2 ml of BCl_3 to obtain, after work up (9), 102.5 mg of **6** via column chromatography (6.94 g Si gel eluted with C_6H_6) and recrystallization (yield 53%).

IDENTIFICATION OF **6**.—Mp 89-90°; ^{1}H nmr ($CDCl_3$) 80 MHz δ 3.82 (s, 6H, 3′ and 5′-O*Me*), 3.90 (s, 3H, 4-O*Me*), 5.60 (s, 1H, 3-OH), 6.38 (t, J=2 Hz, 1H, 4′-H), 6.64 (d, J=2 Hz, 2H, 2′ and 6′-H), 6.86-7.35 (complex overlapping, 5H, 3 aromatic and 2 C*H*=C*H*; cims *m/z* 287 (MH^+); Hrms found: 286.1170, calculated for $C_{17}H_{18}O_4$: 286.1205; ir cm^{-1} 3440 (OH), 1580, 1500, 1450, 1410, 1335.

PREPARATION OF ACETATE OF **6**.—24 mg of **6** was acetylated (as described above for the acetate of **5**) and purified over a small Si gel column eluted with hexane-EtOAc (9:1 and 4:1); 9 mg, 32% yield (crystallized from $CHCl_3$/MeOH); mp 85-87°, eims *m/z* 328 (M^+), 286 (M-Ac); cims 329 (MH^+); ^{1}H nmr ($CDCl_3$) 200 MHz 2.33 (s, 3H, acetate), 3.82 (s, 6H, 2 O*Me* at 3′ and 5′), 3.85 (s, 3H, 4-O*Me*), 6.38 (t, J=2.3 Hz, 1H), 6.62 (d, J=2.3 Hz, 2H, 2′ and 6′-H), 6.88-6.99 (complex overlapping, 5 H, 3 aromatic H and C*H*=C*H*); BS inactive (LC_{50}>1000 ppm).

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