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ANTINEOPLASTIC AGENTS, 103. THE ISOLATION AND STRUCTURE OF HYPOESTESTATINS 1 AND 2 FROM THE EAST AFRICAN *HYPOËSTES VERTICILLARIS*[1]

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ABSTRACT.—The East African shrub *Hypoëstes verticillaris* representing a heretofore chemically unexplored Acanthaceae genus has been found to contain two new cell growth inhibitory (murine P-388 lymphocytic leukemia) phenanthroindolizidine alkaloids termed hypoestestatin 1 (**1a**) and hypoestestatin 2 (**1b**). Both substances were found to markedly inhibit growth of the murine P-388 cell line ($ED_{50} = 10^{-5}$ μg/ml). The structures of hypoestestatins 1 and 2 were assigned primarily on the basis of results from extensive spectral studies.

Most species (~2500) of the relatively large Acanthaceae family grow primarily in tropical areas as shrubs or herbs among 250 genera of considerable biological variety (climbing to marsh and xerophytes types). Although plants of the Acanthaceae have found application in African and Asian primitive medicine (2) for problems as diverse as the need for an aphrodisiac, to a treatment for cancer (3), heart disease, gonorrhea, and snake-bite, only a small number of species have received any prior chemical investigation. Such studies of chemical constituents have led to the isolation of lignans (4) and alkaloids of the quinazoline (5-7) and quinolylimidazole (8,9) classes. At least 15 Acanthaceae species representing ten genera have been employed in the primitive treatment of cancer (3). As part of an alkaloid evaluation, one of these plants, *Adhatoda vasica* Nees, was found to yield members of the quinazoline group (6).

Because of the U.S. National Cancer Institute (NCI) exploratory plant evaluation program, an extract of *Hypoëstes verticillaris* Soland. ex Roem. and Schult. from East Africa was screened in 1966, against the KB cell line from a human nasopharynx carcinoma and found (in the NCI laboratories) to provide a confirmed level (ED_{50} 8.8 to 0.001 μg/ml) of cell growth inhibition. Interestingly, this shrub has been used by the Swahili for chest diseases (2), but no *Hypoëstes* species appears to have been used for cancer treatment or subjected to a prior chemical constituent investigation. Later (1969-1971) extracts of *H. verticillaris* from Ethiopia and Kenya were shown in the NCI collaborative program with Dr. M. Wall[2] at the Research Triangle Institute to give a confirmed level (T/C 131%-155% at 400 mg/kg) of activity against the NCI P-388 murine lymphocytic leukemia (PS system). In the fall of 1979, we undertook a detailed bioassay (PS system) guided evaluation of *H. verticillaris*'s antineoplastic constituents employing a 1969 Kenya collection of the complete plant (including flowers). Research was continued and, as summarized in the sequel, brought to a conclusion employing a 1980 recollection from the same location.

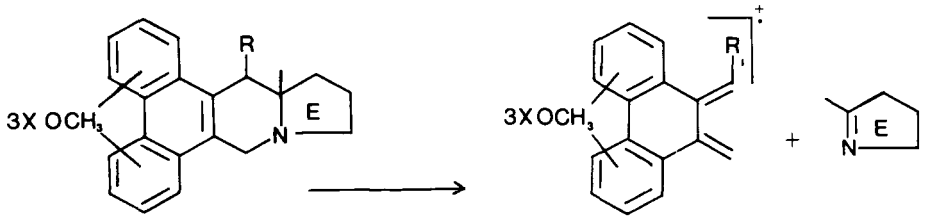
Because the most active (PS) antineoplastic constituents (**1a**,**1b**) of *H. verticillaris* were found to be quite air- and acid-sensitive and to occur in exceptionally small quantities, their isolation and characterization proved to be challenging. Prior to uncovering the successful methods outlined in the Separation Scheme, a variety of approaches to the isolation problem were explored and found unsatisfactory. The key procedures utilized here were developed as part of our parallel studies of marine invertebrate anticancer constituents (cf. 10). A synopsis now follows.

[1]For contribution 102 refer to Reference 1.

[2]We are pleased to thank Drs. Wall and H. Taylor for informing us of the results from their preliminary solvent separations of extracts prepared from *H. verticillaris*.

Extraction of the complete plant (45 kg), including flowers, employing the MeOH-CH_2Cl_2 (1:1) procedure (10) followed by addition of H_2O gave a CH_2Cl_2 extract that was successively partitioned between MeOH-H_2O (9:1→4:1→1:1) with hexane→CCl_4→CH_2Cl_2. The CCl_4 and CH_2Cl_2 fractions were separately partitioned between MeOH-H_2O (4:1) with hexane and MeOH-H_2O (1:4) with EtOAc→*n*-BuOH. The resulting highly active (PS cell line, see Separation Scheme 1) *n*-BuOH fractions were further individually purified by gel permeation (steric exclusion) and partition chromatography on Sephadex LH-20, followed by chromatography on the macroreticular XAD-2 resin. Final purification was achieved by semipreparative hplc on silica gel[3] to afford two new antineoplastic alkaloids designated hypoestestatins 1 (**1a**) and 2 (**1b**) in 2×10^{-5} (8.8 mg) and 1.2×10^{-5} (5.3 mg) % yields, respectively. Alkaloids **1a** and **1b** failed to yield crystals suitable for X-ray crystal structure determination, and a number of attempts to prepare (microtechniques) derivatives suitable for this purpose were also unrewarding. Consequently, the structures for both new anticancer agents were tentatively assigned on the basis of chemical and spectral evidence.

Hypoestestatins 1 and 2 exhibited very similar uv spectra characteristic of the tylophora alkaloids (11-17), namely, a phenanthroindolizidine nucleus with three methoxy groups in the 2,3,6- or 3,6,7-positions. Further evidence for the proposed structures was provided by interpretation of the mass and nmr spectra. The high resolution mass spectra indicated molecular formulas corresponding to $C_{24}H_{27}NO_3$ for hypoestestatin 1 and $C_{24}H_{27}NO_4$ for hypoestestatin 2. Prominent peaks occurred at *m/z* 294 and 310 in the electron impact mass spectra of alkaloids **1a** and **1b**, respectively, corresponding to a loss of 83 mass units from each molecule. The loss was rationalized by a retro-Diels Alder fragmentation process characteristic of such alkaloids (14-16, see Figure 1) and indicated the presence of an unsubstituted E-ring in both compounds. The loss of 83 mass units, compared to that of 69 mass units observed in the case of previously reported phenanthroindolizidine alkaloids' mass spectra (14), indicated the presence of either an additional methyl group or a six-membered ring E. The ^{1}H-nmr spectra of both alkaloids **1a** and **1b** showed a three-proton singlet at 1.25 ppm, thereby confirming the presence of an angular methyl group at position 13a. Interestingly, phenanthroindolizidine alkaloids bearing an angular methyl group have not been isolated previously. The ^{1}H-nmr spectra also confirmed the presence of three methoxyl groups. Because the signal at δ 4.69 ppm was found in close agreement with that (4.65) reported for the C-14-H in tylophorinidine (**2**) (15), the nonphenolic (uv



1a R=H
1b R=OH

m/z 294 (R=H)
m/z 310 (R=OH)

m/z 83

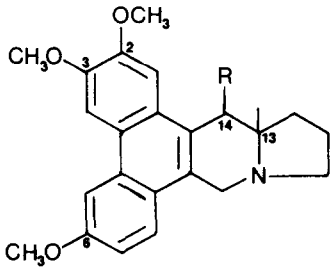
FIGURE 1

[3]Experience with the isolation of hypoestestatins 1 and 2 demonstrated their extreme sensitivity to air and acid exposure. Hypoestestatins 1 and 2 can be purified by rapid chromatography (hplc) on normal silica. Interestingly, both alkaloids (**1a** and **1b**) decompose extensively on reverse phase (C18-silica) hplc columns using MeOH-H_2O mixtures as eluents. Similar decomposition was noted using an authentic sample of cryptopleurine kindly provided by Prof. E. Gellert. Initially, the specimen of cryptopleurine was found to be quite pure using routine silica gel column hplc techniques. Therefore, caution needs to be exercised in the isolation of such alkaloids.

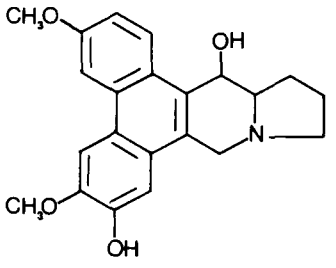
spectrum unaffected by addition of base) hydroxyl group in hypoestestatin 2 (**1b**) was placed at position 14.

Assignment of the angular methyl group configuration at C-13a was achieved by comparison of the cd spectra of alkaloids **1a** and **1b** with the cd spectrum reported for tylocrebine (**3**) (17). Alkaloids **1a** and **1b** both exhibited negative Cotton effects ($[\theta]_{260}$-7180 and $[\theta]_{252}$-11004] in compliance with the negative Cotton effect ($[\theta]_{252}$-4370] requirement of the tylocrebine (**3**) absolute configuration. Members of the R-series display positive Cotton effects (17). The larger molecular ellipticity values recorded for tertiary amines **1a** and **1b** compared to tylocrebine probably result from the C-13a angular methyl group contribution in contrast to hydrogen at that position.

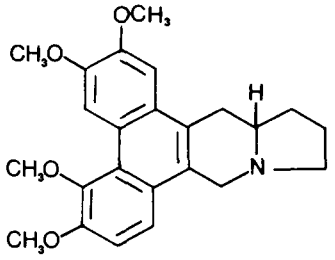
Configuration of the C-14-hydroxyl group of hypoestestatin 2 (**1b**) was tentatively deduced from results of a molecular-rotation difference comparison of hypoestestatins 1 and 2 with the known (e.g., 19) 14α- and 14β-hydroxyphenanthroindolizidine alkaloids. Desoxypergularinine (**4a**) possesses a molecular rotation of −49.4° while the 14α-hydroxy derivative, *O*-methyltylophorinidine (**4b**), and its 14β-hydroxy isomer, pergularinine (**4c**), exhibit +439.6° and −60.6°, respectively (18). The molecular rotations of hypoestestatins 1 and 2 correspond to −137° and −314°. The negative contribution of the C-14-hydroxyl group in alkaloid **1b** suggested a β-configuration in agreement with the negative direction of the molecular rotation difference between desoxypergularinine and pergularinine. The enhanced negative molecular ellipticity in the circular dichroism spectrum of alcohol **1b**, compared to hypoestestatin 1 (**1a**), was attributed to the 14β-hydroxyl group contribution.



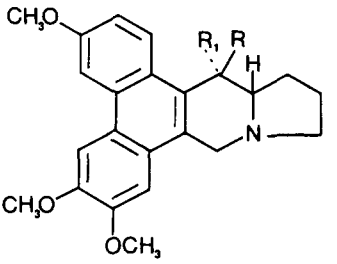
1a R=H, Hypoestestatin 1
1b R=OH, Hypoestestatin 2



2 Tylophorinidine



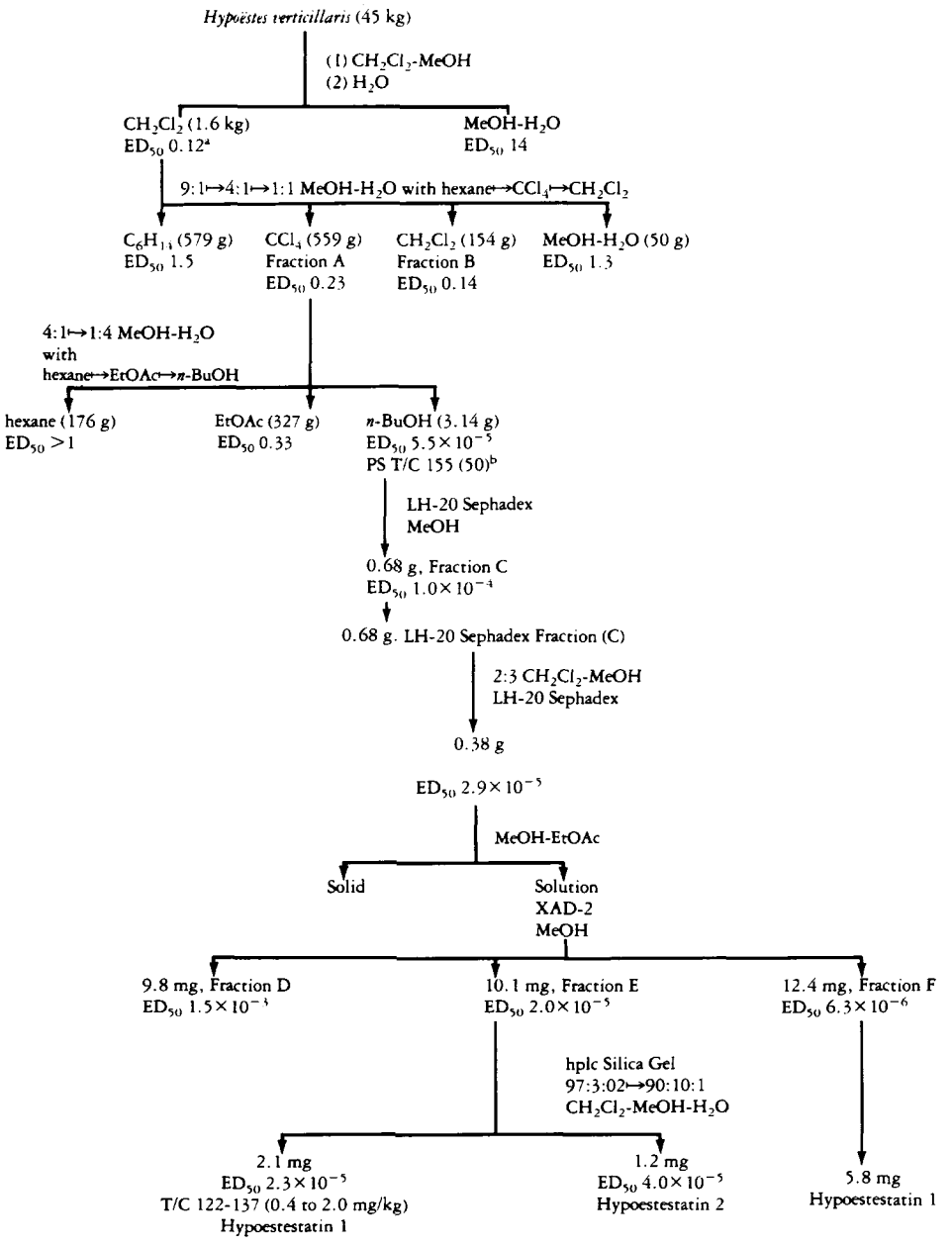
3 Tylocrebine



4a R=R_1=H, Desoxypergularinine
4b R=H, R_1=OH, O-Methyltylophorinidine
4c R=OH, R_1=H, Pergularinine

The remaining structure assignments for hypoestestatins 1 and 2 involved placement of the three methoxyl groups on the aromatic nucleus. As noted above, the uv spectra indicated either a 2, 3, 6- or 3, 6, 7-trimethoxy arrangement. Comparison of the ^{1}H-nmr chemical shift values for the aromatic protons of hypoestestatins 1 and 2 with those of known 2, 3, 6- and 3, 6, 7-trimethoxy phenanthroindolizidine alkaloids

(15,16) favored the 2,3,6-arrangement. Thus, structures **1a** and **1b** were assigned, respectively, to hypoestestatins 1 and 2. Unequivocal structural assignments will require an eventual X-ray crystallographic study when sufficient quantities of hypoestestatins 1 and 2 become available for further chemical and biological investigations. However, structural evidence now in hand seems important for a variety of reasons. For example, hypoestestatins 1 and 2 appear to be the first members of the phenanthroindolizidine alkaloids (usually from the Asclepiadaceae) to be isolated from an Acanthaceae species.



SEPARATION SCHEME. Isolation of Hypoestestatins 1 and 2

Fraction B treated as outlined for Fraction A↦8.8 mg of Hypoestestatin 1 and 5.3 mg of Hypoestestatin 2 as total yields from A and B.

[a]PS cell line expressed in μg/ml

[b]PS in vivo results T/C (mg/kg)

Certain phenanthroindolizidine and phenanthroquinolizidine alkaloids possess significant anticancer activity (20), and the Acanthaceae may provide a source for new and potentially important members. Also, in 1966, a phase I, clinical trial of tylocrebine (**3**) uncovered the prospect of central nervous system (CNS) toxicity (ataxia and disorientation) and further clinical study was discontinued (21). As suggested by Suffness (21), more polar derivatives might decrease the CNS penetration and prove more useful if the antineoplastic activity were retained. In this respect, hypoestestatin 2 and more polar derivatives would be of interest for further extensive antineoplastic evaluations. Presently, preliminary PS in vivo data is only available for hypoestestatin 1 up to a 2 mg/kg dose level (37% life extension, see Separation Scheme). Additional quantities of hypoestestatin 1 will be required for higher dose studies and of hypoestestatin 2 to begin an in vivo evaluation.[4]

EXPERIMENTAL

Each solvent employed for chromatography was redistilled. Sephadex LH-20 (Particle size 25-100 μm) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Amberlite XAD-2 resin was supplied by Rohm and Haas Chemical Co., Philadelphia. Preparative hplc was conducted with Silica gel (10 μm) columns (50 cm×10 mm) using Altex (Model 110A) solvent metering pumps. Fraction collection was partially automated using a Gilson microfractionator. Tlc 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. Analytical hplc was carried out using a Waters Associates liquid chromatograph employing 25 cm×4.6 mm Lichrosorb Si-60 (10 μm) and μC18 (10 μm) columns.

All melting points are uncorrected and were observed utilizing a Koefler-type melting point apparatus. Optical rotations were determined in CH_2Cl_2 solution employing a Perkin-Elmer Model 241 polarimeter. Cd spectra were recorded in MeOH solution using a Jasco model ORD/UV-5 spectrophotometer. Uv spectra were measured in MeOH solution with a Hewlett-Packard model 8450A spectrophotometer. Ir spectra were recorded with a Nicolet MX-1 FT-IR Spectrophotometer. The ^{1}H-nmr spectra at 100, 90, and 400 MHz were recorded using Varian XL-100, Bruker WH-90, and WH-400 spectrophotometers, respectively. The ^{13}C-nmr spectra were determined at 22.63 MHz with a Bruker WH-90 spectrophotometer. TMS was used as internal reference and δ values are reported. Mass spectra were obtained using a MAT 312 mass spectrometer equipped with electron impact (ei) and fast atom bombardment (fab) inlet systems.

PLANT COLLECTION.—The *H. verticillaris* was originally (1965-69) collected and recollected in Ethiopia, Kenya, and Tanzania as part of the joint NCI-USDA exploratory botany programs directed by Drs. J.L. Hartwell and R.E. Perdue. The 1980 Kenya recollection that corresponds to this report was obtained through the collaborative NCI-USDA botanical studies directed by Drs. M.I. Suffness and J.A. Duke. Voucher specimens [PR54377 and 54731 (B634131)] are at the Economic Botany Laboratory, Agricultural Research Center-East, Beltsville, MD.

PLANT EXTRACTION.—The whole (roots and aerial portions including flowers) plant corresponding to the 1980 recollection of *H. verticillaris* (45 kg dry wt) was ground and extracted (10) with CH_2Cl_2-MeOH (1:1, 160 liters) at ambient temperature. Addition of 25% H_2O caused separation into CH_2Cl_2 and aqueous phases. The CH_2Cl_2 fraction (1.573 kg) was found to be active (ED_{50} 1.2×10^{-1} μg/ml) in the PS in vitro system. The aqueous phase was essentially inactive (PS, ED_{50} 1.4×10^{1} μg/ml) and not investigated further.

SOLVENT PARTITION SEQUENCE.—Successive partitioning of the CH_2Cl_2 fraction (1.573 kg) between MeOH-H_2O (9:1) with hexane, MeOH-H_2O (4:1) with CCl_4 and MeOH-H_2O (1:1) with CH_2Cl_2 followed by removal of solvents from the hexane, CCl_4, CH_2Cl_2, and MeOH-H_2O solutions gave, respectively, 579 g (PS, ED_{50} 1.5 μg/ml), 559 g (fraction A, PS, ED_{50} 2.3×10^{-1} μg/ml), 154 g (fraction B, PS, ED_{50} 1.4×10^{-1} μg/ml) and 50 g (PS, ED_{50} 1.3 μg/ml) fractions.

The CCl_4 solvent partition fraction (A, 559 g) was repartitioned successively between MeOH-H_2O (4:1) with hexane and MeOH-H_2O (1:4) with EtOAc followed by *n*-BuOH. Removal of solvents from the hexane, EtOAc, and *n*-BuOH solutions gave, respectively, 176 g (PS, ED_{50} >1.0 μg/ml), 327 g (PS, ED_{50} 3.3×10^{-1} μg/ml), and 3.14 g (PS, ED_{50} 5.5×10^{-4} μg/ml) fractions. Next, the CH_2Cl_2 solvent

[4]Here it is important to consider that the mechanism and site of action of *Tylophora* alkaloids have been found recently to resemble the emetine alkaloids (22,23). Since the latter alkaloids are less toxic and more abundant, future studies will probably be focused on this group (24).

partition fraction (B, 154 g) was repartitioned as just summarized for fraction A to afford hexane (10 g, PS, ED_{50} >1.0 μg/ml), EtOAc (120 g, PS, ED_{50} 2.4×10^{-1} μg/ml), and *n*-BuOH (0.69 g, PS, ED_{50} $<1.0\times10^{-4}$ μg/ml) fractions.

ISOLATION OF HYPOESTESTATINS 1 (**1a**) AND 2 (**1b**).—The *n*-BuOH solvent partition fraction (3.14 g) prepared from fraction A was subjected to gel permeation chromatography in MeOH on Sephadex LH-20 (600 g, 160×4 cm). Elution volumes 1.7-2.5 liter gave the most active fraction (C, 0.68 g, PS, ED_{50} 1.0×10^{-4} μg/ml). The active fraction (C) was partitioned on a column of Sephadex LH-20 (90 g, 75×3 cm) using CH_2Cl_2-MeOH (2:3) as eluent. The active material (0.38 g, PS, ED_{50} 2.9×10^{-5} μg/ml) was concentrated between volumes 400-950 ml. After dissolving the 0.38 g fraction in MeOH, excess EtOAc was added. The insoluble solid (13 mg, PS, ED_{50} $>1.0\times10^{-2}$) was collected and the solution was subjected to chromatography on a column of XAD-2 (50 g, 28×2.8 cm) macroreticular resin (25) using MeOH as eluent. Elution between volumes 540-750 ml, 751-1125 ml, and 1126-2625 ml gave the three most active fractions D (9.8 mg, PS, ED_{50} 1.5×10^{-3} μg/ml), E (10.1 mg, PS, ED_{50} 2.0×10^{-5} μg/ml), and F (12.4 mg, PS, ED_{50} 6.3×10^{-6} μg/ml), respectively. Fraction E was subjected to preparative hplc on silica gel[3] using CH_2Cl_2-MeOH-H_2O 97:3:0.2 (300 ml)→95:5:0.2 (300 ml)→90:10:1 (440 ml) as eluent. Elution between volumes 360-640 ml afforded pure hypoestestatin 1 (**1a**, 2.1 mg, PS, ED_{50} 2.3×10^{-5} μg/ml) as a brownish solid. Elution between volumes 840-940 ml gave pure hypoestestatin 2 (**1b**, 1.2 mg, PS, ED_{50} 4.0×10^{-5} μg/ml) as another brownish solid. By an analogous hplc procedure fraction D afforded 1.3 mg of pure hypoestestatin 2 while the same hplc procedure applied to fraction F afforded 5.8 mg of pure hypoestestatin 1 (**1a**). Treatment of the second *n*-BuOH solvent partition fraction (0.69 g, from fraction B above) in the same way gave additional quantities of hypoestestatin 1 (0.9 mg) and 2 (2.8 mg). Thus the total yields[5] of hypoestestatin 1 and 2 amounted to 8.8 mg (0.0000196%) and 5.3 mg (0.0000118%), respectively.

A chromatographically pure specimen of hypoestestatin 1 (**1a**) was obtained as a pale yellow solid and exhibited the following physical properties: mp 191-197°; $[\alpha]^{31}D$ −36.36° (589 nm), −40.00 (578 nm), −45.45 (546 nm) and c=0.55 in CH_2Cl_2; cd: negative cotton effect at 260 nm with $[\theta]_{260}$=7180; hrms *m/z* 377.1973 ($C_{24}H_{27}O_3N$ requires 377.1991); uv λ max (log ε) 258 (4.45), 286 (4.19), 342 (2.94), and 360 (2.71) nm; ir ($CDCl_3$) ν max 2952, 2928, 1726 (very weak), 1613, 1589, 1513, 1468, 1422, 1256, 1205, 1125, 1042, and 790 cm^{-1}; eims *m/z* 377, 294, 279, 251, 189, 165, 84, 55; ^{1}H-nmr (100 MHz in CD_3OD)[6] δ 8.00 (2H, s, H-4,5), 7.76 (1H, m, H-8), 7.25 (2H, m, H-1,7), 4.07 (3H, s), 4.03 (3H, s), 4.0 (3H, s) (3×OCH_3), ~2.0 (bm), 1.3 (3H, s, 13a-CH_3); (90 MHz in $CDCl_3$) δ 7.87 (2H, s, H-4,5), 7.65 (1H, m, H-8), ~7.25, 4.10 (3H, s), 4.05 (3H, s), 4.01 (3H, s) (3×OCH_3), ~2.0 (bm), 1.26 (3H, s, 13a-CH_3). ^{13}C-nmr (in CD_3OD) 159.97, 151.46, 150.91, 132.16, 126.57, 125.69, 125.01, 123.90, 123.51, 121.40, 117.18, 105.74, 105.74, 105.45 (aromatic C_5), 59.86 (C-9), 56.74, 56.54, 56.15 (3×OCH_3), 54.88 (C-13a), 32.85 (C-11), 32.37, 30.77, 24.99 (C-12,13,14), 23.33 (13a-CH_3).

An analogous pure sample of hypoestestatin 2 (**1b**) was found to show mp 206-212°; $[\alpha]^{31}D$ −80.00 (589 nm), −83.81° (578 nm), −99.05 (546 nm) and c=0.525 in CH_2Cl_2; cd negative cotton effect at 262 nm with $[\theta]_{262}$=11004; hrms *m/z* 393.1928 ($C_{24}H_{27}O_4N$ requires 393.1940); uv λ max (log ε) 259 (4.62), 2.85 (4.41), 340 (3.2036) nm; ir (KBr) ν max 3466, 2931, 2856, 1659, 1611, 1527, 1513, 1470, 1416, 1384, 1259, 1038, and 1016 cm^{-1}; eims *m/z* 393, 377, 310, 294, 267, 224, 197, 165, 152, 84, 55; ^{1}H-nmr (90 MHz in CD_3OD)[6] δ 7.95 (2H, H-4,5), 7.58 (1H, H-1), 7.11 (1H, d, *J*=7.5 Hz), 7.09 (1H, d, *J*=7.5 Hz) (H-7,8), 4.06 (3H, s), 4.01 (6H, s) (3×OCH_3), 1.25 (3H, s, 13a-CH_3); (400 MHz in $CDCl_3$) δ 7.87 (1H, s, H-4), 7.82 (1H, s, H-5), 7.79 (1H, d, H-1), 6.86, 6.77 (2H, AB quartet, *J*=7.5 Hz, H-7,8), 4.69 (1H, s, H-14), 4.13 (3H, s), 4.07 (3H, s), 4.01 (3H, s) (3×OCH_3), 1.2 (3H, s).

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[5]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.

[6]Tentative assignments of ^{1}H-nmr data have been made on the basis of comparison with that published for known compounds (14,15).

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