JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Activity-Guided Isolation of Resveratrol Oligomers from a Grapevine-Shoot Extract Using Countercurrent Chromatography

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ABSTRACT: An activity-guided isolation of bioactive stilbenes has been carried out with the grapevine-shoot extract Vineatrol 30. After hexane precipitation of the polymeric constituents, the stilbene mixture was separated on a preparative scale using low-speed rotary countercurrent chromatography (LSRCCC). The antiproliferative activity of the separated LSRCCC fractions was then screened in the human cancer cell line A-431, and *trans*-resveratrol, *trans*-ε-viniferin, r-2-viniferin, hopeaphenol, and miyabenol C were identified as active principles. In addition, a new class of stilbene derivatives, which exhibit a γ-lactam ring structure and exert a weak growth-inhibiting activity in A-431 cells, has been identified.

KEYWORDS: *Vitis vinifera, grapevine, oligomeric stilbenes, resveratrol, antiproliferative activity, low-speed rotary countercurrent chromatography*

INTRODUCTION

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a well-studied bioactive stilbene that is present in a variety of plant species.[1] One of the main sources of this compound is *Vitis vinifera*, in which it acts as a phytoalexin.[2] A wide range of biological activities has been described;[3−7] for an overview see ref 8.

During recent years the biological activities of so-called resveratrol oligomers, which are formed by an oxidative coupling reaction,[9,10] have attracted increasing scientific attention. For instance, the stilbene tetramer vaticanol C induces apoptosis in different colon cancer cell lines in significantly lower doses than resveratrol itself.[11,12] The same can be said about the grapevine-shoot extract Vineatrol 30, which was the subject of the present study and for which preliminary data indicating a significant in vitro anticancer activity exist.[13,14] Vineatrol 30 is a standardized ethanolic extract of grapevine shoots from the Bordeaux region with a stilbene content of at least 30%. The major stilbenes contained therein are *trans*-resveratrol (7.7%) and the resveratrol dimer *trans*-ε-viniferin (14.6%). In addition, a mixture of higher oligomeric stilbenes containing up to four resveratrol subunits is known to be present in the extract.

To identify the active principles in the extract, an activity-guided isolation of antiproliferative active stilbenes has been carried out for the first time using large-scale fractionation of the extract by preparative countercurrent chromatography[15] and screening of the separated fractions employing the human cancer cell line A-431. Tumor cell growth inhibition was monitored by using the sulforhodamine B (SRB) colorimetric assay.[16]

In the 1970s Ito developed high-speed countercurrent chromatography (HSCCC), which is nowadays widely used for the separation and purification of various natural products.[17,18] The main benefits of this all liquid chromatographic technique are the gentle operation conditions, which reduce the risk of artifact formation and hence guarantee the total recovery of the injected sample.[19] Both aspects are very important for the isolation and further investigation of bioactive natural compounds. Although large-scale isolation with HSCCC has been reported before,[20] scaling up is limited by the relatively high rotational speed, which is required to retain the stationary phase in the column.[21] One possibility for a scale-up is the use of the hydrodynamic technique of low-speed rotary countercurrent chromatography (LSRCCC), which is already in use for the semi-industrial isolation of bioactive compounds from crude plant extracts.[22] This technique has now successfully been used in the present study for the isolation of antiproliferative active stilbenes in the grapevine-shoot extract Vineatrol 30.

MATERIALS AND METHODS

Solvents. Solvents for HPLC analysis included methanol, HPLC gradient grade (VWR, Darmstadt, Germany); methanol, LC-MS quality (Fisher, Schwerte, Germany); water (deionized, Nanopure, Werner, Leverkusen, Germany); and acetic acid, HPLC quality (AppliChem, Darmstadt, Germany). Solvents for CCC and solvent precipitation included ethyl acetate, p.a. (Roth, Karlsruhe, Germany); methanol (distilled, industrial quality); *n*-hexane (distilled, industrial quality); water (deionized, Nanopure); and ethanol (distilled, industrial quality). The NMR solvent was d_6-acetone, 99.96% (Deutero GmbH, Kastellaun, Germany).

Extract. The grapevine-shoot extract Vineatrol 30 was kindly provided by Breko GmbH (Bremen, Germany) and ACTIchem S.A. (Montauban, France).

Solvent Precipitation. Twenty-five grams of Vineatrol 30 was dissolved in 1000 mL of ethanol. The solution was stirred, and 2000 mL of *n*-hexane was added dropwise.

The precipitate was removed by filtration and rinsed two times with 1000 mL of an ethanol/*n*-hexane mixture (1:2, v/v). The combined

Received: July 16, 2012
Revised: November 14, 2012
Accepted: November 17, 2012
Published: November 17, 2012

dx.doi.org/10.1021/jf3030584 | J. Agric. Food Chem. 2012, 60, 11919−11927

clear filtrates were then evaporated under vacuum, dissolved in water, and lyophilized.

Low-Speed Rotary Countercurrent Chromatography (LSRCCC). Separations by LSRCCC were carried out with a prototype low-speed countercurrent chromatograph (Pharma-Tech Research Corp., Baltimore, MD, USA) equipped with a multilayer single coil column (diameter of chemically inert PTFE tubing = 8.2 mm, total volume = 5.5 L, revolution speed = 48 rpm). An amount of 15 g of the lyophilized filtrate of the *n*-hexane precipitation procedure was dissolved in 250 mL of a 1:1 mixture of upper and lower phase of the two-phase solvent system *n*-hexane/ethyl acetate/methanol/water (1:2:1:2, v/v/v/v). After injection through a sample loop, the LSRCCC separation was performed with the solvent system mentioned above at a flow rate of 5.0 mL/min (HPLC Pump 64, Knauer, Berlin, Germany). The upper organic phase acted as the mobile phase. After an elution volume of 7800 mL, the elution mode was switched to extrusion by pumping the aqueous lower phase into the column without changing flow rate, flow direction, or rotation speed. The separation was monitored at λ 280 nm with a Knauer UV−vis detector (Berlin, Germany) and recorded by a plotter (BBC Goerz Metrawatt SE 120, Vienna, Austria). Fractions of 60 mL were collected by a Super Frac fraction collector (Pharmacia LKB, Bromma, Sweden).

High-Speed Countercurrent Chromatography (HSCCC). A high-speed countercurrent chromatograph model CCC-1000 manufactured by Pharma-Tech Research Corp. was equipped with three preparative coils of PTFE tubing (2.6 mm i.d., 160 m length), connected in series. The revolution speed of the apparatus was set at 900 rpm. A flow rate of 3.0 mL/min was used (Biotronik HPLC pump BT 3020, Jasco, Gross-Umstadt, Germany). The two-phase solvent system was composed of *n*-hexane/ethyl acetate/methanol/water (2:5:2:5, v/v/v/v). The aqueous lower phase was applied as the mobile phase in the head to tail elution mode. All samples were dissolved in a 1:1 mixture of upper and lower phase and injected into the system by loop injection (20 mL). The amount of sample injected was 700 mg. During the HSCCC separation, UV absorbance of the effluent was monitored by a Knauer UV−vis detector at λ 280 nm and recorded by a BBC Goerz SE 120 plotter (3 cm/h). Fractions of 12 mL were collected.

Preparative HPLC. A HPLC system from Knauer consisting of a Smartline 1000 HPLC pump, SmartlineManager 5000 solvent organizer and degasser, Wellchrom K-2600 UV detector, Rheodyne 7125 injector (250 μL), and ChromGate version V3.1.7 software was used. A Luna C18, 5 μm, 250 × 15.0 mm preparative HPLC column (Phenomenex, Aschaffenburg, Germany) was used for the fractionation of LSRCCC fraction 3 and the isolation of hopeaphenol and ampelopsin A, whereas an Aqua C-18, 5 μm, 250 × 21.2 mm preparative HPLC column (Phenomenex) was used for the isolation of the four new stilbene derivatives from LSRCCC fraction 8. Collection of HPLC subfractions was corresponding to the elution of the peaks. Water (solvent A) and methanol (solvent B) were used as solvent system. The flow rate was 4.0 mL/min. For the fractionation of LSRCCC fraction 3 the gradient was as follows: 0 min (37% B), 10 min (37% B), 60 min (50% B), 90 min (50% B), and 120 min (100% B). The gradient used for the isolation of hopeaphenol and ampelopsin A was 0 min (30% B), 20 min (50% B), 30 min (60% B), 40 min (70% B), 46 min (70% B), and 47 min (100% B), and that for the isolation of the four new stilbene derivatives was 0 min (50% B), 60 min (50% B), and 65 min (100% B).

TLC Analysis. Evaluation of the CCC fractions was performed by thin-layer chromatography on normal phase silica gel plates (60 F_{254}, Merck, Darmstadt, Germany) with chloroform/ethyl acetate/methanol/water (25:55:5:1, v/v/v/v) as solvent system. Visualization of the stilbenes was achieved by means of an anisaldehyde−sulfuric acid−glacial acid universal spray reagent according to the method of Stahl.[23]

High-Performance Liquid Chromatography−Photodiode Array (HPLC-PDA). A HPLC system from Jasco (Gross-Umstadt, Germany), with a PU-980 pump combined with a degasser (DG-98050), ternary gradient unit (LG-98002), and a photodiode array detector (MD-910) was used. HPLC separation was achieved on a Kromasil 100-5-C18 column (250 mm × 4.6 mm, 5 μm, Eka Chemicals AB, Bohus, Sweden) protected with a guard column of the same material (10 mm × 4 mm). The mobile phases consisted of 1% aqueous acetic acid (v/v) (A) and methanol (B). The separation was carried out at room temperature, under the following conditions: 0 min (20% B), 5 min (30% B), 15 min (30% B), 18 min (37% B), 29 min (37% B), 35 min (50% B), 57 min (50% B), 58 min (100% B), 71 min (100% B), 72 min (20% B), and 75 min (20% B). Detection was carried out at 280 nm with a flow rate of 0.8 mL/min.

High-Performance Liquid Chromatography−Electrospray Ionization Multiple Mass Spectrometry (HPLC-ESI-MS^n). A HPLC system (pump 1100 series, autosampler 1200 series) from Agilent Technologies (Böblingen, Germany) was connected with an HCT Ultra ETD II LC-ESI-MS/MS ion-trap system from Bruker Daltonics (Bremen, Germany). Mass spectra were recorded in the negative ionization mode, with the capillary voltage set at 3500 V, the end plate at −500 V, and the capillary exit at −115.0 V. Drying gas was nitrogen at 330 °C, and the flow rate was 11.0 L/min. The nebulizer pressure was set to 60 psi, the target mass at m/z 400, and the scan range from m/z 100 to 3000. Compass Hystar Software (Bruker Daltonics) was used for analysis and data collection. HPLC conditions were the same as described above.

High-Resolution ESI-MS. High-resolution ESI-MS spectra were recorded on a Thermo Science LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Mass spectra were recorded in the positive ionization mode, with the spray voltage variably set between 2300 and 2800 V, the source temperature at 180 °C, and a flow rate of 11.0 L/min. The target mass was set at m/z 400 and the scan range from m/z 40 to 2000. Electrospray measurements were performed in direct infusion mode using a custom-made microspray-device mounted on a Proxeon nanospray ion source. The microspray device allows for the sample infusion through a stainless steel capillary (90 μm i.d.). Accurate mass measurements in the orbitrap were performed using the lock mass option of the instrument control software and the cation of tetradecyltrimethylammonium bromide (256.29988 amu) as internal mass reference.

Proton and Carbon Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C, DEPT-135, ^{1}H−^{1}H COSY, ^{1}H−^{1}H phase-sensitive NOESY, HSQC, and HMBC experiments were performed at 22 °C on a Bruker Avance II-600 spectrometer equipped with a CPTCI cryo-probe head (Bruker Biospin, Rheinstetten, Germany) at 600.13 and 150.90 MHz, respectively. Samples were dissolved in acetone-d_6 (Deutero GmbH, Kastellaun, Germany). Chemical shifts were referenced to the residual solvent signals (δ 2.05 for ^{1}H and δ 30.83 for ^{13}C).

***trans*-Piceatannol (1).** ^{1}H NMR: δ 7.07 (d, J = 2.0 Hz, H-2), 6.80 (d, J = 8.1 Hz, H-5), 6.90 (dd, J = 8.5/2.0 Hz, H-6), 6.95 (d, J = 15.5 Hz, H-7), 6.82 (d, J = 15.5 Hz, H-8), 6.52 (d, J = 2.0, H-10/14), 6.26 (t, J = 6.26 Hz, H-12). ^{13}C NMR: δ 131.7 (C-1), 114.9 (C-2), 147.1 (C-3), 147.2 (C-4), 117.3 (C-5), 120.9 (C-6), 130.4 (C-7), 127.8 (C-8), 141.8 (C-9), 106.6 (C-10/14), 160.6 (C-11/13), 103.6 (C-12).

Miyabenol C (5). ^{1}H NMR: δ 7.16 (d, J = 8.5 Hz, H-2a/6a), 6.80 (d, J = 8.6 Hz, H-3a/5a), 5.36 (d, J = 5.2 Hz, H-7a), 4.63 (d, J = 5.3 Hz, H-8a), 6.15 (s, H-10a/14a), 6.20 (t, J = 2.1 Hz, H-12a), 6.48 (d, J = 8.5 Hz, H-2b/6b), 6.54 (d, J = 8.5 Hz, H-3b/5b), 5.19 (d, J = 1.0 Hz, H-7b), 4.30 (d, J = 1.3 Hz, H-8b), 6.28 (d, J = 2.1 Hz, H-12b), 6.06 (d, J = 2.0 Hz, H-14b), 7.11 (d, J = 8.5 Hz, H-2c/6c), 6.72 (d, J = 8.6 Hz, H-3c/5c), 6.87 (d, J = 16.3 Hz, H-7c), 6.60 (d, J = 16.3, H-8c), 6.33 (d, J = 1.9 Hz, H-12c), 6.65 (bs, H-14c). ^{13}C NMR: δ 134.5 (C-1a), 128.8 (C-2a/6a), 117.4 (C-3a/5a), 159.2 (C-4a), 95.5 (C-7a), 58.1 (C-8a), 148.6 (C-9a), 107.9 (C-10a/14a), 161.3 (C-11a/13a), 103.5 (C-12a), 134.3 (C-1b), 128.4 (C-2b/6b), 116.6 (C-3b/5b), 158.6 (C-4b), 93.2 (C-7b), 55.9 (C-8b), 144.4 (C-9b), 119.6 (C-10b), 163.4 (C-11b), 97.3 (C-12b), 161.3 (C-13b), 108.6 (C-14b), 130.4 (C-1c), 129.7 (C-2c/6c), 117.6 (C-3c/5c), 159.4 (C-4c), 132.1 (C-7c), 123.8 (C-8c), 137.0 (C-9c), 122.3 (C-10c), 163.1 (C-11c), 98.2 (C-12c), 160.5 (C-13c), 105.3 (C-14c).

***trans*-ω-Viniferin (6).** ^{1}H NMR: δ 7.03 (d, J = 8.3 Hz, H-2a/6a), 6.62 (d, J = 8.6 Hz, H-3a/5a), 5.87 (d, J = 8.0 Hz, H-7a), 4.70 (d, J = 8.0 Hz, H-8a), 5.81 (d, J = 2.1 Hz, H-10a/14a), 5.97 (t, J = 2.2, H-12a), 7.23 (d, J = 8.5 Hz, H-2b/6b), 6.76 (d, J = 8.6 Hz, H-3b/5b),

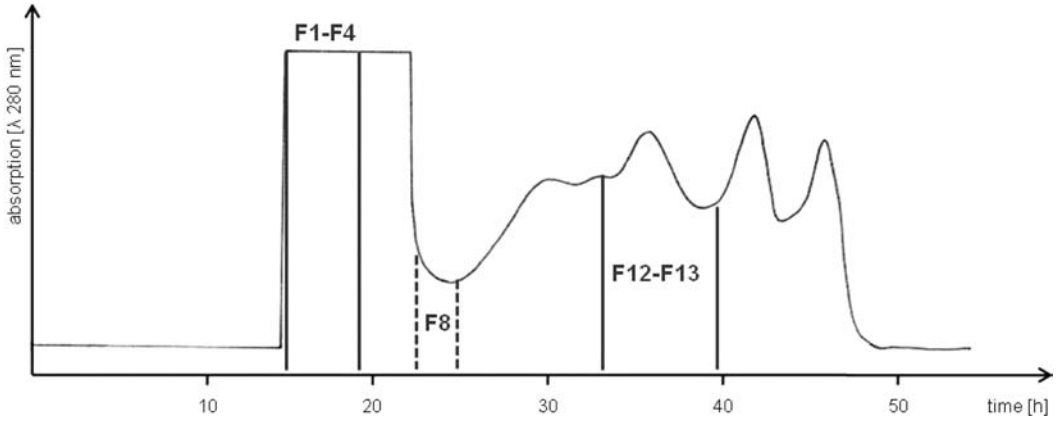

Figure 1. Chromatogram of the LSRCCC separation of the freeze-dried filtrate of the *n*-hexane precipitation monitored at λ 280 nm with marked active fractions.

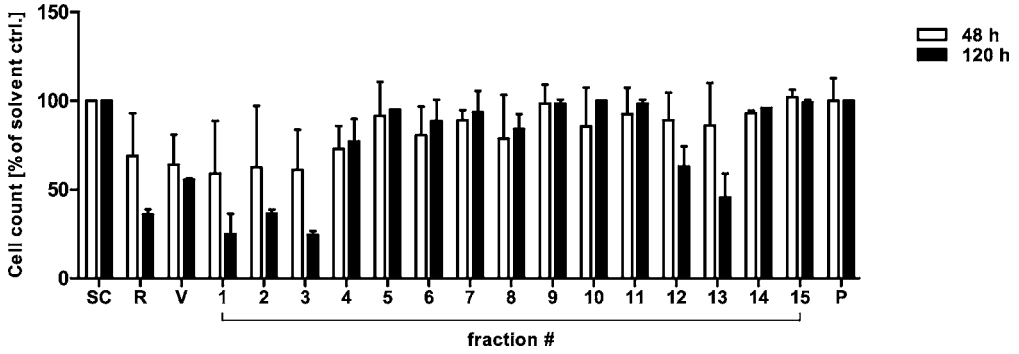

Figure 2. Antiproliferative effect of LSRCCC fractions in cell line A-431 as determined by the SRB assay. Shown are the mean ± standard deviation of two independent experiments; 0.1% DMSO was used as solvent control. SC, solvent control; R, 100 μM resveratrol; V, 23.0 μg/mL Vineatrol 30; P, precipitate.

6.96 (d, *J* = 16.4 Hz, H-7b), 6.77 (d, *J* = 16.6 Hz, H-8b), 6.38 (d, *J* = 2.0 Hz, H-12b), 6.73 (d, *J* = 2.0 Hz, H-14b). ^{13}C NMR: δ 130.2 (C-1a), 130.0 (C-2a/6a), 116.1 (C-3a/5a), 158.3 (C-4a), 91.4 (C-7a), 53.9 (C-8a), 144.3 (C-9a), 109.6 (C-10a/14a), 159.8 (C-11a/13a), 102.7 (C-12a), 130.9 (C-1b), 129.7 (C-2b/6b), 117.3 (C-3b/5b), 159.2 (C-4b), 131.4 (C-7b), 124.6 (C-8b), 137.3 (C-9b), 122.8 (C-10b), 163.3 (C-11b), 98.1 (C-12b), 160.2 (C-13b), 105.8 (C-14b).

Hopeaphenol (7). ^{1}H NMR: δ 7.14 (d, *J* = 8.6 Hz, H-2a/6a), 6.79 (d, *J* = 8.6 Hz, H-3a/5a), 5.75 (d, *J* = 12.1 Hz, H-7a), 4.23 (d, *J* = 12.2 Hz, H-8a), 6.54 (d, *J* = 1.9, H-12a), 6.28 (bs, H-14a), 6.90 (d, *J* = 8.7 Hz, H-2b/6b), 6.56 (d, *J* = 8.7 Hz, H-3b/5b), 5.81 (bs, H-7b), 3.92 (bs, H-8b), 5.72 (d, *J* = 2.1 Hz, H-12b), 5.17 (d, *J* = 2.2 Hz, H-14b). ^{13}C NMR: δ 132.0 (C-1a), 131.3 (C-2a/6a), 117.0 (C-3a/5a), 159.4 (C-4a), 89.2 (C-7a), 50.7 (C-8a), 141.5 (C-9a), 122.1 (C-10a), 159.8 (C-11a), 102.1 (C-12a), 158.2 (C-13a), 107.3 (C-14a), 136.2 (C-1b), 130.3 (C-2b/6b), 116.2 (C-3b/5b), 156.6 (C-4b), 42.1 (C-7b), 49.2 (C-8b), 143.4 (C-9b), 119.6 (C-10b), 160.2 (C-11b), 96.2 (C-12b), 158.1 (C-13b), 112.2 (C-14b).

Ampelopsin A (8). ^{1}H NMR: δ 7.10 (d, *J* = 8.5 Hz, H-2a/6a), 6.76 (d, *J* = 8.6 Hz, H-3a/5a), 5.75 (d, *J* = 11.4 Hz, H-7a), 4.14 (d, *J* = 11.5 Hz, H-8a), 6.41 (d, *J* = 2.0, H-12a), 6.21 (bs, H-14a), 6.87 (d, *J* = 8.7 Hz, H-2b/6b), 6.62 (d, *J* = 8.7 Hz, H-3b/5b), 5.43 (d, *J* = 4.8 Hz, H-7b), 5.40 (d, *J* = 4.9 Hz, H-8b), 6.14 (d, *J* = 2.2 Hz, H-12b), 6.59 (d, *J* = 2.1 Hz, H-14b). ^{13}C NMR: δ 131.9 (C-1a), 131.0 (C-2a/6a), 117.9 (C-3a/5a), 159.5 (C-4a), 89.5 (C-7a), 50.5 (C-8a), 144.1 (C-9a), 119.4 (C-10a), 158.3 (C-11a), 102.5 (C-12a), 159.9 (C-13a), 106.5 (C-14a), 133.7 (C-1b), 129.7 (C-2b/6b), 116.5 (C-3b/5b), 157.1 (C-4b), 44.9 (C-7b), 72.1 (C-8b), 141.5 (C-9b), 119.9 (C-10b), 161.2 (C-11b), 98.1 (C-12b), 159.9 (C-13b), 111.5 (C-14b).

Determination of the Antiproliferative Effect Exerted by the Isolated Fractions and Compounds. A potential antiproliferative effect of the isolated fractions and compounds was determined in the human epidermoid carcinoma cell line A-431[24] by means of the sulforhodamine B (SRB) assay. This cell line was chosen because of its sensitivity toward Vineatrol 30 (Michael T. Empl, personal communication). The cells were cultured at 37 °C in an atmosphere containing 5% CO_2. The cell culture medium consisted of DMEM (Biochrom AG, Berlin, Germany) supplemented with 10% FBS (Biochrom), 2 mM glutamine (Biochrom), and 100 IU/mL, respectively, 100 μg/mL penicillin/streptomycin (Biochrom).

Briefly, 2500 cells per well were seeded in a 96-well microtiter plate (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and incubated for 48 h at 37 °C. Thereafter, fresh medium containing the solvent control (0.1% DMSO; Carl Roth GmbH & Co. KG, Karlsruhe, Germany), *trans*-resveratrol (dissolved in DMSO at a concentration of 100 μM), Vineatrol 30, or the individual fractions (both dissolved in DMSO at a concentration of 23.0 μg/mL) were added, and the cells were incubated for another 48−120 h. After this step, the cells were fixed to the bottom of the microtiter plate by pipetting 50 μL of a 3 M trichloroacetic acid solution (Carl Roth) into each well and incubated for 45 min at 4 °C. The plates were then left to dry for 2−3 h. In a next step 70 μL of SRB solution (0.4% w/v SRB in 1% acetic acid; SRB from Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany; acetic acid from Carl Roth) was added to each well, and the microtiter plate was incubated for 15 min at room temperature. The unbound dye was removed by washing the plates five times with 1% acetic acid, the plates were dried for 2 h, and 100 μL of a 10 mM Tris buffer (pH 10.4; Carl Roth) was added to each well to dissolve the cell protein-bound SRB. Ten minutes later the absorption at 540 nm was measured in a microtiter plate reader (Infinite M200; Tecan Deutschland GmbH, Crailsheim, Germany), and the values of the treated wells (Vineatrol 30, fractions), which are directly proportional to the cell number in each well, were plotted relative to the values of the solvent control.

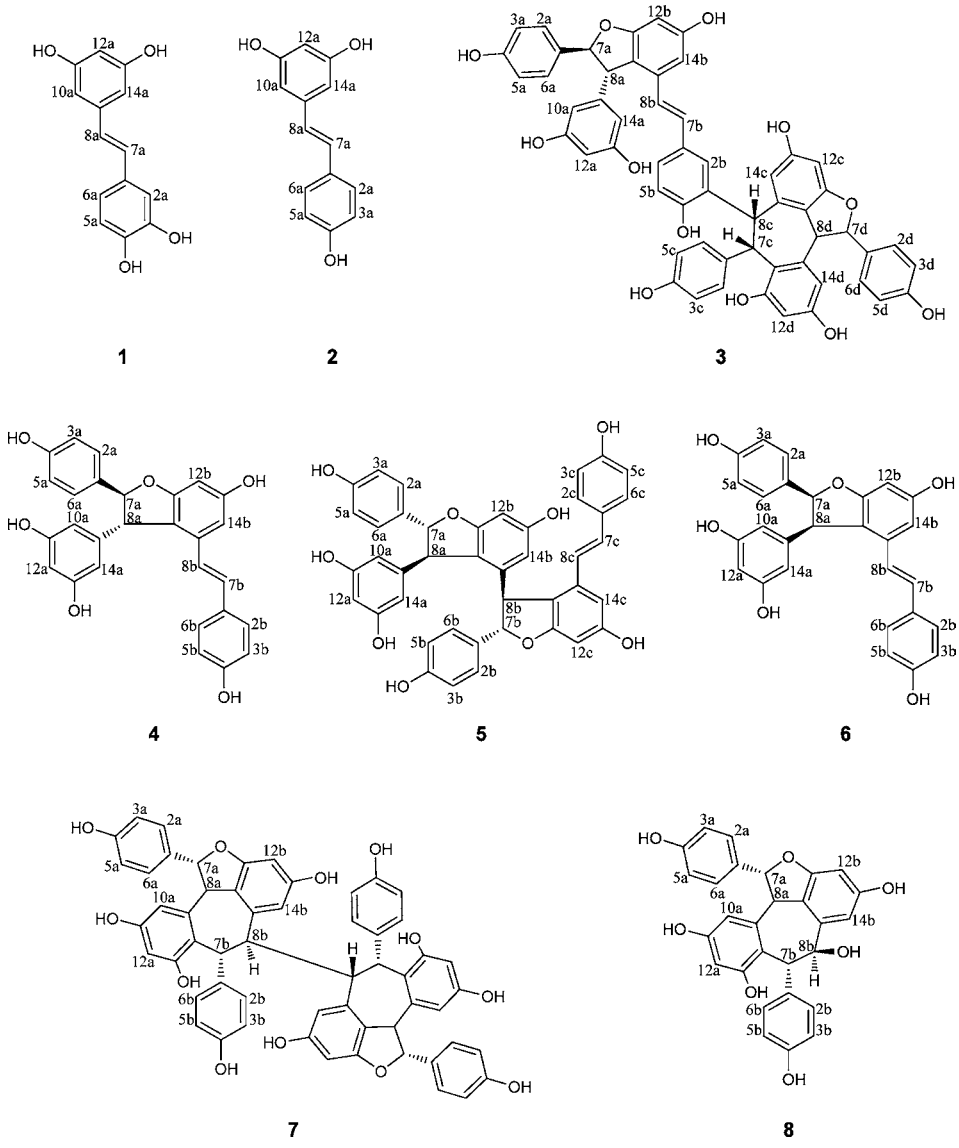

Figure 3. Chemical structures of *trans*-piceatannol (**1**), *trans*-resveratrol (**2**), *r*-2-viniferin (**3**), *trans*-ε-viniferin (**4**), miyabenol C (**5**), *trans*-ω-viniferin (**6**), hopeaphenol (**7**), and ampelopsin A (**8**).

RESULTS AND DISCUSSION

The grapevine-shoot extract under investigation (Vineatrol 30) contains approximately 7.7% *trans*-resveratrol and a complex mixture of oligomeric stilbene derivatives. To correlate the known antiproliferative activity of this extract with its stilbene composition, an activity-guided isolation by using the human epidermoid carcinoma cell line A-431 in combination with the SRB assay was carried out. Prior to the separation by LSRCCC, polymeric surface-active constituents that may hamper the liquid–liquid partition process were removed by *n*-hexane precipitation. In preliminary experiments with low amounts of the extract, different ratios of ethanol/*n*-hexane were tested (1:1, 1:2, 1:3, 1:4, and 1:5), and the 1:2 mixture was found to yield the best result without losing the bioactive compounds of the extract. The precipitate showed no antiproliferative effect in the SRB assay. The freeze-dried filtrate was then used for further studies.

Fractionation by LSRCCC. LSRCCC was performed in the elution–extrusion mode[25] with the solvent system *n*-hexane/ethyl acetate/methanol/water (1:2:1:2, v/v/v/v). Figure 1 shows the chromatogram of the LSRCCC separation recorded at 280 nm. LSRCCC separation yielded 15 fractions. As mentioned above, an aliquot (23.0 μg/mL) of each fraction was tested in the human cancer cell line A-431 (Figure 2). The LSRCCC fractions 1–4, 12, and 13 showed strong antiproliferative effects, whereas fraction 8 exerted only a minor activity.

To further purify these fractions, additional separation steps including HSCCC, silica column chromatography, and preparative HPLC were performed. An amount of 358 mg of LSRCCC fraction 3 was separated by preparative HPLC, and six subfractions with a total weight of 133 mg were obtained. Subfraction 1 consisted of 8.5 mg of *trans*-piceatannol (**1**, Figure 3), a compound that does not induce an antiproliferative effect in A-431 cells.[26] The results for the remaining subfractions (2–6) are depicted in Figure 4. The strongest antiproliferative activity was exerted by subfractions 2–4. In contrast, subfractions 5 and 6 showed only a moderate antiproliferative activity. Structural characterization of the compounds in subfractions 2–6 was performed by LC-MS and NMR.

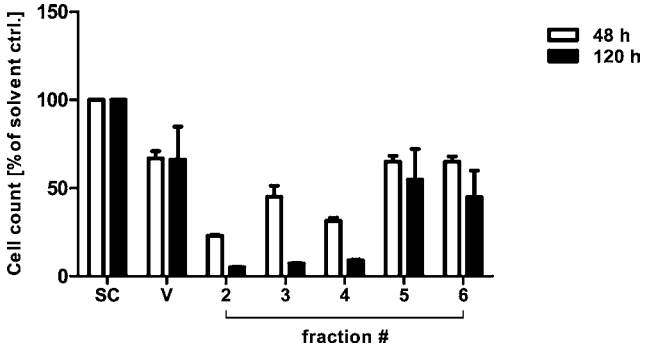

Figure 4. Antiproliferative effect of HPLC subfractions of LSRCCC fraction 3 in cell line A-431 as determined by the SRB assay. Shown are the mean ± standard deviation of two independent experiments; 0.1% DMSO was used as solvent control. SC, solvent control; V. 23.0 μg/mL Vineatrol 30.

Subfraction 2 (30 mg) contained, besides the main component of the extract *trans*-resveratrol (**2**; 96%, chromatographic purity [λ 280 nm]), 4% of the resveratrol tetramer r-2-viniferin (**3**). Subfraction 4 (42.1 mg) contained the other main component of the extract, namely *trans*-ε-viniferin (**4**). These stilbenes were unambiguously identified with the help of authentic reference compounds.[27] From subfraction 3 was isolated 28 mg of the resveratrol trimer miyabenol C (**5**). Consequently, the strong antiproliferative activity of subfrations 2−4 can be explained by the presence of the stilbenes **2**−**5**, the antiproliferative activity of which has previously been described.[26,28] Subfraction 6 contained 12.8 mg of the dimer *trans*-ω-viniferin (**6**). NMR data of stilbenes **1**, **5**, and **6** are in good agreement with previously published data.[29−31] Fraction 5 contained 11.6 mg of a yet unidentified trimer (*m*/*z* 679 $[M - H]^-$) and a further unknown compound with a quasi-molecular ion at *m*/*z* 427 $[M - H]^-$ in the ESI-MS negative mode. Both compounds are likely to be responsible for the antiproliferative effect of subfraction 5, which is not as pronounced as that of subfractions 2−4.

For the further separation of the LSRCCC fractions 12−13, HSCCC and the solvent system *n*-hexane/ethyl acetate/methanol/water in the ratio 2:5:2:5 (v/v/v/v) were used. This separation yielded 10 fractions, which were also tested regarding their growth-inhibiting effect in A-431 cells. The results of these tests are depicted in Figure 5. In this separation the antiproliferative activity was centered on subfractions 7 and 8, whereas the other fractions of this separation exerted no

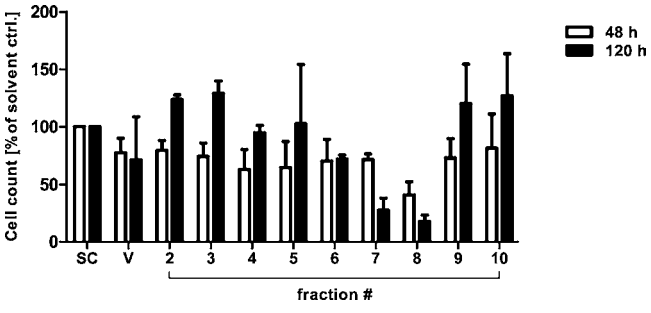

Figure 5. Antiproliferative effect of the subfractions of the HSCCC separation of LSRCCC fraction 13 in cell line A-431 as determined by the SRB assay. Shown are the mean ± standard deviation of two independent experiments; 0.1% DMSO was used as solvent control. SC, solvent control; V, 23.0 μg/mL Vineatrol 30.

antiproliferative effect at all in the cells. In HSCCC subfractions 7 and 8 the resveratrol tetramer hopeaphenol (**7**) could be detected with chromatographic purities [λ 280 nm] of 72 and 83%, respectively. Hopeaphenol is known for its strong growth-inhibiting activity in different cancer cell lines.[32,33] Furthermore, we detected the dimer ampelopsin A (**8**) with a chromatographic purity [λ 280 nm] of 15% in fraction 4, 39% in fraction 5, 50% in fraction 6, and 11% in fraction 7. However, with the exception of fraction 7, these fractions showed only a slight effect in the SRB assay. In accordance with the results of this study, stilbene **8** showed a 6 times lower antiproliferative effect in cell line A-431 than stilbene **7** in a previous study.[34] Stilbenes **7** and **8** were identified and verified by their LC-MS and NMR data after isolation by preparative HPLC. The NMR spectra are in good accordance with literature data.[35]

Fraction 8 of the LSRCCC separation (330 mg), which showed only a slight antiproliferative effect, was fractionated using preparative HPLC. Seven subfractions with a total weight of 157 mg were obtained. Each of the subfractions 4−7 contained new stilbene derivatives (Figure 6). Two of these compounds are diastereomers (**9a** and **9b**; subfractions 4 and 7), have the core structure of *trans*-ε-viniferin, and are substituted by a γ-lactam ring system. The remaining two are constitutional isomers with the basic structure of *trans*-resveratrol and also carry the γ-lactam ring system at C12 (**10**) (subfraction 6) and C14 (**11**) (subfraction 5), respectively. Recently, a similar stilbene exhibiting a γ-lactam ring system was isolated from *Artocarpus nitidus*,[36] but to the best of our knowledge these four stilbene derivatives have not yet been described in the literature. Interestingly, these compounds showed no significant antiproliferative effect in cell line A-431 (Figure 7). In this separation the antiproliferative activity was centered on subfractions 1 and 2, which, on the basis of the obtained MS data, contained two dimers (fraction 1) as well as a dimer and a tetramer (fraction 2). Due to the small amount of these fractions it was not possible to isolate and characterize these trace compounds.

Structure Elucidation of the New Stilbene Derivatives 9−11. The structures **9a**, **9b**, **10**, and **11** were determined by means of low- and high-resolution electrospray ionization mass spectrometry (ESI-MS) and ^1H, ^{13}C, DEPT, and 2D-NMR spectroscopy analysis, including HSQC, HMBC, COSY, and NOESY.

9a and **9b** were obtained as a brown amorphous powder, with high-resolution quasi-molecular ions at *m*/*z* 538.1861 $[M + H]^+$ (**9a**) and *m*/*z* 538.1860 $[M + H]^+$ (**9b**) corresponding to the molecular formula $C_{32}H_{27}O_7N$. The ^1H and ^{13}C NMR data of both compounds were identical (Table 1). The ^1H NMR spectrum of **9a** showed two sets of AA′XX′ type ortho-coupled aromatic hydrogens at δ 6.82 (2H, d, *J* = 8.6 Hz, H-3a/5a) and 7.17 (2H, d, *J* = 8.4 Hz, H-2a/6a) for ring A_1, 6.72 (2H, d, *J* = 8.6 Hz, H-3b/5b) and 7.13 (2H, d, *J* = 8.6 Hz, H-6b/2b) for ring B_2, one set of AX_2 type meta-coupled aromatic hydrogens at δ 6.13 (2H, d, *J* = 2.2 Hz, H-10a/14a) and 6.22 (1H, t, *J* = 2.2 Hz, H-12a) for ring A_2, one single aromatic proton at δ 6.47 (1H, s, H12b) for ring B_1, two aliphatic hydrogens at δ 5.39 (1H, d, *J* = 4.8 Hz, H-7a) and 4.34 (1H, d, *J* = 4.8 Hz, H-8a) for the 7*a*,8*a*-dihydrofuran system, and two coupled doublets at δ 6.37 (1H, d, *J* = 16.6 Hz, H-7b) and 6.76 (1H, d, *J* = 16.6 Hz, H-8b) for a *trans*-configured double bond, for the γ-lactam ring system a methine proton at δ 5.24 (1H, dd, *J* = 5.3/2.5 Hz, H-1′) and four methylene protons at δ 2.30/2.43 (2H, m, H-2′) and at δ 2.22/2.43 (2H, m, H-3′). The correlations of the ring

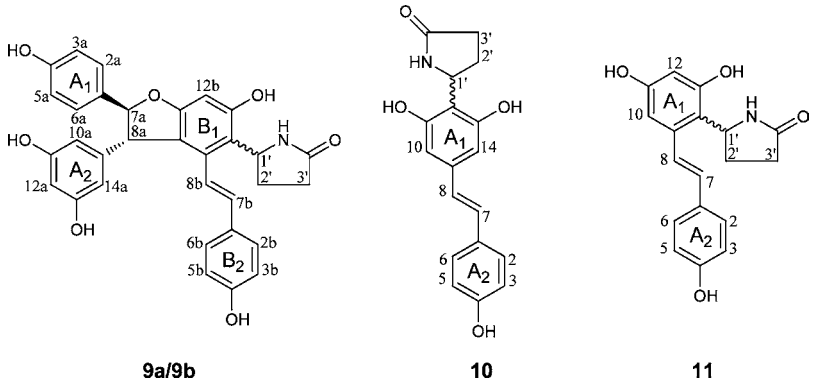

Figure 6. Chemical structures of the new stilbenes **9a**/**9b**, **10**, and **11** with the γ-lactam ring system.

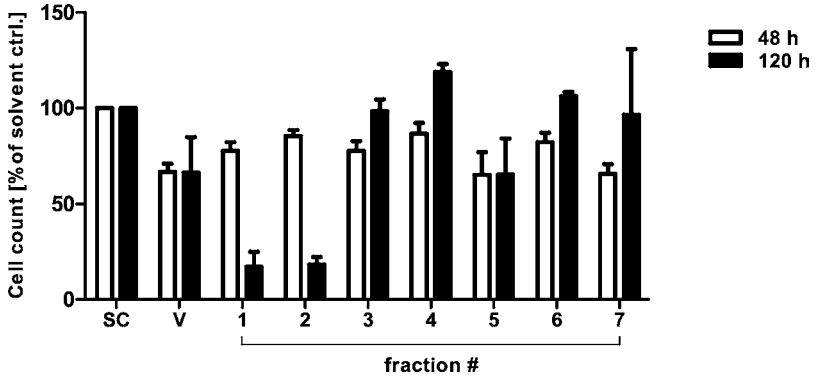

Figure 7. Antiproliferative effect of the fractions of the preparative HPLC separation of LSRCCC fraction 8 in cell line A-431 as determined by the SRB assay. Shown are the mean ± standard deviation of two independent experiments; 0.1% DMSO was used as solvent control. SC, solvent control; V, 23.0 μg/mL Vineatrol 30.

systems, double bond, and aliphatic hydrogens were confirmed from correlations in the 2D $^{1}H-^{1}H$ COSY spectrum. The ^{13}C spectrum of **9a** exhibited five aliphatic carbons at δ 94.5 (C-7a), 59.0 (C-8a), 53.7 (C-1′), 32.5 (C-3′), and 28.4 (C-2′), besides 22 aromatic and olefinic carbons between δ 98.4 and 161.8 plus one amide carbonyl carbon at δ 179.2. All protonated carbons were assigned from the DEPT-135 and the one-bond CH correlations in the HSQC spectrum. Supporting evidence for the proposed structure of **9a** was obtained from HMBC measurements. Important CH long-range correlations from the HMBC spectrum, over two or three bonds, were found between H-7a and six carbon signals [δ 59.0 (C-8a), 134.8 (C-1a), 128.7 (C-2a/6a), 148.4 (C-9a), 121.1 (C-10b), 161.8 (C-11b)], between H-8a and seven carbon signals [δ 134.8 (C-1a), 94.5 (C-7a), 148.4 (C-9a), 107.9 (C-10a/14a), 138.9 (C-9b), 121.1 (C-10b), 161.8 (C-11b)], between H-12b and four carbon signals [δ 121.1 (C-10b), 161.8 (C-11b), 159.4 (C-13b), 120.8 (C-14b)], and between H-8b and four carbon signals [δ 130.7 (C-1b), 135.8 (C-7b), 121.1 (C-10b), 120.8 (C-14b)]. The structure and the attachment of the γ-lactam ring were deduced from the CH long-range correlations between H-1′ and six carbon signals [δ 138.9 (C-9b), 159.4 (C-13b), 120.8 (C-14b), 28.4 (C-2′), 32.5 (C-3′), 179.2 (C-4′)]. The closed ring structure is confirmed by the CH long-range correlations from H-1′ to 179.2 (C-4′) and by the addition of water under acid treatment. **9a** with *m*/*z* 538 $[M + H]^+$ was treated overnight at room temperature with a few drops of concentrated hydrochloric acid in aqueous solution. The monitoring of the reaction by LC-ESI-MS resulted in a new signal at *m*/*z* 556 $[M + H]^+$ with a Δ*m*/*z* 18 mass increase clearly indicating the opening of the γ-lactam ring.

The *trans*-orientation of the aryl pair (A_1/A_2) present in the 2,3-dihydrofuran system is deduced from nuclear Overhauser effects (NOE) between H-7a and the ortho protons of ring A_2 and between H-8a and the ortho protons of ring A_1.[37] These NOE correlations were determined in both stilbenes **9a** and **9b**. Differences in the NOE spectra were observed by the correlations between the ortho protons of ring B_2 and the methine and methylene protons of the γ-lactam ring system. However, these NOESY spectral data gave no further stereochemical informations for the chiral C-1′ due to the planar character of the resveratrol based molecules. The absolute stereochemistry of both compounds can be clarified only by X-ray crystallographic analysis. Currently, this is not possible as these compounds do not crystallize.

Two *trans*-resveratrol derivatives (**10** and **11**) with a γ-lactam substitution pattern such as observed in **9a** and **9b** were obtained as a pale white amorphous powder, with high-resolution quasi-molecular ions at *m*/*z* 334.1050 $[M + Na]^+$ (**10**) and *m*/*z* 334.1049 $[M + Na]^+$ (**11**) corresponding to the molecular formula $C_{18}H_{17}O_4N$. The ^{1}H and ^{13}C NMR spectral data of **10** and **11** are shown in Table 2. Typically, for resveratrol-related compounds, the ^{1}H NMR spectrum of **10** showed a set of AA′XX′ type ortho-coupled aromatic hydrogens at δ 6.83 (2H, d, *J* = 8.7 Hz, H-3/5) and 7.40 (2H, d, *J* = 8.5 Hz, H-2/6) for ring A_2, one set of aromatic hydrogens at δ 6.58 (2H, s, H-10/14), and two coupled doublets at δ 6.95 (1H, d, *J* = 16.2 Hz, H-7) and 6.84 (1H, d, *J* = 15.8 Hz, H-8) for a *trans*-configured double bond.

Table 1. ^{1}H and ^{13}C NMR Data of 9a and 9b Measured in d_6-Acetone

	9a		9b	
position	^{1}H	^{13}C	^{1}H	^{13}C
1a		134.8		134.9
2a/6a	7.17 d (8.4)	128.7	7.17 d (8.5)	128.7
3a/5a	6.82 d (8.6)	117.1	6.81 d (8.6)	117.1
4a		159.1		159.1
7a	5.39 d (4.8)	94.5	5.40 d (4.6)	94.5
8a	4.34 d (4.8)	59.0	4.31 d (4.6)	59.0
9a		148.4		148.7
10a	6.13 d (2.2)	107.9	6.14 d (2.1)	107.9
11a		160.7		160.8
12a	6.22 t (2.2)	102.8	6.24 t (2.0)	102.8
13a		160.7		160.8
14a	6.13 d (2.2)	107.9	6.14 d (2.1)	107.9
1b		130.7		130.7
2b/6b	7.13 d (8.6)	129.6	7.13 d (8.6)	129.6
3b/5b	6.72 d (8.6)	117.2	6.73 d (8.6)	117.2
4b		159.1		159.1
7b	6.37 d (16.6)	135.8	6.30 d (16.6)	136.0
8b	6.76 d (16.6)	123.9	6.78 d (16.6)	123.8
9b		138.9		138.7
10b		121.1		121.1
11b		161.8		161.7
12b	6.47 s	98.4	6.46 s	98.4
13b		159.4		159.4
14b		120.8		120.7
1′	5.24 dd (5.3, 2.5)	53.7	5.24 dd (5.7, 2.7)	53.5
2′	2.4/2.3 m	28.4	2.4/2.3	28.7
3′	2.4/2.2 m	32.5	2.4/2.3	32.5
4′		179.2		179.2

Table 2. ^{1}H and ^{13}C NMR Data of 10 and 11 Measured in d_6-Acetone

	10		11	
position	^{1}H	^{13}C	^{1}H	^{13}C
1		130.8		131.1
2/6	7.40 d (8.5)	129.7	7.42 d (8.6)	129.7
3/5	6.83 d (8.7)	117.4	6.84 d (8.6)	117.4
4		159.3		159.3
7	6.95 d (16.0)	130.1	6.80 d (16.0)	132.6
8	6.84 d (16.0)	127.3	7.42 d (16.1)	125.7
9		140.1		141.6
10	6.58 s	107.2	6.57 d (2.4)	107.1
11		158.5		159.1
12		116.5	6.39 d (2.4)	104.3
13		158.5		158.9
14	6.58 s	107.2		119.0
1′	5.33 dd (5.4, 3.2)	50.0	5.39 dd (6.7, 1.9)	52.5
2′	2.41/2.25 m	28.1	2.42/2.25 m	28.9
3′	2.42/2.23 m	32.4	2.41/2.33 m	32.5
4′		179.0		179.1

The ^{1}H resonances of the four methylene protons of the γ-lactam ring system appeared very similar to compounds **9a**/**9b** with δ 2.25/2.41 (2H, m, H-2′) and at δ 2.23/2.42 (2H, m, H-3′). Solely, the methine proton H-1′ was downfield shifted to δ 5.33 (1H, dd, J = 5.4/3.2 Hz). The ^{13}C spectrum of **10** exhibited 14 aromatic and olefinic carbons between δ 107.2 and 159.3, and the γ-lactam ring was detected by the signals at δ 50.0 (C-1′), 32.4 (C-3′), and 28.1 (C-2′) and at δ 179.0 (C-4′). Unambiguously, the backbone of *trans*-resveratrol was corroborated from correlations in the 2D $^{1}H-^{1}H$ COSY spectrum and the CH long-range correlations from the HMBC spectrum between H-2/6 and four carbon signals [δ 129.7 (C-2/6), 117.4 (C-3/5), 159.3 (C-4), 130.1 (C-7)], between H-7 and four carbon signals [δ 130.8 (C-1), 129.7 (C-2/6), 127.3 (C-8), 140.1 (C-9)], between H-8 and four carbon signals [δ 130.8 (C-1), 130.1 (C-7), 140.1 (C-9), 107.2 (C-10/14)], and between H-10/14 and five carbon signals [δ 127.3 (C-8), 140.1 (C-9), 107.2 (C-10/14), 158.5 (C-11/13), 116.5 (C-12)]. The structure of the γ-lactam ring was derived from the same CH long-range correlations as for **9a** and **9b**. The attachment of the γ-lactam partial structure to position C-12 of ring A_1 was clearly identified by $^{2,3}J$-CH correlations from H-1′ to C-11/13 (δ 158.5) and C-12 (δ 116.5).

The ^{1}H NMR spectrum of **11** showed partly an identical set of resonances as observed for **10** (cf. Table 2) such as the a set of AA′XX′ type ortho-coupled hydrogens of ring A_2. Interestingly, the signals for the two doublets of the *trans*-configured double showed a slight upfield shift Δδ 0.15 for H-7 to δ 6.80 (d, J = 16.0 Hz). An opposite effect was observed for the proton H-8 closer to ring A_1, which was strongly downfield shifted by Δδ 0.58 to δ 7.42 (d, J = 16.1 Hz) compared to substance **10**. As indication that **11** is a positional isomer of **10**, two meta-coupling aromatic hydrogens at δ 6.57 (1H, d, J = 2.4, H-10) and 6.39 (1H, d, J = 2.4, H-12) were detected for ring A_1. Besides the resonance of H-1′ at δ 5.39 (dd, J = 6.7/1.9 Hz) of the γ-lactam ring system, the ppm values of four methylene proton resonances (δ 2.25/2.42, 2H, m, H-2′; δ 2.33/2.41, 2H, m, H-3′) remained unchanged. The basic structure of **11** as a *trans*-resveratrol derivative was confirmed by $^{1}H-^{1}H$ COSY and HMBC spectroscopy. The attachment of the γ-lactam to position C-14 of ring A_1 was deduced from the $^{2,3}J$-CH correlation signals observed between H-1′ and 141.6 (C-9), C-13 (δ 158.9), and C-14 (δ 119.0) in the HMBC spectrum.

The absolute stereochemistry for compounds **10** and **11** could be determined only by X-ray analysis. NOESY experiments were run but gave no additional stereochemical informations due to the planar molecular appearance of the *trans*-resveratrol structures.

The origin of these nitrogen-containing stilbenes remains unknown at the present time. These structures were also detected as minor compounds in Vineatrol 30, a fact that excludes a secondary formation during the isolation procedure described above. Jang et al., who isolated γ-lactam-substituted flavan-3-ols from the roots of *Actinidia arguta*, suggested that they could be formed by a condensation reaction with 5-hydroxypyrrolidin-2-one under acidic conditions.[38] However, one should also consider a microbial synthesis during the storage of the grapevine shoots as an alternative pathway for the formation of these compounds. In either case, this issue needs further investigation.

In conclusion, it can be said that the antiproliferative activity of the grapevine-shoot extract Vineatrol 30 in the epidermoid cancer cell line A-431 can mainly be attributed to *trans*-resveratrol, *trans*-ε-viniferin, hopeaphenol, r-2-viniferin, and miyabenol C. Moreover, this study confirms that CCC combined with bioassays is a suitable tool for the isolation and identification of bioactive compounds in complex plant extracts.

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Funding

We thank the German Federal Ministry of Education and Research for funding this study (Project 0315373D).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to Dr. Ulli Papke (Organic Chemistry Department, Technische Universität Braunschweig) for HR-ESI-MS/MS measurements. We thank Anika Damm, Sandra Ulbricht, and Marion Gröschel for their support.

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