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Isobutylhydroxyamides from the Pericarp of Nepalese *Zanthoxylum armatum* Inhibit *NF1*-defective Tumor Cell Line Growth

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Abstract

A neurofibromatosis type 1 (*NF1*) based bioassay-guided phytochemical investigation on *Zanthoxylum armatum* collected in Nepal led to the isolation of four new timuramides A-D (**1-4**) and six known sanshools (**5-10**). The structures of all compounds were established by using modern spectroscopic techniques, including 1D and 2D NMR analysis and comparison with previously reported data. Most of the compounds inhibited growth of an *Nf1*- and *p53*-deficient mouse glioma cell line at non-cytotoxic concentrations.

Defects in the tumor suppressor gene *NF1* have been recognized as important factors in the development of both sporadic and familial cancers.¹ Both direct genetic inactivation of *NF1* and excessive proteasomal degradation of its gene product, neurofibromin, can lead to the development of glioblastomas^{2,3} while inherited defects in *NF1* lead to higher rates of astrocytoma, malignant peripheral nerve sheath tumors (MPNST), and neuroblastoma, as well as gastrointestinal stromal tumors, breast cancer, pheochromocytoma, and other tumors.¹

Two recent studies^{4,5} have found preparations made from the spice of Asian *Zanthoxylum* spp. to be active against xenografted MDA-MB-231 breast cancer cells, which are known to be deficient in *NF1*,⁶ as well as against cultured MPNST cells from an *NF1* patient. This stimulated us to isolate, characterize and evaluate *NF1* related activity of the compounds obtained from *Zanthoxylum* spp. using a recently published bioassay system in which the mouse *NF1* homolog, *Nf1*, as well as the gene encoding the tumor suppressor *p53* (*Trp53*) are deleted.⁷ For this study, *Zanthoxylum armatum* DC (Rutaceae) of Nepalese origin, locally known as Timur, was selected. *Z. armatum*, an important medicinal and spice plant, is a small tree distributed throughout the Himalayas, predominantly in Nepal, India and Bhutan up to 2,500 m elevation.⁸ In traditional folk medicine, *Zanthoxylum* plants are referred to as 'toothache trees' because their anesthetic or counter-irritant properties render

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ASSOCIATED CONTENT

Supporting Information

NMR spectra for compounds **1-4** are available free of charge via the internet at <http://pubs.acs.org>.

them useful in the treatment of pain.⁹ The characteristic biting or numbing taste of the pericarp of the dry seeds makes it an indispensable spice among the people of South and South East Asia. The stem bark and pericarps are extensively used in indigenous systems of medicine as a carminative, stomachic and anthelmintic.¹⁰ In Nepal, pericarps of *Z. armatum* are used for abdominal pain, cold and cough, tonsillitis, fever, altitude sickness, diarrhea and dysentery.^{11,12} Bioassay-guided fractionation of the pericarp extract of *Z. armatum* led to the isolation of four new (**1-4**) and six known (**5-10**) isobutylhydroxyamides classified as sanshools. More than 50 sanshool structures have been reported from *Zanthoxylum* species, some of which possess antioxidant and anthelmintic properties.^{13,14} Compounds **1** and **2** contain a novel endoperoxide ring. This is the first report of *NFI*-related and glioma-related activity from this class of compounds.

RESULTS AND DISCUSSION

Dried pericarps of *Z. armatum* were extracted in MeOH to yield four new (**1-4**) and six known (**5-10**) isobutylhydroxyamides using diol batch elution, LH-20 Sephadex chromatography and C₁₈-HPLC. All compounds possessed maximal UV absorption near 250 nm, consistent with the presence of a conjugated amide.¹⁵ The IR spectra also showed characteristic bands for the amidocarbonyl group near 3300 cm⁻¹.¹³

Compound **1** was isolated as a colorless oil. The molecular formula, deduced to be C₁₆H₂₅NO₄ by HREIMS, was further supported by ¹³C NMR data showing five double bond equivalents. The ¹H and ¹³C NMR spectra (Tables 1 and 2, respectively) showed the polyunsaturated fatty acid amide nature of compound **1** bearing an *N*-isobutylhydroxyl moiety. In the ¹H NMR spectrum, a 6H singlet resonating at δ_H 1.22 was assigned to H-3' and H-4'. A broad 2H doublet at δ_H 3.31 (J = 6.6 Hz, H-1') showed a ¹H-¹H COSY correlation with δ_H 6.02 (NH) and HMBC correlations with δ_C 167.2 (C-1), 71.3 (C-2') and 27.5 (C-3' and C-4') consistent with the presence of an *N*-isobutylhydroxyl moiety attached to the amide carbonyl. The ¹H NMR spectrum also showed signals for one pair of protons on *trans*-olefinic carbons at δ_H 5.85 (d, J = 15.4, H-2) and δ_H 6.75 (dt, J = 15.4, 6.5 Hz, H-3) and another pair of protons on *cis*-olefinic carbons at δ_H 5.62 as overlapped doublet and triplet (d and t, J = 10.7, 6.9 Hz, H-6) and 5.47 (dd, J = 11.1, 10.5 Hz, H-7) separated by two methylenes at C-4 (δ_C 31.6) and C-5 (δ_C 26.7).¹⁶ The ¹H-¹H COSY correlations for all consecutive protons from H-2 to H-12 (Figure 2) demonstrated an extended spin system in the molecule. The combined analysis of the NMR spectra, molecular formula and degree of unsaturation suggested the presence of an endoperoxide ring containing one olefinic bond in compound **1**. The *cis*-olefinic protons of the ring resonated at δ_H 5.88 (dt, J = 10.1, 2.1 Hz, H-9) and δ_H 5.78 (dt, J = 10.2, 2.5 Hz, H-10). Similarly, two methine protons of the ring geminal to the peroxide resonated at δ_H 5.19 (brd, J = 7.7 Hz, H-8) and δ_H 4.62 (m, H-11). The 11.1 and 10.5 Hz couplings of H-7 indicated *cis*-vicinal protons at C-6 and C-8. In the HMBC spectrum (Figure 2), the correlation of H-8 with C-7 (δ_C 127.0) and C-10 (δ_C 126.6), H-9 with C-8 (δ_C 74.3), C-10 and C-11 (δ_C 74.6), H-10 with C-8 and C-11, H-11 with C-10 and C-12 (δ_C 18.6) and H-12 (δ_H 1.28) with C-9 (δ_C 129.3), C-10 and C-11 were observed. In the ROESY spectrum, H-8 showed a correlation with H-11, indicating that these protons were on the same face of the ring.

We attempted to assign the absolute configuration of compound **1** by converting it into a secondary diol (**1a**) by hydrogenolysis of the peroxide with Lindlar's catalyst.¹⁷ The reaction of **1a** with (*R*)- and (*S*)-methoxytrifluoromethylphenylacetyl chloride (MTPA-Cl) produced the corresponding (*S*)- and (*R*)-MTPA diesters **1b** and **1c** (Figure 1), however, we were unable to assign the configurations due to lack of consistent observed shift changes, presumably due to the close proximity of the two MTPA moieties to each other. While the absolute configuration of 1,2-; 1,3-; and 1,4-saturated diols have been assigned using a sign

distribution model,^{18,19} such assignments for unsaturated diols have not yet been explored. Thus the lack of supporting literature data and limited experimental evidence does not allow us to assign the absolute configuration.

Very few endoperoxide compounds are reported from terrestrial plants, mainly from *Artemisia* spp.,²⁰ however no compounds of the sanshool series have been previously reported to have an endoperoxide ring, making compound **1** the most novel structure of this series. According to the above data, the structure of **1** was assigned as (2*E*,6*Z*,9*Z*)-8,11-endoperoxy-*N*-(2-hydroxy-2-methylpropyl)-2,6,9-dodecatrienamide, trivially named timuramide A.

Compound **2** was obtained as a colorless oil. The molecular formula, deduced to be C₁₆H₂₅NO₄ by HREIMS, was further supported by ¹³C NMR data, and was isomeric with **1**. The ¹H and ¹³C NMR spectra of compound **2** (Tables 1 and 2, respectively) showed distinct similarities with compound **1** except that it contained a C-6/C-7 *trans*-olefinic bond in contrast to the *cis*-configured bond in **1**. The C-6/C-7 *trans*-olefinic bond in **2** was confirmed by the ¹³C-NMR values of the adjacent carbons C-5 and C-8, which resonated at δ_C 31.1 and 78.6, significantly higher²¹ than in compound **1**. Similarly, in the ¹H NMR spectrum, the H-5 methylene protons and H-8 methine proton resonated at δ_H 2.20 (m) and 4.79 (brd, *J* = 5.2 Hz), noticeably lower than in compound **1**. Like that of **1**, the ¹H-¹H COSY correlations between H-2 through H-12 in compound **2** indicated a long spin system in the molecule. In the ROESY spectrum, H-8 showed correlation with H-11, indicating that they were on same face of the ring. According to the above data, the structure of **2** was assigned as (2*E*,6*E*,9*Z*)-8,11-endoperoxy-*N*-(2-hydroxy-2-methylpropyl)-2,6,9-dodecatrienamide, trivially named timuramide B.

Compound **3** was obtained as a colorless oil. The molecular formula C₁₄H₂₁NO₄ for this compound was deduced from HREIMS. The ¹H and ¹³C NMR spectra of compound **3** (Tables 1 and 2) showed many similarities with compounds **1** and **2** except that it lacked an endoperoxide moiety and a terminal carboxylic acid functionality at C-10 appeared in **3**. The carbonyl group of the carboxylic acid was supported by IR (1730 cm⁻¹) and ¹³C NMR (δ_C 174.7). In the ¹H NMR spectrum, a pair of *trans*-olefinic protons resonated at δ_H 7.41 (dd, *J* = 15.0, 11.6 Hz) and δ_H 5.90 (d, *J* = 15.1 Hz) and were assigned to H-8 and H-9, respectively. In the HMBC spectrum, correlations of H-8 with C-7 (δ_C 129.3), C-9 (δ_C 128.2) and C-10, H-9 with C-8 (δ_C 136.9) and C-10 were observed. Like that of compounds **1** and **2**, compound **3** also exhibited ¹H-¹H COSY correlations supporting an extended spin system from H-2 to H-9. The above data show the structure of **3** to be (2*E*,6*Z*,8*E*)-*N*-(2-hydroxy-2-methylpropyl)-2,6,8-decatrienamide-10-oic acid, trivially named timuramide C.

Compound **4** was obtained as a colorless oil. The molecular formula C₁₀H₁₇NO₄ for this compound was deduced from HREIMS. The ¹H and ¹³C NMR spectra of compound **4** (Tables 1 and 2) showed that this compound was a shorter chain sanshool distinctly similar to compound **3** except that it lacked both the C-6/C-7 and C-8/C-9 pairs of olefinic carbons. The terminal carboxylic acid functionality of compound **4** was supported by IR (1738 cm⁻¹) and ¹³C NMR (δ_C 180.7). In the ¹H NMR spectrum, a pair of *trans*-olefinic protons resonated at δ_H 6.00 (d, *J* = 15.4 Hz) and δ_H 6.81 (dt, *J* = 15.1, 6.6 Hz) and were assigned to H-2 and H-3, respectively. The methylene protons appeared at δ_H 2.46 (q, *J* = 6.9 Hz, H-4) and δ_H 2.30 (t, *J* = 7.2 Hz, H-5), showing HMBC correlations with C-6 and C-3 (δ_C 145.5). Compound **4** possessed an extended spin system between H-2 and H-5. According to the above data, the structure of **4** is proposed as (2*E*)-*N*-(2-hydroxy-2-methylpropyl)-2-hexenamide-6-oic acid, trivially named timuramide D.

Together with four new timuramides A-D, six known compounds, namely ZP-amide C (**5**)²², ZP-amide D (**6**)²², hydroxy- α -sanshool (**7**),¹⁶ hydroxy- β -sanshool (**8**),²¹ ZP-amide A (**9**)²² and ZP-amide E (**10**)²² were also isolated and characterized by comparing their spectroscopic data with literature values.

All of the purified compounds were tested in an *NFI*- and *p53*-defective mouse malignant glioma tumor cell line engineered to express a dual reporter, in which cell viability and growth inhibition are reported separately in the same cells using filtered red and green luminescence, respectively (Table 3). Loss of cell viability is measured using a red luminescent reporter of all living cells comparing compound-treated cell in growth media relative to untreated controls in growth-retarding media. Growth inhibition is measured using a green luminescent reporter only expressed in actively dividing cells comparing compound treated cells in growth media to vehicle treated cells in growth media. High growth inhibition scores indicate that cells stop dividing in the presence of the compound.

All compounds exhibited moderate cytotoxicity, however inhibition of cell growth varied at identical concentrations. Compound **7** showed the best activity among the series of compounds, with cell growth inhibition percentage of 53 percent, at 2 $\mu\text{g/mL}$, without observed toxicity. The novel endoperoxide **1**, which possesses the *cis*-configuration at C-6, showed superior activity to compound **2** the corresponding *trans*-geometric isomer at C-6. Similarly, *cis*-isomer **7** was superior to its *trans*-isomer **8**. This is similar to the pattern observed with the pungency of the compounds as the *trans*-amides are tasteless, while the *cis*-amides are strongly pungent.²³ Testing of compounds **1**, **3**, and **4** in the NCI 60 cell screening assay, however, found negligible potency and selectivity when tested at a 10 μM single concentration.

Our results support the original observation that compounds found in *Z. armatum* can inhibit tumor cells mutant for *NFI*, but do not preclude the possibility that these compounds may also have activity against tumors cells lacking the *p53* signaling pathway. Future studies will examine the specificity of these compounds for different tumor suppressor mutations important in human cancer.

EXPERIMENTAL SECTION

General Experimental Procedures

Optical rotations ($[\alpha]_D$) were measured on a Perkin-Elmer 241 polarimeter in a 100×2 mm cell (units $10^{-1} \text{ deg cm}^2\text{g}^{-1}$). UV absorption spectra were obtained using a Varian Cary 50 Bio UV-visible spectrophotometer. IR spectra were measured using a Perkin Elmer FT-IR Spectrometer, SPECTRUM 2000. LCMS were obtained using a Hewlett Packard Series 1100 MSD, whereas HRMS were acquired on an Agilent 6520 Accurate Mass Q-TOF instrument with internal reference masses at 121.05087 and 922.00979, both within 5 ppm. The NMR experiments were performed on a Bruker 600 MHz NMR spectrometer. ^1H and ^{13}C spectra were referenced to deuterated solvent peaks. The diol DIO Spe-ed SPE cartridge and Sephadex LH-20 columns attached to a model UA-6 UV detector and Foxy 200 fraction collector (Teledyne Isco) were used for fractionation of the extract, whereas purification of the compounds was performed using a Varian ProStar 210/215 HPLC equipped with a Varian ProStar 325 UV-Vis detector, operating under Star 6.41 chromatography workstation software. All solvents and chemicals were of analytical grade.

Plant Material

The pericarp of *Z. armatum* (Rutaceae) was collected from Khara of Kaski District, Nepal on November, 2010 at an altitude of 1600 m. A voucher specimen No. KD 109/2010 was

deposited in the Central Department of Botany, Tribhuvan University, Kathmandu, Nepal and identified by taxonomist Prof. Dr. Krishna K. Shrestha of the same department.

Extraction and Isolation

Dried pericarp of *Z. armatum*, 25.0 gm, was ground and soaked in 2 L MeOH overnight. It was then heated up to 70 °C for 5 minutes and filtered. The filtrate was concentrated in a vacuum and yielded 3.2 g of crude extract N192131. The extract, 2.0 g, was loaded on ten diol DIO Spe-ed SPE cartridge (200.0 mg in each cartridge) and eluted with 6.0 mL each of hexane/CH₂Cl₂ (9:1), CH₂Cl₂/EtOAc (20:1), EtOAc, EtOAc/MeOH (5:1) and MeOH to give five fractions A-E of 815.8 mg, 181.6 mg, 75.9 mg, 224.8 mg and 253.8 mg, respectively. The activity against *NFI*-deficient cells of fractions B and C was higher than other fractions, thus these were selected for further fractionation and isolation of pure compounds. Fraction B (180.0 mg) was chromatographed on a 2.5 × 40 cm Sephadex LH-20 column and eluted with hexane/CH₂Cl₂/MeOH (2:5:1, v/v), with 250 drop fractions collected in each tube. Based upon the UV-absorption (254 nm), twenty four fractions A2-X2 were collected. Among those, fractions G2 and H2 showed activity and were further purified by HPLC. HPLC separation was performed on 18.0 mg of fraction G2 dissolved in 2.4 mL of MeOH and injected in 200 µL aliquots (approx. 1.5 mg/injection) onto a 250 × 10 mm Varian Dynamax Microsorb 60-8 C₁₈ HPLC column. The detector wavelength was 225 nm and solvent flow rate was 4.0 mL/min. Elution began with 70% MeOH/H₂O, isocratic for 3 minutes, then a linear gradient of 70% to 100% MeOH at 25 minutes. The column was flushed with 100% MeOH for 5 minutes. Three major UV-absorbing peaks were collected and evaporated in vacuo to yield compound **1** (4.9 mg), compound **2** (2.2 mg) and compound **8** (1.4 mg) eluting at approx. 6.4, 6.1 and 12.1 minutes, respectively. Similar HPLC was employed for fraction H2 (24.0 mg) to yield compounds **7** (2.8 mg) and **8** (4.7 mg) eluting at approx. 11.2 and 12.0 minutes, respectively.

Fraction C (75.0 mg) was chromatographed on a 2.5 × 40 cm Sephadex LH-20 column and eluted with CH₂Cl₂/MeOH (1:1), with 200 drop fractions collected in each tube to give eight fractions A3-H3. Among the eight fractions, fraction B3 and C3 were found to have activity. HPLC purification was performed on 21.0 mg of fraction B3 dissolved in 2.8 mL of MeOH and injected in 200 µL aliquots (approx. 1.5 mg/injection) onto a 250 × 10 mm Varian Dynamax Microsorb 60-8 C₁₈ HPLC column. The detector wavelength was 225 nm and solvent flow rate was 4.0 mL/min. Elution began with 40% MeOH/H₂O, isocratic for 3 minutes, then a linear gradient of 40% to 100% MeOH at 25 minutes. The column was flushed with 100% MeOH for 5 minutes. Four major UV-absorbing peaks were collected and evaporated in vacuo yielded compounds **9** (1.1 mg), **10** (0.9 mg), **5** (0.9 mg) and **6** (1.2 mg) eluting at approx. 7.8, 8.1, 9.7 and 11.3 minutes, respectively. Similar HPLC of fraction C3 (8.0 mg) yielded compounds **4** (1.2 mg) and **3** (1.8 mg) eluting at approx. 5.5 and 10.8 minutes, respectively.

Timuramide A (1), NSC#764085: colorless oil; $[\alpha]_D^{20}$ -20.6 (*c* 0.2, MeOH); UV (MeOH) $\lambda_{\max}$ (log *e*) 215 (3.2), 230 (4.1) and 250 (4.5) nm; IR (CHCl₃) $\nu_{\max}$ 3308, 1670 and 1632 cm⁻¹; ¹H (600 MHz, CDCl₃) and ¹³C (150 MHz, CDCl₃) NMR data, see Tables 1 and 2; HREIMS $[M + H]^+$ at *m/z* 296.1859 (calcd. for C₁₆H₂₆NO₄ 296.1856).

Hydrogenolysis of 1 with Lindlar's Catalyst

Compound **1** was reduced by hydrogenolysis using Lindlar's catalyst (5% Pd/CaCO₃, lead poisoned). A 20 mL RB flask containing a magnetic stir bar was charged with Lindlar's catalyst (2.0 mg). Two mL of THF was added to compound **1** (3.5 mg, 0.0118 mmole). The solution was then transferred to a RB flask in a dropwise manner with constant stirring. The flask was flushed with argon and fitted with a balloon of hydrogen gas. It was then stirred

vigorously with constant supply of hydrogen gas (1 atm.) at room temperature. After 12 h, the mixture was filtered through a plug of silica gel and flushed with 50 mL of CH_2Cl_2 . The residue was loaded in a 250×10 mm Varian Dynamax Microsorb 60-8 C_{18} HPLC column and eluted with gradient of 70–100% MeOH/ H_2O . Eluting at approx. 7.15 minutes was a diol product (**1a**, 2.1 mg, 69%): ^1H NMR (CDCl_3 , 600 MHz), δ 6.73 (1H, dt, J = 15.3, 6.5 Hz, H-3), 5.84 (1H, dt, J = 9.8, 2.2 Hz, H-9), 5.82 (1H, d, J = 15.3 Hz, H-2), 5.79 (1H, dt, J = 9.9, 2.4 Hz, H-10), 5.59 (1H, dd, J = 10.7, 6.9 Hz, H-6), 5.44 (1H, dd, J = 11.0, 10.5 Hz, H-7), 5.15 (1H, t, J = 7.4 Hz, H-8), 4.85 (1H, ddd, J = 6.5, 4.5, 2.1 Hz, H-11), 3.32 (2H, brd, J = 6.1 Hz, H-1'), 2.19 (2H, m, H-4), 2.28 (2H, m, H-5), 1.25 (3H, d, J = 6.6 Hz, H-12), 1.22 (6H, s, H-3'); HREIMS $[\text{M} + \text{Na}]^+$ at m/z 320.1828 (calcd. for $\text{C}_{16}\text{H}_{27}\text{NNaO}_4$ 320.1832).

Preparation of (S)- and (R)-MTPA Esters (**1b** and **1c**) of **1a**

To a solution of compound **1a** (1.0 mg, $3.3 \mu\text{mol}$) in pyridine (0.1 mL) was added (R)-MTPA-Cl ($3 \mu\text{L}$, $13.2 \mu\text{mol}$) and a small crystal of DMAP. The resultant mixture was stirred for 12 h at room temperature. The reaction mixture was worked up by passing through a small silica column and flushing with EtOAc. The crude product was then injected on a 250×10 mm Varian Dynamax Microsorb 60-8 C_{18} HPLC column and eluted with gradient of 70–100% MeOH/ H_2O . The elution at approx. 17.08 minutes yielded the corresponding (S)-MTPA ester **1b** (0.4 mg): partial ^1H NMR data of H-6 to H-12 (CDCl_3 , 600 MHz), δ 6.5399 (1H, dd, J = 11.2, 10.4 Hz, H-7), 5.8574 (1H, dt, J = 10.0, 2.3 Hz, H-9), 5.4952 (1H, dd, J = 10.4, 6.6 Hz, H-6), 5.3740 (1H, dt, J = 9.3, 2.5 Hz, H-10), 5.2312 (1H, ddd, J = 6.4, 4.0, 2.1 Hz, H-11), 1.2342 (3H, d, J = 6.6 Hz, H-12); HREIMS $[\text{M} + \text{H}]^+$ at m/z 730.2813 (calcd. for $\text{C}_{36}\text{H}_{42}\text{F}_6\text{NO}_8$ 730.2809). Treatment of **1a** (1.0 mg) in similar way with (S)-MTPA chloride in pyridine and DMAP gave the corresponding (R)-MTPA ester **1c** (0.3 mg): partial ^1H NMR data of H-6 to H-12 (CDCl_3 , 600 MHz), δ 6.5569 (1H, dd, J = 11.2, 10.3 Hz, H-7), 5.8391 (1H, dt, J = 9.9, 2.2 Hz, H-9), 5.5026 (1H, dd, J = 10.4, 6.6 Hz, H-6), 5.3686 (1H, dt, J = 9.2, 2.5 Hz, H-10), 5.2181 (1H, ddd, J = 6.3, 4.1, 2.0 Hz, H-11), 1.2515 (3H, d, J = 6.6 Hz, H-12); HREIMS $[\text{M} + \text{H}]^+$ at m/z 730.2813 (calcd. for $\text{C}_{36}\text{H}_{42}\text{F}_6\text{NO}_8$ 730.2809).

Timuramide B (2): colorless oil; $[\alpha]_D^{20} + 14.18$ (c 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 225 (2.7), 230 (3.8) and 250 (4.0) nm; IR (CHCl_3) ν_{max} 3333, 1668 and 1633 cm^{-1} ; ^1H (600 MHz, CDCl_3) and ^{13}C (150 MHz, CDCl_3) NMR data, see Tables 1 and 2; HREIMS $[\text{M} + \text{H}]^+$ at m/z 296.1859 (calcd. for $\text{C}_{16}\text{H}_{26}\text{NO}_4$ 296.1856).

Timuramide C (3), NSC#764082: colorless oil; UV (MeOH) λ_{max} (log ϵ) 230 (3.8) and 250 (4.4) nm; IR (CHCl_3) ν_{max} 3307, 1730, 1671 and 1636 cm^{-1} ; ^1H (600 MHz, MeOD) and ^{13}C (150 MHz, MeOD) NMR data, see Tables 1 and 2; HREIMS $[\text{M} + \text{H}]^+$ at m/z 268.1557 (calcd. for $\text{C}_{14}\text{H}_{22}\text{NO}_4$ 268.1543).

Timuramide D (4), NSC#764084: colorless oil; UV (MeOH) λ_{max} (log ϵ) 220 (2.7) nm; IR (CHCl_3) ν_{max} 3321, 1738, 1675 and 1632 cm^{-1} ; ^1H (600 MHz, MeOD) and ^{13}C (150 MHz, MeOD) NMR data, see Tables 1 and 2; HREIMS $[\text{M} + \text{H}]^+$ at m/z 216.1236 (calcd. for $\text{C}_{10}\text{H}_{18}\text{NO}_4$ 216.1231).

Bioassay

A previously described assay⁷ was used to assess the ability of samples to arrest growth and induce toxicity in *Nf1*-null and *Trp53*-null murine astrocytoma cells. Briefly, *Nf1*-/-; *Trp53*-/- KR158 astrocytoma cells expressing two luciferase constructs, one (resulting in green luminescence) driven by the human E2F1 promoter and the other (resulting in red luminescence) driven by the CMV promoter. Cells were plated at 1000 cell/well in white 384-well plates. After attachment (4 h), extracts, fractions, or pure compounds were added

and red and green luminescence assessed using Promega ChromoGlo reagent 2 d later. Inhibition of the green luminescence signal (E2F1) reflects growth inhibition while inhibition of the red signal (CMV) indicates loss of cell viability.⁷

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

$\Delta\delta$ values ($\delta_S - \delta_R$) in ppm x 1000 (**1d**) for the two MTPA esters **1b** and **1c**.

Figure 2.
Important HMBC and COSY correlations in compound 1

Table 1¹H NMR Data (600 MHz) for Compounds **1**, **2** (CDCl₃), and **3-4** (CD₃OD)

Position	1	2	3	4
1	-	-	-	-
2	5.85, d (15.4)	5.80, brs	6.04, d (15.4)	6.00, d (15.4)
3	6.75, dt (15.4, 6.5)	6.78, dt (15.4, 6.5)	6.79, dt (15.1, 6.7)	6.81, dt (15.1, 6.6)
4	2.25, m	2.26, m	2.31, m	2.46, q (6.9)
5	2.33, m	2.20, m	2.46, m	1.30, t (7.2)
6	5.62, dt (10.7, 6.9)	5.71, ddd (15.4, 8.9, 6.3)	5.71, dd (10.3, 7.7)	-
7	5.47, dd (11.1, 10.5)	5.56, dd (15.5, 7.0)	6.14, t (11.1)	-
8	5.19, brd (7.7)	4.79, brd (5.2)	7.41, dd (15.0, 11.6)	-
9	5.88, dt (10.1, 2.1)	5.87, dt (8.4, 1.8)	5.90, d (15.1)	-
10	5.78, dt (10.2, 2.5)	5.83, dt (7.8, 2.3)	-	-
11	4.62, m	4.64, dddd (8.5, 6.6, 4.3, 1.8)	-	-
12	1.28, d (6.6)	1.23, d (6.6)	-	-
1'	3.31, brd (6.1)	3.29, brd (6.2)	3.25, brs	3.24, brs
3' & 4'	1.22, s	1.21, s	1.17, s	1.17, s
NH	6.02, brs	5.96, brs	ND	ND

ND: Not detected

Table 2¹³C NMR Data (δ , 150 MHz) for Compounds **1**, **2** (CDCl₃), and **3-4** (CD₃OD)

Position	1	2	3	4
1	167.2	167.1	169.1	169.3
2	125.4	124.2	125.4	124.7
3	143.4	144.0	144.8	145.5
4	31.6	31.4	33.0	30.1
5	26.7	31.1	27.8	37.4
6	134.2	135.2	137.6	180.7
7	127.0	128.0	129.3	-
8	74.3	78.6	136.9	-
9	129.3	129.5	128.2	-
10	126.6	126.2	174.7	-
11	74.6	74.5	-	-
12	18.6	18.3	-	-
1'	50.7	50.6	51.2	51.2
2'	71.3	71.2	71.8	71.8
3' & 4'	27.5	27.5	27.3	27.3

Table 3

Cell Viability and Growth Inhibition (%) in *Nf1*- and *p53*-defective Mouse CNS Tumor Cell line by Compounds **1-10** at 2.0 $\mu\text{g/mL}$.

Compound	Cell Viability (<i>untreated controls</i> = 100)	% Growth Inhibition
1	97	38
2	65	13
3	66	47
4	70	42
5	79	31
6	66	56
7	106	53
8	56	65
9	75	52
10	67	41