

Identification of Chromomoric Acid C-I as an Nrf2 Activator in *Chromolaena odorata*

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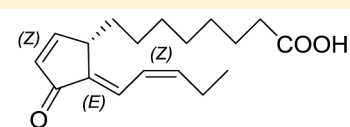
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S Supporting Information

ABSTRACT: Activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) contributes to several beneficial bioactivities of natural products, including induction of an increased cellular stress resistance and prevention or resolution of inflammation. In this study, the potential of a crude leaf extract of *Chromolaena odorata*, traditionally used against inflammation and skin lesions, was examined for Nrf2 activation. Guided by an Nrf2-dependent luciferase reporter gene assay, the phytoprostane chromomoric acid C-I (**1**) was identified as a potent Nrf2 activator from *C. odorata* with a CD (concentration doubling the response of vehicle-treated cells) of 5.2 μ M. When tested at 1–10 μ M, **1** was able to induce the endogenous Nrf2 target gene heme oxygenase 1 (HO-1) in fibroblasts. Between 2 and 5 μ M, compound **1** induced HO-1 in vascular smooth muscle cells (VSMC) and inhibited their proliferation in a HO-1-dependent manner, without eliciting signs of cytotoxicity.



Chromomoric acid C-I (**1**)

- Nrf2 activation
- Induction of HO1
- Inhibition of VSMC proliferation

Nrf2 (nuclear factor-erythroid 2-related factor 2), a ubiquitously expressed mammalian transcription factor, is a key component in the cellular defense against harmful stressors. Under unstressed conditions, the activity of the Nrf2 protein is kept low by complex formation between Kelch-like ECH-associated protein (Keap1) and Nrf2. Keap1, an adapter for ubiquitin ligases, facilitates constant proteasome-dependent degradation of Nrf2. Upon exposure to oxidative or electrophilic agents, the Nrf2/Keap1 complex dissociates, and Nrf2 is stabilized, is able to bind to ARE (antioxidant response element) consensus sequences (TCAG/CXXXGC) in promoters of Nrf2-regulated genes, and also initiates transcription. The resulting gene products are involved mainly in drug metabolism and in the oxidative stress response and detoxification.^{1,2} Activation of Nrf2 by small natural molecules results in an ameliorating effect in several in vivo and in vitro models for diseases that are associated with inflammation or increased oxidative stress, including cancer, diabetes, and atherosclerosis.^{3–5}

Chromolaena odorata (L.) R.M. King & H. Rob. (formerly known as *Eupatorium odoratum* L., Asteraceae) is a perennial herb, native to South and Central America and later introduced into tropical regions of Asia, Africa, and the Pacific.⁶ In the traditional medicine of Vietnam, extracts of the fresh leaves or decoctions of *C. odorata* are used for the treatment of leech

bites, soft tissue wounds, burns, skin infections, and dento-alveolitis.⁷ Functional or target-based pharmacological investigations have revealed antidiabetic, anticataract, antifungal, antibacterial, antioxidant, hemostatic, cytotoxic, and anti-inflammatory activities by constituents of *C. odorata*, as well as inhibition of nuclear factor- κ B (NF- κ B) and activation of peroxisome proliferator (PPAR) γ .^{8–15} Phytochemical analysis has uncovered so far the presence of fatty acids,^{13,14} phenolic acids,⁷ flavonoids,^{12,13,15} alkaloids,¹⁶ diterpenoids,¹⁷ anthraquinones,¹² and essential oil¹⁸ in *C. odorata*.

On the basis of the obvious overlap between the outcome of Nrf2 activation and the reported bioactivities of *C. odorata*, constituents of this species that activate Nrf2 were investigated. This work has provided an additional molecular explanation for the traditional use of *C. odorata* and complements the previously known pharmacological profile of this species.

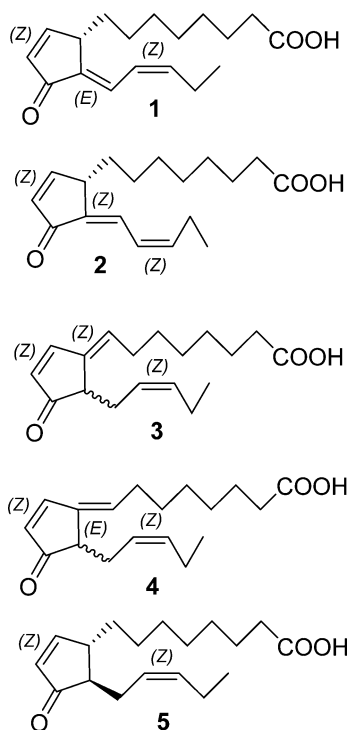
RESULTS AND DISCUSSION

Chromomoric Acid C-I (1**) as an Nrf2 Activator in *Chromolaena odorata*.** To get a first hint of the activation of

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Nrf2 by *C. odorata*, a crude methanol extract of the leaves was tested in an Nrf2-dependent ARE-driven luciferase reporter gene assay. Significant induction of luciferase was observed at a concentration of 30 $\mu\text{g/mL}$ (Figure S1A, Supporting Information). The extract was further fractionated by liquid-liquid extraction with solvents of increasing polarity. The diethyl ether fraction elicited potent concentration-dependent activation of Nrf2 (Figure S1B, Supporting Information), comparable to the extent of the positive control, 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazole (CDDO-IM, 100 nM, for structure refer to Figure S1),¹⁹ and was therefore chosen for further bioassay-guided fractionation.

Out of the most active fractions, 13 flavonoids, one phenolic compound, and five phytoprostanes were isolated. These compounds were identified by means of mass spectrometry and 1D- and 2D-NMR spectroscopy as well as by comparison of their physical and spectroscopic data with those of reference values reported in the literature, as cystosiphonin,²⁰ scutellarein tetramethyl ether,²¹ 6-methoxyhesperetin,²² hesperetin,²² naringenin,²² acacetin,²² 6-methoxyacacetin,²² salvigenin,²² ombuin,²² kaempferol 4'-methyl ether,²² betuletol,²³ kaempferol,²² aromadendrin 7-methyl ether,²² 4-hydroxybenzoic acid,²² and five phytoprostanes. The latter included chromomoric acid C-I²⁴ (**1**), chromomoric acid C-IV²⁴ (**2**), (8Z)-chromomoric acid G^{13,25,26} (**3**), (8E)-chromomoric acid G^{13,26} (**4**), and (9S,13R)-12-oxophytodienoic acid¹³ (**5**).

In contrast to several reports on Nrf2 activation by flavonoids,^{27,28} none of the flavonoids isolated from *C. odoratum* exerted Nrf2 activation in the reporter gene assay used (data not shown). Possibly, the concentrations tested, 10 to 30 μM , were too low to activate Nrf2 under the assay conditions utilized. Phytoprostanes (prostaglandin-like fatty acids), first identified from *Chromolaena morrii*²⁹ and some other *Chromolaena* species,³⁰ are bioactive plant lipids and, from the chemical point of view, nonenzymatic radical initiated peroxidation products of α -linoleic acid.³¹ Among the five phytoprostanes (**1**–**5**) isolated, compounds **4** and **5** have

previously been found in the chloroform extract of *C. odorata*,¹³ while the others (**1**–**3**) were identified in this plant for the first time. Of these, compound **1** (chromomoric acid C-I), which made up 0.014% w/w of the dried plant material (Supporting Information, Figure S2), showed a promising activation of Nrf2 at 10 μM . Despite its structural similarity with the other isolated phytoprostanes, only **1** was capable of markedly activating Nrf2-driven gene expression (Figure 1A). Testing **1** at different concentrations revealed that already 5.2 μM suffices to elicit a 2-fold activation of Nrf2-driven luciferase expression compared to vehicle control cells. Compound **1** shows high structural similarity to the human 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂, a known Nrf2 activator,³² which launches a concentration-dependent activation of Nrf2 highly comparable to **1** (Figure 1B). Therefore, it is conceivable that **1** undergoes an electrophilic attack of cysteine residues of Keap1 and thereby activates Nrf2 signaling as shown for 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (PGJ₂).³² Thiol reactivity of **1** is underlined by a weaker Nrf2 activation when the compound is added to an excess of extracellular glutathione (Figure 1B). 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J₂ also interferes with proinflammatory NF- κ B signaling, at least in part via electrophilic attack and inactivation of proteins within this pathway.³³ By analogy, **1** inhibited the NF- κ B activity in the respective luciferase reporter gene assay with an IC₅₀ of 6.9 μM , whereas the other isolated phytoprostanes were obviously less active (Figure 1C). These data suggest that **1** possesses optimized structural features, i.e., an exocyclic *trans*-configured double bond ($\Delta^{13,14}$) conjugated to the carbonyl group, favoring a putative electrophilic attack of intracellular cysteine residues, a hypothesis deserving further investigation and confirmation in the future.

Compound 1 Activates the Endogenous ARE-Dependent Heme Oxygenase (HO)-1 Promoter. In the next step of this study, the newly identified Nrf2 activator **1** was tested to see if it could activate endogenous Nrf2-dependent promoters (in contrast to the artificial simplified ARE-luciferase promoter in the reporter gene assay). For this purpose, mouse embryonic fibroblasts were treated with different concentrations of **1**, and the expression of HO-1 was examined by immunoblot analysis. HO-1 is a cytoprotective Nrf2-dependent target gene and degrades heme to carbon monoxide, biliverdin, and ferrous iron (Fe²⁺). A strong and concentration-dependent induction of HO-1 was observed in wild-type mouse embryonic fibroblasts, which was less evident in Nrf2^{−/−} isogenic cells, clearly demonstrating Nrf2 dependency (Figure 2A). The induction of HO-1 in Nrf2^{−/−} cells by 10 μM **1** suggests that the compound at this concentration also activates transcription factors other than Nrf2 that are involved in the induction of HO-1, such as PPAR γ or hypoxia-inducible factor 1 α .^{34,35}

Compound 1 Induces HO-1 in VSMC and Thereby Inhibits Proliferation. Vascular smooth muscle cells (VSMC) usually contract and dilate blood vessels to facilitate circulation. Under atherosclerotic conditions, however, VSMC omit their contractile phenotype and start to migrate and proliferate, leading to narrowing and occlusion of the vessel. Inhibition of VSMC proliferation is considered as a valid approach in the prevention of atherosclerotic events. Notably, the Nrf2→HO-1 axis has been linked to an antiproliferative influence on VSMC in many studies.^{36–38} This and the considerable Nrf2-dependent induction of HO-1 by **1** in mouse embryonic fibroblasts prompted the testing of compound **1** also in primary VSMC. As seen in Figure 2B, 3 μM of this substance markedly induced HO-1 in VSMC. Moreover, **1** was able to inhibit

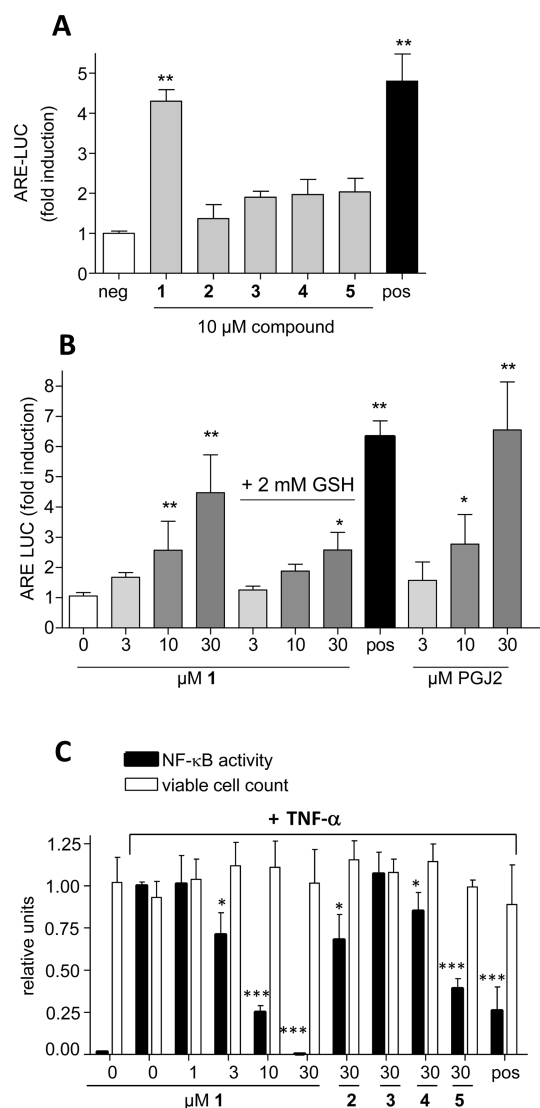


Figure 1. Activation of Nrf2 and inhibition of NF- κ B by phytoprostanes of *C. odorata*. (A) CHO-ARE Luc cells were treated with DMSO (0.1%, negative control, neg), 100 nM CDDO-IM (positive control, pos), and 10 μ M of the isolated phytoprostanes (1–5) for 18 h, as indicated. Luciferase expression was assessed, normalized to the cell count, and expressed as a fold induction of the negative DMSO control. The bar graph depicts compiled data of three independent experiments (means + SD, ** p < 0.01, ANOVA). (B) CHO-ARE Luc cells were treated with DMSO (0.1%, negative control, neg), 100 nM CDDO-IM (positive control, pos), and different concentrations of 1 and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 (PGJ₂), respectively. When indicated, the medium was supplemented with 2 mM glutathione (GSH) prior to addition of 1. Then, 18 h later luciferase expression was assessed, normalized to the cell count, and expressed as a fold induction of the negative DMSO control. The bar graph depicts the compiled data of three independent experiments (means + SD, * p < 0.05 ** p < 0.01, ANOVA). (C) After staining with Cell Tracker Green CMFDA (2 μ M) for 1 h, HEK293/NF- κ B-luc cells were seeded in 96-well plates (4 \times 10⁴ cells/well) overnight and pretreated for 30 min with the indicated concentration of the phytoprostanes 1–5, solvent vehicle (0.1% DMSO), and 5 μ M parthenolide [as positive control for NF- κ B inhibition (pos)]. Afterward, the cells were incubated for 4 h and lysed for luminescence (NF- κ B signal) and fluorescence (cell count) determination. The data represent means + SD (n = 3; * p < 0.05, *** p < 0.001, vs vehicle control; ANOVA/Bonferroni).

VSMC proliferation triggered by platelet-derived growth factor (PDGF), the most potent mitogen for VSMC in the vasculature, without eliciting signs of cytotoxicity, as evident by the absent release of lactate dehydrogenase (LDH) (Figure 2C,D). Co-incubation with the HO-1 inhibitor tin protoporphyrin IX abolished the antiproliferative effect of 1, demonstrating causality between HO-1 induction and inhibition of proliferation (Figure 2E).

Overall, phytoprostane 1 was identified for the first time as an Nrf2-activating principle of *C. odorata* leaves. Nrf2 activation by 1 may add to and/or synergize with the bioactivities of other constituents of *C. odorata* and finally contribute its share to the use of this plant in traditional medicine. Pure compound 1 induced HO-1, an endogenous Nrf2 target gene, in mouse embryonic fibroblasts and VSMC. Induction of HO-1 in VSMC led to inhibition of proliferation by 1. Notably, phytoprostanes are signals of oxidative stress in plants and trigger an increased stress resistance by induction of the plant detoxification machinery.³¹ This picture is highly reminiscent of the Nrf2-mediated detoxification response in mammalian cells upon exposure to 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2, a prostaglandin found under conditions of inflammation and redox stress. The homology between the plant and the mammalian stress response is underlined by the fact that phytoprostanes can activate the stress-sensing mammalian transcription factor Nrf2. This suggests the existence of a common molecular antistress language in plant and mammalian cells using a comparable vocabulary that may be exploited to boost a deficient mammalian detoxification capacity by appropriate plant metabolites, as exemplified with 1 in activated VSMC in this study.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotations were determined with a Perkin-Elmer 341 polarimeter (Wellesley, MA, USA) at 20 °C. 1D- and 2D-NMR experiments were recorded on a Bruker DRX 300 (Bruker Biospin Rheinstetten, Germany) or Bruker Advance II 600 NMR spectrometer; NMR solvents: MeOH-*d*₄/CDCl₃/DMSO-*d*₆/with 0.03% TMS (Eurisotop Gif-Sur-Yvette, France), which was used as internal standard. LC analyses were carried out using an HP 1050 system (Agilent, Waldbronn, Germany) equipped with autosampler, DAD, and column thermostat. Separations were performed on a Phenomenex Aqua 125A (4.6 mm (i.d.) \times 250 mm, 5 μ m) and a Merck (VWR, Darmstadt, Germany) LiChroCART 4-4 guard column with LiChrospher 100 RP18 (5 μ m) packing. A mobile phase consisting of 0.5% FA + 1% 1-BuOH + 1% THF in H₂O (v/v) (solvent A) and MeOH (solvent B) was employed with gradient elution (0 min, 55:45 (A:B); 50 min, 20:80; 51 min, 2:98; 60 min, 2:98). The detection wavelength was 280 nm, and the thermostat was set at 45 °C. The injection volume was 10 μ L; the flow rate was 0.5 mL/min. ESIMS were obtained on an Esquire 3000plus mass spectrometer (Bruker Daltonics, Bremen, Germany), using the following parameters: alternating mode; spray voltage, 4.5 kV, 350 °C; dry gas, 10.0 L/min; nebulizer 30 psi; full scan mode, m/z 100–1500.

Fast centrifugal partition chromatography (FCPC) was carried out on apparatus (Kromaton, France) equipped with a Gilson 302/803C pump system model 302 (Villiers-la-Bel, France). Column chromatography was performed with Sephadex LH-20 (Pharmacia Biotech AB, Stockholm, Sweden) and silica gel 60 (0.040–0.063 mm; Merck, VWR, Darmstadt, Germany) as stationary phases. TLC was carried out on silica gel 60 F254 plates (VWR, Darmstadt, Germany). Semi-preparative HPLC was performed with a Dionex Ultimate 3000-preparative HPLC system with Chromeleon software. A Heto Powerdry 6000 freeze-dryer was used for water-containing fractions.

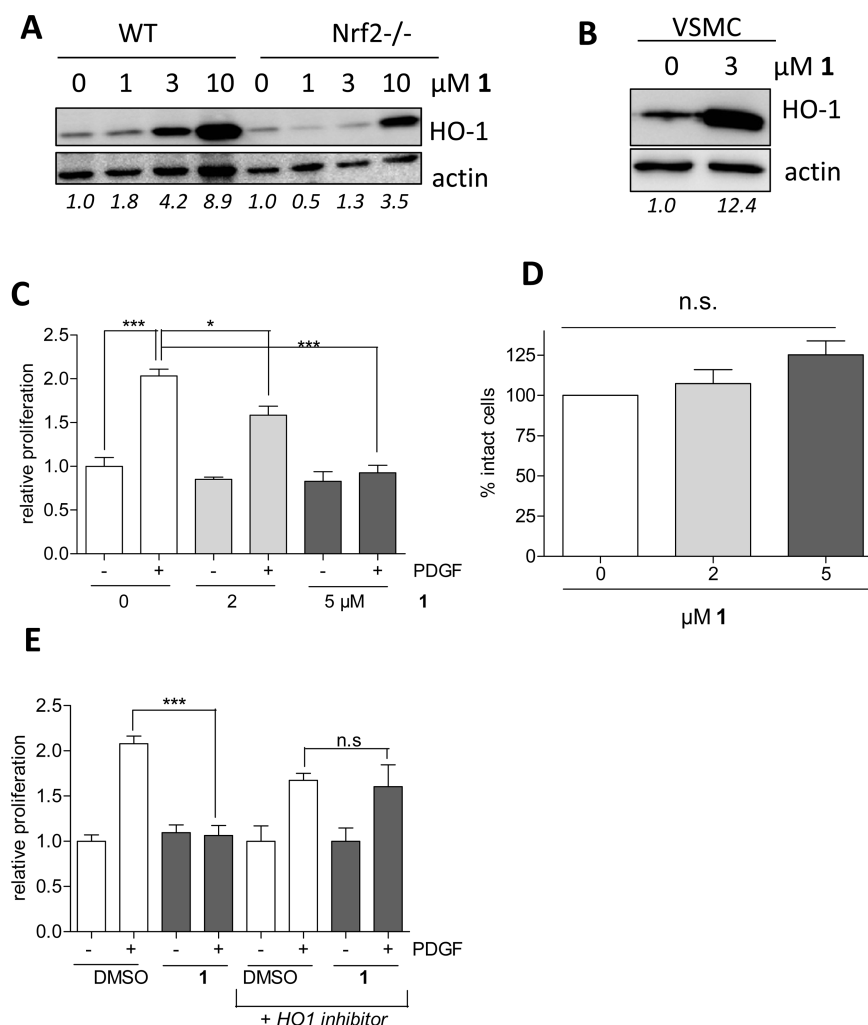


Figure 2. Compound **1** induces HO-1 in a Nrf2-dependent manner and inhibits VSMC proliferation via HO-1 induction. WT and isogenic Nrf2^{-/-} mouse embryonic fibroblasts (A) as well as VSMC (B) were treated with **1** at the indicated concentration for 18 h before total cell lysates were subjected to immunoblot analysis for HO-1 and actin as loading control. Representative blots out of three independent experiments are shown. The numbers below the blots indicate the compiled densitometric analysis of HO-1/actin [referred to the vehicle (0.1% DMSO) control, which is set at 1] of all performed experiments. (C) Quiescent VSMC were treated with **1** at the indicated concentration for 30 min and then stimulated with 20 ng/mL PDGF for 48 h. Proliferation was assessed based on resazurin conversion as described in the Experimental Section. The bar graph depicts compiled data of three independent experiments (means + SD, * $p < 0.05$; *** $p < 0.001$, ANOVA). (D) Quiescent VSMC were treated with **1** at the indicated concentration for 48 h before their release of LDH as readout for cell membrane disintegration and cytotoxicity was determined. The bar graph depicts compiled results of three independent experiments. (E) Antiproliferative activity of 5 μM **1** in the absence and presence of 10 μM tin protoporphyrin IX (HO-1 inhibitor) was assessed as in (C). The bar graph depicts compiled data of three independent experiments (means + SD, *** $p < 0.001$, ANOVA).

Primary rat VSMC were purchased from Lonza (Braine-L'Alleud, Belgium). Chinese hamster ovary cells (CHO-K1) were obtained from LGC (Wesel, Germany), and the stable CHO-ARE-Luc clones thereof were established in our laboratory as described previously.³⁹ Wild-type and isogenic Nrf2^{-/-} mouse embryonic fibroblasts were kindly provided by Dr. T. Kensler, University of Pittsburgh.⁴⁰ PDGF was from Bachem (Weilheim, Germany). CDDO-IM was a kind gift from Dr. M. Sporn, Dartmouth Medical School. The HO-1 inhibitor tin protoporphyrin IX dichloride was from Enzo Life Sciences (Lausen, Switzerland), and all other chemicals were obtained from Sigma-Aldrich (Vienna, Austria). The anti-HO-1 antibody was from Stressgene (via Enzo, Lausen, Switzerland), the anti-actin antibody was from mpbio (Eschwege, Germany), and the secondary horseradish-peroxidase-coupled antibodies came from Cell Signaling (Heidelberg, Germany).

All solvents used for isolation were purchased from VWR International (Darmstadt, Germany). Solvents for HPLC were obtained from Merck (Darmstadt, Germany). Ultrapure water was

produced by a Sartorius Arium 611 UV water purification system (Göttingen, Germany).

Plant Material. The leaves of *Chromolaena odorata* were collected in Chua Chan Mountains, DongNai, Vietnam, in May 2011 and identified by Prof. Tran Hung (Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy of HoChiMinh City, Vietnam). A voucher specimen (DN108) is stored at the Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy of HoChiMinh City.

Extraction and Isolation. Extraction was carried out with 8 kg of the milled and air-dried leaves, which were percolated with 96 L of MeOH at room temperature. The solution obtained was evaporated to dryness at 35 °C, yielding 1356.1 g of crude extract. The initial separation was performed by means of liquid–liquid extraction; 210 g of crude extract was suspended in 1.0 L of water and extracted with *n*-hexane (500 mL × 6), diethyl ether (500 mL × 6), and ethyl acetate (500 mL × 4) followed by *n*-butanol (500 mL × 6). Each of the combined organic layers as well as the aqueous layer was evaporated to

dryness, affording *n*-hexane (32.0 g), diethyl ether (53.2 g), ethyl acetate (4.5 g), *n*-butanol (20.4 g), and water (89.1 g) fractions.

The obtained diethyl ether fraction, which showed the most promising pharmacological effects, was subjected to bioassay-guided isolation. An aliquot (45.0 g) of this fraction was subjected to silica gel CC (petroleum ether–EtOAc, 10:0 to 4:6, v/v), to obtain 15 fractions (A1 to A15). Fraction A11 (1.7 g), which showed the most potent activity in the Nrf2 assay, was applied to FCPC (*n*-heptane–EtOAc–MeOH–H₂O, 3:5:5:3, lower phase: mobile phase) to afford 16 fractions (A11-1 to A11-16). The insoluble part of A11 in the FCPC solvent system was separated by Sephadex CC (MeOH) to obtain acacetin (17.4 mg) and ombuin (13.6 mg). Among the 16 subfractions of A11, fractions A11-6 to A11-9, A11-15, and A11-16 exhibited Nrf2 activation. Fractions A11-7, A11-8, and A11-9 were purified by Sephadex CC (MeOH) to afford cystosiphonin (14.4 mg), scutellarein tetramethyl ester (20.5 mg), 6-methoxyacacetin (15.6 mg), and salvigenin (5.4 mg). Fraction A11-15 was further chromatographed on a Sephadex column (MeOH, followed by CH₂Cl₂–acetone; 85:15, v/v) to yield 6-methoxyhesperetin (10.3 mg), hesperetin (16.4 mg), naringenin (20.9 mg), kaempferol (7.0 mg), and aromadendrin 7-methyl ether (54.8 mg). Fraction A11-16 was separated by Sephadex CC (MeOH) to yield 4-hydroxybenzoic acid (5.6 mg).

The subfractions of A11 containing fatty acid derivatives showed the most potent activities in the Nrf2 assay; however, when separated from the flavonoid constituents, these fractions were found to be unstable when stored at room temperature. Therefore, further isolation was conducted with fraction A10, which contained the identical fatty acid components when analyzed by HPLC. Fraction A10 was divided into two parts. The first part, A10-F (1.7 g), was worked up with the same work flow as applied to fraction A11, using FCPC (*n*-heptane–EtOAc–MeOH–H₂O, 5:3:3:3, lower phase: mobile phase) to provide 11 fractions. Fractions A10-F3 and A10-F4 were purified by Sephadex CC (MeOH) to yield fatty acid-containing fractions: A10-F3-2, A10-F4-2, A10-F5-1. Fraction A10-F3-2 (136.5 mg) was rechromatographed by silica gel CC (CH₂Cl₂–EtOAc, 10:0 to 94:6, v/v), followed by Sephadex CC (CH₂Cl₂–acetone, 85:15, v/v) to obtain compound 5 (15.5 mg). Fraction A10-F3-2-4 was applied to semipreparative HPLC (Phenomenex Aqua 5 μ m C₁₈ 125 Å; 250 \times 10.0 mm, isocratic MeCN–H₂O + 0.02% TFA, 68:32; flow rate 2.0 mL/min) to afford compounds 3 (3.2 mg) and 4 (6.1 mg). Fraction A10-F5-1 (44.3 mg) was purified by silica gel CC (CH₂Cl₂–EtOAc, 10:0 to 94:6, v/v) to yield compound 1 (8.8 mg). Subfractions A10-F5-9 and A10-F4-6 were combined (8.7 mg) and separated by semipreparative HPLC (X-Terra Prep C₁₈ 125 Å; 100 \times 7.8 mm, isocratic MeOH–water, 75:25, v/v; flow rate 1.0 mL/min), to afford beturetol (4.6 mg), which was present only in a small amount in the active fraction A11-9. The second part of fraction A10 (A10-S, 1.21 g) was separated by Sephadex CC, to obtain a fraction containing fatty acid derivatives (A10-S2, 90.2 mg) and kaempferol 4'-methyl ether (15.8 mg), which was also present in active fraction A11-9. Fractions A10-F4-2 and A10-S2 were combined (328 mg) and applied to silica gel CC (CH₂Cl₂–EtOAc, 10:0 to 94:6, v/v) to produce nine fractions and compound 1 (23 mg). Fraction A10-S2-6 was purified by semipreparative HPLC (Phenomenex Aqua 5 μ m C₁₈ 125 Å; 250 \times 10.0 mm, isocratic MeOH–H₂O + 0.02% TFA, 80:20, flow rate 2.0 mL/min) to yield compound 2 (7.2 mg).

Cell Culture. CHO-ARE-Luc and mouse embryonic fibroblasts were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum and 2 mM glutamine and, for CHO-ARE-Luc, additionally with 4 μ g/mL puromycin. VSMC were cultivated in DMEM–F12 (1:1) supplemented with 20% fetal calf serum and gentamycin. HEK293/NF- κ B-luc cells (Panomics, RC0014) were maintained in DMEM with 100 μ g/mL hygromycin B, 100 U/mL benzylpenicillin, 100 μ g/mL streptomycin, 2 mM glutamine, and 10% serum.

Luciferase Reporter Gene Assay. The ARE- and NF- κ B-dependent luciferase assays were performed as described previously.^{39,41} CDDO-IM (100 nM) served as positive control in the ARE-dependent luciferase assay and parthenolide (5 μ M) in the NF- κ B luciferase assay. Equal concentrations of the solvent vehicle (0.1%

DMSO) were present in all treatment groups and served as negative control in both assays. Cell Tracker Green CMFDA (Invitrogen) staining was used to monitor the cell viability.⁴¹ For quantification of NF- κ B activities HEK293/NF- κ B-luc cells were pretreated for 30 min as indicated and afterward stimulated with 2 ng/mL TNF- α for 4 h. The cells were lysed with a luciferase lysis buffer (Promega; E1531), and afterward luminescence and fluorescence were quantified on a Genios Pro plate reader (Tecan, Grödig, Austria).

Immunoblot Analysis. Cells (MEF or VSMC) were seeded onto six-well plates ((3–4) \times 10⁵ cells/well). The next day, they were treated with DMSO (0.1%) or 1 at the indicated concentrations for 18 h. Then, cells were lysed and protein extracts were subjected to SDS-PAGE electrophoresis and immunoblot analysis, as described previously.³⁵

VSMC Proliferation. Cell proliferation was assessed based on metabolic conversion of resazurin to fluorescent resorufin that correlates with the cell number, as described previously.⁴² Briefly, serum-starved cells were pretreated for 30 min with 0.1% DMSO, 1, or tin protoporphyrin IX as indicated and subsequently stimulated for 48 h with PDGF (20 ng/mL). Then, cells were washed with phosphate-buffered saline and incubated in serum-free medium containing 10 μ g/mL resazurin for 45 min. Samples were measured by monitoring the increase in fluorescence at a wavelength of 590 nm using an excitation wavelength of 535 nm in a 96-well plate reader (Tecan GENios Pro).

Assessment of Cytotoxicity. As a readout for potential cytotoxicity, membrane integrity was assessed and the amount of LDH released from VSMC determined after a 48 h treatment with 1. For this, Promega's CytoTox nonradioactive cytotoxicity assay was used according to the manufacturer's instructions.

Statistics. Data are presented as means and standard deviation (SD) from three independent experiments unless stated otherwise. Statistical significance was determined by ANOVA and Dunnett's or Bonferroni's post-test using GraphPad Prism software. Results with *p* < 0.05 were considered significant.

■ ASSOCIATED CONTENT

● Supporting Information

¹H and ¹³C NMR data of compounds 1 and 2, HPLC profiles of investigated extracts, activation of Nrf2 by extracts of *Chromolaena odoratum*, as well as quantification of 1 are available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

Dedicated to Prof. Dr. Otto Stichler, of ETH-Zurich, Zurich, Switzerland, for his pioneering work in pharmacognosy and phytochemistry.

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