Published in final edited form as:

Free Radic Biol Med. 2013 April ; 57: 141–153. doi:10.1016/j.freeradbiomed.2012.12.019.

Seaweed extracts and unsaturated fatty acid constituents from the green alga *Ulva lactuca* as activators of the cytoprotective Nrf2–ARE pathway

Rui Wang^a, Valerie J. Paul^b, and Hendrik Luesch^{a,*}

Hendrik Luesch: luesch@cop.ufl.edu

^aDepartment of Medicinal Chemistry, University of Florida, Gainesville, FL 32610, USA

^bSmithsonian Marine Station, Fort Pierce, FL 34949, USA

Abstract

Increased amounts of reactive oxygen species (ROS) have been implicated in many pathological conditions, including cancer. The major machinery that the cell employs to neutralize excess ROS is through the activation of the antioxidant-response element (ARE) that controls the activation of many phase II detoxification enzymes. The transcription factor that recognizes the ARE, Nrf2, can be activated by a variety of small molecules, most of which contain an α,β -unsaturated carbonyl system. In the pursuit of chemopreventive agents from marine organisms, we built, fractionated, and screened a library of 30 field-collected eukaryotic algae from Florida. An edible green alga, *Ulva lactuca*, yielded multiple active fractions by ARE–luciferase reporter assay. We isolated three monounsaturated fatty acid (MUFA) derivatives as active components, including a new keto-type C18 fatty acid (**1**), the corresponding shorter chain C16 acid (**2**), and an amide derivative (**3**) of the C18 acid. Their chemical structures were elucidated by NMR and mass spectrometry. All three contain the conjugated enone motif between C7 and C9, which is thought to be responsible for the ARE activity. Subsequent biological studies focused on **1**, the most active and abundant ARE activator isolated. C18 acid **1** induced the expression of ARE-regulated cytoprotective genes, including NAD(P)H:quinone oxidoreductase 1, heme oxygenase 1, thioredoxin reductase 1, both subunits of the glutamate–cysteine ligase (catalytic subunit and modifier subunit), and the cystine/glutamate exchange transporter, in IMR-32 human neuroblastoma cells. Its cellular activity requires the presence of Nrf2 and PI3K function, based on RNA interference and pharmacological inhibitor studies, respectively. Treatment with **1** led only to Nrf2 activation, and not the increase in production of *NRF2* mRNA. To test its ARE activity and cytoprotective potential in vivo, we treated mice with a single dose of a *U. lactuca* fraction that was enriched with **1**, which showed ARE-activating effects similar to those observed in vitro. This could be owing to this fraction's ability to stabilize Nrf2 through inhibition of Keap1-mediated Nrf2 ubiquitination and the subsequent accumulation and nuclear translocation of Nrf2. The induction of many ARE-driven antioxidant genes in vivo and most prominently in the heart agreed with the commonly recognized cardioprotective properties of MUFAs. A significant increase in *Nqo1* transcript levels was also found in other mouse tissues such as the brain, lung, and stomach. Collectively, this study provides new insight into why consumption of dietary seaweed may have health benefits, and the identified compounds add to the list of chemopreventive dietary unsaturated fatty acids.

Keywords

Oxidative stress; Reactive oxygen species; Nrf2; Keap1; Antioxidant-response element; Antioxidants; Edible seaweeds; Cytoprotective enzymes; NQO1; *Ulva lactuca*; Monounsaturated fatty acids; Fatty acid amides; Glutathione; Free radicals

Introduction

In aerobes, reactive oxygen species (ROS)¹ are produced during cellular respiration and energy metabolism [1]. In a healthy cell, the level of ROS is tightly regulated by the antioxidant defense system. However, upon environmental stress or cellular damage, the cell cannot readily detoxify the ROS generated and may thereby suffer from oxidative stress, which is implicated in the pathogenesis of many age-related diseases, such as cancer, Alzheimer disease, and stroke, and aging itself [2].

One of the major defense systems employed by the cell to counteract oxidative insult is the nuclear factor E2-related factor 2 (Nrf2)–antioxidant-response element (ARE) signaling pathway. Under normal conditions, the Kelch-like ECH-associated protein 1 (Keap1) sequesters Nrf2 in the cytoplasm and targets it for proteasomal degradation. When the oxidative stress sensor Keap1 encounters reactive oxygen species or electrophilic chemicals, Nrf2 is stabilized and released. The transcriptional activator (Nrf2) translocates to the nucleus, where it binds to the ARE, initiating the expression of cytoprotective enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione S-transferase [3–5].

Many naturally occurring small-molecule inducers of the Nrf2–ARE pathway have been identified and explored as chemo-preventive or therapeutic agents. For example, curcumin [6], the active ingredient in a traditional herbal remedy and the dietary spice turmeric (*Curcuma longa*), is currently in clinical trials for multiple conditions, including several cancers and Alzheimer disease [7]. The skin of red grapes (*Vitis vinifera*) is rich in resveratrol [8,9], which was found to be responsible for an inverse relationship between grape consumption and breast cancer occurrence in an epidemiologic study [10]. In a clinical setting, resveratrol was observed to induce the reexpression of tumor suppressor genes in a group of women who were at increased risk of breast cancer [11]. The detoxification enzyme inducer sulforaphane [12] was found in many cruciferous vegetables. It has been shown that a daily regimen of hot water infused with 3-day-old broccoli sprouts has promising results in cancer chemoprevention in healthy individuals [13]. Broccoli sprouts (*Brassica oleracea italica*) contain high levels of the precursor of sulforaphane, glucoraphanin [14], which can be enzymatically converted to sulforaphane in the gastrointestinal tract after ingestion [15].

In addition to terrestrial organisms, the marine environment has proven to be a rich source of potent compounds with diverse therapeutic properties [16,17]. Our group has focused on the discovery and development of drug leads from marine cyanobacteria, which resulted in several molecules with anticancer activities [18–20]. In an effort to expand our research area to disease (including cancer) prevention, we started investigating marine eukaryotic algae, or seaweeds, because the consumption of edible seaweeds has been postulated to account for a longer life span and low cancer occurrence [21,22]. We hypothesized that certain “fruits and vegetables” from the sea, like their terrestrial counterparts, could also activate the ARE and protect the cell from potential oxidative damage [23]. In fact, individual compounds with antioxidant activities have been identified from marine algae. For example, the free radical scavenger fucoxanthin, a carotenoid from a common edible seaweed, *Hijikia fusiformis*

[24], was found to activate the antioxidant defense system (Nrf2–ARE) in mouse liver cells during the course of our project [25].

In this study, we aimed to discover other small-molecule antioxidants from seaweeds. We screened a collection of Floridian marine eukaryotic algae for their ability to activate the Nrf2–ARE pathway by using an ARE–luciferase reporter gene assay that is amenable to high-throughput screening [2,26,27]. An edible green alga, *Ulva lactuca*, was highly potent in activating the ARE-luc reporter. Three keto-fatty acid-based compounds were subsequently isolated from the lipophilic extract through bioassay- and NMR-guided fractionation. Their chemical structures were elucidated and the biological mechanism of the actions of the most active compound was characterized.

Material and methods

Chemicals and reagents

Unless otherwise indicated, solvents for extraction and chromatography were purchased from Fisher Scientific, and all other chemicals were from Sigma.

General instrumentation

HPLC was carried out on a Shimadzu Prominence Series. ^1H and 2D NMR data in CDCl_3 were recorded on a Bruker Avance II 600 MHz NMR spectrometer using a 5-mm TXI cryogenic probe. The ^{13}C NMR data were recorded at 125 MHz on a Bruker 500-MHz NMR spectrometer. All spectra were referenced to residual solvent signals (δ_{H} 7.26 ppm, δ_{C} 77.16 ppm). UV absorbance was measured in a submicro (50 μl) quartz cuvette (Starna cells) using a SpectraMax M5 (Molecular Devices) spectrophotometer. Accurate mass (HRESIMS) data were obtained on an Agilent 6210 TOF LC-TOF mass spectrometer equipped with an APCI/ESI multimode ion source detector.

Eukaryotic algae library preparation

The algae species were hand-collected in the period of 2006–2009 in Florida waters (see Supplementary Table S1). They were immediately frozen and then stored at $-20\text{ }^{\circ}\text{C}$ until lyophilized. The dried samples were first extracted with agitation in $200 \times (\text{w/v})$ nonpolar solvent mixture (50% EtOAc in methanol (MeOH), v/v) twice for two consecutive 24-h periods and then in $200 \times (\text{w/v})$ polar solvent mixture (50% ethanol (EtOH) in H_2O , v/v) for another two consecutive 24-h periods. At the end of each extraction, the solvents were removed by a Rotavapor (BüCHI Labortechnik AG), and the resulting extracts were stored at $4\text{ }^{\circ}\text{C}$ until analyzed. Approximately 20 mg of each nonpolar extract was dissolved in CH_2Cl_2 , loaded onto a silica SPE column (1000 mg/8 mlx; Alltech Extract-Clean), and eluted with increasing percentage of *i*-PrOH (0, 2, 5, 10, 20%) in CH_2Cl_2 followed by 100% MeOH. Approximately 20 mg of each polar extract was dissolved in 50% EtOH/ H_2O (v/v), loaded onto a C18 SPE column (1000mg/8ml; Alltech Extract-Clean), and eluted with increasing percentage of MeOH (0, 20, 40, 60, and 80%) in H_2O followed by 100% MeOH. All fractions were dried under N_2 gas and stored at $4\text{ }^{\circ}\text{C}$ ($< 48\text{ h}$) until tested.

Fractionation and isolation from *U. lactuca*

U. lactuca was collected from Shark Island (N $27^{\circ} 28.024$, W $80^{\circ} 19.163$) near Fort Pierce, Florida, on 25 July 2006 and 3 June 2009. The initial (2006) nonpolar extract of *U. lactuca* (2.3 g) was separated on a silica-gel column into seven fractions by eluting with increasing percentage of *i*-PrOH (0, 2, 5, 8, 10, and 20%) in CH_2Cl_2 followed by 100% MeOH. Each fraction was concentrated to dryness and stored at $-20\text{ }^{\circ}\text{C}$. The 8% *i*-PrOH fraction (fraction 4, 81.0 mg) was chromatographed on a semipreparative HPLC column (Phenomenex; Synergi Hydro-RP, $250 \times 10\text{ mm}$, $4\text{ }\mu\text{m}$; flow rate, 2.0 ml/min) by HPLC. The HPLC

method consisted of increasing percentage of CH₃CN in H₂O (10–100% in 0–40 min, 100% in 41–60 min). HPLC fraction 19 (*t_R*, 41.2 min, 1.9 mg) was subjected to analytical HPLC (Restek; Allure C18, 250 × 4.6 mm, 5 μm; flow rate, 1.0 ml/min) using a gradient of increasing percentage of CH₃CN in H₂O with 0.1% HCOOH as a modifier (10–100% in 0–20 min, 100% in 20–25 min) to afford the C18 acid (**1**) (*t_R*, 21.9 min). The C16 acid (**2**) (*t_R*, 19.7 min) was isolated from an adjacent HPLC fraction (14, *t_R*, 37.0 min), the C18 amide (**3**) (*t_R*, 20.4 min) from an adjacent silica gel fraction (fraction 5, HPLC fraction 11, *t_R*, 42.8 min). All three compounds were later identified in one or multiple other silica gel fractions (3–6) and isolated using the same procedure. The total amount reported for each compound is the combined weight from all isolations and purifications. The compounds were isolated as follows:

7(*E*)-9-keto-octadec-7-enoic acid (**1**), 0.5 mg, colorless, amorphous solid; UV (EtOH) λ_{max} (log ε) 210 (2.46); ¹H NMR, ¹³C NMR, and 2D NMR data, see Table 1; HRESIMS *m/z* 319.2247 for [M+Na]⁺ (calcd for C₁₈H₃₂O₃: 319.2244);

7(*E*)-9-keto-hexadec-7-enoic acid (**2**), 0.2 mg, colorless, amorphous solid; UV (EtOH) λ_{max} (log ε) 212 (2.41); ¹H NMR, ¹³C NMR, and 2D NMR data, see Supplementary Table S2; HRESIMS *m/z* 291.1942 for [M+Na]⁺ (calcd for C₁₆H₂₈O₃: 291.1931);

7(*E*)-9-keto-octadec-7-enamide (**3**), 0.1 mg, colorless, amorphous solid; UV (EtOH) λ_{max} (log ε) 209 (2.46); ¹H NMR, ¹³C NMR, and 2D NMR data, see Table 1; HRESIMS *m/z* 318.2417 for [M+Na]⁺ (calcd for C₁₈H₃₃NO₂: 318.2404).

For the in vivo studies, a **1**-containing fraction (fraction 3*) from a recollection of *U. lactuca* (2009) was used because of the exhaustion of the originally collected material. The recollected sample was handled in a slightly different fashion in an attempt to concentrate the amount of fatty compounds. After lipophilic extraction as described above, the nonpolar extract was partitioned with EtOAc and H₂O. The EtOAc portion was dried down and separated by silica-gel chromatography to yield six fractions. Fraction 3* (25% hex in EtOAc, v/v) was the only fraction containing **1** by HPLC (see above) trial injections. The identity of **1** in this fraction was confirmed by HPLC co-injection, NMR, and mass spectrometry.

Cell culture

IMR-32 (human neuroblastoma) cells (ATCC) were cultured in Eagle's minimum essential medium (ATCC) supplemented with 10% (v/v) fetal bovine serum (HyClone) and 1% (v/v) antibiotic–antimycotic (Invitrogen). The cells were maintained at 37 °C in a humidified 5% carbon dioxide (CO₂) atmosphere.

Reporter gene assays

IMR-32 cells (33,000 cells/well) were transiently transfected with ARE-luc plasmids (ARE-luc-wt and ARE-luc-mt [28]) or AP-1-(PMA)-luc plasmid [29] (100 ng/well) using FuGENE HD (Roche Diagnostics) as a transfection reagent (1:3, w/v). The cells were dispensed into each well of a 96-well plate and incubated for 24 h. They were then treated with solvent control (dimethyl sulfoxide (DMSO) or EtOH, 1%, v/v) or individual extracts/fractions/compounds for 24 h before luciferase activities were detected by using BriteLite detection reagent (PerkinElmer). For assays in the presence of an antioxidant, the cells were pretreated with excess *N*-acetylcysteine (NAC; 1 mM, pH 7.50) for 2 h before they were treated with solvent control or the compounds (see above). For cells transfected with AP-1-(PMA)-luciferase plasmids, 1 μg/ml PMA (phorbol 12-myristate 13-acetate) was used as a

positive control to stimulate the reporter expression 6 h before detection. Relative fold activation for the treated samples over solvent control was reported.

Whole-cell lysate preparation

IMR-32 cells (800,000 cells/well) were seeded into each well of six-well plates. Twenty-four hours later, the cells were treated with solvent control (EtOH, 0.5%, v/v) or fractions/compounds. After another 24 h, whole-cell lysates were collected in PhosphoSafe buffer (Novagen) following the manufacturer's protocol and stored at -80°C until analyzed. For kinase inhibitor assays, the cells were pretreated with the previously established active concentrations of the corresponding inhibitor, LY294002 (25 μM) or PD098059 (50 μM) [26], for 30 min before being treated with the solvent control or **1**.

Immunoblot analysis

For each sample, the protein concentration was measured using a BCA assay kit (Pierce). Samples containing equal amounts of total protein were separated by SDS-PAGE (NuPAGE Novex 4-12% Bis-Tris mini gels; Invitrogen), transferred onto a polyvinylidene difluoride membrane, and incubated overnight with the indicated primary antibodies at 4°C on an orbital shaker. The membranes were then incubated with the corresponding horseradish peroxidase (HRP)-linked secondary antibodies for 1 h at room temperature and detected with a Supersignal Femto Western blotting kit (Pierce). Anti-NQO1 (mouse) and anti-Nrf2 (rabbit) antibodies were from Abcam; anti-hemagglutinin (HA) (mouse) was from Covance; anti- β -actin (rabbit), anti- α -tubulin (rabbit), anti-mouse-HRP, and anti-rabbit-HRP antibodies were from Cell Signaling Technology; anti-Oct-1 (C-21), anti-Gal4 (DBD), and anti-Keap1 (E-20) were from Santa Cruz Biotechnology; and anti-goat-HRP was from Chemicon (Millipore).

RNA extraction, cDNA synthesis, and quantitative PCR (qPCR) analysis

Cultured cells were treated in the same way as described for the immunoblot experiments. After 12 h, total RNA was extracted using a commercial kit (RNeasy mini kit; Qiagen). For mouse tissues, total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's standard protocol.

cDNA synthesis and qPCR experiments for both cultured cells and mouse tissues were performed following the same procedure. Each RNA sample (2 μg) was reverse-transcribed into cDNA, which served as a template for TaqMan gene expression assays (Applied Biosystems). For each qPCR run, a total reaction volume of 25 μl was prepared consisting of 12.5 μl of TaqMan 2 $\times$ Universal Master Mix, 1.25 μl of a 20 $\times$ TaqMan gene expression assay probe, 1 μl of cDNA, and 10.25 μl of RNase-free sterile water. The reactions were dispensed into 96-well optical reaction plates (Applied Biosystems) and detected in an Applied Biosystems 7300 Real-Time PCR system. The thermocycler program consisted of 2 min at 50°C , 10 min at 95°C , and 40 cycles of 95°C for 15 s and 60°C for 1 min. For normalization, GAPDH expression was used as an internal control for human cells (IMR-32) and β -actin for mouse tissue samples.

RNA interference experiments

IMR-32 cells (600,000 cells/well) were seeded in six-well plates and incubated at 37°C for 24 h. The medium was then carefully aspirated and replaced with a transfection mixture containing small interfering RNAs (siRNAs; 50 nM) and siLentFect lipid transfection reagent (Bio-Rad Laboratories) in fresh medium (2 ml/well). Forty-eight hours after transfection, the cells were treated with solvent control (EtOH, 0.5%, v/v) or **1** for 24 h. The treated cells were then lysed in PhosphoSafe buffer and whole-cell lysates analyzed by

immunoblot (see above). The siRNAs, siGENOME nontargeting siRNA pools and siGENOME SMARTpool (human NFE2L2), were purchased from Dharmacon.

Glutathione assays

Glutathione assays were performed following the manufacturer's protocol (Sigma). Briefly, IMR-32 cells (800,000 cells/well) were seeded into six-well plates and incubated at 37 °C. The cells were then treated with solvent control (EtOH, 0.5%, v/v) or **1** for the indicated periods of time. At the end of each treatment, the cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS), harvested in 200 µl of DPBS, and pelleted at 600g for 10 min at 4 °C. After removal of DPBS, the cell pellet was deproteinized and resuspended in three volumes of 5% sulfosalicylic acid solution (v/v). The suspension was frozen (liquid N₂) and thawed (37 °C water bath) twice and then incubated at 4 °C for 5 min. The cell debris was spun down at 10,000 g for 10 min at 4 °C. The supernatant was transferred to fresh tubes and used as glutathione stock. The concentration of the total glutathione (GSH + GSSG) was measured and compared with a standard curve of reduced glutathione (GSH).

Preparation of cytosolic and nuclear extracts

IMR-32 cells (2,000,000 cells/well) were seeded into 6-cm dishes and then treated with a solvent control (EtOH, 0.5%, v/v) or fraction 3* for the indicated periods of time. After treatment, the cytosolic and nuclear portions of the cells were separated by using a commercial kit (NE-PER nuclear and cytoplasmic extraction reagents; Pierce). Briefly, cells were first rinsed with DPBS, collected in 1 ml of DPBS, and pelleted at 500g for 30 min at 4 °C. The pellet was then resuspended by vortexing in ice-cold buffer CER I containing 1% cOmplete, EDTA-free protease inhibitor cocktail (v/v; Roche Diagnostics). Ice-cold buffer CER II was then added to the resuspended cells, which were again vortexed vigorously for 5 s and incubated on ice for 1 min. After an additional vortexing, the sample was centrifuged at 16,100g for 5 min at 4 °C. The supernatant was transferred to an ice-cold fresh tube and used as cytosolic stock. The remaining insoluble portion was washed and centrifuged twice with ice-cold DPBS and then resuspended in ice-cold buffer NER supplemented with 1% protease inhibitor cocktail (v/v). The suspension was vortexed vigorously for 15 s and incubated on ice for 10 min. This step was repeated four times. Subsequently, the sample was centrifuged and the supernatant was used as nuclear extract stock.

Ubiquitination assay

Using Fugene HD as a transfection reagent, HEK293 cells (1,600,000 cells/dish) were transiently cotransfected with Gal4-Neh2 (0.7 µg/dish), HA-Ub (0.6 µg/dish), and Keap1-CBD (0.7 µg/dish) expression vectors [30] and seeded into 6-cm dishes. Forty-three hours after transfection, the cells were treated with solvent control (EtOH, 0.5%, v/v) or serial dilutions of fraction 3* for 5 h. Then, the cells were washed twice in ice-cold DPBS and collected in 2% SDS (v/v) in TBS (10 mM Tris-HCl, pH 8.0, and 150 mM NaCl). The lysates were boiled at 95 °C for 10 min to inactivate endogenous ubiquitin hydrolase. This lysing and inactivation process also disrupted any noncovalent interactions including Keap1-Neh2. The samples were then sonicated for 20 s in an ice bath and diluted with 8 volumes of 1% Triton X-100 (v/v) in TBS. The protein of interest in the sample was purified immediately by immunoprecipitation (see below).

Immunoprecipitation

Equal amounts of the whole-cell lysates (measured by BCA assay) were first precleared by incubating with 20 µl of protein A/G-agarose beads (Santa Cruz Biotechnology) for 30 min at 4 °C. The beads were pelleted at 800g for 30 s at 4 °C. The supernatants were transferred to fresh tubes and incubated with an anti-Gal4 antibody (2 µg/sample; Santa Cruz

Biotechnology) for 1 h at 4 °C. After this conjugation step, 20 µl of protein A/G–agarose beads (Santa Cruz Biotechnology) was added to each sample, and these were then incubated at 4 °C overnight with agitation. Afterward, the beads were pelleted at 800 *g* for 30 s at 4 °C and washed once with ice-cold 0.5 M LiCl in TBS and then twice with ice-cold TBS. The washed beads were then boiled in 2 × electrophoresis sample buffer (Santa Cruz Biotechnology) at 95 °C for 2.5 min to elute the immunoprecipitated protein.

Mouse studies

All animal experiments conducted were approved by the Institutional Animal Care & Use Committee at the University of Florida. The transgenic mice (B6C3-ARE-Tg) were obtained from Professor J. Johnson [31], bred by the animal care services at the University of Florida, and genotyped at Professor D. Borchelt's lab. The mice were housed in a pathogen-free environment and inspected daily. Three male mice (3–4 months of age) were used in each treatment group. Fraction 3* was prepared in a vehicle consisting of 10% DMSO (v/v), 10% Cremophor-EL (v/v) in PBS as a 10 mg/ml suspension. A single dose of 200 µl (50 mg/kg) was administered by oral gavage. No apparent toxicity was observed for any animal. After 12 h, the mice were euthanized in 100% CO₂. The tissues were harvested immediately, frozen on dry ice, and kept at –80 °C until analyzed.

Human placental alkaline phosphatase (hPAP) assay

The frozen tissues were thawed on ice and lysed in buffer (0.05 M Tris-HCl, 0.005 M mgCl₂, 0.1 M NaCl, 1% Chaps (w/v), pH 7.0 [32]). The homogenized samples were centrifuged at 16,100*g* for 2 min at 4 °C. The supernatants were transferred to fresh tubes and used as hPAP stock. HPAP activity in each sample was determined by using a commercial kit (Phospha-Light system). Briefly, the protein concentration in each sample was measured using the BCA assay. One hundred microliters of protein (300 µg) was diluted with an equal volume of 1 × assay buffer and incubated at 65 °C for 30 min to inactivate any endogenous alkaline phosphatase activity. The heated samples were then cooled on ice to room temperature and dispensed into 96-well plates (50 µl/well). Fifty microliters of assay buffer (room temperature) was added to each well and incubated for 5 min before addition of reaction buffer (50 µl/well, room temperature). The reaction was incubated for another 20 min and detected for luminescence (0.25 s/well).

The average hPAP activity of a set of mice (n=3) that did not carry the transgene was used as an internal control for normalization and subtracted from each value obtained for transgenic mice.

Results

ARE activity profiling of various seaweeds and the identification of *U. lactuca* as a rich source of antioxidants

Using an ARE luciferase reporter assay [28], we tested the bioactivities of 30 marine algae. The seaweeds were collected in Florida waters, frozen immediately on site, and then lyophilized and extracted consecutively with nonpolar (50% EtOAc in MeOH, v/v) and polar (50% EtOH in H₂O, v/v) solvent mixtures. Afterward, a 20-mg sample of each crude extract was fractionated over normal and reversed-phase (C18) SPE cartridges to generate six or seven fractions. All of the extracts and fractions were screened for ARE-luc activity in a high-throughput format in IMR-32 neuroblastoma cells, a standard cell type for oxidative stress-related studies [26,27]. Each sample was tested at two concentrations, 10 and 100 µg/ml. An arbitrary prioritization system was employed. An extract was considered sufficiently active if one of its SPE fractions had more than 10-fold activity or more than three of the fractions had 3-fold activity at any of the two concentrations. For comparison, a standard

ARE activator, *tert*-butylhydroquinone (tBHQ), normally activates the ARE-luc to 15- to 30-fold at 10 mM in the reporter assay in IMR-32 cells [26,28]. Whereas 58% of the extracts and/or fractions from the green algae and 45% from the red algae activated the reporter, fewer (25%) from the brown algae appeared to be active at the concentrations tested (Supplementary Table S1 and Fig. 1A). One particular species, *U. lactuca* (Chlorophyta), stood out as yielding highly active polar and nonpolar extracts (Fig. 1A). Subsequently, the remainder of the more active lipophilic extract was further fractionated by large-scale silica gel chromatography and the resulting fractions were retested in the ARE-luc reporter assay (Fig. 1B).

Bioassay-guided fractionation, isolation, and structure determination of three ARE activators

To identify the chemical components responsible for the ARE activity, we further separated the active silica gel fraction that had the predominant weight (fraction 4, Fig. 1B) by reversed-phase HPLC, which indicated that this fraction was still a very complex mixture of many minor constituents. The bioactivities of each of the HPLC fractions were tested again at two concentrations (10 and 100 $\mu\text{g/ml}$) using the reporter gene assay. The fractions that had greater than 10-fold activity at 10 $\mu\text{g/ml}$ and major weight (> 1 mg) were purified to afford three fatty acid-type compounds, **1-3** (Fig. 2A).

For compound **1**, 7(*E*)-9-keto-octadec-7-enoic acid, the HRE-SIMS spectrum showed a pseudomolecular ion $[\text{M}+\text{Na}]^+$ of 319.2247, suggesting a molecular formula of $\text{C}_{18}\text{H}_{32}\text{O}_3$, with 3 degrees of unsaturation. The chemical structure was elucidated by 1D (^1H and ^{13}C) and 2D (COSY, TOCSY, HSQC, and HMBC) NMR techniques (Table 1). The ^1H NMR spectrum was indicative of a fatty acid-type molecule, with signals for a methylene envelope (δ_{H} 1.30, 12H, C12–C17) and a poorly defined triplet due to a terminal $-\text{CH}_3$ (δ_{H} 0.88, H₃-18) with virtual coupling (Fig. 2B). The two olefinic protons between δ_{H} 6 and 7 ppm (δ_{H} 6.83, H-7, and δ_{H} 6.08, H-8) appeared to have HMBC correlations to a ketone carbonyl carbon at δ_{C} 201.24 (C9), constituting an α,β -unsaturated carbonyl system. The *trans* configuration of the double bond was deduced based on the large coupling constant (15.8 Hz) between the olefinic protons (H-7 and H-8). The third degree of unsaturation was accounted for by a carboxylic acid carbonyl group at δ_{C} 177.20 (C1).

Compound **2**, 7(*E*)-9-keto-hexadec-7-enoic acid, possessed a ^1H NMR spectrum very similar to that of **1**, indicating that it is also a functionalized fatty chain (Fig. 2B). HRESIMS analysis showed a pseudomolecular ion $[\text{M}+\text{Na}]^+$ of 291.1942, with a 28-amu ($2 \times \text{CH}_2$) difference from compound **1**. The molecular formula was calculated as $\text{C}_{16}\text{H}_{28}\text{O}_3$. The structure was determined by NMR (Supplementary Table S2). It was shown that compound **2** has the same exact functionalities as compound **1**, including a terminal carboxylic acid group (C1) and an α,β -unsaturated carbonyl system (C7–C9), but with a truncated fatty chain (C10–C16). This acid had previously been isolated from a laboratory-cultured marine diatom, *Skeletonema marinoi* [33].

Compound **3**, 7(*E*)-9-keto-octadec-7-enamide, showed a pseudomolecular ion $[\text{M}+\text{Na}]^+$ of 318.2417 by HRESIMS, with only 1-amu difference from acid **1**. Its molecular formula was calculated as $\text{C}_{18}\text{H}_{33}\text{NO}_2$, also with 3 degrees of unsaturation. The structure was determined again by NMR (Table 1). Although the same α,β -unsaturated carbonyl system (C7–C9) and the fatty chain (C10–C18) were observed in the ^1H NMR spectrum, there was an additional pair of broad, low-field proton signals at δ_{H} 5.39 and δ_{H} 5.24. These two protons were suggestive of a primary amide moiety at the C1 position in place of the carboxylic acid group present in the other two compounds (Fig. 2B).

Biological activity of the purified compounds

The ARE activities of the three compounds in IMR-32 cells were compared by dose–response analysis using the reporter gene assay (Fig. 3A). At 10 $\mu\text{g/ml}$, **1** showed remarkable ARE activation (17.6-fold), which was six times higher than that of the C16 acid (**2**; 2.8-fold), four times higher than that of the fatty acid amide (**3**; 4.5-fold), and even slightly higher than that of the positive control (tBHQ; 14.9-fold). At the higher concentration tested, 32 $\mu\text{g/ml}$, **1** showed signs of cytotoxicity, whereas the other two strongly activated the reporter. At 32 $\mu\text{g/ml}$, **2** was over two times more efficacious than **3** (30.3-fold vs 13.2-fold, respectively).

We compared the bioactivities and chemical structures of the three compounds with our previous studies of other fatty acids and predicted that the electrophilic Michael acceptor motif is essential for the potent ARE activities of the three compounds; related fatty acids that do not have this exact motif showed less or no ARE-luc activation [26]. The carboxylic acid functional group seemed to contribute to the activity because the amide, **3**, was less efficacious than the acids, **1** (4.5-fold vs 17.6-fold, respectively, at 10 $\mu\text{g/ml}$) and **2** (13.2-fold vs 30.3-fold, respectively, at 32 $\mu\text{g/ml}$). Furthermore, the length of the fatty tail also seemed to play a role because with identical acid and enone groups, the short-chain acid (**2**, C16) was less potent than the long-chain acid (**1**, C18) (EC_{50} 6.0 $\mu\text{g/ml}$ vs. 17.7 $\mu\text{g/ml}$, respectively; Fig. 3B). Because **1** proved to be the most active in the series, we focused on this compound for further biological studies.

ARE sequence specificity and induction of multiple cytoprotective genes and glutathione levels by **1** in IMR-32 cells

The ARE–luciferase reporter gene construct used for the bioassay-guided fractionation and purification contains the core ARE sequence of human NQO1. Within this sequence, one copy of the activator protein-1 (AP-1) binding site (the TPA-responsive element (TRE)) was identified [28] (Supplementary Fig. S1A). Studies have shown that although some NQO1 inducers function through the activation of AP-1 and its subsequent binding to the TRE, others require the binding to the full length of the core ARE sequence for their induction activity [34]. To test if the previously observed ARE-luc reporter gene bioactivity of **1** is dependent on the full ARE core sequence or only the AP-1 binding sequence, we conducted two sets of experiments. First, the reporter gene activity of the wild-type ARE-luc was compared to that of an ARE-luc mutant (GC $\rightarrow$ AT) that still contained the intact TRE (Supplementary Fig. S1A). The induction of the luciferase reporter gene was completely abolished in the presence of the mutant (Supplementary Fig. S1B), suggesting that **1** may require the full-length ARE core sequence for its bioactivity. Second, to test if **1** can activate AP-1 in IMR-32 cells, an additional AP-1-specific reporter gene construct (AP-1-(PMA)-luc [29]) was employed. No significant luciferase activity was detected when IMR-32 cells transfected with the AP-1-(PMA)-luc reporter gene were treated with **1** (Supplementary Fig. S1C), indicating that **1** may not activate AP-1 in IMR-32 cells. Taken together, activation of AP-1 may not play a key role in the ARE-luc activity of **1**.

We next assessed the levels of endogenous *NQO1* mRNA in IMR-32 cells upon exposure to **1**. Similar to the ARE-luc reporter gene profile, the induction of *NQO1* was dose-dependent. Higher than basal levels of *NQO1* transcripts were detected starting from 1 $\mu\text{g/ml}$ (1.5-fold) and were much more pronounced at the higher nontoxic concentration (19.9-fold, 10 $\mu\text{g/ml}$) (Fig. 4A). Consistent with the AP-1-(PMA)-luc reporter gene results, **1** did not induce *FOS*, a representative AP-1 target gene (Supplementary Fig. S2) [35].

GSH, a thiol-containing tripeptide, is the major intracellular antioxidant. The sulfhydryl group serves as an electron donor and reacts with ROS and unwanted electrophilic

xenobiotics, maintaining a reducing cellular environment. Certain small molecules, e.g., diethyl maleate (DEM), activate the ARE by temporarily depleting the endogenous pool of free thiols, primarily GSH [36,37], leading to a disrupted redox status of the cellular environment. Multiple responses, all of which are ARE-dependent, have been proposed to be utilized by the cell to restore its GSH levels. Other than increased extracellular cystine uptake [38], the redox sensor Keap1 may release Nrf2 to activate the ARE, thereby initiating the expression of ARE-regulated glutathione biosynthetic genes [39]. We examined the levels of endogenous genes responsible for both events upon treatment with the highest active concentration of **1** (10 μ g/ml) in IMR-32 cells. Nrf2-regulated genes involved in both cystine uptake (*SLC7A11*, cystine/glutamate exchange transporter [40]) and GSH biosynthesis (*GCLC*, glutamate–cysteine ligase, catalytic subunit, and *GCLM*, glutamate–cysteine ligase, modifier subunit) were induced (Fig. 4B). Moreover, the levels of two other antioxidant genes that we have monitored, *TXNRD1* (thioredoxin reductase 1) and *HMOX1* (heme oxygenase 1), were also increased.

To test if **1** is thiol-reactive rather nonspecifically, we first compared the ARE activities in a model system, i.e., the presence and absence of an exogenously supplied, cell-permeative small-molecule antioxidant, NAC [2,37]. In the presence of 1 mM NAC, the ARE-luc activity of the fatty acid was reduced by 70% (Fig. 4C), suggesting that it may indeed have a propensity to react with small-molecule thiols and intracellular glutathione.

Next, the levels of endogenous glutathione in response to **1** were directly examined in IMR-32 cells. The result was similar to our previous observation for another ARE-activating keto-MUFA, 12,13-epoxy-9-keto-10-(*trans*)octadecenoic acid [26]. A transient decrease was observed until 8 h (<30%), suggesting that **1** may have caused a temporary redox imbalance in the cell, which would have required the consumption of GSH to restore. The level of glutathione increased and overcompensated by more than 50% of the basal level at 16 h and stayed above baseline at least up to 24 h (Fig. 4D), which is consistent with both increased GSH biosynthesis via biosynthetic gene transcription and increased substrate uptake (cystine/glutamate exchange transporter) (Fig. 4B) [40].

Nrf2 and phosphoinositide 3-kinase (PI3K) are required for ARE activation by **1 in IMR-32 cells**

ARE activation is tightly regulated by Nrf2 and Keap1. In a healthy cell, Nrf2 is targeted by its cytoplasmic repressor, Keap1, for proteasomal degradation. When Keap1 senses environmental stress or harmful chemicals, it releases Nrf2, which translocates to the nucleus, where it binds to the ARE and activates expression of downstream cytoprotective genes.

As shown in Fig. 4A, the levels of *NRF2* mRNA remained unchanged at the effective doses (1–10 μ g/ml) of **1**, indicating that the induction of ARE-regulated genes was not due to an elevated *NRF2* transcript level. To confirm the involvement of Nrf2 in **1**-induced ARE activation, we used siRNAs to knock down endogenous *NRF2* transcripts in IMR-32 cells [26]. The Nrf2-depleted cells (~75% depletion, Fig. 5A) were then treated with **1** and analyzed for the expression of the ARE-regulated NQO1 levels (Figs. 5A and 5B). The expression of NQO1 at the active concentration (10 μ g/ml) was strongly reduced compared with control cells at both the transcript (Fig. 5A) and the protein levels (Fig. 5B). The residual NQO1 levels (~25% for mRNA and protein) can be attributed to incomplete siRNA-mediated knockdown of Nrf2 (~25% remaining). These data suggest that Nrf2 is essential for **1**-induced cytoprotective gene expression.

Further dissection of the Nrf2–ARE mechanism had previously revealed that certain protein kinases may be involved in the activation of Nrf2 by phosphorylation and facilitate its

stabilization and nuclear translocation [41–43]. Two kinase signaling cascades in particular, the PI3K and mitogen-activated protein kinase (MAPK) pathways, have been extensively investigated [41,44]. Their requirement for ARE activity seems to be cell-type dependent. For example, the model ARE activator, tBHQ, requires the presence of PI3K, but not MAPK, to activate the ARE in IMR-32 cells [42], whereas the opposite was found in HepG2 cells [45]. We performed loss-of-function experiments to investigate the kinase requirements for **1**-induced ARE activation. IMR-32 cells were pretreated with pharmacological inhibitors of either the PI3K or the MAPK pathway for 30 min, before being exposed to **1** at its active concentration (10 μ g/ml). After 24 h, the cells were lysed and analyzed by Western blot. Expression of NQO1 was reduced by half in the presence of a MAPK inhibitor (MEK1) and completely diminished in the presence of a PI3K inhibitor (Fig. 5C). These data indicated that the MAPK signaling pathway may be partially required, whereas the PI3K pathway is fully required for the induction of ARE-regulated genes by **1** in IMR-32 cells.

C18 acid 1-enriched extract of *U. lactuca* induces cytoprotective genes in vitro and in vivo

To determine if the in vitro cell culture results translate into in vivo activity, we wanted to test the activity of a **1**-containing *U. lactuca* fraction in mice. However, materials from a recollection of *U. lactuca* (2009) had to be used because of the exhaustion of the original collection (2006). The fractionation scheme was also updated to potentially better concentrate the fatty acid contents in the lipophilic fractions (see Material and methods). Fraction 3* (25% hexane in EtOAc, v/v) was found to be the only one that contained **1**. To ensure that the bioactivity of fraction 3* was comparable to that of **1**, the endogenous expression of the ARE-regulated gene *NQO1* was assessed at the transcript and protein levels in IMR-32 cells. It was shown that fraction 3* induced high levels of NQO1 in a dose-dependent manner. Higher than baseline levels of *NQO1* mRNA were detected starting from 3.2 μ g/ml (1.4fold) and continued to increase until 32 μ g/ml (23.0-fold; Fig. 6A); a similar trend was observed at the level of protein expression (Fig. 6B). Fraction 3* was also found to comparably induce the levels of additional cytoprotective genes in IMR-32 cells (Fig. 6C).

Moreover, to further verify that Nrf2 is involved in fraction 3*-mediated ARE-regulated gene induction, we examined the stabilization and nuclear translocation of Nrf2 upon treatment with fraction 3* (Fig. 6D). Increased levels of Nrf2 in the nucleus were detected starting from 1 h after treatment. It has been suggested that certain small-molecule inducers stabilize Nrf2 by inhibiting the otherwise constant ubiquitin labeling of Nrf2 and its subsequent proteasomal degradation [30]. To test if fraction 3* can inhibit Nrf2 ubiquitination, a cellular ubiquitination system was reconstructed by overexpression of mammalian expression vectors encoding Keap1 (Keap1-CBD), Neh2 (Gal4-Neh2, Keap1-interactive domain of Nrf2), and ubiquitin (HA-Ub) in HEK293 cells (Fig. 6E). Fraction 3* indeed inhibited Neh2 ubiquitination at its highest active concentration (32 μ g/ml), comparable with the observed effect of the positive control (tBHQ) that was previously reported [27].

Having confirmed its in vitro ARE activities, we administered a single dose (50 mg/kg) of the **1**-containing fraction 3* by oral gavage to mice carrying a transgene that has the ARE-containing promoter region of the rat *Nqo1* gene coupled with the hPAP reporter [31,46]. After 12 h, the mice were euthanized and various tissues harvested. Each tissue was uniformly divided into two parts and analyzed for the increase in endogenous *Nqo1* mRNA levels and the enzymatic activities of the transgene-encoded hPAP protein. Although changes in hPAP activity were not statistically significant ($P > 0.05$, Supplementary Fig. S3), significant induction ($P < 0.05$) of endogenous *Nqo1* was detected in more than half of the seven tissues tested (Fig. 7A). Significantly elevated levels of *Nqo1* were found in the

lung (4.8-fold) and the brain (1.3-fold). Other than, expectedly, the stomach, the heart tissues showed the highest significant *Nqo1* induction (30.3-fold). In addition, we also found that multiple other Nrf2-regulated cytoprotective genes were significantly induced in the heart tissues (Fig. 7B). Borderline significant induction was found in the liver (9.3-fold, $P=0.06$). Lack of statistical significance for *Nqo1* induction was calculated for the kidney ($P=0.37$) and small intestine ($P=0.14$) tissues (Supplementary Fig. S4) despite an average of 1.8- and 4.8-fold induction, respectively, which may be due to our limited sample size ($n=3$) or physicochemical properties of components in fraction 3* affecting tissue-specific bioavailability.

Discussion

The ARE has gained much attention in oxidative stress-related research since it was found to govern the transcriptional activation of cytoprotective enzymes in 1990 [47]. The identification of the two cytoplasmic regulators of the ARE, the transcriptional activator Nrf2 [48,49] and its repressor Keap1 [50], delineated the antioxidant signaling pathway that provided a promising target for antioxidant drug discovery [39,51]. Numerous natural and synthetic activators of the Keap1–Nrf2–ARE pathway have since been discovered and reported. Consistent with our hypothesis, extracts of various marine algae were identified as activating the Nrf2–ARE pathway. In particular, we found potent *in vitro* and *in vivo* ARE activities from the lipophilic silica gel fractions of an edible green alga, *U. lactuca*, which had previously been reported to have anti-inflammatory [52], antiviral [53], antiperoxidative, and antihyperlipidemic [54] properties.

Three active compounds from *U. lactuca* were isolated, including a new monounsaturated keto-fatty acid (**1**; C18:1(n-11)) and two structural analogs, a second keto-fatty acid (**2**; C16:1(n-9)) and a fatty acid amide (**3**). These fatty compounds share a common structural feature, the conjugated α,β -unsaturated enone motif, which is capable of alkylating reactive thiol groups in cysteine residues. As mentioned before under Results, the Michael acceptor motif is a common denominator among many ARE activators [55,56] and must be responsible for the ARE activity by comparison to previously published data for related fatty acids [26]. Given the same α,β -unsaturated enone system, it seems quite puzzling that substantial differences in the biological activities exist among the three compounds. The variation in bioactivities may be attributed to the other functional groups on the molecules. First, fatty acid amides are known to have poor aqueous solubility [57], leading to their poor bioavailability, which could potentially explain why fatty acid amide **3** is less potent and efficacious than the two acids. Second, the shortening of the chain by two carbons had two effects on the bioactivity. It shifted the EC₅₀ from 6.0 (**1**) to 17.7 $\mu\text{g/ml}$ (**2**), and the maximum efficacy from 17.6- (**1**, 10 $\mu\text{g/ml}$) to 30.3-fold (**2**, 32 $\mu\text{g/ml}$). Both phenomena may again be explained by bioavailability. But this time, the possible causes might be the less efficient movement across the phospholipid bilayer forming the cell membrane by the shorter chain (C16) acid [58].

C18 acid **1** may exert a dual biological mechanism of action that culminates in ARE activation. It transiently lowered the level of the intracellular guard (GSH) via direct chemical reaction with sulfhydryl groups to cause a transient redox imbalance, generating a feedback to activate the glutathione biosynthetic machinery (*SLC7A11*, *GCLC*, and *GCLM*, Fig. 4B) [36,40], which is governed by the Nrf2–ARE signaling pathway [59]. Additionally, the α,β -conjugated enone may directly alkylate reactive cysteine residues on Keap1 [60], leading to Nrf2 release and the subsequent ARE activation, which induced a broad spectrum of cytoprotective genes including *NQO1*, *HMOX1*, and the above-mentioned GSH biosynthesis, recycling, and transport genes (Figs. 4A and 4B), which are key players in cellular detoxification of harmful agents [59].

Expectedly, Nrf2 is essential for the cytoprotective gene induction by both **1** itself and a **1**-enriched fraction (fraction 3*) in cultured human cells. The mRNA levels of *NRF2* remained unchanged, suggesting that the stabilization of transcription factor Nrf2 protein and not the upregulation of its own transcription led to the downstream cytoprotective gene induction. Furthermore, **1** is probably a monofunctional inducer of phase II enzymes because *NRF2* mRNA levels are induced via the aryl hydrocarbon receptor pathway that controls phase I enzyme induction [61,62].

A single acute dosing study (12 h) in mice produced a strong functional response of *Nqo1* mRNA in a variety of tissues, most prominent being in the heart tissues. More than a 30-fold increase in *Nqo1* mRNA and increased transcript levels of multiple other Nrf2–ARE-regulated cytoprotective genes in the heart were found 12 h after oral gavage, suggesting sufficient bioavailability of the active ingredient(s) and great cytoprotective potential through upregulation of the antioxidant defense genes. This is also consistent with literature reports that a MUFA-containing diet reduces the risk for cardiovascular diseases [63,64]. Nevertheless, we cannot rule out the possibility that other factors may have contributed to the induction of the cytoprotective gene expression in vivo. For example, based on HPLC and NMR analysis, there were many other compounds in the particular fraction that was used; some of them also activate the ARE and/or cooperate with the identified active compounds. Unfortunately, sufficient quantities could not be isolated for further studies.

Conclusions

Our data emphasize that more studies are warranted to explore the biomedical potential of certain seaweeds. We have shown that they appear to be a rich source of small molecules with antioxidant properties. Specifically, we identified potent inducers of the ARE–Nrf2 antioxidant defense system from an edible seaweed, *U. lactuca*, which by themselves and/or in combination showed activity in vitro and in vivo. Further studies are ongoing to identify other potentially beneficially active compounds in seaweeds and evaluate their bioactivities, to better utilize marine algae as a chemopreventive dietary source to protect from cancer and other diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the National Institutes of Health, Grants R21CA133681 and R01EY020825. We are grateful to the Florida Institute of Oceanography for providing the R/V Bellows and to the National Park Service for granting the collection permits for the Dry Tortugas National Park. We thank the crew of the R/V *Bellows*, K. Arthur, F. Gurgel, T. Meickle, S. Reed, R. Ritson-Williams, S. Matthew, and K. Taori, for assistance with algae collections and S. Gunasekera for extracting and fractionating the recollected *U. lactuca* samples. We also thank Professor J. Johnson for ARE-luc reporter constructs and ARE reporter mice; Professor S. Chanda for AP-1-(PMA)-luc plasmids; Professors N. Gray and D. Zhang for Keap1-CBD, Gal4-Neh2, and HA-Ub plasmids; Professor R. Tsien for sharing pcDNA3-mRFP through Addgene; Professor D. Borchelt and S. Fromholt for mouse genotyping; and Y. Liu for mouse oral gavaging and tissue dissection.

References

1. Halliwell B. Biochemistry of oxidative stress. *Biochem Soc Trans.* 2007; 35:1147–1150. [PubMed: 17956298]
2. Liu Y, Kern JT, Walker JR, Johnson JA, Schultz PG, Luesch H. A genomic screen for activators of the antioxidant response element. *Proc Natl Acad Sci USA.* 2007; 104:5205–5210. [PubMed: 17360324]

3. Zhang DD. Mechanistic studies of the Nrf2–Keap1 signaling pathway. *Drug Metab Rev.* 2006; 38:769–789. [PubMed: 17145701]
4. Kobayashi M, Yamamoto M. Nrf2–Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. *Adv Enzyme Regul.* 2006; 46:113–140. [PubMed: 16887173]
5. Dinkova-Kostova AT, Holtzclaw WD, Kensler TW. The role of Keap1 in cellular protective responses. *Chem Res Toxicol.* 2005; 18:1779–1791. [PubMed: 16359168]
6. Balogun E, Hoque M, Gong P, Killeen E, Green CJ, Foresti R, Alam J, Motterlini R. Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. *Biochem J.* 2003; 371:887–895. [PubMed: 12570874]
7. Hatcher H, Planalp R, Cho J, Torti FM, Torti SV. Curcumin: from ancient medicine to current clinical trials. *Cell Mol Life Sci.* 2008; 65:1631–1652. [PubMed: 18324353]
8. Langcake P, Pryce RJ. Production of resveratrol by *Vitis-vinifera* and other members of Vitaceae as a response to infection or injury. *Physiol Plant Pathol.* 1976; 9:77–86.
9. Rubiolo JA, Mithieux G, Vega FV. Resveratrol protects primary rat hepatocytes against oxidative stress damage: activation of the Nrf2 transcription factor and augmented activities of antioxidant enzymes. *Eur J Pharmacol.* 2008; 591:66–72. [PubMed: 18616940]
10. Levi F, Pasche C, Lucchini F, Ghidoni R, Ferraroni M, La Vecchia C. Resveratrol and breast cancer risk. *Eur J Cancer Prev.* 2005; 14:139–142. [PubMed: 15785317]
11. Zhu W, Qin W, Zhang K, Rottinghaus GE, Chen YC, Kliethermes B, Sauter ER. Trans-resveratrol alters mammary promoter hypermethylation in women at increased risk for breast cancer. *Nutr Cancer.* 2012; 64:393–400. [PubMed: 22332908]
12. Kensler TW, Egner PA, Agyeman AS, Visvanathan K, Groopman JD, Chen JG, Chen TY, Fahey JW, Talalay P. Keap1-Nrf2 signaling: a target for cancer prevention by sulforaphane. *Top Curr Chem.* 2013; 329:163–177. [PubMed: 22752583]
13. Kensler TW, Chen JG, Egner PA, Fahey JW, Jacobson LP, Stephenson KK, Ye L, Coady JL, Wang JB, Wu Y, Sun Y, Zhang QN, Zhang BC, Zhu YR, Qian GS, Carmella SG, Hecht SS, Benning L, Gange SJ, Groopman JD, Talalay P. Effects of glucosinolate-rich broccoli sprouts on urinary levels of aflatoxin–DNA adducts and phenanthrene tetraols in a randomized clinical trial in He Zuo township, Qidong, People's Republic of China. *Cancer Epidemiol Biomarkers Prev.* 2005; 14:2605–2613. [PubMed: 16284385]
14. Farnham MW, Stephenson KK, Fahey JW. Glucoraphanin level in broccoli seed is largely determined by genotype. *Hortscience.* 2005; 40:50–53.
15. Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc Natl Acad Sci USA.* 1992; 89:2399–2403. [PubMed: 1549603]
16. Newman DJ, Cragg GM. Marine natural products and related compounds in clinical and advanced preclinical trials. *J Nat Prod.* 2004; 67:1216–1238. [PubMed: 15332835]
17. Montaser R, Luesch H. Marine natural products: a new wave of drugs? *Future Med Chem.* 2011; 3:1475–1489. [PubMed: 21882941]
18. Taori K, Paul VJ, Luesch H. Structure and activity of largazole, a potent antiproliferative agent from the Floridian marine cyanobacterium. *Symploca sp J Am Chem Soc.* 2008; 130:1806–1807.
19. Hong J, Luesch H. Largazole: from discovery to broad-spectrum therapy. *Nat Prod Rep.* 2012; 29:449–456. [PubMed: 22334030]
20. Chen QY, Liu Y, Luesch H. Systematic chemical mutagenesis identifies a potent novel apratoxin A/E hybrid with improved *in vivo* antitumor activity. *ACS Med Chem Lett.* 2011; 2:861–865. [PubMed: 22081789]
21. Hoshiyama Y, Sekine T, Sasaba T. A case–control study of colorectal cancer and its relation to diet, cigarettes, and alcohol consumption in Saitama Prefecture, Japan. *Tohoku J Exp Med.* 1993; 171:153–165. [PubMed: 8128484]
22. Hoshiyama Y, Sasaba T. A case–control study of single and multiple stomach cancers in Saitama Prefecture, Japan. *Jpn J Cancer Res.* 1992; 83:937–943. [PubMed: 1429203]
23. Surh YJ. Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer.* 2003; 3:768–780. [PubMed: 14570043]

24. Yan X, Chuda Y, Suzuki M, Nagata T. Fucoxanthin as the major antioxidant in *Hijikia fusiformis*, a common edible seaweed. *Biosci Biotechnol Biochem*. 1999; 63:605–607. [PubMed: 10227153]
25. Liu CL, Chiu YT, Hu ML. Fucoxanthin enhances HO-1 and NQO1 expression in murine hepatic BNL CL.2 cells through activation of the Nrf2/ARE system partially by its pro-oxidant activity. *J Agric Food Chem*. 2011; 59:11344–11351. [PubMed: 21919437]
26. Wang R, Kern JT, Goodfriend TL, Ball DL, Luesch H. Activation of the antioxidant response element by specific oxidized metabolites of linoleic acid. *Prostaglandins Leukotrienes Essent Fatty Acids*. 2009; 81:53–59.
27. Hur W, Sun Z, Jiang T, Mason DE, Peters EC, Zhang DD, Luesch H, Schultz PG, Gray NS. A small-molecule inducer of the antioxidant response element. *Chem Biol*. 2010; 17:537–547. [PubMed: 20534351]
28. Moehlenkamp JD, Johnson JA. Activation of antioxidant/electrophile-responsive elements in IMR-32 human neuroblastoma cells. *Arch Biochem Biophys*. 1999; 363:98–106. [PubMed: 10049503]
29. Chanda SK, White S, Orth AP, Reisdorph R, Miraglia L, Thomas RS, DeJesus P, Mason DE, Huang Q, Vega R, Yu DH, Nelson CG, Smith BM, Terry FJ, Linford AS, Yu Y, Chirn GW, Song C, Labow MA, Cohen D, King FJ, Peters EC, Schultz PG, Vogt PK, Hogenesch JB, Caldwell JS. Genome-scale functional profiling of the mammalian AP-1 signaling pathway. *Proc Natl Acad Sci USA*. 2003; 100:12153–12158. [PubMed: 14514886]
30. Zhang DD, Hannink M. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol Cell Biol*. 2003; 23:8137–8151. [PubMed: 14585973]
31. Johnson DA, Andrews GK, Xu W, Johnson JA. Activation of the antioxidant response element in primary cortical neuronal cultures derived from transgenic reporter mice. *J Neurochem*. 2002; 81:1233–1241. [PubMed: 12068071]
32. Kraft AD, Johnson DA, Johnson JA. Nuclear factor E2-related factor 2-dependent antioxidant response element activation by tert-butylhydroquinone and sulforaphane occurring preferentially in astrocytes conditions neurons against oxidative insult. *J Neurosci*. 2004; 24:1101–1112. [PubMed: 14762128]
33. Fontana A, d'Ippolito G, Cutignano A, Romano G, Lamari N, Massa Gallucci A, Cimino G, Miralto A, Ianora A. LOX-induced lipid peroxidation mechanism responsible for the detrimental effect of marine diatoms on zooplankton grazers. *ChemBioChem*. 2007; 8:1810–1818. [PubMed: 17886321]
34. Wang B, Williamson G. Transcriptional regulation of the human NAD(P)H:quinone oxidoreductase (NQO1) gene by monofunctional inducers. *Biochim Biophys Acta*. 1996; 1307:104–110. [PubMed: 8652659]
35. Shen G, Jeong WS, Hu R, Kong AN. Regulation of Nrf2, NF-kappaB, and AP-1 signaling pathways by chemopreventive agents. *Antioxid Redox Signaling*. 2005; 7:1648–1663.
36. Deneke SM, Fanburg BL. Regulation of cellular glutathione. *Am J Physiol*. 1989; 257:L163–173. [PubMed: 2572174]
37. Lee JM, Moehlenkamp JD, Hanson JM, Johnson JA. Nrf2-dependent activation of the antioxidant responsive element by *tert*-butylhydroquinone is independent of oxidative stress in IMR-32 human neuroblastoma cells. *Biochem Biophys Res Commun*. 2001; 280:286–292. [PubMed: 11162512]
38. Bannai S. Transport of cystine and cysteine in mammalian cells. *Biochim Biophys Acta*. 1984; 779:289–306. [PubMed: 6383474]
39. Jung KA, Kwak MK. The Nrf2 system as a potential target for the development of indirect antioxidants. *Molecules*. 2010; 15:7266–7291. [PubMed: 20966874]
40. Sasaki H, Sato H, Kuriyama-Matsumura K, Sato K, Maebara K, Wang H, Tamba M, Itoh K, Yamamoto M, Bannai S. Electrophile response element-mediated induction of the cystine/glutamate exchange transporter gene expression. *J Biol Chem*. 2002; 277:44765–44771. [PubMed: 12235164]
41. Feng J, Zhang P, Chen X, He G. PI3K and ERK/Nrf2 pathways are involved in oleanolic acid-induced heme oxygenase-1 expression in rat vascular smooth muscle cells. *J Cell Biochem*. 2011; 112:1524–1531. [PubMed: 21328610]

42. Lee JM, Hanson JM, Chu WA, Johnson JA. Phosphatidylinositol 3-kinase, not extracellular signal-regulated kinase, regulates activation of the antioxidant-responsive element in IMR-32 human neuroblastoma cells. *J Biol Chem*. 2001; 276:1–20016. [PubMed: 11134063]
43. Surh YJ, Kundu JK, Na HK. Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. *Planta Med*. 2008; 74:1526–1539. [PubMed: 18937164]
44. Na HK, Kim EH, Jung JH, Lee HH, Hyun JW, Surh YJ. (—)-Epigallocatechin gallate induces Nrf2-mediated antioxidant enzyme expression via activation of PI3K and ERK in human mammary epithelial cells. *Arch Biochem Biophys*. 2008; 476:171–177. [PubMed: 18424257]
45. Zipper LM, Mulcahy RT. Erk activation is required for Nrf2 nuclear localization during pyrrolidine dithiocarbamate induction of glutamate cysteine ligase modulatory gene expression in HepG2 cells. *Toxicol Sci*. 2003; 73:124–134. [PubMed: 12657749]
46. Yates MS, Tauchi M, Katsuoka F, Flanders KC, Liby KT, Honda T, Gribble GW, Johnson DA, Johnson JA, Burton NC, Guilarte TR, Yamamoto M, Sporn MB, Kensler TW. Pharmacodynamic characterization of chemopreventive triterpenoids as exceptionally potent inducers of Nrf2-regulated genes. *Mol Cancer Ther*. 2007; 6:154–162. [PubMed: 17237276]
47. Rushmore TH, Pickett CB. Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene: characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. *J Biol Chem*. 1990; 265:14648–14653. [PubMed: 2387873]
48. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun*. 1997; 236:313–322. [PubMed: 9240432]
49. Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc Natl Acad Sci USA*. 1994; 91:9926–9930. [PubMed: 7937919]
50. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev*. 1999; 13:76–86. [PubMed: 9887101]
51. Jeong WS, Jun M, Kong AN. Nrf2: a potential molecular target for cancer chemoprevention by natural compounds. *Antioxid Redox Signaling*. 2006; 8:99–106.
52. Margret RJ, Kumaresan S, Ravikumar S. A preliminary study on the anti-inflammatory activity of methanol extract of *Ulva lactuca* in rat. *J Environ Biol*. 2009; 30:899–902. [PubMed: 20143726]
53. Chiu YH, Chan YL, Li TL, Wu CJ. Inhibition of Japanese encephalitis virus infection by the sulfated polysaccharide extracts from *Ulva lactuca*. *Mar Biotechnol (NY)*. 2012; 14:468–478. [PubMed: 22193590]
54. Sathivel A, Raghavendran HR, Srinivasan P, Devaki T. Anti-peroxidative and anti-hyperlipidemic nature of *Ulva lactuca* crude polysaccharide on D-galactosamine induced hepatitis in rats. *Food Chem Toxicol*. 2008; 46:3262–3267. [PubMed: 18706469]
55. Talalay P, De Long MJ, Prochaska HJ. Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. *Proc Natl Acad Sci USA*. 1988; 85:8261–8265. [PubMed: 3141925]
56. Dinkova-Kostova AT, Massiah MA, Bozak RE, Hicks RJ, Talalay P. Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. *Proc Natl Acad Sci USA*. 2001; 98:3404–3409. [PubMed: 11248091]
57. Craig C. Kirk-Othmer Encyclopedia of Chemical Technology and Ullmann's Encyclopedia of Industrial Chemistry [Review]. *Issues Sci Technol Librarian-ship*. 2006; 46:6–6.
58. Calder PC, Bond JA, Harvey DJ, Gordon S, Newsholme EA. Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. *Biochem J*. 1990; 269:807–814. [PubMed: 2117922]
59. Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res*. 2002; 62:5196–5203. [PubMed: 12234984]

60. Kansanen E, Jyrkkanen HK, Levonen AL. Activation of stress signaling pathways by electrophilic oxidized and nitrated lipids. *Free Radic Biol Med*. 2012; 52:973–982. [PubMed: 22198184]
61. Kohle C, Bock KW. Coordinate regulation of phase I and II xenobiotic metabolisms by the Ah receptor and Nrf2. *Biochem Pharmacol*. 2007; 73:1853–1862. [PubMed: 17266942]
62. Miao W, Hu L, Scrivens PJ, Batist G. Transcriptional regulation of NF-E2 p45-related factor (NRF2) expression by the aryl hydrocarbon receptor–xenobiotic response element signaling pathway: direct cross-talk between phase I and II drug-metabolizing enzymes. *J Biol Chem*. 2005; 280:20340–20348. [PubMed: 15790560]
63. Gillingham LG, Harris-Janz S, Jones PJ. Dietary monounsaturated fatty acids are protective against metabolic syndrome and cardiovascular disease risk factors. *Lipids*. 2011; 46:209–228. [PubMed: 21308420]
64. Abeywardena MY, Patten GS. Role of ω 3 long-chain polyunsaturated fatty acids in reducing cardio-metabolic risk factors. *Endocr Metab Immune Disord Drug Targets*. 2011; 11:232–246. [PubMed: 21831036]

Abbreviations

ARE	antioxidant-response element
AP-1	activator protein-1
DEM	diethyl maleate
GCLC	glutamate–cysteine ligase, catalytic subunit
GCLM	glutamate–cysteine ligase, modifier subunit
GSH	glutathione
GSTA4	glutathione S-transferase Alpha 4
HMOX1	heme oxygenase 1
hPAP	human placental alkaline phosphatase
Keap1	Kelch-like ECH-associated protein 1
MAPK	mitogen-activated protein kinase
MUFA	monounsaturated fatty acid
NAC	<i>N</i> -acetylcysteine
NQO1, NAD(P)H	quinone oxidoreductase 1
Nrf2	nuclear factor E2-related factor 2
PI3K	phosphoinositide 3-kinase
PMA	phorbol 12-myristate 13-acetate
ROS	reactive oxygen species
SLC7A11	solute carrier family 7 (cationic amino acid transporter, y+ system)
SPE	solid-phase extraction
tBHQ	<i>tert</i> -butylhydroquinone
TXNRD1	thioredoxin reductase 1

Fig. 1.

ARE-luciferase activity profiling of the eukaryotic algae collection and distribution of activities in each phylum in IMR-32 cells. (A) The algae extracts were fractionated on a small scale (~20 mg) by normal or reversed-phase (C18) silica-gel-based SPE columns to generate 372 fractions. The ARE-luc activity of each fraction was tested in duplicate at two concentrations, 10 and 100 μg/ml. Each dot represents the average fold activation of one fraction at one concentration. The enlarged dots represent the active fractions from *U. lactuca* (Fort Pierce, FL, 2006). (B) ARE-luc activities of the large-scale silica gel chromatography fractions from 2006 *U. lactuca* ($n=2$) in IMR-32 cells. The fractions were eluted with CH₂Cl₂ (fraction 1, 7.2 mg), 2% *i*-PrOH in CH₂Cl₂ (fraction 2, 6.9 mg), 5% *i*-PrOH in CH₂Cl₂ (fraction 3, 10.9 mg), 8% *i*-PrOH in CH₂Cl₂ (fraction 4, 81.0 mg), 10% *i*-PrOH in CH₂Cl₂ (fraction 5, 21.1 mg), 20% *i*-PrOH in CH₂Cl₂ (fraction 6, 14.0 mg), and 100% MeOH (fraction 7, 1726.5 mg). Fractions 5 and 6 also show significant ARE activity, but they were not chromatographed initially because of relatively lower amounts. The ARE-luc results for the large-scale fractions are shown as fold activation ± the standard error of the mean. *Observed toxicity.

Fig. 2. Structural information of the three compounds isolated from *U. lactuca*. (A) Proposed chemical structures. (B) ^1H NMR characteristic signals for compounds 1–3 in CDCl_3 recorded at 600 MHz.

Fig. 3. Structure–activity relationship analysis for **1**. (A) Dose-dependent ARE-luc activities of the three compounds in IMR-32 cells ($n=3$). (B) The proposed structure–activity relationships for **1**. The results for the ARE-luc assay are shown as fold activation $\pm$ SEM. *Observed toxicity.

Fig. 4.

C18 acid **1** induces cytoprotective genes in IMR-32 cells. (A) Treatment with **1** led to dose-dependent increase in *NQO1*, but not *NRF2*, mRNA levels after 12 h ($n=3$). (B) At the highest active concentration (10 $\mu\text{g/mL}$) **1** induced multiple other ARE-Nrf2-regulated antioxidant genes ($n=3$), including *GCLC* (glutamate–cysteine ligase, catalytic subunit), *GCLM* (glutamate–cysteine ligase, modifier subunit), *SLC7A11* (solute carrier family 7 (cationic amino acid transporter, y+ system), member 11), *TXNRD1* (thioredoxin reductase 1), and *HMOX1* (heme oxygenase 1). (C) At the active concentration (10 $\mu\text{g/mL}$), **1**'s ARE-luc activity was reduced in the presence of an antioxidant, *N*-acetylcysteine ($n=3$). After transfection, IMR-32 cells were incubated for 24 h and then pretreated with 1 mM NAC for 2 h before they were exposed to 10 $\mu\text{g/mL}$ **1**. (D) **1** induced a net increase in glutathione levels at its active concentration ($n=3$). After decreasing ($\sim 30\%$) until 8 h, GSH levels started to increase and peaked at 16 h and then dropped back but stayed above the basal level up to at least 24 h. The results for the ARE-luc assay are shown as fold activation $\pm$ SEM; the qPCR and GSH results are fold activation $\pm$ standard deviation.

Fig. 5.

C18 acid **1** requires Nrf2 and PI3K for the induction of ARE-regulated genes in IMR-32 cells. (A, B) Nrf2 is essential for **1**-induced NQO1 expression at both (A) the transcript and (B) the protein levels. The cells were incubated for 48 h after siRNA transfection and then treated with **1** for (A) 12 or (B) 24 h before whole-cell lysates were collected. Levels of NQO1 in lane 5 (siNEG, 10 µg/ml) vs lane 8 (siNRF2, 10 µg/ml) represent 100% vs 25% as quantified by densitometry using ImageJ software (NIH). (C) PI3K activity is required for induction of NQO1 expression by **1**. 50 µM PD098059 (a MEK1 inhibitor) or 25 µM LY294002 (a PI3K inhibitor) was used to pretreat the cells for 30 min before they were exposed to 10 µg/ml **1**. After another 24 h of incubation, whole-cell lysates were prepared and assessed for NQO1 expression by immunoblot analysis.

Fig. 6.

A 1-containing *U. lactuca* fraction (fraction 3*, see Material and methods) comparably induced the cytoprotective genes through the Nrf2–ARE pathway. (A) Fraction 3* increased endogenous *NQO1* mRNA levels in human IMR-32 cells after 12 h of treatment ($n=3$). (B) Fraction 3* dose-dependently induced expression of *NQO1* at the protein level after 24 h of treatment. (C) Fraction 3* (32 μ g/ml) induced multiple other Nrf2–ARE-regulated antioxidant genes ($n=3$), including *GCLC* (glutamate–cysteine ligase, catalytic subunit), *GCLM* (glutamate–cysteine ligase, modifier subunit), *SLC7A11* (solute carrier family 7 (cationic amino acid transporter, y+ system), member 11), *TXNRD1* (thioredoxin reductase 1), and *HMOX1* (heme oxygenase 1). (D) At the highest active concentration, fraction 3* induced Nrf2 nuclear translocation in IMR-32 cells starting from 1 h. (E) Treatment with fraction 3* at its highest active concentration (32 μ g/ml) inhibited Nrf2 ubiquitination (mock plasmid: pcDNA3-mRFP). The results for qPCR assays are fold activation $\pm$ SD.

Fig. 7.

Fraction 3* induced cytoprotective genes in vivo. (A) Fraction 3* induced endogenous *Nqo1* expression in multiple tissues in mice. A strain of transgenic mice (B6C3-ARE-Tg) was used in this study. The mice ($n=3$) were fed by oral gavage and the tissues were collected after 12 h. Each tissue was divided into two identical portions and one set of was analyzed for *Nqo1* mRNA level (for small intestine vehicle mice, $n=2$). (B) Fraction 3* induced other Nrf2-regulated genes in the heart tissues. *** $P < 0.05$, ** $P = 0.06$. The results are shown as fold activation $\pm$ SEM.

Table 1
NMR data for 7(E)-9-keto-octadec-7-enoic acid (1) and 7(E)-9-keto-octadec-7-enamide (3) in CDCl₃

C/H No.	1	3				
	δ_c^a	δ_H (J, Hz) ^b	HMBC ^c	δ_c^d	δ_H (J, Hz) ^b	HMBC ^c
NH	—	—	—	—	5.39 br s	—
NH	—	—	—	—	5.24 br s	—
1	177.20 qC	—	—	175.36 qC	—	—
2	33.78 CH ₂	2.34 t (7.1)	3, 1	35.22 CH ₂	2.21 t (6.5)	3, 1
3	24.72 CH ₂	1.63 m	1, 2	24.68 CH ₂	1.63 m	1, 2
4	31.71 CH	1.31 m	3	28.44 CH	1.33 m	3
5	27.92 CH ₂	1.46 m	4, 6	27.48 CH ₂	1.46 m	4, 6
6	32.49 CH ₂	2.21 dt (6.9, 7.0)	5, 7, 8	31.92 CH ₂	2.19 dt (6.9, 7.3)	5, 7, 8
7	146.46 CH	6.83 dt (15.8, 6.9)	5, 6, 9	147.58 CH	6.81 dt (15.9, 6.9)	5, 6, 9
8	130.87 CH	6.08 d (15.8)	6, 9	130.26 CH	6.07 d (15.9)	6, 9
9	201.24 qC	—	—	200.80 qC	—	—
10	40.24 CH ₂	2.52 td (7.4, 1.4)	11, 9, 12	39.43 CH ₂	2.50 td (7.4, 1.6)	11, 9, 12
11	24.34 CH ₂	1.60 m	10, 9, 12	23.56 CH ₂	1.60 m	10, 9, 12
12–17	29.19 CH ₂	1.30 m	—	28.71 CH ₂	1.31 m	—
18	14.11 CH ₃	0.88 m	17	13.50 CH ₃	0.88 m	17

^a¹³C NMR data were recorded at 125 MHz.

^b¹H NMR data were recorded at 600 MHz.

^cLong-range heteronuclear correlations between the indicated carbon and the protons at 600 MHz.

^d¹³C data were deduced from HMBC and HSQC.