Published in final edited form as:

J Med Chem. 2017 September 28; 60(18): 7850–7862. doi:10.1021/acs.jmedchem.7b00990.

New Mandelalides Expand a Macrolide Series of Mitochondrial Inhibitors

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Abstract

Mandelalides A–D (**1–4**) are macrocyclic polyketides known to have an unusual bioactivity profile influenced by compound glycosylation and growth phase of cultured cells. The isolation and characterization of additional natural congeners, mandelalides E–L (**5–12**), and the supply of synthetic compounds **1** and **12**, as well as seco-mandelalide A methyl ester (**13**), have now facilitated mechanism of action and structure-activity relationship studies. Glycosylated mandelalides are effective inhibitors of aerobic respiration in living cells. Macrolides **1** and **2** inhibit mitochondrial function similar to oligomycin A and apoptolisin A, selective inhibitors of the mammalian ATP synthase (complex V). **1** inhibits ATP synthase activity from isolated mitochondria and triggers caspase-dependent apoptosis in HeLa cells, which are more sensitive to inhibition by **1** in the presence of the glycolysis inhibitor 2-deoxyglucose. Thus, mandelalide cytotoxicity depends on basal metabolic phenotype; cells with an oxidative phenotype are most likely to be inhibited by the mandelalides.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website: NMR spectra and chemical shift tables for natural products, ¹H and ¹³C NMR spectra for synthetic products, cancer and non-cancer cytotoxicity, caspase-3/7 activation and complex V concentration-response profiles, NCI-60 cancer cell line panel results; CSV file with SMILES codes for all compounds.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval for the final version of the manuscript. Jeffrey Serrill and Xuemei Wan contributed equally to this work as co-second authors.

The authors declare no competing financial interest.

INTRODUCTION

Sessile, marine filter-feeding organisms, such as urochordate tunicates and invertebrate sponges, have been targeted as valuable sources of new biologically active natural products.¹ Initially, this was because tunicates and sponges are macro-organisms that persist successfully on densely populated, highly diverse coral reefs thronged by myriads of potential predators. More recently these sessile macro-organisms have been targeted as hosts to specific microbial consortia, which are the biogenetic source of complex biologically active natural products.² Clinically approved anticancer agents, pharmaceutical lead compounds and molecular probes for studying disease mechanisms continue to be isolated directly or originate from these organisms.³ In pursuit of new biologically active marine natural products from South African tunicates we discovered four complex polyketide macrolides named mandelalides A–D from a rare new *Lissoclinum* species.⁴ Glycosylated mandelalides A (**1**) and B (**2**) displayed low nanomolar cytotoxicity against neuroblastoma and lung cancer cell lines, although the paucity of material prevented biological testing of the pure aglycones, mandelalides C (**3**) and D (**4**). In 2014, the Ye research group reported the first total synthesis of **1** and reassigned the absolute structure to a configuration where all five stereocenters in the northern hemisphere are revised.⁵ Subsequently, total syntheses of the revised structure of **1**, were reported by the research groups of Fürstner,⁶ Altmann,⁷ Carter,⁸ and Smith.⁹ with several investigators noting weak or disappointing biological activity against human cancer cells. These inconsistent results reported for the cytotoxic efficacy of synthetic **1**, and recollection of the rare source tunicate in 2013, prompted our further investigation.¹⁰ Our biological evaluation of synthetic **1** from the Ye,⁵ Carter,⁸ and Smith⁹ groups confirmed the potent activity originally reported for the natural product,⁴ and also revealed cell density to be a critical determinant of mandelalide action.¹⁰ Remarkably, actively proliferating NCIH460 lung cancer and Neuro-2a neuroblastoma cells, seeded at low starting density, were relatively resistant to **1** yet more confluent cultures of these and other cell types seeded at high starting density, remained sensitive to **1** and **2** with clear evidence of structure-activity relationships.¹⁰ The re-isolation of **1–3** and identification of new mandelalide E (**5**) permitted further evaluation that demonstrated a dramatic loss of activity for the aglycone **3** relative to glycosylated **1** and **2**, and 100-fold loss in activity when the saccharide hydroxyl groups at C-3' and C-4' are esterified as in **5**.¹⁰ These insights into the mechanistic basis for mandelalide selectivity are expanded here with the discovery that cytotoxic mandelalides inhibit mitochondrial function and induce apoptotic cell death, in a manner consistent with metabolic inhibition of the mammalian ATP synthase complex. ATP synthase has long been known as a target of natural products, including

phenolic compounds from plants and antimicrobial cationic peptides from animals,¹¹ as well as the macrocyclic polyketide oligomycins and apoptolidins.¹² Here, we report new congeners **6–12** in the mandelalide series of macrocyclic polyketides. Their structure-activity relationships are presented in the context of three different macrocycle motifs associated with the prototype structures of mandelalide A ("A-type", a macrocycle with a regular lactone connection), mandelalide B ("B-type", a butyrolactone-containing macrocycle), and mandelalides C/D ("C-type", a 23-hydroxy butyrolactone-containing macrocycle).

RESULTS AND DISCUSSION

Additional quantities of mandelalides A – D (**1 – 4**), originally isolated in sub-milligram amounts, were needed for further biological investigation and as authentic standards for comparison with synthetic products. Therefore, the producing *Lissoclinum* tunicate was finally located again and re-collected in 2013 from Whitesands Reef, Algoa Bay, South Africa. The lyophilized tunicate was extracted and fractionated as reported previously.⁴ Briefly, the organic extract (2:1 DCM-MeOH) was fractionated on Sephadex LH-20 (DCM-MeOH, 1:3), and provided two consecutively eluting fractions containing MS peaks indicative of mandelalide-type compounds, which were combined and subjected to RP₁₈ SPE. Exhaustive HPLC separations of the MS-targeted SPE fraction yielded the desired compounds **1 – 4**, **5**,¹⁰ and seven new congeners, named mandelalides F–L (**6–12**), in sufficient quantities for chemical characterization and investigation of the mechanistic basis for mandelalide action. Notably, there was also evidence in the natural product fractions of the ring-expanded 24-*O*-macrolactone congener (isomandelalide A) of **1**, the total synthesis of which has been achieved.⁸ The latter minor component could be dereplicated by LC-MS comparison to the synthetic product, on the basis of LC-MS retention time and characteristic mass peak.

Structure Elucidation of New Natural Products

Mandelalide F (**6**) yielded an $[M + H]^+$ ion at m/z 795.4175 by HRMS (ES^+), and a low resolution $[M+Na]^+$ ion at m/z 817.2, for a molecular formula of $C_{41}H_{62}O_{15}$. Compared to

published data for **1–5**, this molecular mass and formula suggested a glycosylated “C-type” macrocycle for **6**, with a greater oxidation state than **5** (C₄₁H₆₄O₁₅). This inference was consistent with multiple additional signals in the midfield regions of both the ¹H and ¹³C NMR spectra for **6**, and ¹³C shifts for a ketone carbonyl (d_C 196.8), as well as for three ester carbonyls (δ_C 174.4, 173.2, 172.3). Careful comparison of the 1D NMR data for **6** with those for **3**^{4, 10} and **5**¹⁰ revealed a close match between the chemical shifts for the aglycone atoms of **6** and **3** (Table S1A), which varied only in the ¹³C shifts for C-7 (glycosylated in **6**), C-6 and C-8. An HMBC correlation from the H-3 doublet (δ_H 5.49) to the butyryl carbonyl C-1'' signal (δ_C 173.2) for **6** confirmed a 3-*O*-butyryl substituent as in **3**, rather than a free 3-OH and the dibutyrylated monosaccharide as in **5**. Placement of the keto group in the monosaccharide was supported by HMBC correlations to the carbonyl shift (δ_C 196.8) from COSY-correlated H-1' (δ_H 5.32) and H-2' (δ_H 4.00) shifts, as well as COSY-correlated H-4' (δ_H 4.87) and H-5' (δ_H 4.11). The relatively downfield shift of H-4' and its HMBC correlation to an ester carbonyl shift at d_C 173.2 located the second butyryl group at C-4' on the monosaccharide. The absolute structure of the aglycone in **6** is consistent with that for **3**, as evidenced by the essentially identical ¹H (within 0.05 ppm) and ¹³C (within 0.3 ppm) NMR signals for almost all aglycone atoms in **6** and **3**, and their similar optical rotations ([α]_D²⁵ –17 for **6** and [α]_D²¹ –9 for **3**).¹⁰ The only significant chemical shift differences, observed for C-6, C-7, C-8, H₂-6 and H-8_{ax} (Table S1A), were consistent with the presence of a monosaccharide substituent at C-7 in **6**, but not in **3**. To date there are no published total syntheses of any butyrolactone-containing mandelalides (e.g. **2–4**). However, previously, we used molecular mechanics conformational searches, quantum mechanical NMR chemical shift predictions, and DP4 analyses to support revised absolute structures of **1–4**,¹³ with **1** being used as a reference model after its earlier revision by total synthesis.⁵ Thus, we propose the absolute configuration of the macrocycle in **6** as shown. The relative configuration of the new butyrylated keto-sugar in **6**, formally 5-(butyryloxy)-2-hydroxy-3-methoxy-6-methyl-4-oxotetrahydro-2H-pyran, can be proposed from homonuclear coupling constants and ROESY correlations. Specifically, a large coupling between H-4' and H-5' (³J_{H-4'/H-5'} = 10.0 Hz) oriented these protons axially in a trans relationship. In agreement with this, the chemical shift for H₃-6' (δ_H 1.31 ppm) was closer to those for H₃-6' in the rhamnose moiety of **1** and **12** (δ 1.27 and 1.29 ppm, respectively), compared to H₃-6' chemical shifts in the proposed 4'-epimer (talose) of other butyrolactone-containing mandelalides **5**, **7**, **9–11** (1.16 – 1.18 ppm). ROESY correlations between H-5' and both H-8_{eq} and H-7, indicated the orientation of H-5' toward the macrolide THP moiety. ROESY correlations between H-5, H-7 and H-9 indicated their 1,3-diaxial interaction and an equatorial C-7 glycosidic bond. An axial C-1' glycosidic bond is consistent with a stabilizing anomeric effect in the pyranose, leaving the equatorial position for H-1'. A small coupling between H-1' and H-2' (³J_{H-1'/H-2'} = 4.3 Hz) combined with a ROESY correlation between H-2' and H-7 also suggested an equatorial position for H-2', oriented toward the aglycone THP moiety. Thus, the absolute configuration of the keto-sugar in **6** is proposed as 1'*S*, 2'*S*, 4'*S*, 5'*S*. Degradation of the macrolide would allow comparison of the released monosaccharide with authentic monosaccharide standards (requiring synthesis) for confirmation of the absolute configuration. However, our decision to conserve the small amount of **6** available, as an authentic standard for future chemical and biological investigations, precluded degradation of any amount of **6**.

HRMS (ES^+) analysis for mandelalide G (**7**) provided m/z 889.4553 $[\text{M}+\text{Na}]^+$, for a molecular formula of $\text{C}_{45}\text{H}_{70}\text{O}_{16}$. This relatively large molecular mass of 866.5 Da was 300 mass units ($\text{C}_{15}\text{H}_{24}\text{O}_6$) greater than the butyrylated aglycone **3**, and 70 mass units ($\text{C}_4\text{H}_6\text{O}$) greater than **5**, which is butyrylated only on the monosaccharide. While no 1D ^{13}C NMR spectrum could be acquired for **7** (Table S2), the ^1H NMR spectrum revealed signals that closely matched those for aglycone **3**. Of the remaining ^1H chemical shifts not assigned to the macrocycle, relatively deshielded oxymethine signals at δ_{H} 5.19 and 5.18 were consistent with two esterified positions on the monosaccharide, and a close match of ^1H and ^{13}C NMR data between the monosaccharides for **7** and **5**¹⁰ was confirmed from 2D NMR data (Table S2). Hence mandelalide G (**7**) is a 3',4'-*O*-dibutyrylglycoside of **3**. In addition to the closely similar 1D NMR shifts and signal multiplicities, the optical rotation for **7** ($[\alpha]_{\text{D}}^{25} -12$) was consistent with those for the mandelalide series, including **3** ($[\alpha]_{\text{D}}^{25} -12$) and **5** ($[\alpha]_{\text{D}}^{23} -2.1$). Furthermore, homonuclear coupling constants and ROESY correlations (Table S2) supported the same absolute configuration for the butyrolactone-containing macrocycle in **7** as for **2–6**. For the 3',4'-*O*-dibutyrylated monosaccharide, the overlapping chemical shifts for H-3' and H-4' (δ 5.19 and 5.18 ppm) impeded the measurement of coupling constants and distinguishing ROESY correlations. However, broad singlets for H-1' and H-2', and apparent 3.5 Hz splitting in the overlapped triplets for H-3' and H-4', as well as a small coupling (< 2 Hz) between H-4' and H-5', indicated gauche relationships for consecutive adjacent protons in the monosaccharide spin system. Together with a diaxial ROESY correlation between H-3' and H-5', ROESY correlations of H-1' and H-2' with H-7, suggested a 2-*O*-methyl-6-dehydro- α -L-talose moiety. Thus, the absolute configuration of the pyranose in **7** is proposed as 1'*S*, 2'*S*, 3*R*, 4'*R*, 5'*S*, as was previously assigned for **5**.

Mandelalide H (**8**) was designated as an aglycone from HRMS (ES^+) data for $[\text{M}+\text{Na}]^+$ at m/z 687.3701, which was consistent with a relatively deoxygenated molecular formula of $\text{C}_{36}\text{H}_{56}\text{O}_{11}$. Comparison of the 1D NMR data for **8** (Table S3) with those for **3** and **4** revealed a very close match with the data for the 3, 24-*O*-dibutyrylated aglycone **4** ($\text{C}_{34}\text{H}_{52}\text{O}_{11}$). The additional C_2H_4 could be accounted for by signals for two additional methylenes in the shielded regions of the ^1H and ^{13}C NMR spectra for **8**. COSY and HMBC data placed these methylenes in a hexanoyl spin system, which was positioned at *O*-3 on the basis of an HMBC correlation from H-3 (δ_{H} 5.58) to hexanoyl C-1' (δ_{C} 173.2). Hence mandelalide H (**8**) was assigned as a 3-*O*-hexanoyl analogue of **4**. Again, a negative optical rotation value ($[\alpha]_{\text{D}}^{25} -41$), and NMR shifts closely similar to those for **4**, implied an

absolute configuration conserved with that for the other butyrolactone-containing mandelalides.

HRMS (ES^+) data for mandelalide I (**9**) yielded a molecular formula of $C_{45}H_{70}O_{15}$ ($[M+Na]^+$ m/z 873.4640), suggesting a glycosylated and esterified congener. The deshielded region of the ^{13}C NMR spectrum for **9** (Figure S22) displayed ^{13}C shifts for four ester carbonyls and one acetal carbon (δ_C 96.4). After accounting for four additional oxymethine and one methoxy saccharide ^{13}C shifts, the nine remaining oxymethine ^{13}C shifts (δ_C 69.1–81.9) suggested a butyrolactone-containing macrocycle. As expected, 1H and ^{13}C NMR data (Table S4) comprised shifts that closely matched those for the macrocyclic portion of **2** (including a 3-*O*-butyryl substituent). Comparison of the remaining chemical shifts with the dibutyrylated talose moiety of **5** and analysis of 2D NMR data led to the assignment of mandelalide I (**9**) as the 3',4'-*O*-dibutyryl analogue of **2**.

Mandelalide J (**10**) yielded a molecular mass that was 42 Da greater than that for **9** for a molecular formula of $C_{47}H_{72}O_{16}$ (HRMS, ES^+ , $[M+Na]^+$, m/z 915.4700), indicating an additional C_2H_2O unit in **10** compared to **9**. The ^{13}C NMR spectrum for **10** contained five, rather than four, ester carbonyl ^{13}C shifts (Figure S29), with the relatively shielded δ_C 170.4 shift consistent with an acetyl as the additional unit. Comparison of the 1H and ^{13}C NMR data for **10** (Table S5) with those for **9** (Table S4) revealed an additional 1H shift at δ_H 5.16 and absence of a shift at δ_H 3.78 (H-21), as well as slight shielding of the ^{13}C shifts for C-18 to C-22 in the spectra for **10**. This suggested acetylation at H-21 in **10**, which was confirmed by assignment of 2D NMR data. Thus, mandelalide J (**10**) is the 21-*O*-acetyl-3',4'-*O*-dibutyryl analogue of **2**.

HRMS (ES^+) data for mandelalide K (**11**, m/z 887.4375, $C_{45}H_{68}O_{16}Na$) indicated another glycosylated mandelalide with a highly substituted macrocycle, although 28 Da (C_2H_4) less than **10**. Of the five ester carbonyl shifts in the ^{13}C spectrum for **11** (Figure S36), two were relatively shielded (δ_C 169.9 and 170.4) and implied two acetate substituents. Comparison of the 1D NMR data for **10** (Table S5) and **11** (Table S6), and assignment of 2D NMR data, confirmed mandelalide K (**11**) as a 3, 21-*O*-diacetyl-3',4'-*O*-dibutyryl analogue of **2**.

Comparison of the optical rotation values and NMR data, including ROESY, for **9–11** with each other and with those for **2**⁴ indicated a conserved absolute configuration around the closely related macrocycles. Comparison of the NMR data for the dibutyrylated monosaccharide units in **9–11** with those for the same moiety in **5** and **7** (Table S7), also

indicated a conserved configuration between the monosaccharide substituents. Thus, the absolute configuration of the sugar substituent in **9–11** was also assigned as 1'*S*, 2'*S*, 3*R*, 4'*R*, 5'*S*.

Only a small amount of mandelalide A (**1**) was detected for re-isolation in the extract of the recollected tunicate, which was dominated by the butyrolactone-containing (B- and C-type) mandelalides. The ring-expanded 24-*O*-macrolactone congener (isomandelalide A) of **1**, was also detected, and could be identified, on the basis of HPLC retention time and characteristic mass peak (m/z 647.3), by LC-MS comparison to the reported synthetic product.⁸ The latter constitutional isomer was a minor component of the natural product fraction, present in insufficient amounts in the natural product fraction for isolation as a pure compound.

One other new analogue of **1** ("A-type" mandelalide) could be purified for structure elucidation and biological testing. This compound, named mandelalide L (**12**), was distinguished by two additional deshielded ¹H shifts (δ_H 5.97 and 6.97) and six olefinic ¹³C shifts in its 1D NMR spectra (Figures S42, S43), consistent with the additional ² unsaturation in **1**. HRMS (ES⁺) for **12** provided a molecular formula of C₄₁H₆₆O₁₂ ([M + Na]⁺ m/z 773.4430), for an additional C₈H₁₄O compared to the atomic composition of **1**. A second carbonyl ¹³C shift (δ_C 173.5) indicated one esterification (Figure S43), and an octanoyl moiety was consistent with a ¹H methyl triplet (d_H 0.88) and additional signals in the methylene envelope region of the ¹H NMR spectrum (Figure S42). The ¹H NMR spectrum for this congener also displayed two relatively deshielded, coupled ¹H double doublets (δ_H 4.37 and 4.07), and was missing the ¹H double doublets at δ_H 3.81 and 3.61 (H₂-24) present in the ¹H NMR spectrum for **1**. Full assignment of 2D NMR data (Table S8) and HMBC correlations from δ_H 4.37 and 4.07 to the ester carbonyl resonance (δ_C 173.5) led to assignment of **12** as 24-*O*-octanoylmandelalide A (a.k.a. mandelalide L). This (-)-mandelalide L structure for **12** was validated via total synthesis in the Smith laboratory.⁹ The same synthetic sequence (Scheme 1) developed to achieve **1** was employed, however, orthogonal protection of the 24-OH at an early stage, permitted deprotection and acylation at this position in a penultimate, otherwise TBS-protected, mandelalide A precursor.

Chemical Synthesis

Exploiting the reliable Types I and II Anion Relay Chemistry union tactics,^{14–16} both the northern [(–)-**12b**] and southern [(–)-**12c**] hemispheres of **12** were synthesized starting from commercially available and/or readily prepared simple building blocks (**14–20**). Union of (–)-**12b** and (–)-**12c** was achieved by Yamaguchi esterification,¹⁷ to furnish advanced intermediate (–)-**12d** in 89% yield. Removal of the PMB protecting group, followed by Kahne glycosylation¹⁸ with known sulfoxide (+)-**12f**⁵ provided (–)-**12g** in 85% yield over the two steps. Ring-closing Heck reaction^{19–21} and selective cleavage of the butyryl ester side chain with K₂CO₃ in MeOH then provided (–)-**12i**, possessing the free hydroxy group at C₂₄. A small amount of known methyl ester (–)-**13a**⁸ was obtained as a side-product during the ester saponification and was subjected to global desilylation to yield seco-mandelalide A methyl ester (–)-**13**. Installation of the octanoyl ester side chain on (–)-**12i** and global desilylation completed the total synthesis of (–)-**12**. The synthetic material displays spectral properties identical to those of the natural product.

Biological Evaluation

We sought to address the mechanistic basis of mandelalide action in mammalian cells using mandelalides A–C (**1–3**) as chemical probes to represent A-type (regular lactone connection), B-type (butyrolactone-containing) and C-type (23-hydroxy butyrolactone-containing) macrocycles, respectively. Cell density has been shown to influence the action of several clinically relevant cancer chemotherapeutic agents in both negative or positive ways in vitro. For example, a negative effect of cell density has been described for anti-microtubule agents such as paclitaxel,²² whereas the efficacy of platinum-based drugs can be positively influenced by the formation of functional gap junctions as cell confluency increases to permit intercellular signaling.^{6, 23, 24} It became clear, however, that differences

in the metabolic phenotype of the cells could provide an alternate explanation for the observed cell density-dependent action of mandelalides. Cancer cells have varied basal metabolic phenotypes as a result of inconsistent influences of the Warburg effect.²⁵ This phenomenon describes the ability of cancer cells to favor production of ATP by aerobic glycolysis, to gain a survival advantage, rather than mitochondrial oxidative phosphorylation. It has been noted that cancer cells growing in culture also have the capacity to shift metabolic routes and will take on a more oxidative phenotype as proliferation rates slow and nutrients become depleted.²⁶ The Sulikowski laboratory recently tested this hypothesis with apoptolidin A, a macrolide for which basic screening assay conditions have also proved difficult to establish.²⁷ They observed clear cell density-dependent cytostasis and cytotoxicity to H292 lung cancer cells.²⁸ To investigate the potential of mandelalide A to function as a metabolic inhibitor we seeded HeLa cells at three different starting densities (2,000, 6,000 and 10,000 cells per well) and tested cell viability after exposure to increasing concentrations of the glycolytic inhibitor 2-DG or **1** for 48 h using standard end-point assays. We found low-density cultures (2,000 cells per well) to be the most sensitive to 2-DG but least sensitive to **1** (Figure 1A). Lower concentrations of 2-DG (ranging from 0.25 μ M to 1 μ M) induced a 50% reduction in HeLa cell viability relative to vehicle-treated cultures, while **1** induced statistically significant but relatively modest 25 % decreases at the concentrations tested (100 nM – 1 μ M). Higher-density cultures (6,000 and 10,000 cells per well) revealed the opposite trend. Under these conditions, HeLa cells were most sensitive to **1**, with cell viability reduced to 85% of control (100 nM – 1 μ M), but were resistant to 2-DG (1 μ M) (Figures 1B and 1C). This shift in the sensitivity of HeLa cells to 2-DG is consistent with previous reports demonstrating that cultures of the same cell type can display different metabolic phenotypes as a function of cell confluency.²⁶ Together these results extend our earlier observations that low density (glycolytic) HeLa cell cultures are less sensitive to **1** than high density (more oxidative) cultures.¹⁰

To investigate the potential of the mandelalides to inhibit aerobic respiration in living cells we explored the oxygen consumption rate (OCR) of H292 lung cancer cells in response to natural products **1–3** and known site-specific inhibitors of mitochondrial function. H292 cells were chosen as a model cell type for these studies as we have previously found them to have a higher basal OCR/ECAR ratio than NCI-H460 cells,²⁷ indicating that H292 cells have a basal metabolic phenotype that favors oxidative phosphorylation over glycolysis. Using a metabolic flux analyzer, that permits sequential delivery of compounds in real-time, we observed a rapid decrease in the OCR of H292 cells in response to the addition of either oligomycin A (Figure 2A) or **2** (Figure 2B). The OCR remained at 25–30% that of control until the addition of the electron uncoupling agent FCCP to uncouple ATP production from oxygen consumption. The further addition of rotenone and antimycin A, to inhibit electron transport chain complexes I and III, respectively, caused an immediate decrease in OCR reflecting oxygen consumption due to non-mitochondrial respiration. We systematically substituted **2** for either FCCP (Figure 2C), or rotenone and antimycin A (Figure 2D) but observed no change in OCR under either of these conditions.

With a focus on ATP-dependent respiration, we next compared the ability of **1–3** to modulate basal OCR in H292 cells (Figure 3). In this series of assays **1** and **2** induced a

rapid decline in OCR and were functionally indistinguishable from each other, and from the control ATP synthase inhibitor oligomycin A (Figure 3A–C). In contrast, **3** produced no significant change in basal OCR, which remained relatively stable until the addition of FCCP at 85 min (Figure 3D). Taken together, these results indicated that the intracellular target of **1** and **2** is likely the mammalian ATP synthase complex, also known as mitochondrial complex V.

We therefore tested the ability of **1** to inhibit the complex V activity of isolated bovine heart mitochondria in a coupled enzymatic reaction that measures the rate of NADH oxidation (Figure 4). In these cell-free assays, synthetic **1** induced a potent and concentration-dependent reduction in ATPase activity (IC_{50} 2.2 nM). Maximal inhibition of complex V activity by **1** (30–100 nM) was comparable to inhibition of ATPase activity measured in the presence of saturating concentrations of the control inhibitors apoptolisin A or oligomycin A (Figure 4). Natural **3** also inhibited complex V activity in isolated mitochondria but showed over a 1,000-fold loss in activity (IC_{50} 3.4 μ M) relative to **1** (Figure S63). These results are consistent with the observation that the aglycone (**3**) failed to decrease the basal OCR in living cells (Figure 3D), and indicate that **3** is a weaker inhibitor of complex V activity, as well as potentially having limited ability to access the mitochondrial inner membrane. Moreover, the potent nanomolar activity of **1** in cell-free assays supports our conclusion that the cytotoxic action of mandelalides **1** and **2** in sensitive cell types is explained by inhibition of mitochondrial ATP synthase rather than binding to an ectopically-expressed ATP synthase on the plasma membrane.²⁹

During the course of these studies natural products **1** and **2** were also submitted for testing against the NCI60 human tumor cell line panel. Although mandelalides **1** and **2** did not meet the NCI selection criteria for progression to the 5 dose-response screen, standard one-dose (10 μ M) testing in the full panel, reported as a mean graph of the percent growth of treated cells, revealed a pattern of differential sensitivity (Figure S64). Individual cell lines tended to respond to **1** and **2** in the same way, indicating a common mechanism of action, with most cell types showing growth inhibition at 10 μ M (values between 0 and 100) and some showing lethality (values less than 0). Subsequent comparisons of these data showed that the pattern of anti-proliferative responses for **1** and **2** are similar to the NCI60 panel results for previous testing of well-characterized ATP synthase/complex V inhibitors, oligomycin A and apoptolisin A.^{6, 12, 30}

As the intrinsic apoptosis signaling pathway originates in the mitochondria,³¹ we next investigated whether sustained inhibition of mitochondrial function by mandelalides is sufficient to trigger cell death. For these studies, we exposed HeLa cells to **1** over a lower range of concentrations (1–300 nM), than that used by the NCI, and observed statistically significant activation of major downstream executioner caspases-3 and -7 by 24 h (Figure S65A). Lysates from HeLa cells treated with or without **1** also revealed time- and concentration-dependent expression of an 89 kDa band corresponding to the caspase-3-cleaved form of PARP1 together with expression of a major cleavage product of caspase-3 (Figure S65B). These results demonstrate the ability of **1** to induce cell death in HeLa cells via a caspase-dependent pathway. As **1** inhibits ATP-dependent respiration in living cells, we also studied the effects of exposure to **1** on overall cell viability in the presence of a

glycolytic inhibitor. HeLa cells became generally more sensitive to 2-DG alone if treatment times were extended to 72 h (Figure S66), illustrating the time-sensitive action of metabolic inhibition. Moreover, the effects of **1** on cell viability were additive when cells were treated concurrently with 2-DG (Figure S66). Finally, as the molecular target of **1** and **2** is found in normal cells we undertook the first test of synthetic **1** in a non-cancer cell line. HEK 293T cells were seeded at two different starting densities and treated with or without increasing concentrations of **1** or oligomycin A. As anticipated for a mitochondrial inhibitor, **1** decreased the viability of HEK293T cells in a concentration-dependent manner, although it was 13- to 30-fold less potent than oligomycin A, depending on assay cell density (Figure S67).

While the influence of macrocycle glycosylation for biological activity was established previously,¹⁰ the isolation of a suite of mandelalides with varying acylation around both the monosaccharide and the macrocycle offered the opportunity to investigate the theory that nonspecific acylation of the primary mandelalide biosynthetic products comprises a potential detoxification mechanism carried out by co-existing microbes, the host tunicate and/or the mandelalide-producing bacterium, for self-resistance and/or cellular transport. Towards this end, the cytotoxic potential of all new analogues was tested against human HeLa and NCI-H460 lung carcinoma cells grouped according to macrocycle type and relative to at least one other macrocycle type. HeLa and NCI-H460 cells were chosen for comparative screening of new natural product analogues as these cell types were previously found to be most sensitive to glycosylated **1**, **2** and **5**.¹⁰ In the regular macrolactone (A-type) series, **1** and its 24-*O*-octanoyl congener **12** display similar potency and cytotoxic efficacy against HeLa and NCI-H460 cells (Figure 5; Table 1). The cytotoxicity profile of high purity synthetic **12** (Figure S68) was also consistent with that of natural product **12** (Figure 5). Given that **1** and **12** possess the same monosaccharide (not acylated), these results imply that derivatization of the macrocycle at 24-OH does not interfere with the ability of the **12** to access its cellular target, or that the octanoate ester is cleaved to yield **1**, either spontaneously or by nonspecific cellular esterases.

The structural diversity of the new C-type mandelalides **6–8** also permitted a comparative assessment of the contributions of both the monosaccharide and macrocycle acylation to their biological activity. No C-type mandelalides possessing a monosaccharide without acylations were isolated. The most cytotoxic C-type mandelalide was **6**, with a 3-*O*-butyryl substituent and monobutyrylated ketosugar (Figure 5). The relatively modest (10- to 100-fold) reduction in cytotoxic potency of **6** relative to **1** and **2**, against HeLa (EC₅₀ 50 nM) and NCI-H460 (EC₅₀ 270 nM) cells suggests that an accessible H-bond acceptor on the monosaccharide (the ketone oxygen of **6**) contributes to cytotoxicity. By comparison, the dibutyryl monosaccharide-containing mandelalide **7** was 10- to 100-fold less potent than **6** (EC₅₀ >3 μM), yet retained cytotoxic efficacy. This result for **7**, which differs from **5** only in an additional 3-*O*-butyryl substituent is consistent with the low micromolar cytotoxicity obtained previously for **5**.¹⁰ For the C-type aglycones, some role of 24-*O*-butyrylation is implied when considering that **3** (3-*O*-butyryl substituent only) displayed weak (HeLa) to no (NCI-H460) activity at the concentrations tested (EC₅₀ > 3 μM), whereas **4** (3, 24-*O*-dibutyryl substituents) revealed sub to low micromolar activity (EC₅₀ 660 nM, HeLa; 1.7

μM , NCI-H460). Aglycone **8**, which possesses 3-*O*-hexanoyl and 24-*O*-butyryl substituents, was the most active of the aglycones against HeLa cells (EC_{50} 330 nM), although considerably less active against NCI-H460 cells (EC_{50} 2.6 μM). It may be speculated that this partial recovery of cytotoxicity for aglycones with increasingly numerous and/or longer chain acyl groups, is due to modifications in transport to the mandelalide site of action. While the glycosylated mandelalides may be transported across membranes by a mechanism(s) that recognizes the monosaccharide substituent, it is conceivable that acylated mandelalide aglycones may become sufficiently lipophilic to pass through membranes by passive diffusion.

The new B-type mandelalides **I** (**9**), **J** (**10**) and **K** (**11**), which all possess dibutyrylated monosaccharides, were significantly less potent than **2** against HeLa and NCI-H460 cells, although they retained cytotoxic efficacy against both cell lines. These analogues displayed high nanomolar to low micromolar activity (Figure 6; Table 1), in agreement with our proposal¹⁰ that butyrylation of the monosaccharide abrogates mandelalide cytotoxicity. Interestingly, NCI-H460 lung cancer cells appeared more sensitive to these B-type mandelalides, in particular 3-*O*-butyrylated mandelalides **9** and **10**, which differ only in 21-*O*-acetylation of **10**. Comparing the 21-*O*-acetylated **10** and **11**, exchange of the 3-*O*-butyryl (in **10**) for 3-*O*-acetyl (in **11**) resulted in a 3-fold reduction in cytotoxicity to NCI-H460 cells.

The potential susceptibility of mandelalides to esterases led us to question whether the macrolactone of **1** is necessary for the observed cytotoxicity. Thus, seco-mandelalide A methyl ester (**13**) was synthesized in one step from a known intermediate⁹ and tested in parallel with synthetic **1** (Figure 7). Consistent with the activity of the natural product, synthetic **1** induced a dramatic concentration-dependent decrease in the viability of HeLa cells relative to vehicle-treated cultures. This potent nanomolar activity was absent, however, in response to the seco acid **13**, which produced no significant change in cell viability under the same conditions (Figure 7). These data are thus consistent with a requirement for structural organization provided by macrocyclization, and/or a pharmacophoric lactone moiety to impart cytotoxicity. Moreover, generation of chemical probes for further analysis of the mandelalide binding target may be achievable by additional functionalization of the 24-OH.

CONCLUSIONS

In summary, we demonstrate here that the mandelalide natural product series represent two distinct macrocycle types (regular/simple lactone and butyrolactone-containing macrocycles) that are natural product inhibitors of mitochondrial ATP synthase. Our studies indicate that acute exposure to **1** or **2** results in a dramatic decrease in the aerobic respiratory capacity of living mammalian cells. The ability of these compounds to target oxidative phosphorylation was confirmed in cell-free assays where **1** was found to be a potent inhibitor of complex V activity in isolated mitochondria. Thus, in addition to glycosylation status, it is predicted that the cytotoxic potential of these structures will be based primarily on the basal metabolic phenotype of the cell, rather than histological selectivity or genetic background. These findings, together with independent confirmation of the pattern of cancer cell selectivity by

the NCI, provide a reasonable explanation for all previous reported disparities with respect to biological testing of synthetic mandelalide A (**1**) products. We conclude that cells with an oxidative phenotype, and/or compromised adaptive survival responses, are most likely to exhibit mandelalide-induced changes in proliferation rate and activation of cell death signaling in vitro. The variety of short chain fatty acid esterifications and the variability in substitution pattern suggests that these are biotransformations that occur post-biosynthesis, perhaps as a detoxification mechanism implemented by the host tunicate, or spontaneously in the extracellular environment. In particular, acylation of the monosaccharide warrants more detailed biological studies of the requirements for glycosylation of the mandelalides.

EXPERIMENTAL SECTION

General Experimental Procedures

For isolated natural products, optical rotations were measured on a Jasco P-1010 polarimeter. UV spectra were measured on a SpectraMax190 (Molecular Devices). NMR data were acquired in CDCl₃ referenced to internal TMS signals (0.00 ppm) on a Bruker Avance III 700 MHz spectrometer equipped with a 5mm ¹³C cryogenic probe. HRMS (ES⁺) was performed on a AB Sciex Triple TOF 5600. LC-MS (ES⁺) data were obtained on an ABSciex 3200 QTrap mass spectrometer. HPLC was performed using a Shimadzu dual LC-20AD solvent delivery system with a Shimadzu SPD-M20A UV/VIS photodiode array detector. For synthetic products, all moisture-sensitive reactions were performed using syringe-septum cap techniques under an inert atmosphere of N₂. All glassware was flame dried or dried in an oven (140 °C) for at least 4 h prior to use. Reactions were magnetically stirred unless otherwise stated. THF, DCM, diethyl ether (Et₂O) and toluene were dried by passage through alumina in a Pure Solve™ PS-400 solvent purification system. THF was degassed vigorously via freeze-pump-thaw before being employed in Anion Relay Chemistry protocols. Unless otherwise stated, solvents and reagents were used as received. Analytical thin layer chromatography was performed on pre-coated silica gel 60 F-254 plates (particle size 40–55 micron, 230–400 mesh) and visualized by a UV lamp or by staining with PMA (2 g phosphomolybdic acid dissolved in 20 mL absolute ethanol), KMnO₄ (1.5 g of KMnO₄, 10 g of K₂CO₃ and 2.5 mL of 5% aq. NaOH in 150 mL H₂O), or CAM (4.8 g of (NH₄)₆Mo₇O₂₄·4H₂O and 0.2 g of Ce(SO₄)₂ in 100 mL of a 3.5 N H₂SO₄ solution). Column chromatography was performed using silica gel (Silacyle Silaflash®) P60, 40–63 micron particle size, 230–300 mesh) and compressed air pressure with commercial grade solvents. Yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. NMR spectra were recorded at 500 MHz/125 MHz (¹H / ¹³C) on a Bruker Avance III 500 MHz spectrometer at 300 K. Chemical shifts are reported relative to residual protonated chloroform (δ 7.26), acetone (δ 2.05), methanol (δ 3.31), or benzene (δ 7.16) for ¹H NMR and chloroform (δ 77.16), acetone (δ 29.84), methanol (δ 49.00), or benzene (δ 128.06) for ¹³C NMR. Optical rotations were measured on a Jasco P-2000 polarimeter. Melting points were determined using a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were measured on a Jasco FT/IR 480 plus spectrometer. High-resolution mass spectra were obtained at the University of Pennsylvania on a Waters GCT Premier spectrometer. Immediately before biological testing at OSU, all natural and synthesized products were re-purified by HPLC,

dried and weighed. Purity of 95% for all natural and synthesized products was confirmed by LC-MS before biological testing.

Collection and Identification

The ascidian, *Lissoclinum mandelai*.⁴ (Ascidacea, Aplousobranchia, Didemnidae) was collected by hand using SCUBA at a depth of 18 m (July 7, 2013) from White Sands Reef in Algoa Bay, Eastern Cape Province, South Africa (33:59.916S, 25:42.573W). The type specimen for this ascidian species is housed at the South African Institute for Aquatic Biodiversity (SAIAB), Grahamstown, South Africa.

Extraction and Isolation

The freeze-dried organism (74.4 g) was extracted with 2:1 DCM-MeOH to yield 1.6 g of organic extract. This organic extract was fractionated on Sephadex LH-20 (DCM-MeOH, 1:3) to give 50 fractions, of which combined fractions 17 and 18 (42 mg) were subjected to RP₁₈ SPE using a stepped gradient of 55 and 100% MeCN in H₂O. The 55% MeCN-H₂O and 100% MeCN fractions were further separated by RP HPLC (Synergi Hydro-RP, Phenomenex, 250 mm × 10 mm, 7:3 MeCN in H₂O), to yield mandelalides B (**2**, 0.5 mg), C (**3**, 0.8 mg), E (**5**, 0.8 mg), G (**7**, 0.4 mg), I (**9**, 0.7 mg), J (**10**, 0.7 mg), K (**11**, 0.6 mg), and L (**12**, 0.9 mg).

RP₁₈ SPE of the combined LH20 fractions 19–23 (208 mg) using 100% MeOH, followed by RP₁₈ HPLC (Synergi Hydro-RP, Phenomenex, 250 mm × 10 mm, 7:3 MeCN in H₂O), yielded mandelalides A (**1**, 1.7 mg), B (**2**, 2.5 mg), D (**4**, 2.5 mg), F (**6**, 1.2 mg), H (**8**, 2.0 mg), and L (**12**, 2.3 mg).

Mandelalides A–E (1–5)—Amorphous solids; $[\alpha]_D^{25}$ values, UV, ¹H and ¹³C NMR, and LRMS data matched those reported in the literature.^{4, 10}

Mandelalide F (6)—Amorphous solid; $[\alpha]_D^{25} - 17$ ($c = 0.30$, MeOH); HRMS (ES⁺) [M + H]⁺ m/z 795.4175, (calcd for C₄₁H₆₃O₁₅, 795.4167); ¹H and ¹³C NMR, COSY, HMBC, TOCSY, ROESY (Table S1).

Mandelalide G (7)—Amorphous solid; $[\alpha]_D^{25} - 12$ ($c = 0.25$, MeOH); HRMS (ES⁺) [M + Na]⁺ m/z 889.4553, (calcd for C₄₅H₇₀O₁₆Na, 889.4562); ¹H and ¹³C NMR, COSY, HMBC, TOCSY, ROESY (Table S2).

Mandelalide H (8)—Amorphous solid; $[\alpha]_D^{25} - 41$ ($c = 0.40$, MeOH); HRMS (ES⁺) [M + Na]⁺ m/z 687.3701, (calcd for C₃₆H₅₆O₁₁Na, 687.3720); ¹H and ¹³C NMR, COSY, HMBC, TOCSY, ROESY (Table S3).

Mandelalide I (9)—Amorphous solid; $[\alpha]_D^{25} - 5.2$ ($c = 0.25$, MeOH); HRMS (ES⁺) [M + Na]⁺ m/z 873.4640, (calcd for C₄₅H₇₀O₁₅Na, 873.4612); ¹H and ¹³C NMR, COSY, HMBC, TOCSY, ROESY (Table S4).

Mandelalide J (10)—Amorphous solid; $[\alpha]_D^{25} - 6.0$ ($c = 0.30$, MeOH); HRMS (ES⁺) [M + Na]⁺ m/z 915.4700, (calcd for C₄₇H₇₂O₁₆Na, 915.4718); ¹H and ¹³C NMR, COSY, HMBC, TOCSY, ROESY (Table S5).

Mandelalide K (11)—Amorphous solid; $[\alpha]_D^{25} - 6.5$ ($c = 0.30$, MeOH); HRMS (ES⁺) [M + Na]⁺ m/z 887.4375, (calcd for C₄₅H₆₈O₁₆Na, 887.4405); ¹H and ¹³C NMR, COSY, HMBC, TOCSY, ROESY (Table S6).

Mandelalide L (12)—Amorphous solid; $[\alpha]_D^{25} - 19$ ($c = 0.80$, MeOH); HRMS (ES⁺) [M + Na]⁺ m/z 773.4430, (calcd for C₄₁H₆₆O₁₂Na, 773.4452); ¹H and ¹³C NMR, COSY, HMBC, TOCSY, ROESY (Table S8).

Synthesis of mandelalide L (12) and seco-mandelalide A methyl ester (13)

Compounds **12a** and **12c** were synthesized according to the reported procedure.⁹

Compound 12b—A solution of diol **12a** (25.0 mg, 0.053 mmol) in 2 mL DCM at room temperature was added sequentially a solution of 2,3,5-collidine (38.8 mg/mL in DCM, 1.05 mL, 0.333 mmol) and a solution of butyryl chloride (22.8 mg/mL in DCM, 1.05 mL, 0.224 mmol) via syringe. The resulting mixture was stirred at room temperature for 7 h. The solution was quenched with saturated aqueous NH₄Cl. The resulting mixture was extracted with EtOAc. The combined organic layers were washed with brine, dried with Na₂SO₄, and concentrated *in vacuo*. The crude product was purified by flash chromatography on silica gel (10% EtOAc/hexanes) to afford the desired product **12b** as a colorless oil (27.2 mg, 0.0504 mmol, 95%): $[\alpha]_D^{20} -25.46$ (c 0.44, DCM); IR (film, cm⁻¹) 3466, 2957, 2932, 2852, 1738, 1461, 1386, 1254, 1181, 1092, 999, 837, 778; ¹H NMR (500 MHz, CDCl₃) δ 6.35-6.29 (m, 1 H), 6.29-6.25 (m, 1 H), 4.12-4.04 (m, 2 H), 4.01-3.84 (m, 4 H), 3.33 (bs, 1 H), 2.41-2.34 (m, 1 H), 2.33 (t, $J = 7.4$ Hz, 2 H), 2.27-2.22 (m, 2 H), 2.06-1.99 (m, 1 H), 1.74-1.62 (m, 3 H), 1.59-1.52 (m, 1 H), 1.30-1.22 (m, 1 H), 0.98 (d, $J = 6.9$ Hz, 3 H), 0.95 (t, $J = 7.4$ Hz, 3 H), 0.89 (s, 9 H), 0.11 (s, 6 H); ¹³C NMR (125 MHz, CDCl₃) δ 173.9, 138.9, 83.7, 81.4, 80.2, 73.3, 68.7, 67.0, 37.1, 36.6, 36.2, 35.9, 35.8, 26.1, 18.6, 18.3, 15.5, 13.8, -4.1, -4.7; HRMS (ES⁺) m/z (M+Na)⁺: Calcd for C₂₂H₄₁O₅NaSi: 563.1666, found: 563.1667.

Compound 12d—A solution of carboxylic acid **12c** (39.0 mg, 0.104 mmol) in 0.70 mL toluene was added a solution of Et₃N (13% v/v in toluene, 1.00 mL, 0.937 mmol) and a solution of 2,4,6-trichlorobenzoylchloride (4.6% v/v in toluene, 0.700 mL, 0.208 mmol) via syringe dropwise. The solution was stirred at room temperature for 5 h, at which time a solution of alcohol **12b** (37.5 mg, 0.0694 mmol) in 2.5 mL toluene was added via cannula. A solution of DMAP (25.4 mg, 0.208 mmol) in 0.700 mL toluene was added and the resulting mixture was stirred for 24 h at room temperature. The solution was then quenched with saturated aqueous NH₄Cl (5 mL) and the resulting mixture was extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with brine, dried with Na₂SO₄, and concentrated *in vacuo*. The crude product was purified by flash chromatography on silica gel (10% EtOAc/hexanes) to afford the desired product **12d** as a colorless oil (55.4 mg, 0.0618 mmol, 89%): $[\alpha]_D^{20} -22.71$ (c 0.48, DCM); IR (film, cm⁻¹) 2956, 2927, 2853, 1744, 1722, 1654, 1613, 1513, 1463, 1355, 1249, 1172, 1088, 836; ¹H NMR (500 MHz, CDCl₃) δ 7.25

(d, $J = 8.5$ Hz, 2 H), 7.01-6.93 (m, 1 H), 6.87 (d, $J = 8.5$ Hz, 2 H), 6.34-6.29 (m, 1 H), 6.28-6.24 (m, 1 H), 5.88 (d, $J = 15.7$ Hz, 1 H), 5.77-5.67 (m, 1 H), 5.26-5.19 (m, 1 H), 4.99-4.87 (m, 2 H), 4.49 (s, 2 H), 4.3 (dd, $J = 11.8, 3.7$ Hz, 1 H), 4.12 (dd, $J = 11.9, 5.4$ Hz, 1 H), 3.96-3.90 (m, 1 H), 3.80 (s, 3 H), 3.80-3.74 (m, 2 H), 3.57-3.49 (m, 1 H), 3.41-3.34 (m, 1 H), 3.34-3.27 (m, 1 H), 2.52-2.44 (m, 1 H), 2.38-2.20 (m, 7 H), 2.06-1.96 (m, 3 H), 1.91-1.84 (m, 1 H), 1.69-1.55 (m, 4 H), 1.37-1.12 (m, 4 H), 1.01-0.96 (m, 6 H), 0.93 (t, $J = 7.4$ Hz, 3 H), 0.87 (s, 9 H), 0.04 (s, 3 H), 0.02 (s, 3 H); ^{13}C NMR (125 MHz, CDCl_3) δ 173.4, 165.9, 159.3, 145.9, 144.6, 138.9, 130.7, 129.3, 123.1, 114.0, 112.6, 83.7, 81.6, 80.2, 74.4, 74.2, 73.7, 71.0, 69.4, 69.1, 65.5, 55.4, 42.7, 39.0, 38.11, 38.07, 37.1, 36.2, 35.7, 35.2, 34.5, 34.1, 26.1, 20.0, 18.5, 18.3, 15.5, 13.8, -3.8, -4.8; HRMS (ES^+) m/z ($\text{M}+\text{H}$) $^+$: Calcd for $\text{C}_{44}\text{H}_{70}\text{O}_9\text{Si}$: 897.3834, found: 897.3843.

Compound 12e—A solution of **12d** (46.0 mg, 0.0513 mmol) in 1.3 mL DCM and 0.275 mL pH 7 phosphate buffer (0.5 M) was added a solution of DDQ (recrystallized over CHCl_3 , 34.9 mg, 0.154 mmol) in 2.6 mL DCM dropwise at 0 °C. After stirring at 0 °C for 2.5 h, the mixture was then quenched with pH 7 phosphate buffer (10 mL) and the resulting mixture was extracted with DCM (3×30 mL). The combined organic layers were washed with brine, dried with Na_2SO_4 , and concentrated *in vacuo*. The crude product was purified by flash chromatography on silica gel (10–20% EtOAc/hexanes) to afford the desired product **12e** as a colorless oil (38.7 mg, 0.0498 mmol, 97%): $[\alpha]_D^{20}$ -29.36 (c 0.60, DCM); IR (film, cm^{-1}) 3449, 3069, 2962, 2929, 2854, 1723, 1656, 1467, 1364, 1310, 1256, 1172, 1090, 1046, 987, 906, 837, 778; ^1H NMR (500 MHz, CDCl_3) δ 7.00-6.92 (m, 1 H), 6.34-6.24 (m, 2 H), 5.88 (d, $J = 15.7$ Hz, 1 H), 5.76-5.65 (m, 1 H), 5.25-5.19 (m, 1 H), 4.98-4.88 (m, 2 H), 4.30 (dd, $J = 11.8, 3.7$ Hz, 1 H), 4.11 (dd, $J = 11.7, 5.4$ Hz, 1 H), 3.97-3.90 (m, 1 H), 3.85-3.72 (m, 3 H), 3.44-3.30 (m, 2 H), 2.50-2.43 (m, 1 H), 2.38-2.21 (m, 7 H), 2.03-1.83 (m, 4 H), 1.70-1.52 (m, 5 H), 1.38-1.23 (m, 2 H), 1.21-1.07 (m, 2 H), 1.01-0.96 (m, 6 H), 0.93 (t, $J = 7.4$ Hz, 3 H), 0.86 (s, 9 H), 0.04 (s, 3 H), 0.01 (s, 3 H); ^{13}C NMR (125 MHz, CDCl_3) δ 173.4, 165.9, 145.8, 144.5, 138.9, 123.2, 112.7, 83.7, 81.6, 80.2, 74.1, 73.7, 70.9, 69.1, 68.2, 65.5, 42.6, 41.1, 41.0, 38.8, 37.1, 36.2, 35.6, 35.2, 34.5, 34.1, 26.1, 20.0, 18.5, 18.3, 15.4, 13.8, -3.8, -4.8; HRMS (ES^+) m/z ($\text{M}+\text{H}$) $^+$: Calcd for $\text{C}_{36}\text{H}_{62}\text{O}_8\text{Si}$: 777.3259, found: 777.3242.

Compound 12g—A flask containing activated 4A MS powder (700 mg) was added sugar donor **12f**⁵ (165.8 mg, 0.322 mmol) in 2.2 mL Et_2O and 2,6-di-*t*-butyl-4-methylpyridine (141.7 mg, 0.375 mmol) in 1.1 mL Et_2O and the resulting mixture was stirred at room temperature for 1 h. The flask was cooled to -78 °C and a solution of Tf_2O (46.4 μL , 0.276 mmol) in 0.56 mL Et_2O was added dropwise and the mixture was stirred at -78 °C for 20 min. A solution of alcohol **12e** (35.7 mg, 0.046 mmol) in 4.5 mL Et_2O was added via cannula dropwise. The resulting mixture was stirred at -78 °C for 1 h, then at -40 °C for 2 h and at -35 °C for 1 h. The flask was then cooled to -78 °C and the reaction was quenched with deionized H_2O (15 mL), diluted with EtOAc (20 mL) and allowed to warm to room temperature. The reaction mixture was filtered, extracted with EtOAc (3×40 mL). The combined organic layers were washed with brine, dried with Na_2SO_4 , and concentrated *in vacuo*. The crude product was purified by flash chromatography on silica gel (5% EtOAc/Hexanes) to afford the desired product **12g** as a colorless oil (47.2 mg, 0.0405 mmol, 88%):

$[\alpha]_D^{20}$ -41.94 (*c* 0.60, DCM); IR (film, cm^{-1}) 2956, 2928, 2854, 1725, 1657, 1460, 1254, 1165, 1096, 1052, 837, 777; ^1H NMR (500 MHz, CDCl_3) δ 7.01-6.93 (m, 1 H), 6.34-6.24 (m, 2 H), 5.88 (d, *J* = 15.7 Hz, 1 H), 5.77-5.68 (m, 1 H), 5.25-5.19 (m, 1 H), 4.99-4.85 (m, 3 H), 4.30 (dd, *J* = 11.8, 3.5 Hz, 1 H), 4.12 (dd, *J* = 11.8, 5.3 Hz, 1 H), 3.96-3.90 (m, 1 H), 3.87-3.83 (m, 1 H), 3.82-3.69 (m, 3 H), 3.60-3.52 (m, 2 H), 3.45 (s, 3 H), 3.42-3.30 (m, 3 H), 2.49-2.41 (m, 1 H), 2.38-2.21 (m, 7 H), 2.04-1.92 (m, 3 H), 1.91-1.83 (m, 1 H), 1.68-1.53 (m, 4 H), 1.35-1.13 (m, 7 H), 1.02-0.96 (m, 6 H), 0.95-0.90 (m, 12 H), 0.89 (s, 9 H), 0.86 (s, 9 H), 0.11-0.09 (m, 9 H), 0.08 (s, 3 H), 0.04 (s, 3 H), 0.02 (s, 3 H); ^{13}C NMR (125 MHz, CDCl_3) δ 173.4, 165.9, 145.9, 144.5, 138.9, 123.1, 112.6, 83.7, 81.6, 80.2, 74.1, 73.7, 73.4, 73.3, 70.9, 70.5, 69.2, 65.5, 58.9, 42.7, 39.3, 39.0, 37.6, 37.1, 36.2, 35.7, 35.2, 34.5, 34.0, 26.4, 26.2, 26.1, 19.9, 18.8, 18.5, 18.3, 18.2, 15.5, 13.8, -2.7, -3.6, -3.8, -4.0, -4.1, -4.8; HRMS (ES^+) *m/z* ($\text{M}+\text{H}^+$): Calcd for $\text{C}_{55}\text{H}_{102}\text{O}_{12}\text{Si}_3\text{I}$: 1165.5724, found: 1165.5740.

Compound 12h—To a solution of **12g** (45.0 mg, 0.0386 mmol) in 5 mL anhydrous DMF (degassed via freeze-pump-thaw) was added a solid mixture of $\text{Pd}(\text{OAc})_2$ (15.6 mg, 0.0695 mmol) and Cs_2CO_3 (25.2 mg, 0.0772 mmol), followed by a solution of Et_3N (8.1 μL , 0.058 mmol) in 0.40 mL DMF. The resulting solution was stirred at room temperature for 2 days. The reaction was quenched with deionized H_2O (20 mL), extracted with EtOAc (3×40 mL). The combined organic layers were washed with brine, dried with Na_2SO_4 , and concentrated *in vacuo*. The crude product was filtered through a short pad of silica gel and concentrated to obtain ca. 42.0 mg of crude **12h**, which was used directly in the next step without further purification.

Compound 12i—To a solution of 13.6 mg of the crude **12h** in 7.0 mL THF at 0 °C was added solid K_2CO_3 (7 mg). The resulting solution was stirred at 0 °C for 7 h. The reaction was then quenched with 10 mL sat. aq. NH_4Cl at 0 °C. The resulting mixture was then warm to room temperature and the organic phase was extracted with DCM (3×30 mL). The combined organic layers were dried with Na_2SO_4 , and concentrated *in vacuo*. The crude product was purified by flash chromatography on silica gel (20–40% EtOAc /hexanes) to afford the desired product **12i** as a colorless oil (7.9 mg, 0.0081 mmol, 65% over 2 steps): $[\alpha]_D^{20}$ -27.35 (*c* 0.20, DCM); IR (film, cm^{-1}) 3449, 2961, 2927, 2856, 1720, 1657, 1462, 1362, 1254, 1095, 1046, 897, 837, 777, 668; ^1H NMR (500 MHz, CDCl_3) δ 7.02-6.94 (m, 1 H), 6.27 (dd, *J* = 14.9, 11.3 Hz, 1 H), 6.05-5.97 (m, 2 H), 5.48 (dd, *J* = 14.9, 8.7 Hz, 1 H), 5.29 (td, *J* = 10.5, 5.2 Hz, 1 H), 5.14-5.08 (m, 1 H), 4.89 (d, *J* = 2.6 Hz, 1 H), 4.01-3.95 (m, 1 H), 3.88-3.84 (m, 1 H), 3.82-3.73 (m, 3 H), 3.65-3.52 (m, 4 H), 3.45 (s, 3 H), 3.44-3.40 (m, 1 H), 3.37-3.30 (m, 2 H), 2.47-2.32 (m, 5 H), 2.07-1.99 (m, 1 H), 1.98-1.88 (m, 3 H), 1.80-1.72 (m, 1 H), 1.64-1.49 (m, 2 H), 1.32-1.19 (8 H), 1.01 (d, *J* = 6.9 Hz, 3 H), 0.95-0.91 (m, 12 H), 0.89 (s, 9 H), 0.86 (s, 9 H), 0.13-0.09 (m, 9 H), 0.08 (s, 3 H), 0.04 (s, 3 H), -0.03 (s, 3 H); ^{13}C NMR (125 MHz, CDCl_3) δ 167.1, 146.8, 140.6, 130.3, 128.0, 124.9, 123.3, 82.8, 81.8, 81.4, 73.8, 73.5, 73.3, 73.2, 73.0, 72.8, 70.6, 66.1, 58.9, 43.5, 39.7, 39.3, 38.0, 36.5, 36.3, 35.6, 33.5, 31.3, 29.9, 26.4, 26.22, 26.16, 18.9, 18.8, 18.5, 18.3, 18.2, 14.8, -2.8, -3.7, -4.0, -4.1, -5.0; HRMS (ES^+) *m/z* ($\text{M}+\text{Na}^+$): Calcd for $\text{C}_{51}\text{H}_{94}\text{O}_{11}\text{Si}_3\text{Na}$: 989.6002, found: 989.6014.

Compound 12j—A solution of **12i** (3.0 mg, 0.0031 mmol) in 1.0 mL DCM at room temperature was added sequentially a solution of 2,3,5-collidine (11.0 mg/mL in DCM, 1.10 mL, 0.100 mmol) and a solution of octanoyl chloride (10.0 mg/mL in DCM, 1.10 mL, 0.068 mmol) via syringe. The resulting mixture was stirred at room temperature for 13 h. The solution was quenched with saturated aqueous NH_4Cl . The resulting mixture was extracted with EtOAc. The combined organic layers were washed with brine, dried with Na_2SO_4 , and concentrated *in vacuo*. The crude product was purified by flash chromatography on silica gel (20% EtOAc/hexanes) to afford the product **12j** contaminated with octanoic acid, which was used directly in the global deprotection step.

Mandelalide L (12)—To a solution of the product mixture of **12j** contaminated with octanoic acid in 0.5 mL THF in a polypropylene tube at 0 °C was added 0.5 mL pyridine and 0.5 mL HF.Pyridine complex (70% HF) dropwise via Eppendorf pipette. The resulting solution was stirred at 0 °C for 5 min. The cold bath was then removed and the resulting solution was stirred at room temperature for another 38 h. The reaction was then quenched with 20 mL sat. aq. NaHCO_3 solution slowly and stirred at room temperature for 30 min. The organic phase was extracted with DCM (3 × 30 mL). The combined organic layers were dried with Na_2SO_4 , and concentrated *in vacuo*. The crude product was purified by RP₁₈ HPLC (10–90% MeCN- H_2O , 0.05% formic acid) to afford the natural product **12** as colorless amorphous solid (1.7 mg, 0.0023 mmol, 74% over 2 steps): $[\alpha]_D^{20}$ –52.25 (*c* 0.07, MeOH); IR (film, cm^{-1}) 3434, 2954, 2925, 2871, 1721, 1465, 1378, 1285, 1264, 1147, 1109, 1069, 737; ^1H NMR (600 MHz, CDCl_3 , residual solvent peak set at 7.24 ppm) δ 6.95 (ddd, *J* = 15.1, 10.4, 4.6 Hz, 1 H), 6.26 (dd, *J* = 14.7, 11.4 Hz, 1 H), 6.05 (t, *J* = 10.6 Hz, 1 H), 5.95 (d, *J* = 15.4 Hz, 1 H), 5.44 (dd, *J* = 14.7, 9.9 Hz, 1 H), 5.35 (d, *J* = 11.0 Hz, 1 H), 5.27 (td, *J* = 10.5, 5.7 Hz, 1 H), 5.02 (s, 1 H), 4.35 (dd, *J* = 11.7, 3.7 Hz, 1 H), 4.06 (dd, *J* = 11.7, 4.0 Hz, 1 H), 3.98 (t, *J* = 9.9 Hz, 1 H), 3.86–3.79 (m, 1 H), 3.72–3.60 (m, 3 H), 3.46 (s, 3 H), 3.43–3.28 (m, 5 H), 2.61–2.48 (m, 2 H), 2.43–2.21 (m, 8 H), 2.06–1.98 (m, 2 H), 1.92–1.85 (m, 2 H), 1.74–1.68 (m, 1 H), 1.63–1.16 (m, 19 H), 1.03 (d, *J* = 6.6 Hz, 3 H), 0.86 (t, *J* = 6.6 Hz, 3 H), 0.85 (d, *J* = 6.2 Hz, 3 H); ^{13}C NMR (125 MHz, CDCl_3 , residual solvent peak set at 77.0 ppm) δ 173.5, 165.8, 146.6, 141.4, 131.0, 126.6, 123.6, 122.7, 93.9, 82.9, 80.8, 80.5, 74.0, 73.7, 72.8, 72.6, 72.3, 71.4, 67.9, 67.7, 65.1, 58.9, 42.7, 39.4, 38.6, 37.3, 37.1, 36.6, 34.1, 34.0, 33.9, 31.7, 30.9, 29.0, 28.9, 24.9, 22.6, 18.1, 17.5, 14.3, 14.1; HRMS (ES^+) *m/z* ($\text{M}+\text{Na}^+$): Calcd for $\text{C}_{41}\text{H}_{66}\text{O}_{12}\text{Na}$: 773.4452, found: 773.4452.

Seco-mandelalide A methyl ester (13)—The known compound **13a**⁸ was obtained as a side-product during the conversion of **12h** to **12i** and was subjected to global deprotection to yield seco-mandelalide A methyl ester **13**. To a solution of **13a** (10.0 mg, 0.010 mmol) in 0.8 mL THF in a polypropylene tube at 0 °C was added 0.8 mL pyridine and 0.8 mL HF.Pyridine complex (70% HF) dropwise via Eppendorf pipette. The resulting solution was stirred at 0 °C for 5 min. The cold bath was then removed and the resulting solution was stirred at room temperature for another 16 h. The reaction was then quenched with 20 mL sat. aq. NaHCO_3 solution slowly and stirred at room temperature for 30 min. The organic phase was extracted with DCM (3 × 30 mL). The combined organic layers were dried with Na_2SO_4 , and concentrated *in vacuo*. The crude product was purified by flash chromatography on silica gel (8% MeOH/DCM) to afford the desired product **13** as

colorless waxy solid (4.0 mg, 0.0061 mmol, 61%): $[\alpha]_D^{20}$ -48.07 (c 0.33, MeOH); IR (film, cm^{-1}) 3391, 2924, 2857, 1721, 1658, 1447, 1373, 1322, 1277, 1106, 1042, 992; ^1H NMR (500 MHz, CDCl_3) δ 6.97 (m, 1 H), 6.26 (dd, J = 14.9, 11.1 Hz, 1 H), 6.04 (t, J = 11.1 Hz, 1 H), 5.89 (d, J = 15.7 Hz, 1 H), 5.59 (m, 1 H), 5.33 (m, 1 H), 5.02 (s, 1 H), 4.07-3.99 (m, 1 H), 3.98-3.91 (m, 1 H), 3.74 (s, 3 H), 3.69-3.60 (m, 4 H), 3.56-3.49 (m, 1 H), 3.48 (s, 1 H), 3.44-3.30 (m, 6 H), 2.51-2.31 (m, 7 H), 2.30-2.22 (m, 1 H), 2.11-2.05 (m, 1 H), 2.00-1.89 (m, 3 H), 1.71-1.50 (m, 4 H), 1.43-1.33 (m, 3 H), 1.30 (d, J = 5.9 Hz, 3 H), 1.19-1.10 (m, 2 H), 1.02 (d, J = 6.3 Hz, 3 H), 0.99 (d, J = 6.9 Hz, 3 H); ^{13}C NMR (125 MHz, CD_3OD) δ 168.6, 147.4, 141.7, 131.0, 127.4, 125.1, 123.7, 96.6, 83.8, 83.0, 82.6, 75.4, 74.9, 74.7, 74.3, 72.2, 71.9, 70.1, 69.9, 68.0, 59.3, 52.0, 44.1, 40.3, 39.6, 38.5, 38.2, 37.04, 36.99, 34.7, 30.6, 20.8, 18.1, 15.6; HRMS (ES^+) m/z ($\text{M}+\text{Na}^+$): Calcd for $\text{C}_{34}\text{H}_{56}\text{O}_{12}\text{Na}$: 679.3669, found: 679.3696.

Chemicals, Reagents and Antibodies for Biological Studies

All compounds were reconstituted in 100% DMSO and stored at -20°C until the day of treatment; final concentrations of DMSO never exceeded 0.1%. 2-DG, rotenone and MTT were from Sigma-Aldrich Corp. (St. Louis, MO). Prepared solutions of oligomycin A, FCCP and rotenone/antimycin A, were purchased from Seahorse Bioscience Inc. (Billerica, MA) as components of the XF Cell Mito Stress Test Kit (#103105-100) to assess mitochondrial function. Apoptolidin A, for complex V activity assays, was a kind gift from Dr. Taifo Mahmud (College of Pharmacy, OSU). All antibodies were from commercial sources and used according to the recommended protocols. Primary antibodies were from Cell Signaling Technology, Inc. (Danvers, MA) as follows: PARP1 (#9532S), cleaved caspase-3 (#9579), GAPDH (#8884) and alpha-tubulin (#2125).

Mammalian Cell Culture

Human H460 lung cancer cells were from the NCI cell line repository (Frederick National Laboratory); H292 lung, HeLa cervical cancer and HEK 293T cells were from ATCC. H460 and H292 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), L-glutamine (2 mM), sodium pyruvate (1 mM), sodium bicarbonate (1.5 g/L) and 1% penicillin/streptomycin. HeLa cells were grown in MEM formulation and 293T cells in DMEM each with 10% FBS, L-glutamine (2 mM), and 1% penicillin/streptomycin. All cells were maintained in a humidified chamber at 37°C with 5% CO_2 and seeded (at the densities indicated) 16–18 h before treatment. General culture supplies were from Thermo Fisher Scientific (Waltham, MA) or Sigma-Aldrich.

Cell viability, caspase activity and immunoblot analysis

Cell viability was assessed by MTT assay with the viability of vehicle-treated cells defined as 100%.¹⁰ Concentration-response relationships were analyzed using GraphPad Prism Software (GraphPad Software Inc., San Diego, CA), and EC_{50} values derived using nonlinear regression analysis fit to a logistic equation. Caspase-3,7 activation was assessed by Caspase-Glo® (Promega Corp., Madison, WI) as described previously.³² For immunoblot analysis cell monolayers were rinsed with PBS, lysed and processed as described previously.³² Statistical significance of cell viability was assessed using a one-way ANOVA followed by a student's t-test to compare control and treatment groups.

Metabolic assays

Real-time measurements of cellular OCR were measured in H292 cells with an XF-24 Extracellular Flux Analyzer (Seahorse Bioscience). Cells were seeded in XF microplates (Seahorse Bioscience) in complete medium and maintained in a humidified chamber at 37 °C with 5% CO₂. Prior to measurements, standard culture medium was replaced with unbuffered XF Assay Media (Seahorse Biosciences) supplemented with 1mM sodium pyruvate and 25 mM D-glucose, pH 7.4, and allowed to equilibrate for 1 h at 37 °C in a non-CO₂ incubator. Cell culture plates were inserted into the instrument and basal OCR measurements were acquired before treating cells were treated with: (1) mandelalides A, B or C (1 μM) or oligomycin A (1 μM) at 32 min, (2) carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) (1 μM) or mandelalide B (1 μM) at 87 min and, (3) rotenone plus antimycin A (0.5 μM) or mandelalide B (1 μM) at 135 min by serial injection. OCR was monitored continuously over time to profile the mitochondrial stress response.

Complex V activity assay

Mitochondrial ATPase activity was measured with a MitoCheck® Complex V Activity Assay kit (#701000) from Cayman Chemical (Ann Arbor, MI) according to the manufacturers instructions. Briefly, the activity of complex V in isolated bovine heart mitochondria was determined in a colormetric assay by measuring the rate of NADH oxidation at 340 nm (captured every 30 seconds for 30 min) at 25°C. The reaction rate was determined by plotting absorbance (y-axis), calculated from the slope for the linear portion of each curve, versus time (x-axis). Complex V activity was calculated as % activity relative to the vehicle control:

$$\text{Complex V Activity(\%)} = \left[\frac{\text{Rate of Sample wells}}{\text{Rate of Vehicle Control}} \right] \times 100$$

IC₅₀ values were derived for each compound using nonlinear regression analysis fit to a logistic equation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the South African government for permission to make this collection; collection permit # RES2013/43 issued by the South African Department of Environmental Affairs. Financial support from the OSU College of Pharmacy is gratefully acknowledged, as well as support of JDS by an American Foundation for Pharmaceutical Education (AFPE) Pre-doctoral Fellowship; KLM and JEI by the NIH, the National Institute of General Medical Sciences, via GM122016-01; ABS by the NIH, the National Cancer Institute, via NCI-29033. We thank the Carter laboratory for provision of synthetic isomandelalide A as an authentic standard.

ABBREVIATIONS USED

2-DG	2-deoxyglucose
ANOVA	analysis of variance, ARC, Anion Relay Chemistry

ATCC	American Type Culture Collection
CAM	ceric ammonium molybdate
ECAR	extracellular acidification rate
ES⁺	positive mode electrospray ionization
FCCP	carbonylcyanide <i>p</i> -trifluoromethoxyphenylhydrazone
FBS	fetal bovine serum
MeCN	acetonitrile
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
OCR	oxygen consumption rate
PMA	phosphomolybdic acid
RP₁₈	reversed phase C ₁₈
SPE	solid phase extraction
TOCSY	total correlation spectroscopy
MEM	Minimal Essential Medium Eagle
DMEM	Dulbecco's Modified Eagle's Medium

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Figure 1.

Cultured HeLa cells show density-dependent changes in sensitivity to the metabolic inhibitors 2-deoxyglucose and mandelalide A (**1**). The metabolic phenotype of HeLa cells is influenced by culture conditions; low confluency cells are more sensitive to 2-deoxyglucose (2-DG), whereas high confluency cultures are more sensitive to **1**. Cells were seeded at 2,000, 6,000 or 10,000 cells/well and exposed to increasing concentrations of 2-DG (0.25 to 10 mM), synthetic **1** (100 to 1000 nM) or vehicle (0.1% DMSO) for 48h. Viability was determined by MTT assay with the viability of vehicle-treated cells defined as 100%. Bars

represent mean viability (** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$), relative to control (0.1 % DMSO) from at least three independent comparisons.

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Figure 2. Mandelalide B (2) inhibits ATP-linked mitochondrial respiration in living cells
 Real-time analysis of OCR in intact H292 lung cancer cells in response to: (A) oligomycin A (1 μ M) or (B) natural 2 (1 μ M), followed by sequential addition of FCCP (1 μ M) and rotenone/antimycin A (0.5 μ M). Natural 2 was also substituted for (C) FCCP (1 μ M) and (D) rotenone/antimycin A (0.5 μ M). Mitochondrial function was assessed using an XF-24 Extracellular Flux Analyzer (Seahorse Bioscience). OCR was measured continuously and compounds were injected automatically and sequentially at the time indicated. Graphs show a representative comparison (n=5 wells per data point) of an assay that was repeated twice with the same result.

Figure 3. Real-time analysis of mitochondrial respiration in response to natural mandelalides A–C and target-specific inhibitors of mitochondrial function

Real-time analysis of OCR in intact H292 lung cancer cells in response to: (A) oligomycin A ($1 \mu\text{M}$), (B) natural 1 ($1 \mu\text{M}$), (C) natural 2 ($1 \mu\text{M}$) or (D) natural 3 ($1 \mu\text{M}$) followed by sequential addition of FCCP ($1 \mu\text{M}$) and rotenone/antimycin A ($0.5 \mu\text{M}$). Mitochondrial function was assessed using an XF-24 Extracellular Flux Analyzer (Seahorse Bioscience). OCR was measured continuously and compounds were injected automatically and sequentially at the time indicated. Graphs show a representative comparison ($n=5$ wells per data point) of an assay that was repeated twice with the same result.

Figure 4. Mandelalide A (1) inhibition of complex V activity in isolated mitochondria

The % rate of ATP-dependent NADH oxidation relative to control was calculated from a series of time-dependent reactions carried out in the presence of increasing concentrations of synthetic **1** using isolated mitochondria from bovine heart as the source of complex V ATP synthase activity. Complex V activity was determined for each reaction by measuring NADH absorbance at 340 nm over time (captured every 30 s for 30 min) at 25°C. The reaction rate was calculated from the slope of each curve and plotted as % activity relative to vehicle control (0.2 % DMSO). Single saturating concentrations of apoptolidin A (1 μ M) and oligomycin A (12.5 μ M) were used as control inhibitors of complex V. Graph shows a representative titration that was repeated twice with the same result.

Figure 5. Comparative Analysis of Cell Viability in Response to New and Known Mandelalides Concentration-response profiles for new mandelalides **6–8** and **12** against human (A) HeLa cervical and (B) NCI-H460 non-small cell lung cancer cells relative to known mandelalides **1–4**. Cells were seeded at high starting density and exposed to increasing concentrations of each mandelalide (ranging from 1 nM to 3 μ M as indicated) or vehicle (0.1% DMSO) for 72 h. Cell viability was determined by MTT assay with the viability of vehicle-treated cells defined as 100%. Graphs represent mean viability $\pm$ SE ($n = 3$ wells per treatment) from at least three independent comparisons.

Figure 7.

Seco-mandelalide A methyl ester (**13**) produces no change in HeLa cell viability.

Concentration-response profiles for seco-mandelalide A methyl ester (**13**) and synthetic **1** against human HeLa cervical cells. Cells were exposed to increasing concentrations of synthetic mandelalides, as indicated, or vehicle (0.1% DMSO) for 48 h. Cell viability was determined by MTT assay with the viability of vehicle-treated cells defined as 100%.

Graphs represent mean viability $\pm$ SE ($n = 3$ wells per treatment) from at least three independent comparisons.

Scheme 1. Enantioselective total synthesis of mandelalide L (12) and seco-mandelalide A methyl ester (13) exploiting Anion Relay Chemistry

- a) (i) 2,3,5-trichlorobenzoyl chloride, Et₃N, toluene, rt, 5 h (ii) DMAP, rt, 2 h; (b) DDQ, DCM, pH7 buffer, 0 °C, 2.5 h; (c) **12f**, 2,6-di-*t*-butyl-4-methylpyridine, Tf₂O, Et₂O, -78 oC to -35 °C, 4 h; (d) Pd(OAc)₂, Cs₂CO₃, Et₃N, DMF, rt, 2 days; (e) K₂CO₃, MeOH, 0 °C, 7 h; (f) 2,3,5-collidine, octanoyl chloride, DCM, rt, 13 h; (g) HF, pyridine, rt.

Table 1

Cytotoxicity of natural mandelalides against human HeLa and NCI-H460 cells.

Macrocyclic Type	Test Compound	Cancer Cell Line; EC ₅₀ (95% C.I.) ^a	
		HeLa cervix (nM)	NCI-H460 lung (nM)
A	Mandelalide A (1)	9.9 (5 – 17)	11 (5.7 – 21)
A	Mandelalide L (12)	2.8 (1.0 – 6.4)	9.8 (2.7 – 32)
B	Mandelalide B (2)	16 (6.2 – 36)	44 (25 – 89)
C	Mandelalide C (3)	> 3,000	> 3, 000
C	Mandelalide D (4)	660 (352 – 1,200)	1,700
C	Mandelalide E (5)	1,900	2,000
C	Mandelalide F (6)	50 (26 – 100)	270 (130 – 620)
C	Mandelalide G (7)	> 3,000	> 3,000
C	Mandelalide H (8)	330 (190 – 660)	2,600
B	Mandelalide I (9)	2,000	910 (420 – 1,700)
B	Mandelalide J (10)	> 3,000	790 (320 – 2,700)
B	Mandelalide K (11)	> 3,000	2,400

^aMaximum concentration tested was 3 μ M; 95% C.I. not indicated for mandelalides with weak activity.