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Discovery of Mandelalide E and Determinants of Cytotoxicity for the Mandelalide Series

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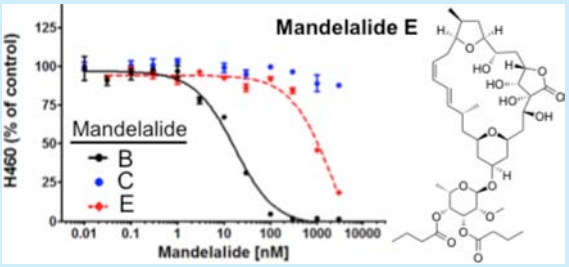
Supporting Information

ABSTRACT: Recollection of the tunicate source of the mandelalides has provided new and known analogues that have facilitated expanded analyses of the disputed cancer cytotoxicity of mandelalide A following a number of recent reported total syntheses. Using newly characterized mandelalide E, reisolated natural mandelalides B and C, and synthetic mandelalide A, the cytotoxicity of the mandelalides is demonstrated to be strongly influenced by compound glycosylation and assay cell density. Glycosylated mandelalides reduced the viability of human cancer cells cultured at a high starting density with a rank order of potency A > B ≫ E, yet display dramatically reduced cytotoxic efficacy against low density cultures.



Over the past decade alone, an average of ∼35 new, structurally diverse alkaloid, peptidic, and polyketide natural products have been documented annually from tunicates, many with important biological properties.[1,2] Numerous polyketide and peptide natural products isolated from tunicates are proposed, or proven, to be produced by their associated microorganisms,[3] which may ultimately prove important for an adequate supply of these natural products for clinical development. However, identification and expression of biosynthetic gene clusters from the metagenomes of filter-feeding sessile marine organisms such as tunicates and sponges is a labor intensive, case-by-case endeavor. Thus, total syntheses of complex natural products to confirm their absolute structures and preliminary biological activity are often pivotal to their further biological investigation/evaluation.[4] The recently described, rare *Lissoclinum* species of South African ascidian[5] has yielded mandelalide E (**1**), belonging to the structural suite published as mandelalides A−D (**2**−**5**) (Figure 1). Glycosylated mandelalides A (**2**) and B (**3**) were reported as cytotoxins with low nanomolar potency, while a paucity of material prevented testing of pure aglycones mandelalides C (**4**) and D (**5**). The relative configurations within the northern and southern hemispheres of the macrolides were assigned using homonuclear and heteronuclear coupling constants as well as ROESY correlations. The absolute configuration of the southern hemisphere of mandelalide A was then assigned by hydrolysis of the mandelalide A glycoside and chiral GC-MS analysis of the released monosaccharide.[5] Unfortunately, this configuration was not translated across the macrocycle to the northern hemisphere successfully. The Ye research group[6] later

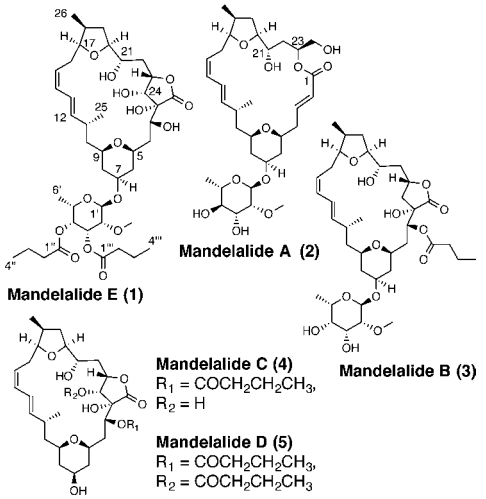


Figure 1. Chemical structures of new mandelalide E (**1**) and known mandelalides A−D (**2**−**5**).[5]

reported the total synthesis of mandelalide A and reassigned the absolute structure to a configuration where all five stereocenters in the northern hemisphere are inverted. Subsequently, Fürstner and co-workers confirmed this revision and predicted similar revisions for mandelalides B−D (**3**−**5**).[7] Most recently, the total synthesis of the revised mandelalide A and results of

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Table 1. Cytotoxic Effects of Mandelalides B, C, and E on Human Cancer Cells

	Cancer Cell Line; EC_{50} (95% C.I.)			
mandelalide	NCI-H460 lung	HeLa cervix	U87-MG glioblastoma	HCT116 colon
B (**3**)	25 nM (10–61)	23 nM (8.6–52)	61 nM (43–88)	54 nM (37–80)
C (4)	**inactive**	**inactive**	**inactive**	**inactive**
E (**1**)	2.0 μM (1.2–3.2)	1.9 μM (1.1–3.3)	>3 μM (2–6.2)	>3 μM (1–12)

cancer cytotoxicity testing have also been reported by the research groups of Altmann[8] and Carter.[9] Early reports of negligible biological activity by the Ye[6] and Fürstner[7] groups for synthetic mandelalide A, and recollection of the rare source tunicate, prompted our further investigation of the biological action of mandelalides A–C (**2**–**4**) and a newly discovered analogue, mandelalide E (**1**), for structure–activity relationships. Herein we report that the cytotoxicity of the mandelalides, besides being cell type-specific,[8] is remarkably influenced by cancer cell density, as well as by mandelalide glycosylation and butyration. These results lay the foundation for extended studies of the molecular mechanism and structure–activity relationships of the mandelalides.

The tunicate source of the mandelalides (*Lissoclinum mandelai*) was successfully recollected in 2013, after extended efforts, to provide material for reisolation of the authentic natural products, as well as for investigation of their biogenetic source. Mandelalides A–C (**2**–**4**) were reisolated and purified in low milligram quantities, which permitted expanded cytotoxicity testing with the major metabolite mandelalide B (**3**) and the first concentration–response analyses for a nonglycosylated analogue, mandelalide C (**4**). It should be noted that **2** isolated here was submitted to the NCI60 panel and, thus, was not tested in parallel with **1**, **3**, and **4**, all of which contain the furanone not present in **2**. Nevertheless, compounds **2** and **3** have been subjected to parallel testing on at least three occasions and **2** is consistently the more active. The relationship of the different macrocycles in **2** and **3** has not been established. The possibility that **2** is (1) a biosynthetic precursor to the furanone-containing mandelalides, (2) a biosynthetic product of an alternate macrocyclization mechanism, or (3) a biocatalyzed rearrangement product of the furanone-containing mandelalides must be considered. These considerations are currently under collaborative investigation.

LC-MS profiling of a chromatographic fraction containing significant amounts of targeted mandelalides **3** and **4** indicated the presence of a component with m/z 797. Purification of this component (mandelalide E, **1**) was pursued on the assumption that it was likely a glycosylated form of mandelalide D (**5**, 696 Da).

HR-ESI-MS data for mandelalide E (**1**) gave a pseudomolecular ion $[M + Na]^+$ at m/z 819.3796, for a molecular formula of $C_{41}H_{64}O_{15}$, and implied 10 degrees of unsaturation. Inspection of ^1H and ^{13}C NMR spectra for **1** (Figures S1, S2) showed signals consistent with a mandelalide-like macrocycle, a methylated sugar moiety, and two additional ester carbonyls with aliphatic carbons for two butyrates. Nevertheless, direct chemical shift comparisons with the data for the 3, 24-dibutyrated **5** revealed a different butyrate substitution for **1** and necessitated the acquisition of comprehensive 2D NMR experiments (Table S1). Surprisingly, COSY and TOCSY data for **1** indicated that two relatively deshielded signals for oxymethine protons (δ_H 5.20, 5.19 ppm) on butyryl-substituted carbons were associated with the monosaccharide, and not the macrocyclic spin system. The latter instead comprised corresponding oxymethine protons (δ_H 3.58–4.41) associated with hydroxylated carbons. This regiochemistry was supported by HMBC data (Table S1). The monosaccharide in **1** was assigned as 2-*O*-methyl-6-dehydro-α-L-talose based on NOESY correlations within the monosaccharide and between the monosaccharide and the macrocycle tetrahydropyran (Figure S8), and comparisons with coupling constant data for **3** (containing 2-*O*-methyl-6-dehydro-α-L-talose) and **2**, in which the epimeric 2-*O*-methyl-α-rhamnose was assigned following sugar hydrolysis and GC-MS analysis.[5] The variation in substitution pattern between mandelalides B–E suggests either abiotic spontaneous transacylation or that butyration is an independent biotransformation used as a means of self-resistance (producing organism) or detoxification (co-occurring organism).

Originally, glycosylated mandelalides A (**2**) and B (**3**) showed low nanomolar cytotoxicity to human lung cancer and mouse neuroblastoma cells while insufficient amounts of pure nonglycosylated mandelalides C (**4**) and D (**5**) prevented their biological testing.[5] With sufficient quantities of pure mandelalides **1**, **3**, and **4** in hand, the biological effect of the monosaccharide substituent was investigated. Natural products **1**, **3**, and **4** were tested in parallel against a panel of four human cell lines (NCI-H460 lung, HeLa cervical, U87-MG glioblastoma, and HCT116 colon cancer cells). Mandelalide B (**3**) was the most potent of these three compounds with EC_{50} values ranging from 22.9 to 61 nM against the four cell lines (Table 1), whereas mandelalide C (**4**) was inactive at concentrations up to 3 μM (Table 1, Figure S11). Mandelalide E (**1**) was at least 100-fold less potent than **3**, against all four cell lines, with EC_{50} values in the μM range (Table 1, Figure S11). However, **1** still produced a concentration-dependent cytotoxic response, especially against NCI-H460 and HeLa cells, and maintained a ranked order of potency against the four cell lines that matched that of **3**: HeLa > NCI-H460 > HCT116 ≥ U87-MG (Table 1, Figure S11). It is known that the presence and type of saccharide substituents can play a critical role in the pharmacological action of natural products.[10] The difference in activity between **1** and **3** also presents a potential explanation for the lack of cytotoxicity in the related madeirolide macrolides,[11] which contain the keto-sugar cinerulose. It is possible that the decreased hydrophilicity of the butyrated talose in **1** and the nonhydroxylated cinerulose of the madeirolides interfere with transport of these molecules into cells.[12,13]

The growth inhibitory or antiproliferative activity of synthetic mandelalide products have reported to be nonexistent,[7] weak,[6] or cell type-specific against a range of human cancer cell lines,[8] including those used in our original screen. Our reisolation of natural mandelalides A (**2**) and B (**3**) and supply of synthetic **2**[6] allowed us to reevaluate the biological activity of mandelalide A. The cytotoxic potential of natural and synthetic **2** was compared against human NCI-H460 lung cancer and mouse neuro-2A neuroblastoma cells under two different assay conditions (Figure S12, Table S2). Cells were seeded into

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standard 96-well plates at low starting density (3000 cells per well), or at high starting cell densities (25 000 cells per well), reflecting assay conditions comparable to those used in our original screen for novel cytotoxic structures. We found mandelalide A (**2**), from synthetic and natural sources, to be a potent, efficacious cytotoxin against cancer cells cultured at high starting density, as used in our original screen, yet was essentially nontoxic against the same cells exposed to **2** at low starting densities. It should be noted that we use "low density" and "high density" as relative terms and that cell seeding density should be optimized to reflect the growth characteristics of each particular primary or cancer cell line. However, lower seeding densities are generally used for antiproliferative screening against validated cancer cells to ensure optimal conditions for a phase of rapid exponential growth (logarithmic phase) during drug treatment.

Altmann et al.[8] recently published the synthesis and testing of mandelalide A against a panel of lung carcinoma lines (A549, H460, and H1299) and the neuroblastoma line SK-N-SH. Although mandelalide A effectively inhibited the growth of all three lung cancer cell lines with low nanomolar potency, the authors noted the absence of a complete cell kill in response to μM concentrations of **2** in any of the cell lines tested. Our present findings are in agreement with those of Altmann et al.,[8] in that H460 and neuro-2a cells were generally resistant to mandelalide A when exposed at low confluency and remained viable at the end of the experiment (Figure 2). In both studies

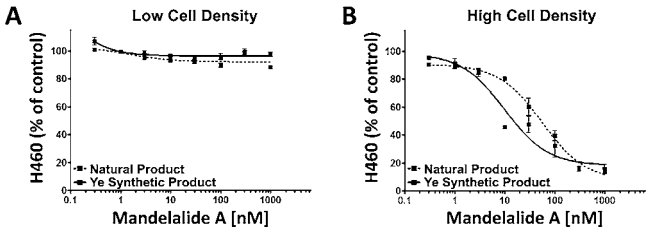

Figure 2. Relationship of cell density to mandelalide A induced cytotoxicity. Concentration–response relationships for natural mandelalide A[5] (dashed line, not repurified) and a synthetic mandelalide A[6] (purified) provided by Prof. Tao Ye (solid line) against human NCI-H460 cells seeded at low (panel A) or high (panel B) density.

mandelalide-induced decreases in cell viability were assessed by measuring a loss in the metabolic capacity of treated cells to reduce tetrazolium (MTT and WST-8, Sikorska et al.[5] and herein) or resazurin (Alamar Blue[8]) relative to control cultures. A loss in metabolic capacity (which, given the design of our original screening assay, we termed cytotoxicity) can result in varied or, with time, a continuum of cellular responses, including reduced proliferation, cytostasis, and cell death. A basic viability assay alone is rarely adequate to distinguish these potential responses to treatment and should be viewed as a starting point to justify further biological evaluation. In the case of the mandelalides an expanded pharmacological evaluation of this structural motif seems warranted. Comparative testing of mandelalide A and actinomycin D by Altmann et al.[8] revealed a distinct concentration–response relationship for each compound. Furthermore, recent testing of the synthetic C-24 macrolactone, isomandelalide A, revealed reproducible loss of cell viability for the ring-expanded isomer against high density cultures of NCI-H460 lung and HeLa cervical cells and contributes to the expanding biological profile of the mandelalides.[9] Our observation that mandelalide action is positively influenced by cell density *in vitro* is in direct contrast to the action of several classic cytotoxic drugs known to target DNA or tubulin such as doxorubicin, daunorubicin, vincristine, and cytosine arabinoside that were shown to have significantly reduced cytotoxic efficacy against leukemic cells as cell density increases.[14,15] This so-called negative effect of cell density *in vitro* has also been described for paclitaxel in adherent cultures,[16] and together these patterns have generally been attributed to both decreases in cellular uptake and/or an inability of the drug to saturate all cellular binding sites as cell density, and number of binding targets, increases.[15,17] Instead, the positive relationship between cytotoxicity and cell density observed for mandelalide A is more consistent with a pattern that has been described for some platin-based chemotherapy drugs. Cells show density-dependent sensitivity to cisplatin and oxaliplatin under conditions that favor increased cell-to-cell contact and the development of functional gap junctions.[18−20] The ability of death signals to then move between adjacent cells via functional gap junctions subsequently enhances the cytotoxic efficacy of cisplatin. In turn, the significance of intercellular communication as a contributing factor in cisplatin-induced toxicity is also highlighted by the finding that reduced gap junctional signaling and varied expression of connexin channels contribute to cisplatin resistance.[18−20] At present it is unclear if the relationship between cell density and mandelalide action corresponds to expression of the as yet unknown mandelalide cellular target(s), or is of relevance to any selective action of a mandelalide-like molecule *in vivo*. Thus, differences in basic assay conditions provide the most reasonable explanation for the reported disparity in potency and cytotoxic efficacy of mandelalide A by several independent research groups.[6−9] Together these findings have important consequences for bioassay-guided natural products drug discovery efforts, as potentially exciting hits may easily be missed with routine end-point bioassays even in common cancer cell lines. Insights gained from the various bioassays performed to date support our current pursuit of the biological mechanism of action of the mandelalides.

In conclusion, the discovery of mandelalide E (**1**) and reisolation of mandelalides A–C (**2**–**4**) permitted comparative biological testing to reveal the necessity for a monosaccharide substituent with free hydroxyls for activity. The new biological test results for synthetic mandelalide A support our original claim that the glycosylated mandelalides are potent cytotoxins and reveal intriguing cell density dependence of their biological activity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b00308.

Experimental section, NMR data tables and spectra for **1**, and concentration–response curves for cytotoxicity assays (PDF)

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Notes

The authors declare no competing financial interest.

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