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Production of Cytotoxic Glidobactins/Luminmycins by *Photorhabdus asymbiotica* in Liquid Media and Live Crickets

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Abstract

Photorhabdus asymbiotica engages in a two-part life cycle that requires adaptation to both symbiotic and pathogenic phases. The genome of *P. asymbiotica* contains several gene clusters, which are predicted to be involved in the biosynthesis of unique secondary metabolites that are hypothesized to enhance the bacterium's pathogenic capabilities. However, recent reports on *Photorhabdus* secondary metabolite production have indicated that many of its genes are silent under laboratory culture conditions. Using a circumscribed panel of media and alternative fermentation conditions, we have successfully achieved the production of a series of new and known glidobactin/luminmycin derivatives from *P. asymbiotica* including glidobactin A (**1**), luminmycin A (**2**) and luminmycin D (**3**). These compounds were also obtained upon infection of live crickets with the bacterium. Luminmycin D showed cytotoxicity against human pancreatic cells (IC_{50} of 0.11 μM), as well as proteasome inhibition (IC_{50} of 0.38 μM).

Photorhabdus spp. live in association with a variety of soil-dwelling entomopathogenic *Heterorhabditis* nematodes.[1] After the nematode host infects an insect, the bacterial symbiont is released, which kills the insect and provides the nematode a rich food source for growth and reproduction.[2] Once the insect food resources are exhausted, the bacteria recolonize the nematode, which reemerges from the insect carcass to begin its search for new prey.[1] These distinct environments, the nematode gut and the insect hemolymph, induce the differentiation of *Photorhabdus* spp. into what are described as two phase variants.[3] Phase I (the pathogenic phase) occurs in the insect hemolymph and is characterized by the production of a variety of proteases, lipases and small molecules. Phase II (the mutualistic phase) occurs in the nematode gut and is categorized by a general lack of production of most phase I extracellular digestive enzymes and other organic substances.[4,5]

Among the many described *Photorhabdus* spp., *Photorhabdus asymbiotica* has received less attention from chemists and biologists compared to its better known sister species *Photorhabdus luminescens* and *Photorhabdus temperata*, as well as closely related species

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Supporting Information

antiSMASH data output, media formulations, NMR, IR and HRESIMS spectroscopic data for **3** and *in vitro* cytoxicity and proteasome inhibition against normal mouse fibroblasts using **1**, **2**, and **3**. This material is available free of charge via the Internet at http://pubs.acs.org

Notes

The authors declare no competing financial interests.

in the genus *Xenorhabdus*.[3] While it is believed that *P. asymbiotica* engages in a life cycle similar to other *Photorhabdus* and *Xenorhabdus*, it is apparent that *P. asymbiotica* has an additional confirmed role as a human pathogen.[6,7] It has been reported that the genome of *P. asymbiotica* contains a large number of virulence loci and secondary metabolite biosynthetic gene clusters.[8] The combination of an intriguing lifecycle and capacity to generate a variety of secondary metabolites makes *P. asymbiotica* an attractive organism to investigate due to its ecological impact and human health concerns.

The genome of *P. asymbiotica* ATCC43949 has been previously sequenced and annotated.[9] Our reexamination of the genome using the secondary-metabolite gene-cluster search tool antiSMASH[10] revealed 19 biosynthetic gene clusters (Supporting Information, Table S1), which included a group of genes that were decidedly similar to the gene cluster attributable to glidobactin production[11] (Supporting Information, Figure S1). A recent publication had supported the hypothesis that a closely related *Photorhabdus* sp. was capable of producing glidobactin-like compounds.[12] Moreover, the same group recently verified the presence of a glidobactin/luminmycin gene cluster in *P. luminescens via* heterologous expression in *Escherichia coli* because this gene cluster was considered to be a silent biosynthetic pathway when the bacterium was cultured in the laboratory.[13] Knowing that the glidobactins/luminmycins are potent proteasome inhibitors,[14] we used a combination of LC-MS and bioassay analysis to test for the presence of these compounds. However, our initial tests growing the bacterium in tryptic soy broth (TSB), a medium in which *P. asymbiotica* grows remarkably well, resulted in no LC-MS evidence for glidobactins/luminmycins and produced extracts that tested negative for cytotoxicity. Expanding on the basic TSB recipe, a variety of supplements and changes in the medium formulation were tested including: altering the amount of dextrose and peptone in the medium; the addition of soil extract, ground invertebrates, nematodes, and sterile blood; and alternative carbon sources (sucrose, glucose and ribose) (Supporting Information, Table S2). However, the ethyl acetate extracts of these cultures also proved negative for glidobactins/luminmycins and cytotoxicity. Consequently, several other media formulations were tested (Supporting Information, Table S2) including Luria broth, potato dextrose broth, and a defined medium used for the induction of secondary metabolites in *Streptomyces*.[15] In each case, the ethyl acetate extracts generated from cultures grown in these media demonstrated cytotoxicity. Moreover, the culture prepared in the defined medium exhibited a golden-red hue that was quite distinctive from the colors of all the other cultures (*e.g.*, pale yellow to cream colored). Analysis of the culture grown in defined medium by LCMS led to the observation of several new peaks (Figure 1), including four substances that were indicative of glidobactins (based on their mass spectrometric and UV spectroscopic data). Subsequent bioassay-guided isolation led to the purification and dereplication of two known compounds, glidobactin A (**1**)[16] and luminmycin A (**2**).[13] In addition, two presumably new (based on mass spectrometric analysis) glidobactin/luminmycin derivatives were also purified resulting in the chemical characterization of a new analogue, luminmycin D (**3**). Unfortunately, the other new metabolite, which eluted from C_{18} between **1** and **2** and exhibited a *m/z* 519 (presumably representing the $[M+H]^+$ quasi-molecular ion) degraded rapidly upon purification before its structure was determined.

Compound **3** was obtained as a white solid. HRESIMS analysis indicated that the metabolite possessed the molecular formula $C_{28}H_{46}N_4O_5$. The ^1H and ^{13}C NMR data for **3** were similar to the data collected for **2**. Specifically, the carbon and proton chemical shifts throughout the 12-membered ring and much of the polyketide tail were nearly identical for the two compounds. However, inspection of the ^1H NMR spectrum revealed a new methyl signal that was not present in **2**. In addition, the H-11" methyl protons in **2** were shifted downfield from 1.25 ppm to 1.50 ppm in **3**, while the ^{13}C NMR signal of its associated carbon atom was shifted from 22.1 ppm to 27.0 ppm. Inspection of the 2D NMR data for **3**

provided unambiguous evidence that the new methyl group was part of a terminal isopropyl unit. The configuration of the three *trans* double bonds were confirmed by analysis of their respective coupling constants (Δ^3 J = 15.4 Hz, $\Delta^{2"}$ J = 15.2 Hz, and $\Delta^{4"}$ J = 15.2 Hz). Comparisons of the chemical shifts and coupling constants of the proton resonances attached to each of the asymmetric carbon atoms in **3** further supported the metabolite's strong similarity with **2**. Since the specific rotation data for the co-occurring metabolites **1** and **2** were identical to those values previously reported,[13, 17] and we presume that compound **3** shares the same biogenic origins, the absolute configuration of the new metabolite was deduced to be the same as the other analogues. Metabolite **3** has been given the trivial name luminmycin D.

The distinct lifecycle variations experienced by *P. asymbiotica* during its symbiotic and pathogenic periods indicates that tight regulation of secondary metabolite production must be essential to the bacterium and the host nematode's survival.[3] Our observations pertaining to the marked variation in the *P. asymbiotica* metabolome in different media types appear to be consistent with the idea that the bacterium requires specific environmental cues to induce secondary metabolite production. Paralleling our studies, a recent report from the Muller group asserted that the biosynthetic genes for glidobactins/luminmycins in *P. luminescens* were silent, which led the authors to pursue a heterologous expression-based approach for their production.[13] The challenges of producing *Photorhabdus* metabolites highlighted in this report, coupled with our successful production of glidobactins/luminmycins *via* fermentation in liquid media, led us to propose that an insect might provide an appropriate trigger for inducing the production of metabolites by *P. asymbiotica*. Accordingly, *P. asymbiotica* cells were grown in a nonglidobactin/ luminmycin-inducing TSB medium and injected into live crickets. After 24 hours post-inoculation, we observed that all of the *P. asymbiotica*-infected crickets were dead. The insect carcasses were ground and extracted with ethyl acetate. Examination of the extracts by LC-MS confirmed that all four of the glidobactin/luminmycin metabolites produced by *P. asymbiotica* in the defined medium were also present in the cricket corpus (Figure 2). These data further reinforce the theory that insects can provide the requisite environment for stimulating glidobactin/luminmycin production as part of *P. asymbiotica*'s phase I growth strategy.

Metabolites **1–3** were evaluated for toxicity against human pancreatic cancer cells to assess their relative potencies (Figure 3A). Compounds **1** and **2** exhibited similar activity profiles, although **1** was more potent with an IC_{50} value of 7.0×10^{-3} μM (95% confidence interval: 0.0044–0.011 μM) compared to an IC_{50} value of 0.018 μM (95% confidence interval: 0.011–0.028 μM) for **2**. In contrast, compound **3** was surprisingly much less active with an IC_{50} value of 0.11 μM (95% confidence interval: 0.066–0.17 μM). The compounds exhibited relatively less toxicity against mouse fibroblasts (Supporting Information, Figure S9).

Since **1** is known to inhibit the proteasomes,[14] all of the compounds were analyzed in a proteasome inhibition assay (Figure 3B). The results of these experiments showed a similar pattern of inhibition compared to the cancer cell cytotoxicity tests with **1** being slightly more active than **2** and much more active than **3** (IC_{50} values of 0.069, 0.88, and 0.38 μM; and 95% confidence intervals of 0.024–0.19, 0.059-0.13, and 0.32–0.44 μM, respectively).

We have used a combination of sequence analysis and culture medium optimization studies to isolate a new member of the luminmycin metabolite family. In addition, we have demonstrated that the production of these compounds is generally suppressed under a variety of common laboratory culture conditions. The results of the *in vivo* experiments involving live crickets serves to further highlight the important roles that specific environmental triggers play in inducing phase variant differentiation and secondary metabolite production in *P. asymbiotica*.

EXPERIMENTAL SECTION

General Experimental Procedures

UV data were collected on a Hewlett Packard 8452A diode array spectrophotometer. IR data were collected on a Shimadzu IRAffinity FTIR. Optical rotation data was determined on an AutopolIII automatic polarimeter. NMR data were collected on Varian VNMR 400 MHz NMR and Varian 500 MHz instruments. Accurate mass data were collected on an Agilent 6538 HRESI QTOF MS coupled with an Agilent 1290 HPLC. Metabolite profiles were obtained on a Shimadzu LCMS 2020 system (ESI quadrupole) coupled to a photodiode array detector. LC-MS samples were separated using a Phenomenex Kinetex column (2.6 μm C_{18} column, 100 A, 75×3.0 mm).

Microorganism and Culture Conditions

Photorhabdus asymbiotica ATCC 43949 was purchased from ATCC (originally deposited as *Xenorhabdus luminescens*). For the media panel tests, starter cultures of the bacterium were prepared in TSB at room temperature on a rotary shaker at 120 rpm for 12 h. Flasks (1 L Erlenmeyer) containing 300 mL of autoclaved medium (refer to Supporting Information, Table S1 for a list of the media tested, their formulations, and their glidobactin/luminmycin induction properties) were seeded with 1 mL of inoculum and allowed to grow for 1 week at 25 °C on a rotary shaker at 120 rpm. For the scale-up metabolite production, a starter culture of the bacterium was prepared in TSB at room temperature on a rotary shaker at 120 rpm. After 12 hours, the starter culture was used to inoculate a 20 L bioreactor containing the defined medium [15] [reported in $g \cdot L^{-1}$: KH_2PO_4 (2.0), NH_4Cl (1.5), $MgSO_4 \cdot 7H_2O$ (0.5), glycerol (10), *myo*-insitol (0.4), monosodium L-glutamate monohydrate (5.0), NaF (0.084), $FeSO_4 \cdot 7H_2O$ (0.025), $ZnSO_4 \cdot 7H_2O$ (0.01), $CoCl_2 \cdot 6H_2O$ (0.01), $CaCO_3$ (0.25), *p*-aminobenzoate (0.001)]. No pH adjustments were performed. The culture was stirred at 20 rpm at 25 °C for 1 week.

Extraction and Isolation

A bioactivity-guided fractionation procedure was followed for metabolite purification. The scale-up culture was removed from the bioreactor and extracted three times with EtOAc (1:1 vol.:vol.). The organic layers were removed, combined, and dried under reduced pressure on a rotary evaporator. The resulting crude extract (3.46 g) was absorbed onto silica and subjected to fractionation by flash chromatography using a hexane-CH_2Cl_2-MeOH gradient. The bioactive fractions (340 mg) were further fractionated by C_{18} HPLC. A MeOH-H_2O gradient (20% MeOH held for 5 minutes, 20–100% MeOH for 50 minutes, 100% MeOH held for 10 minutes) at flow rate of 10 mL/min was used to elute the glidobactin/luminmycin metabolites. This procedure provided an active fraction that was further purified by C_{18} semi-preparative HPLC (60% isocratic MeCN) to provide **1** (3.1 mg), **2** (2.6 mg), and **3** (0.6 mg).

Luminmycin D (3)

white solid; $[\alpha]_D$ (c 0.06, MeOH) −14.5; UV(MeOH) λ_{max} 256 nm (ε 20,511); IR (film) ν_{max} 3421, 1643cm^{-1}, ^1H and ^{13}C NMR, see Table 1; HRESIMS *m/z* 519.3543, $[M+H]^+$ (calcd for $C_{28}H_{47}N_4O_5$, 519.3541).

Production of Glidobactin/Luminmycin in Crickets

Crickets (*Acheta domestica*) were purchased from Timberline Fisheries through a local distributor. Live crickets were inoculated using modifications to a published procedure.[18] The *P. asymbiotica* inoculum was grown in a non-glidobactin/luminmycin-inducing TSB medium. Bacteria were obtained by centrifugation (8000 ×g), washed with PBS, and diluted

to a standardized concentration so that 10^8 cells were delivered in a 2 μL injection.[19] Crickets were chilled on ice until they appeared lethargic and were injected between the third and fourth abdominal segments with *P. asymbiotica*. Control crickets were injected with sterile PBS. The treated crickets were given access to water and food (corn meal) and left at 25 °C in the light. After 24 h, all of the control crickets were alive whereas the crickets treated with *P. asymbiotica* were dead. The crickets from each treatment group were processed separately by flash freezing in liquid nitrogen, crushing with a mortar and pestle, and extracting with EtOAc. The EtOAc was removed under vacuum and the resulting organic residue was suspended in 9:1 MeOH-H_2O at a concentration of 10 mg/mL for LC-MS analysis.

Cell Cytotoxicity Testing

Mammalian cell cytotoxicity assays were performed on pancreatic cancer (MIA PaCa-2) and mouse fibroblast cells (NIH/3T3) by adding 2,000 cells per well into 96-well plates. The cells were allowed to adhere overnight at 37°C in a humidified incubator (5% CO_2 atmosphere). Test compounds were diluted in DMSO and added to the wells so that the final concentration of DMSO per well did not exceed 1% by volume. The plates containing treated and control cells were incubated for 72 hours and cell viability was determined by MTT assay.[20]

20S Proteasome Inhibition Assay

The proteasome inhibition assay was performed using a colorometric test kit purchased from Cayman Chemical (item number 10008041). Briefly, cells were seeded at a density of 50,000 cells/well and incubated overnight at 37°C. Cells are treated with compounds for 90 minutes, the plate centrifuged, assay buffers were added, and the results read with a fluorescence plate reader (the excitation and emission wavelengths were 360 nm and 480 nm, respectively). The assays were performed using pancreatic cancer (MIA PaCa-2) and mouse fibroblast cells (NIH/3T3).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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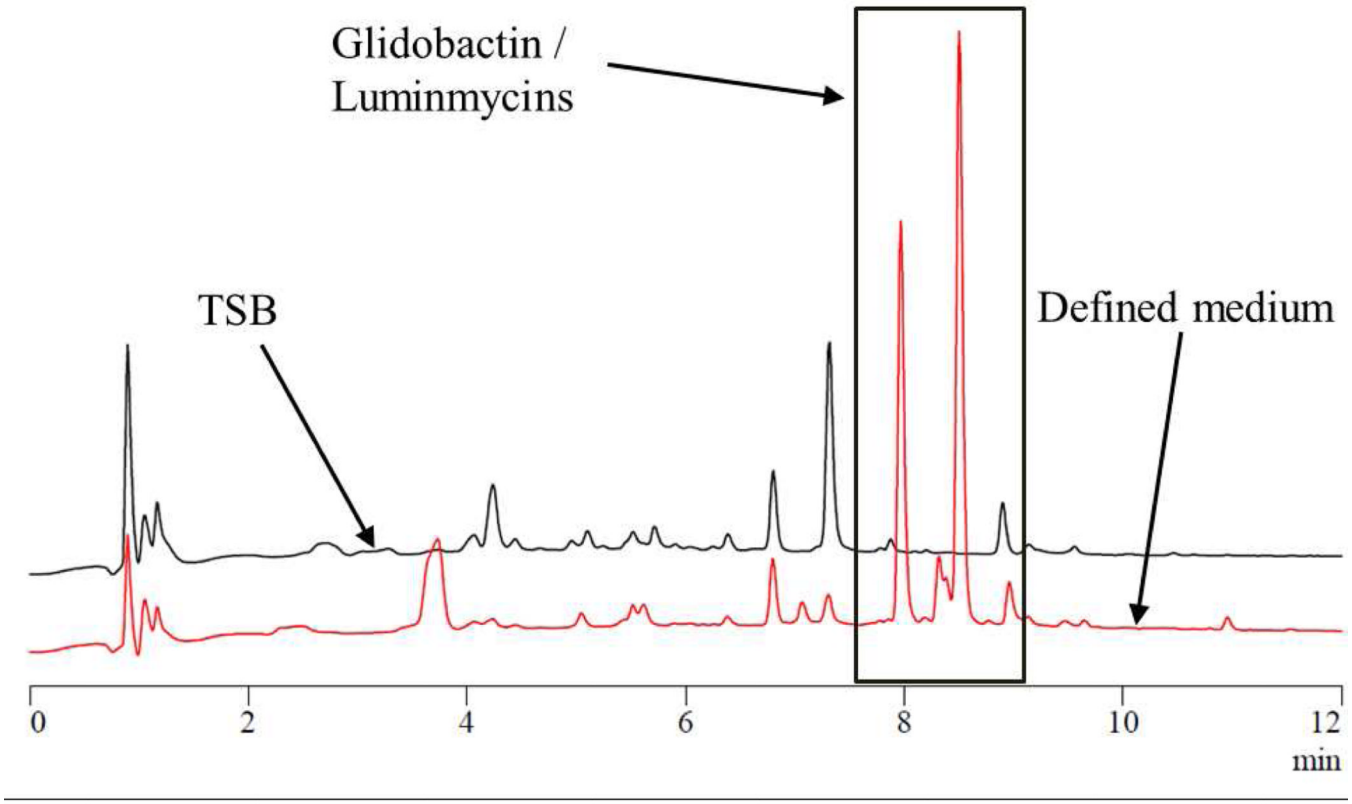


Figure 1.
LC-MS traces (254 nm) of *P. asymbiotica* in TSB (red, top trace) and defined medium (black, lower trace). Note the appearance of glidobactin/luminmycin peaks appearing between 8–9 minutes (indicated by a box) in the defined medium and their absence in TSB. Refer to Figure 2 for an expansion of this region showing the retention times for each glidobactin/luminmycin metabolite.

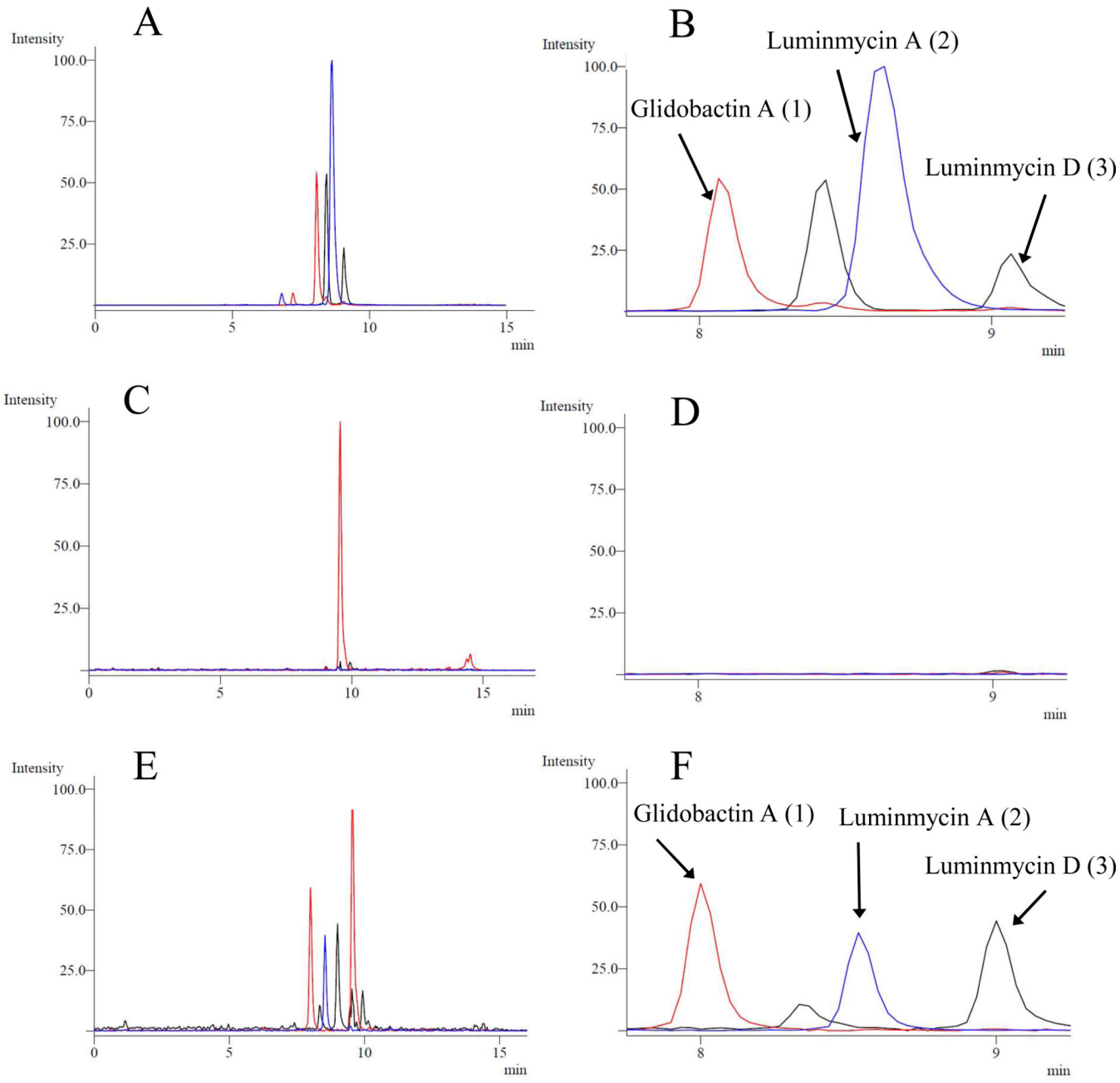


Figure 2.

Compilation of LC-MS selected-ion-trace profiles for glidobactins/luminmycins: *m/z* 521 for **1** (glidobactin A) in red, *m/z* 519 for **3** (luminmycin D) and the unidentified derivative in black, and *m/z* 505 for **2** (luminmycin A) in blue. Panel A illustrates the results from experiments using the defined medium, while panel B provides an expansion of the region containing the glidobactins/luminmycins eluting between 8–9 minutes. Panel C results obtained from the PBS (control) injected cricket (the large peak eluting just prior to 10 minutes is an endogenous cricket compound) with the expansion in panel D showing no glidobactins/luminmycins present in this sample. Panel E shows the LC-MS trace of crickets inoculated with *P. asymbiotica*, while panel F provides an expansion of the chromatogram detailing the glidobactins/luminmycins eluting between 8–9 minutes.

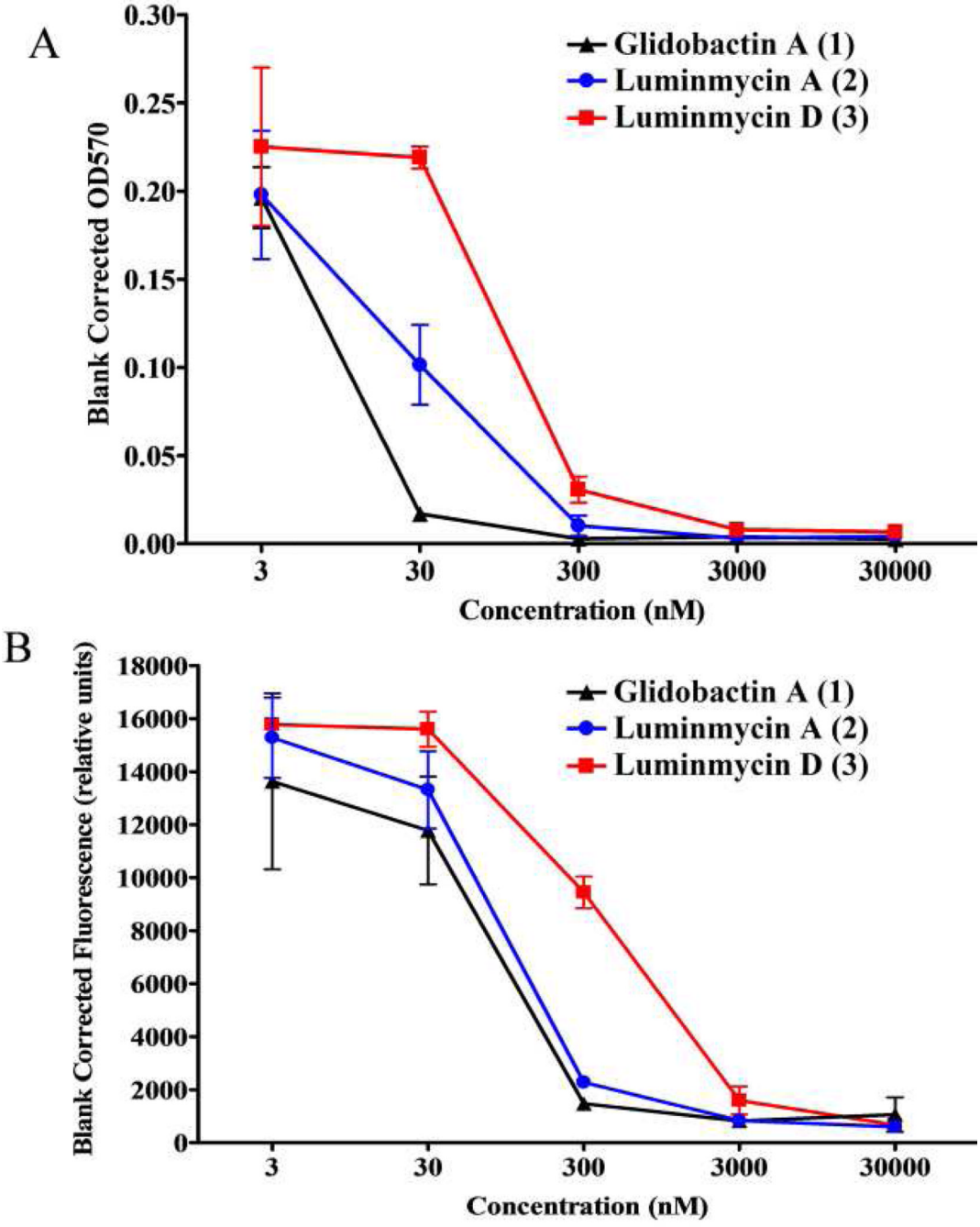
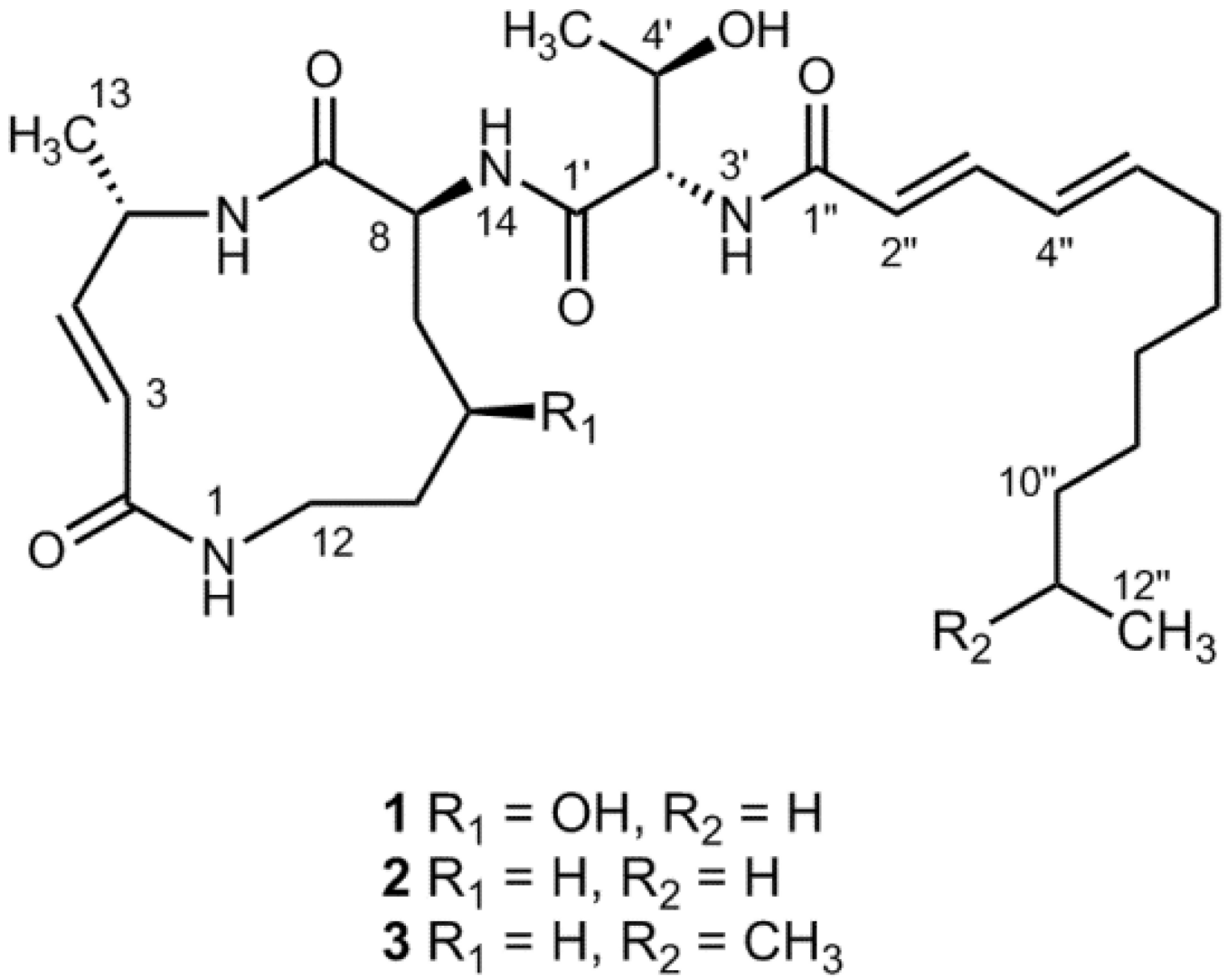


Figure 3.

In vitro human pancreatic cancer cell cytoxicity (panel A) and proteasome inhibition (panel B) for **1**, **2**, and **3**. All compounds were tested in triplicate at each concentration. Date points are shown as the means ± standard deviations.



1 R_1 = OH, R_2 = H
2 R_1 = H, R_2 = H
3 R_1 = H, R_2 = CH_3

Figure 4.

Table 1

^{1}H and ^{13}C NMR data (500 MHz, DMSO-d_6, 25 °C) for luminmycin D (**3**)

	Luminmycin D (3)	
Position	**δ_C, type[a]**	**δ_H (*J* in Hz)**
2	166.5, CH	
3	118.2, CH	6.23, m
4	146.4, CH	6.78, dd (15.4,4.6)
5	45.3, CH	4.41, m
7	171.0, C	
8	51.1, CH	4.51, m
9	29.5, CH_2	2.07, m
10	29.4, CH_2	1.69, m
11	29.5, CH_2	1.44, m
12a	37.7, CH_2	3.20, m
12b		2.94,m
13	18.0, CH_3	1.20, d (7.0)
1'	169.3, C	
2'	57.9, CH	4.32, dd (8.8,3.9)
4'	66.2, CH	3.99, m
5'	19.4, CH	1.02, d (6.4)
1"	165.9, C	
2"	122.7, CH	6.17, m
3"	139.4, CH	7.00, dd (15.2, 11.2)
4"	128.3, CH	6.17, dd (15.2)
5"	141.6, CH	6.09, m
6"	31.9, CH_2	2.13, q (7.0,7.0,7.0)
7"	28.1, CH_2	1.37, m
8"	28.6, CH_2	1.28-1.25, m
9"	28.6, CH_2	1.28-1.25, m
10"	31.0, CH_2	1.28-1.25, m
11"	27.8, CH	1.5, m
12"	23.0, CH_3	0.85, d (6.8)
13"	23.0, CH_3	0.85, d (6.8)
NH (1)		7.34, dd (6.6)
NH (6)		8.45, d (7.8)
NH (14)		7.62, d (7.8)
NH (3′)		8.01, d (8.8)
OH		4.94, d (4.4)

[a]The ^{13}C NMR data were obtained *via* inverse detection ^{1}H-^{13}C HSQC and ^{1}H-^{13}C *g*HMBC experiments.