Antiprotealide Is a Natural Product

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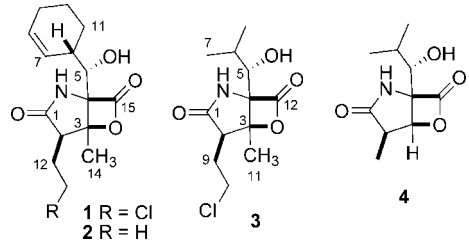
Large-scale fermentation of the marine actinomycete *Salinispora tropica* for production of salinosporamide A (NPI-0052; **1**) clinical trials materials provided crude extracts containing minor secondary metabolites, including salinosporamide B (**2**) and a new congener, **3**. Spectroscopic characterization revealed that **3** is identical to antiprotealide, a molecular hybrid of 20S proteasome inhibitors **1** and omuralide (**4**) not previously described as a natural product. Analysis of crude extracts from shake flask cultures of three wild-type *S. tropica* strains confirmed the production of antiprotealide at 1.1, 0.8, and 3.0 mg/L. Thus, antiprotealide is a natural product metabolite of *S. tropica*.

Small-molecule proteasome inhibitors have fueled the scientific evolution of the ubiquitin-proteasome system, from its recognition as a critical pathway in protein degradation in the cell to its emergence as an important target in cancer chemotherapy.[1−4] While several inhibitors are being developed as therapeutic agents, including bortezomib, which is FDA-approved for the treatment of relapsed and relapsed/refractory multiple myeloma,[5,6] others have maintained the status of research tools that have further fueled drug discovery and development efforts. In fact, representative proteasome inhibitors from diverse structural classes are required to fulfill growing R&D needs, including peptide derivatives (e.g., peptide aldehydes, vinyl sulfones, boronic acids, and epoxyketones) and members of the β-lactone-γ-lactam family.[4] The latter have long been represented by omuralide (**4**),[7−9] which became commercially available to researchers in 1997 from Calbiochem (EMD Chemicals). More recently, the salinosporamide family of β-lactone-γ-lactam proteasome inhibitors was identified from the marine environment, with salinosporamide A (NPI-0052, **1**) in clinical trials for the treatment of cancer.[10,11] Both compounds **1** and **4** have attracted considerable attention from the synthetic organic chemistry community,[12,13] and perhaps it was inevitable that a salinosporamide−omuralide hybrid would be developed. First synthesized and dubbed "antiprotealide" by E. J. Corey,[14] this hybrid synthetic analogue comprises the core structure of salinosporamide A with the omuralide-derived isopropyl group in place of the cyclohexene ring. In an effort to further fulfill the need for potent proteasome inhibitors in a research environment, antiprotealide was made commercially available by Calbiochem in 2005.



Despite the growing number of synthetic strategies toward the salinosporamides, clinical trial materials are being generated by a robust fermentation process using a single-colony isolate of the original wild-type organism *Salinispora tropica*. Large-scale manufacturing of **1** afforded extraneous materials enriched in related substances, including salinosporamide B (**2**).[15] During purification of compound **2** from these materials, we identified a peak by LC-MS for a congener not previously encountered in our laboratory. The compound was quickly found to be identical to antiprotealide. Thus, we report that this hybrid synthetic compound is a natural product, demonstrating for the first time that antiprotealide is produced by the wild-type organism.

The crude extract (72 g) obtained from a large-scale fermentation broth (350 L) was divided into three portions (24 g each), and each portion was purified on a silica 150 L Biotage column using an EtOAc/*n*-heptane solvent system. The fractions eluted in 40% EtOAc/*n*-heptane were monitored by TLC and analytical HPLC, and fractions that were highly enriched in **1** were pooled (pool A) separately from later-eluting fractions (pool B). The combined pool B fractions (3 g) derived from five individual 150 L Biotage columns were subsequently purified by silica flash column chromatography using a step gradient of *n*-heptane/EtOAc to obtain the minor compound **3** along with the major compounds **2** and **1**. In 40% EtOAc in *n*-heptane fractions, compound **3** coeluted with **2**; crystallization of the mixture from 1:1 *n*-heptane/EtOAc gave compound **2**, whereas compound **3** remained in the mother liquor. The mother liquor was further purified by semipreparative RP HPLC using a CH_3CN/H_2O linear gradient to obtain **3** as a pure compound.

Compound **3** was obtained as a white solid. Analysis of the low-resolution mass spectrum showed a characteristic $[M + H + 2]^+$ peak indicative of the presence of one chlorine atom. High-resolution mass spectroscopic analysis suggested a molecular formula of $C_{12}H_{19}{}^{35}ClNO_4$ (calcd for $[M + H]^+$ *m/z* 276.1003, obsd *m/z* 276.0990). The infrared spectrum of **3** showed characteristic bands of amide and β-lactone carbonyl groups (ν_{max} 1704 and 1824 cm^{-1}), which were further confirmed by ^{13}C NMR signals at δ_C 176.6 (C-1) and 167.7 (C-12) ppm, respectively, and an amide proton (δ_H 6.58, 1H, brs). The ^1H and ^{13}C NMR spectra further indicated the presence of an isolated methyl (δ_H 1.79, 3H, s, H_3-11; δ_C 18.3), a methine bearing an oxygen (δ_H 3.8, 1H, brs, H-5; δ_C 71.4), and a chloroethyl substituent [(δ_H 2.25 and 2.11, m, H_2-9, δ_C 28.0) and (δ_H 3.95 and 3.75, m, H_2-10, δ_C 42.3)]. Further inspection of the NMR data suggested the presence of an isopropyl group [2 methyl doublets, δ_H 1.08, (3H, d, J = 7 Hz, H_3-7) and 1.05 (3H, d, J = 7 Hz, H_3-8) that were correlated with a methine proton (δ_H 1.9, 1H, m, H-6) in the $^1H-^1H$ COSY spectrum]. By comparison of the spectroscopic data of **3** with that of salinosporamide A (**1**), it was clear that the cyclohexenyl group in **1** was replaced with an isopropyl group. The collective data indicated that **3** is a molecular hybrid of compounds **1** and **4**. The proposed structure was identical to the synthetic compound antiprotealide. Thus, the spectroscopic data for **3** were compared with those of synthetic antiprotealide[14] and found to be nearly identical, thereby confirming the structure. With respect to configuration, strong NOE correlations were observed between H_3-11 and H-5 as well as from

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Table 1. IC_{50} Values[a] (nM) for Inhibition of Purified Rabbit 20S Proteasome Chymotrypsin-like (CT-L), Trypsin-like (T-L), and Caspase-like (C-L) Activities and Cytotoxicity against Human Multiple Myeloma Cell Line RPMI 8226

compound	CT-L	T-L	C-L	cytotoxicity
1	4 ± 0.4	34 ± 1	364 ± 8	11 ± 2
3 (*S. tropica*)	31 ± 5	262 ± 7	>20 000	283 ± 55
3 (synthetic)	27 ± 2	211 ± 4	>10 000	220 ± 36

[a] IC_{50} values represent the mean ± standard deviation of three independent experiments.

Table 2. Production of Salinosporamides by *Salinispora tropica* Strains CNB440, CNB476, and NPS21184

S. tropica	**3** (mg/L)	**1** (mg/L)	**2** (mg/L)
CNB440	1.1	106	6.7
CNB476	0.8	87	6.8
NPS21184	3.0	277	4.4

H_3-11 to H-2; this compared favorably with published correlations for compound **1**, for which the absolute configurations were established by X-ray.[10] The optical rotation was in agreement with the synthetic compound.[14] These data support the absolute configuration assigned to **3**.

Evaluation of the biological activities of naturally occurring and commercially available synthetic compound **3** revealed comparable IC_{50} values for the *in vitro* inhibition of purified rabbit 20S proteasome chymotrypsin-like, trypsin-like, and caspase-like activities, as well as for inhibiting the cellular proliferation of the human multiple myeloma cell line RPMI 8226 (Table 1). In these assays, **3** was less potent than **1**.

The synthesis of antiprotealide[14] reflects the product of a biosynthesis conjoining elements of the salinosporamide A and lactacystin (omuralide precursor) pathways. Antiprotealide has also been generated as a product of the *S. tropica salX*$^-$ mutant, in which the pathway for the biosynthesis of the cyclohexenyl moiety (L-3-cyclohex-2′-enylalanine) of salinosporamide A has been silenced.[16] The production of antiprotealide by feeding leucine to the *S. tropica salX*$^-$ mutant was ~1 mg/L, and no antiprotealide was produced by the wild-type strain *S. tropica* CNB440.[16] Since we detected the production of antiprotealide in the large-scale fermentation of wild-type strain *S. tropica* NPS21184, this prompted us to examine the production of antiprotealide by three wild-type strains of *S. tropica*, CNB440, CNB476, and NPS21184, in the original shake flask fermentation (100 mL of medium in a 500 mL flask). We detected the production of antiprotealide by the *S. tropica* strains CNB440, CNB476, and NPS21184 in shake flask culture at a concentration of 1.1, 0.8, and 3.0 mg/L, respectively (Table 2). It is now confirmed that the pathway to **3** is active in the wild-type strains of *S. tropica*. The discrepancy in the observation of antiprotealide production by the wild-type strain *S. tropica* CNB440 by McGlinchey et al.[16] and in this study may be due to the different media used.

S. tropica CNB440 is the type strain isolated from a sediment sample by Jensen et al.[17] *S. tropica* strain CNB476 was isolated from another sediment sample by Jensen et al.[17] *S. tropica* NPS21184 is a single-colony isolate derived directly from strain CNB476 without any mutation or genetic modification.[18] The production of antiprotealide (**3**) and salinosporamide A (**1**) by strain NPS21184 is 3-fold higher than by strains CNB440 and CNB476 (Table 2). These three strains have different sensitivities to the medium ionic strength for maintenance of viability and growth;[18] therefore, it is not surprising to observe different productivities exhibited by these three strains. While the Sal B A domain appears to have relatively broad substrate specificity with a preference for aliphatic amino acids,[16] all naturally occurring salinosporamides reported to date bear a cyclohexene ring at C-5.[10,15,19] Identification of **3** as a natural product of *S. tropica* represents the first report of incorporation of an alternative amino acid substrate (i.e., leucine) by the wild-type organism.

Experimental Section

General Experimental Procedures. The melting point was measured on a MEL-TEMP Electrothermal instrument. The optical rotation was measured on a Rudolph Autopol III polarimeter. The UV spectrum was obtained from analytical HPLC analysis of the purified compound using an Agilent HP1100 HPLC equipped with an Agilent PDA detector; the mobile phase was a mixture of acetonitrile (CH_3CN) and H_2O. The IR spectrum was recorded on a Perkin-Elmer 1600 series FT-IR spectrometer. NMR spectra were collected using a 500 MHz Bruker Avance NMR spectrometer that was equipped with a broadband observe probe. The high-resolution mass spectrum was acquired using a Micromass Q-Tof2 mass spectrometer with ES+ ionization. HRESI spectra were referenced using a polyethylene glycol polymer mixture, which was co-injected during acquisition as an internal accurate mass standard. Additional ESIMS and crude extract analysis experiments were collected using an Agilent HP1100 HPLC equipped with an Agilent PDA detector and an 1100 series MSD Agilent mass spectrometer. Preparative-scale purifications were done on a Biotage FLASH 150i purification system using prepacked cartridges (FLASH 150 L KP-Sil). Additional normal-phase flash chromatography was performed using open flash columns (250 cc Si gel, column dimensions 2.5 cm diameter by 35 cm length). Reversed-phase HPLC was performed on a Gilson HPLC equipped with a Gilson 215 fraction collector and Agilent PDA detector with an Eclipse XDB-C18, 5 μm, 150 × 21 mm i.d. column at a flow rate of 14.5 mL/min. Silica gel TLC plates (with fluorescent indicator and aluminum support) were obtained from Sigma.

The human multiple myeloma cell line RPMI 8226 was obtained from ATCC (Manassas, VA). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate at 37 °C, 5% CO_2, and 95% humidified air. Synthetic antiprotealide (**3**) was obtained from Calbiochem (San Diego, CA). Compound **1** was obtained by normal-phase HPLC purification[19] of the crude extract from *S. tropica*.

Fermentation and Extraction. For production in shake flask culture, 5 mL of seed culture of *S. tropica* strains CNB440 (ATCC BAA-916^T), CNB476 (ATCC PTA-5275), and NPS21184 (ATCC PTA-6685) was inoculated into 100 mL of production medium in a 500 mL Erlenmeyer flask. The composition of production medium containing salt formulation I was described by Tsueng et al.[20] The production cultures were incubated at 28 °C and 250 rpm on a rotary shaker. At 24 h of the production cycle, 2 g of Amberlite XAD-7 resin was added to the culture. At 96, 120, and 144 h of the production cycle, aliquots (3.5 mL) of cultures were withdrawn and extracted with an equal volume of EtOAc. The EtOAc extracts were used for the determination of production of salinosporamides and antiprotealide by HPLC (against a standard curve). For production in fermentor culture, 18 to 20 L of seed culture of *S. tropica* NPS21184 was inoculated into 350 L of production medium in a 500 L fermentor. The composition of the production medium was the same as in shake flask culture supplemented with 30 mL of antifoam agent. The production culture was operated at 28 °C incubation temperature, 100 rpm agitation, 125 L/min aeration, and 4.5 psi back-pressure. At 34 to 38 h of the production cycle, 7.9 kg of Amberlite XAD-7 resin was added to the culture. At 96 to 102 h of the production cycle, the resin in the culture was recovered using a pressure filter unit (24 in. diameter) equipped with a 100 mesh stainless steel screen. After washing the resin with water, the resin was extracted 4 to 6 times with 25 to 30 L of EtOAc. The combined extract was dried *in vacuo*. The dried extract was then processed for the recovery of salinosporamides.

Purification of 3. The crude extract (72 g) obtained from the 350 L fermentation broth was divided into three portions (24 g each), and each portion was absorbed on silica gel and loaded onto a silica 150 L Biotage column using a sample injection module (SIM). The column was eluted with 12 column volumes (CV) of 40% EtOAc/*n*-heptane and collected as 2 × 1 CV fractions followed by 1/4 CV fractions. The fractions were monitored by analytical HPLC and TLC. TLC plates were developed with 40% EtOAc/hexanes and visualized after spraying with 5% w/v phosphomolybdic acid/ethanol and heating. Fractions containing **1** were combined as pool A (~2.5 to ~6 CV) and later eluting fractions as pool B (~6 to 12 CV). All pool B fractions obtained

from five individual Biotage 150 L columns (derived from two fermentation extracts) were combined to yield a mixture (3 g) of **2** (major; 43.6% UV area), **1** (12.9% UV area), **3** (minor; 1.5% UV area), and other minor compounds (see Supporting Information for HPLC profile) including salinosporamides D and E[19] (10% UV area). Compound **3** was purified along with the major compounds **2** and **1** by silica flash column using a step gradient of *n*-heptane/EtOAc, increasing in the percentage of EtOAc in steps of 10% (200 mL of solvent per step). Compound **1** was eluted in 30% EtOAc in heptane fractions, whereas **3** coeluted with **2** in 40% EtOAc in *n*-heptane fractions. These two compounds were further separated by crystallization using 1:1 *n*-heptane/EtOAc solvent ratio to obtain compound **2** as a crystalline solid, while compound **3** remained in the mother liquor. The mother liquor was further purified by semipreparative RP HPLC in which the solvent gradient increased linearly from 20% CH_3CN/80% H_2O to 100% CH_3CN over 23 min. Under these conditions **3** eluted at 9 min as a pure compound (12 mg, yield 0.02 mg/L).

Compound 3: white solid; mp 138−140 °C; $[\alpha]^{22}_D$ −27 (*c* 0.3, $CHCl_3$);[21] UV (CH_3CN/H_2O) λ_{max} (log ε) 221 (sh) nm; IR (film) ν_{max} 3240, 2962, 2944, 1824, 1704, 1386, 1051, 832 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 6.58 (1H, brs, NH), 3.95 (1H, m, H-10a), 3.80 (1H, brs, H-5), 3.75 (1H, m, H-10b), 2.80 (1H, t, J = 7.5 Hz, H-2), 2.25 (1H, m, H-9a), 2.11 (1H, m, H-9b), 1.90 (1H, m, H-6), 1.79 (3H, s, H_3-11), 1.08 (3H, d, J = 7.0 Hz, H_3-7), 1.05 (3H, d, J = 7.0 Hz, H_3-8); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 176.6 (C, C-1), 167.7 (C, C-12), 85.7 (C, C-3), 78.3 (C, C-4),71.4 (C, C-5), 44.5 (CH, C-2), 42.3 (CH_2, C-10), 31.2 (CH, C-6), 28.0 (CH_2, C-9), 19.7 (CH_3, C-7), 19.6 (CH_3, C-8), 18.3 (CH_3, C-11); ESIMS *m/z* 276.0 $[M + H]^+$; 298.0 $[M + Na]^+$; HRESIMS *m/z* 276.0990 $[M + H]^+$ (calcd for $C_{12}H_{19}ClNO_4$, 276.1003).

Cellular Proliferation Assay. The assays were performed essentially as described by Mitchell et al.[22] Briefly, RPMI 8226 cells were plated at a density of 2×10^4 cells/well in 96-well plates, treated with serial diluted compounds, and incubated for 48 h. Cell growth was assessed using resazurin, a dye that is reduced by metabolically active cells. The IC_{50} values (the drug concentration at which 50% of the cellular growth is inhibited) were determined using a standard sigmoidal dose−response curve-fitting algoritm (XLfit 3.0, ID Business Solutions Ltd.).

***In Vitro* Purified Rabbit Muscle 20S Proteasome Activity Assays.** The chymotrypsin-like activity of the 20S proteasome was determined essentially as described previously.[23] Briefly, serial diluted compounds were added in duplicate to 1 μg/mL purified rabbit 20S proteasome in assay buffer containing 20 mM HEPES, pH 7.3, 0.5 mM EDTA, 0.05% Triton X-100, and 0.035% SDS and preincubated for 5 min at 37 °C. Reactions were initiated by the addition of the Suc-LLVY-AMC peptide substrate at a final concentration of 20 μM. Fluorescence of the cleaved peptide substrate was measured at λ_{ex} = 390 nm and λ_{em} = 460 nm using a Fluoroskan Ascent 96-well microplate reader (Thermo Electron, Waltham, MA). The IC_{50} values (the drug concentration at which 50% of the maximal relative fluorescence is inhibited) were calculated by Prism (GraphPad Software) using a sigmoidal dose−response, variable slope model. The caspase-like activity of the 20S proteasome was determined as described above except that Z-LLEAMC was used as the peptide substrate. For the evaluation of the trypsin-like activity, the SDS was omitted from the assay buffer, and Boc-LRR-AMC was used as the peptide substrate.

Supporting Information Available: ^1H, ^{13}C NMR and $^1H-^1H$ COSY NMR spectra of compound **3**. HPLC profile of fraction pool B. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Adams J. *Proteasome Inhibitors in Cancer Therapy*; Humana Press: Totowa, NJ, 2004.
(2) Hershko, A.; Ciechanover, A. *Annu. Rev. Biochem.* **1998**, *67*, 425–479.
(3) Orlowski, R. Z.; Kuhn, D. J. *Clin. Cancer Res.* **2008**, *14*, 1649–1657.
(4) Kisselev, A. F.; Goldberg, A. L. *Chem. Biol.* **2001**, *8*, 739–758.
(5) Bross, P. F.; Kane, R.; Farrell, A. T.; Abraham, S.; Benson, K.; Brower, M. E.; Bradley, S.; Gobburu, J. V.; Goheer, A.; Lee, S.-L.; Leighton, J.; Liang, C. Y.; Lostritto, R. T.; McGuinn, W. D.; Morse, D. E.; Rahman, A.; Rosario, L. A.; Verbois, S. L.; Williams, G.; Wang, Y.-C.; Pazdur, R. *Clin. Cancer Res.* **2004**, *10*, 3954–3964.
(6) Richarson, P. G.; Barlogie, B.; Berenson, J.; Singhal, S.; Jagannath, S.; Irwin, D.; Rajkumar, S. V.; Srkalovic, G.; Alsina, M.; Alexanian, R.; Seigel, D.; Orlowski, R. Z.; Kuter, D.; Limentani, S. A.; Lee, S.; Hideshima, T.; Esseltine, D. L.; Kauffman, M.; Adams, J.; Schenkein, D. P.; Anderson, K. C. *N. Engl. J. Med.* **2003**, *348*, 2609–2617.
(7) Fenteany, G.; Standaert, R. F.; Reichard, G. A.; Corey, E. J.; Schreiber, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3358–3362.
(8) Omura, S.; Fujimoto, T.; Otoguro, K.; Matsuzaki, K.; Moriguchi, R.; Tanaka, H.; Sasaki, Y. *J. Antibiot.* **1991**, *44*, 113–116.
(9) Omura, S.; Matsuzaki, K.; Fujimoto, T.; Kosuge, K.; Furuya, T.; Fujita, S.; Nakagawa, A. *J. Antibiot.* **1991**, *44*, 117–118.
(10) Feling, R. H.; Buchanan, G. O.; Mincer, T. J.; Kauffman, C. A.; Jensen, P. R.; Fenical, W. *Angew. Chem., Int. Ed.* **2003**, *42*, 355–357.
(11) Chauhan, D.; Catley, L.; Li, G.; Podar, K.; Hideshima, T.; Velankar, M.; Mitsiades, C.; Mitsiades, N; Yasui, H.; Letai, A.; Ovaa, H.; Berkers, C.; Nicholson, B.; Chao, T.-H.; Neuteboom, S. T. C.; Richardson, P.; Palladino, M.; Anderson, K. C. *Cancer Cell* **2005**, *8*, 407–419.
(12) Shibasaki, M.; Kanai, M.; Fukuda, N. *Chem. Asian J.* **2007**, *2*, 20–38.
(13) Ling, T.; Macherla, V. R.; Manam, R. R.; McArthur, K. A.; Potts, B. C. M. *Org. Lett.* **2007**, *9*, 2289–2292.
(14) Reddy, L. R.; Fournier, J.-F.; Reddy, B. V. S.; Corey, E. J. *J. Am. Chem. Soc.* **2005**, *127*, 8974–8976.
(15) Williams, P. G.; Buchanan, G. O.; Feling, R. H.; Kauffman, C. A.; Jensen, P. R.; Fenical, W. *J. Org. Chem.* **2005**, *70*, 6196–6203.
(16) McGlinchey, R. P.; Nett, M.; Eustaquio, A.; Asolkar, R. N.; Fenical, W.; Moore, B. S. *J. Am. Chem. Soc.* **2008**, *130*, 7822–7823.
(17) Jensen, P. R.; Dwight, R.; Fenical, W. *Appl. Environ. Microbiol.* **1991**, *57*, 1102–1108.
(18) Tsueng, G.; Lam, K. S. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 873–880.
(19) Reed, K. A.; Manam, R. R.; Mitchell, S. S.; Xu, J.; Teisan, S.; Chao, Ta.-H.; Deyanat-Yazdi, G.; Neuteboom, S. T. C.; Lam, K. S.; Potts, B. C. M. *J. Nat. Prod.* **2007**, *70*, 269–276.
(20) Tsueng, G.; Teisan, S.; Lam, K. S. *Appl. Microbiol. Biotechnol.* **2008**, *78*, 827–832.
(21) The optical rotation was measured at a lower concentration compared to the synthetic product[14] because of the limited availability of **3**.
(22) Mitchell, S. S.; Nicholson, B.; Teisan, S.; Lam, K. S.; Potts, B. C. M. *J. Nat. Prod.* **2004**, *67*, 1400–1402.
(23) Stein, R. L.; Melandri, F.; Dick, L. *Biochemistry* **1996**, *35*, 3899–3908.

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