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Psammaplin A, a Chitinase Inhibitor Isolated from the Fijian Marine Sponge *Aplysinella Rhax*

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Abstract—Several brominated tyrosine derived compounds, psammaplins A (**1**), K (**2**) and L (**3**) as well as bisaprasin (**4**) were isolated from the Fijian marine sponge *Aplysinella rhax* during a bioassay guided isolation protocol. Their structures were determined using NMR and MS techniques. Psammaplin A was found to moderately inhibit chitinase B from *Serratia marcescens*, the mode of inhibition being non-competitive. Crystallographic studies suggest that a disordered psammaplin A molecule is bound near the active site. Interestingly, psammaplin A was found to be a potent antifungal agent.

Introduction

Chitinase is widely distributed among plants, microorganisms, marine invertebrates, fish and insects.[1] It plays a variety of important roles in these organisms ranging from nutrition to defence and control of ecdysis in insects. All these roles involve the degradation of chitin [β-(1,4)-linked homopolymer of *N*-acetyl-D-glucosamine units], which is mainly used as a structural polymer by these organisms. The degradation of the chitin molecule is achieved by a variety of chitinolytic enzymes that contribute to the degradation process in different ways. Exochitinases release short oligosaccharides (most often chitobiose) from the ends of the chitin chains. β-*N*-Acetyl glucosaminidases release *N*-acetylglucosamine monomers whereas endochitinases cleave randomly within the chitin chain. The importance of chitinases in many biological processes makes their inhibitors important targets for potential antifungal and insecticidal agents as well as antimalarial agents.[2]

There are few inhibitors of chitinase, the most studied of which is allosamidin, isolated from *Streptomyces* sp., with an IC_{50} of <1 μM.[3] Crystal structures of chitinase–allosamidin complexes give insights into the mode of action of this inhibitor, as well as the mechanism of action of the enzymes.[2,4] The more recently reported argadin, a modified cyclic peptide isolated from the fungus *Clonostachys* sp. FO-7314 has an IC_{50} against *Lucilia cuprina* chitinase of 3.4 nM at 20 °C.[5] Under the same conditions, allosamidin shows an IC_{50} of 0.4 nM. Another fungal strain, *Gliocladium* sp FTD-0668 produced the modified cyclic pentapeptide argifin with an IC_{50} of 100 nM against *L. cuprina* chitinase at 20 °C.[6] Crystallographic studies of a *Serratia marcescens* chitinase in complex with argadin/argifin has revealed ordered binding of these cyclic peptides in the active site. Two less potent inhibitors are also known, Cyclo(L-Arg-D-Pro), a diketopiperazine isolated from a marine bacterium, *Pseudomonas* sp.[7] (17% inhibition at 1 mM) and the styloguanidines isolated from the marine sponge *Stylotella aurantium*.[8]

This paper reports on the bioassay guided isolation of a brominated tyrosine derived natural product, psammaplin A, which moderately inhibits chitinases and displays anti-fungal activity. In addition, two

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†Graham Gooday passed away whilst this manuscript was being refereed. His presence in the chitinase research community will be greatly missed.

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psammaplins not previously reported in the literature are described.

Results and Discussion

Bioassay guided isolation

Small samples of sponges, soft corals (129) and seaweed (9) were collected by free diving from reefs in various locations in the Fiji Islands: Laucala bay, Suva harbour and Verata (Viti Levu); reefs in Wainunu and Dama and in the island of Vanua Levu. A small amount of each sample was extracted with ethanol, and the extracts were used in the preliminary chitinase inhibition bioassay. Out of the 138 samples screened, eight extracts showed between 22 and 50% inhibition versus the control in the preliminary assay. The organisms giving the lower inhibition values were not identified taxonomically. The strongest inhibitory activity was shown by the sponge *Aplysinella rhax* (Order Verongida, Family Druinellidae), giving a 50% inhibition versus the control. Verongid sponges are known to produce bromotyrosine derived metabolites such as the bastadins,[9] aeroplysinins,[10] psammaplysins,[11] and psammaplins,[12] and *A. rhax* is known to produce psammaplins.[13,14]

A larger re-collection of *A. rhax* showed a 50% inhibition compared to the control and was subjected to a bioassay-guided isolation procedure. Solvent partitioning of the crude extract followed by size exclusion chromatography of the dichloromethane fraction gave an active semi-purified fraction which was further purified by reversed-phase HPLC, yielding four pure compounds **1**–**4** (Table 1).

Compound identification

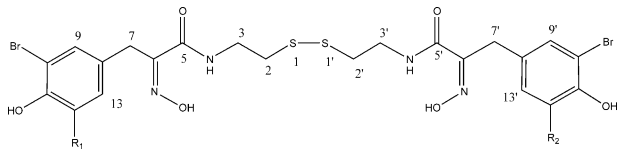
Comparison of spectroscopic experimental data of compound **1**, the predominant compound isolated, with that found in the literature showed this compound to be psammaplin A (Tables 2 and 3).[12,15]

The ^{13}C NMR spectrum of **2** was similar except that nearly every signal was doubled, suggesting that this compound had a similar gross structure, but was not symmetrical. Mass spectrometry indicated that an extra O was present (HRESIMS *m/z* 700.9385 $[M+Na]^+$ Δ −3.4 mmu from calculated for $C_{22}H_{24}S_2{}^{79}Br_2N_4O_7$). The ^{13}C NMR chemical shifts of C8′–C13′ indicated that C12′ was hydroxylated (Table 3). HMBC correlations from C12′ to H13′ and between C8′ to both H13′ and H9′ were observed indicating that the two hydroxyl groups belong to same benzene ring system.

Compound **3** contained only 11 resonances in its ^{13}C NMR spectrum with the chemical shifts of C8/8′–C13/13′ matching those of the dihydroxylated benzene ring chemical shifts C8′–C13′ in **2** (Table 3), suggesting that **3** was a symmetrical version of **2** with two dihydroxylated benzene rings, a fact which was confirmed by HRESIMS (*m/z* 694.9453 $[M+H]^+$ Δ 2.8 mmu calculated for $C_{22}H_{25}S_2{}^{79}Br_2N_4O_8$).

Comparison of spectroscopic experimental data of compound **4**, with that found in the literature showed this compound to be bisaprasin, a dimer of psammaplin A coupled at C12′ (Tables 2 and 3).[16] This was confirmed by the LRESIMS at *m/z* 1345.2 $[M+Na]^+$, consistent with the compound being dimeric. The isotope pattern also indicated that it contained four bromines.

Table 1. Structures and tumour cell IC_{50}'s for compounds **1**–**4**



Compound	R_1	R_2	IC_{50} (μM)	
			A2780 ovarian tumour	K562 leukaemia
1 (Psammaplin A)	H	H	1.2	0.71
2 (Psammaplin K)	H	OH	> 50	> 50
3 (Psammaplin L)	OH	OH	> 50	> 50
4 (Bisaprasin)	H	Dimer	> 50	> 50

Chitinase inhibition

A chitinase inhibition bioassay using *Bacillus* sp. chitinase of compounds **1**–**4** showed psammaplin A (**1**) to be the main active compound in this sponge. Compounds **2**–**4** showed no significant inhibition of this chitinase. The IC_{50} for **1** of 68 μM was moderate when compared to allosamidin, the most potent chitinase inhibitor known. The mode of inhibition was initially determined using the *Bacillus* sp. chitinase. The results showed psammaplin A had a dual mode of inhibition. At higher concentrations psammaplin A was an uncompetitive inhibitor whilst at lower concentrations it inhibited chitinase in a mixed non-competitive way. Psammaplin A was also assayed using the chitinase bioassay described by Gooday.[17] Preliminary results had shown strong inhibitions of endochitinase enzymes, particularly against the bacterial enzymes from *Streptomyces* with an IC_{50} of 50 μM.

More detailed enzyme kinetics studies were conducted with Chitinase B from *Serratia marcescens*. Initial results showed that psammaplin A had an IC_{50} of approximately 100 μM. This is higher than the IC_{50} values obtained for this inhibitor against chitinases from *Bacillus* sp. and *Actinomyces* sp. The mode of inhibition was determined by measuring kinetic parameters and by plotting the data in Lineweaver–Burk plots (Fig. 1). The results clearly show that psammaplin A acts as a non-competitive inhibitor. Calculations using EnzFitter confirmed that the inhibitor primarily affects V_{max} and not K_m. Using the standard reciprocal Michaelis–Menten equation for non-competitive inhibition, the K_i for psammaplin A was calculated to be 148 μM.

Table 2. ^{1}H NMR Data (δ/ppm, CD_3OD, 400 MHz, ppm, multiplicity, *J* in Hz) for compounds **1–4**

H	1	2	3	4
2	2.75 (2H, t, 6.9)	2.77 (2H, t, 6.8)	2.78 (2H, t, 6.6)	2.75 (2H, m)
3	3.49 (2H, t, 6.6)	3.48 (2H, t, 6.8)	3.50 (2H, t, 6.7)	3.49 (2H, m)
7	3.75 (2H, s)	3.68 (2H, s)	3.70 (2H, s)	3.82 (2H, s)
9	7.32 (1H, d, 1.8)	7.32 (1H, d, 2)	6.81 (1H, d, 2.0)	7.35 (1H, d, 2.0)
12	6.70 (1H, d, 8.2)	6.72 (1H, d, 8.0)	—	6.70 (1H, d, 8.5)
13	7.02 (1H, dd, 1.8, 8.4)	7.02 (1H, dd, 2.0,8.0)	6.68 (1H, d, 2.0)	7.02 (1H, m)
2′	2.75 (2H, t, 6.9)	2.77 (2H, t, 6.8)	2.78 (2H, t, 6.7)	2.75 (2H, m)
3′	3.49 (2H, t, 6.6)	3.48 (2H, t, 6.8)	3.50 (2H, t, 6.7)	3.49 (2H, m)
7′	3.75 (2H, s)	3.76 (2H, s)	3.70 (2H, s)	3.74 (2H, s)
9′	7.32 (1H, d, 1.8)	6.81 (1H, d, 2.0)	6.81 (1H, d, 2.0)	7.32 (1H, d, 2.0)
12′	6.70 (1H, d, 8.2)	—	—	—
13′	7.02 (1H, dd, 1.8, 8.4)	6.68 (2H, d, 1.6)	6.68 (2H, d, 2.0)	7.00 (1H, m)

Crystallography

The structure of ChiB soaked with psammaplin A was solved and refined with 1.85 Å diffraction data, to $R = 0.20$, $R_{free} = 0.246$ (Table 4). In the final maps, fragmented density for a large molecule bound to the active site is observed. Although this density is compatible with a model of psammaplin A, repeated attempts at interpreting it failed, possibly due to the great conformational flexibility of the psammaplin A molecule. Features of the ChiB structure indicate that the inhibitor molecule is bound to the active site. Family 18 chitinases contain a characteristic signature sequence in their active site, Asp-X-X-Asp-X-Asp-X-Glu, of which the Glu is the catalytic acid (Glu144 in ChiB). Previous structural work has shown that the Asp before the Glu (Asp142 in ChiB) serves to stabilise a positively charged oxazolinium ion intermediate formed during enzymatic chitinolysis by attack of the *N*-acetyl group in the −1 subsite on the C1 carbon of the same sugar.[2] In all family 18 chitinase apo-structures presently available Asp142 points 'down' towards Asp140 to form a hydrogen bond.[2,18–21] In all presently known structures of chitinases in complex with substrates/inhibitors, however, Asp142 is pointing 'up', towards Glu144 and the *N*-acetyl group[2,4] (Fig. 2). In the ChiB–psammaplin structure, Asp142 is partially pointing upwards, suggesting an interaction with a moiety bound to the −1 site. These results show that psammaplin A interacts with the active site of ChiB, but that it does not bind tightly and precisely as does, for example, allosamidin. Since Psammaplin A acts as a non-competitive inhibitor, one would not expect the compound to bind tightly to the active site, at least not to the subsites occupied by 4MU-$(GlcNAc)_2$.

Table 3. ^{13}C NMR spectral data (δ/ppm, CD_3OD, 100 MHz, ppm, multiplicity) for compounds **1–4**

C	1	2	3	4
2	38.6 (t)	38.2 (t)	38.1 (t)	38.0 (t)
3	39.2 (t)	39.2 (t)	39.2 (t)	39.8 (t)
5	165.5 (s)	165.5 (s)	165.5 (s)	166.0 (s)
6	152.8 (s)	153.8 (s)	152.8 (s)	151.5 (s)
7	28.3 (t)	28.3 (t)	28.5 (t)	28.9 (t)
8	130.2 (s)	130.0 (s)	130.1 (s)	130.8 (s)
9	134.1 (d)	134.1 (d)	124.4 (d)	134.6 (d)
10	110.1 (s)	110.1 (s)	110.1 (s)	110.6 (s)
11	153.3 (s)	153.3 (s)	142.3 (s)	153.8 (s)
12	116.7 (s)	116.7 (d)	146.8 (s)	117.2 (d)
13	130.0 (d)	130.2 (d)	116.1 (d)	132.7 (d)
2′	38.6 (t)	38.1 (t)	38.1 (t)	38.7 (t)
3′	39.2 (t)	39.2 (t)	39.2 (t)	39.7 (t)
5′	165.5 (s)	165.5 (s)	165.5 (s)	166.1 (s)
6′	152.8 (s)	152.8 (s)	152.8 (s)	153.3 (s)
7′	28.3 (t)	28.5 (t)	28.5 (d)	28.9 (t)
8′	130.2 (s)	130.0 (s)	130.1 (s)	129.1 (s)
9′	134.1 (d)	124.4 (d)	124.4 (d)	134.0 (d)
10′	110.1 (s)	110.1 (s)	110.1 (s)	110.6 (s)
11′	153.3 (s)	142.8 (s)	142.3 (d)	153.3 (s)
12′	116.7 (d)	146.8 (s)	146.8 (s)	112.9 (s)
13′	130.0 (d)	116.1 (d)	116.1 (s)	130.6 (d)

Additional biological activity

Psammaplin A has previously been shown to possess potent antibiotic activity against Gram-positive bacteria, especially methicillin-resistant *Staphylococcus aureus* (MRSA).[22] The mechanism of inhibition was believed to be the inhibition of DNA synthesis and DNA gyrase activity. Psammaplin A has also been shown to be cytotoxic and to inhibit the human topoisomerase II enzyme.[23]

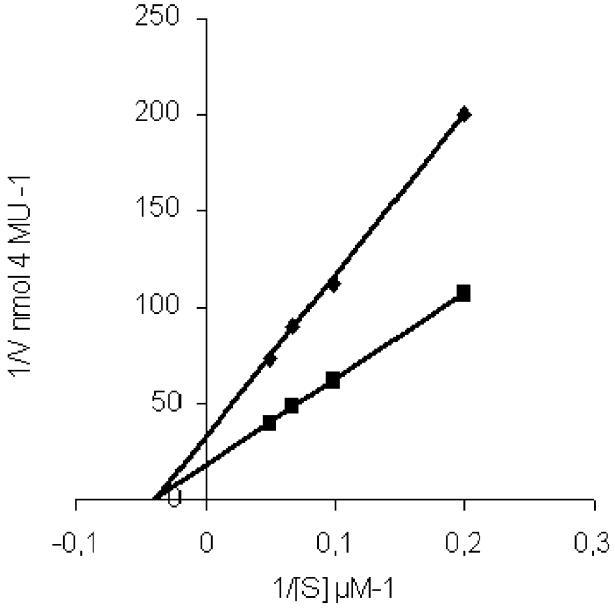


Figure 1. Lineweaver–Burk plots. The lines corresponding to measurements with (◆) and without (■) psammaplin A have correlation coefficients of 0.9984 and 0.9988, respectively. Each point represents the average of three individual measurements, where each measurement was done in duplicate.

Table 4. Details of data collection and structure refinement[a]

Cell dimensions (Å)	$a = 55.75$ $b = 103.59$ $c = 186.07$
Resolution range (Å)	30–1.85 (1.92–1.85)
No. observed reflections	254155
No. unique reflections	83,126 (6368)
Redundancy	3.9 (3.1)
I/σI	10.8 (2.4)
Completeness (%)	89.1 (69.2)
R_{merge} (%)	5.5 (44.0)
R_{cryst} (%)	20.0
R_{free} (%)	24.6
No. protein atoms	7846
No. water molecules	614
RMSd bond ideality (Å)	0.012
RMSd angle ideality (°)	1.6
RMSd B-factors bonds (Å^2)	1.46

[a]Values between brackets are for the highest resolution shell. Crystals were of space group $P2_12_12_1$, and were cryo-cooled to 100 K. All measured data were included in structure refinement.

The crude extract of *A. rhax* was tested for inhibition of cancer cells. Against ovarian cancer cells it showed an IC_{50} of 5.37 μg/mL and against leukaemia cells it showed an IC_{50} of 4.97 μg/mL. Tests on the pure compounds showed psammaplin A to be the main active component. The IC_{50}'s (Table 1) were 1.20 and 0.71 μM for ovarian tumour and leukaemia cells, respectively. This is not unusual as Verongid sponges have been previously reported to yield cytotoxic compounds.[24]

Because chitinases are common in fungi, and crucial to the control of ecdysis in insects, it was felt that investigating the agrochemical potential of psammaplin A was justified.[2] Psammaplin A was submitted to Syngenta Ltd. for antifungal and insecticidal bioassays. The compound was tested in vivo against commercially important fungal species and insect pests. Psammaplin A was found to be particularly effective against vine downy mildew, *Plasmopora viticola* (Table 5). Efficacy against insect pests was shown to be much lower. Only mild efficacy was shown against one species, the diamondback moth, *Plutella xylstella* (Table 6).

Conclusion

This study confirms that marine sponges are a source of potential inhibitors of chitinase enzymes, and that further investigation of the phylum *Porifera* is warranted. The X-ray crystal structure of the psammaplin A/ChiB complex indicates that the psammaplin A interacts with the active site, but, that due to its conformational flexibility, the interaction is not as tight and precise as more potent inhibitors such as allosamidin. The enzyme kinetics studies, showing psammaplin A is a non-competitive inhibitor of ChiB, are consistent with non-specific binding of psammaplin A to the ChiB active site. As might be expected for chitinase inhibitors, psammaplin A shows anti-fungal activity. As filter feeders, sponges are prone to microbial infections, and might have developed these bromotyrosine containing compounds as antifungal agents.

Experimental

General experimental procedures

HPLC separations were carried out using a Spectraphysics P100 isocratic pump and monitored using a Hewlett Packard HP 1050 Series Variable Wavelength UV Detector and a Waters ODS (10×250 mm) column. Low resolution mass spectra were obtained on a Finnigan Masslab Navigator (electrospray) while the high resolution mass data was obtained on a Finnigan Mat-95. ^1H, ^{13}C and all NMR 2D experiments were recorded on a Varian Unity INOVA 400 spectrometer in CD_3OD solution.

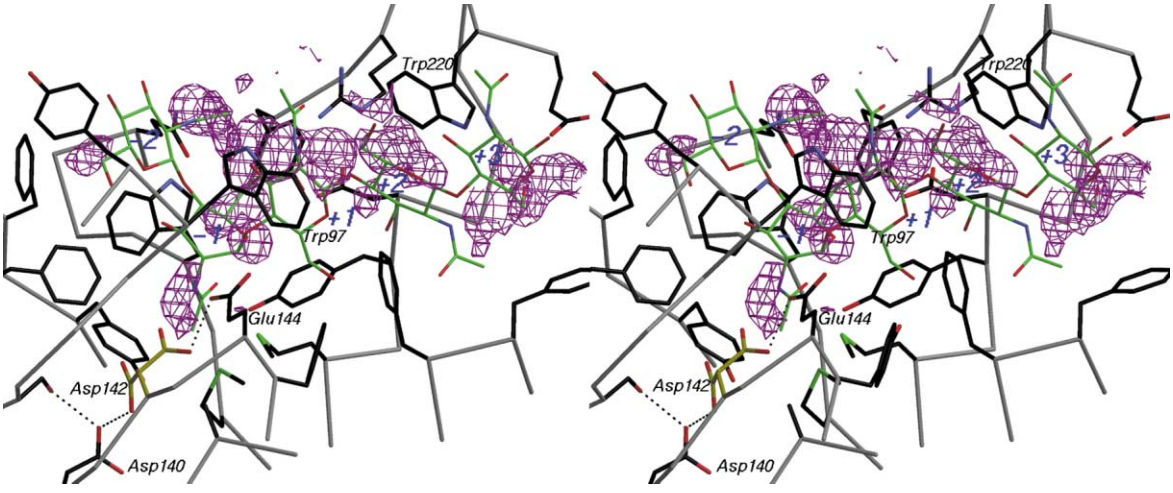


Figure 2. Stereo image of the final model with the remaining difference density. Side chains (carbons in black) surrounding the carbohydrate binding site are shown as sticks, together with relevant stretches of backbone (grey). Some key residues are labeled, the catalytic acid Glu144 and the preceeding Asp140/Asp142 (the latter of which was refined in two alternate conformations, identified with yellow carbons), and the tryptophans important for chitin binding (Trp97/Trp220). The position a NAG5 chitin oligomer would occupy according to a previously published structure[2] are drawn in a stick model with green carbons. The unbiased F_o–F_c map (i.e., prior to inclusion of any ligand atom) is contoured at 2.5 σ (magenta). Some hydrogen bonds discussed in the text are drawn as dotted lines. The sugar binding subsites −2 to +3 are labelled in blue.

Collection

The sample of *A. rhax* (de Laubenfels 1954, Order Verongida, Family Druinellidae), collection number 9712SD130, was collected in December 1997 at a depth of about 5 m by free diving on a wall of *Cakaulevu* reef, Wainunu, Vanua Levu Island, Fiji (17° 2.609′ S; 178° 54.694′ E), and was identified by Dr. John Hooper of the Queensland Centre for Biodiversity, Queensland Museum, Australia. A voucher specimen is preserved at the Regional Herbarium, School of Pure and Applied Sciences, University of the South Pacific, Fiji (voucher no. 9712SD130).

Extraction and isolation

The frozen sample 1300 g (wet weight) was extracted with MeOH (3×) and CH_2Cl_2 (3×), and then the concentrated extracts were combined and shipped to the home laboratory in Aberdeen. The crude extract (30 g) was partioned between water and CH_2Cl_2. The CH_2Cl_2 partion fraction was evaporated to give a crude oil (12 g) that was partioned between hexane and 90% MeOH/H_2O. The MeOH fraction was phase-adjusted to 50% MeOH/H_2O and partitioned with CH_2Cl_2. The CH_2Cl_2 fraction was then subject to Sephadex LH-20 size-exclusion chromatography (1:1 CH_2Cl_2/MeOH). After the pooling together of similar fractions, reverse-phase ODS HPLC using 70% MeOH/H_2O as eluent gave compounds **1**–**4**.

Psammaplin A (1). White powder (100 mg); LRESIMS *m/z* 685.8 $(M+Na)^+$; HRESIMS *m/z* 662.9587 $[M+H]^+$ Δ −0.5 mmu calculated for $C_{22}H_{25}S_2{}^{79}Br_2N_4O_6$; UV (100%, MeOH) λ_{max} 278 nm (∈ 3790). IR 3377–3179, 2920, 2350, 1660, 1538, 1426, 1359, 1285, 1210, 1044, 985.

Psammaplin K (2). light pinkish oil (40 mg); LRESIMS *m/z* 705.9 $[M+Na]^+$; HRESIMS *m/z* 700.9385 $[M+Na]^+$ Δ −3.4 mmu calculated for $C_{22}H_{24}S_2{}^{79}Br_2N_4O_7$. UV (100%, MeOH) λ_{max} 290 (∈ 3438). IR 3377–3185, 2369, 1735, 1701, 1655, 1638, 1525, 1430, 1288, 1222, 986.

Psammaplin L (3). Light pinkish oil (10 mg); HRESIMS *m/z* 694.9453 $[M+H]^+$ Δ 2.8 mmu calculated for $C_{22}H_{25}S_2{}^{79}Br_2N_4O_8$. UV (100%, MeOH) λ_{max} 291.2 (∈ 3139). IR 3575–3211, 2360, 1653, 1636, 1541, 1420, 1362, 1287, 1215, 1044.

Bisaprasin (4). Colourless oil (40 mg); UV (100%, MeOH) λ_{max}. 325 (ε 3230), 289 (ε 3652). IR 3561–3286, 2076, 2927, 2340, 1652, 1624, 1418, 1360, 1207, 1043, 979, 878, 800.

Table 5. In-vivo fungal disease tests[a]

Fungus	% Control
Erysiphe graminis f. sp tritici—Wheat powdery mildew	0
Phytophthora infestans—Tomato late blight	0
Plasmopora viticola—Vine downy mildew	100
Puccinia recondite—Wheat brown rust	67
Septoria nodorum—Wheat leaf spot	0

[a]100 ppm of **1** applied to various fungi on test plants. Activity expressed as % disease control compared to controls.

Table 6. In-vivo insect tests. Insects treated with 500 ppm of **1**

Insect	% Mortality
Tetranychus urticae—Two spotted spider mite	0
Myzus persicae—Peach potato aphid	55*
Heliothis virescens—Tobacco budworm	0
Plutella xylstella—Diamondback moth	55
Drosophila melanogaster—Fruitfly	0
Meloidogyne incognita—Root knot nematode	0

Control mortality = 55%.

Enzyme assays

The preliminary assay method for chitinase enzyme inhibition of crude extracts was developed by Dr. Shizuri's group at the Marine Biotechnology Institute, Japan. Preliminary assays on crude extracts were performed using *Bacillus* sp. chitinase with the synthetic substrate 4-nitrophenyl-β-D-*N*,*N*-diacetylchitobioside. 300 μL of each extract was dried under vacuum at 30 °C after which it was properly diluted and assayed. 1-mL UV cuvettes were filled with 900 μL substrate (0.4 mM) in 50 mM pH 7 phosphate buffer, 50 μL of the extract solution in 75% EtOH and 50 μL of *Bacillus* chitinase (1 mg/mL in distilled water) at 30 °C. In control reactions, 50 μL 75% EtOH was added instead of the extracts. The reaction rate of the enzyme on the synthetic substrate was followed between 20 and 80 s at λ_{max} 405 nm on a UV spectrophotometer. Inhibition became apparent by lowered reaction rates. The degree of inhibition was quantified by expressing the activities as percentage of the activity of the control samples with no extract. The same assay was used for determination of the IC_{50} of purified psammaplin A, using inhibitor concentrations between 0.6 mM and 8.0 μM. To get an impression of the mode of inhibition, assays were conducted in which both substrate concentrations and psammaplin A concentrations were varied. The results were analysed by plotting 1/(% remaining activity) versus 1/[S].

The effect of psammaplin A on the activity of Chitinase B from *Serratia marcescens* was analysed using the synthetic substrate 4-methylumbelliferyl β-D-*N*,*N*-diacetylchitobioside [4-MU-$(GlcNAc)_2$]. This substrate permits accurate determination of kinetic parameters, despite the fact that one has to use relatively low substrate concentrations in the assays.[25] In a standard assay (used for the determination of the IC_{50} value) the reaction mixture (100 μL) contained 1.15 nM enzyme, 12 μM substrate, various amounts of psammaplin A, 50 mM citrate–phosphate buffer, pH 6.3 and 0.1 mg/mL BSA. The reaction was incubated for 10 min at 37 °C, after which the reaction was stopped with 1.9 mL 0.2 M Na_2CO_3. The amount of 4-MU released was determined using a DyNA 200 Fluorimeter (Hoefer Pharmacia Biotech, San Francisco, CA, USA).

Kinetic parameters were determined using substrate concentrations of 5, 10, 15 and 20 μM. Psammaplin A

was added to a final concentration of 125 μM. To check for linearity samples were taken after 0, 5, 10 and 15 min. The production of 4-MU was linear in all cases, thus permitting straightforward calculation of enzyme velocities. The data sets were analysed using Lineweaver–Burk plots as well as the program EnzFitter (Biosoft, Cambridge, UK).

Antifungal assays

The sample, dissolved in acetone was diluted (100 ppm) in sterile de-ionised water was applied on the plants (wheat, grape, vine and tomatoes) as a foliar spray using a hand-held sprayer. The tested compound was also sprayed on the roots of the plants. The plants were then inoculated with a calibrated suspension of fungal spores and maintained under conditions appropriate for disease development. Assessment was done after 10 days by visual inspection of the area covered by the disease and was compared against untreated plants and also against plants treated with commercial agrochemicals fungicides. This was expressed as percentage disease control.

Insecticidal assays

Adult or larval stages of the insects which were maintained on leaf dics were treated with 500 ppm of the compound. Assessment which was based on% mortality was made after 3–5 days.

Crystallography

ChiB was overexpressed and purified as described previously.[25] ChiB was crystallized by vapour diffusion from solutions containing ammonium sulphate, glycerol and HEPES pH 7.[20] Pure psammaplin A was dissolved in 1:1 H_2O/MeOH and used for soaking experiments. A 50-fold molar excess of psammaplin was added to crystallisation drops containing native ChiB crystals. After 12 h of soaking crystals were frozen in a nitrogen stream. Diffraction data (Table 4) were collected at ESRF, Grenoble, on beamline BM14. The structure was refined with CNS[26] using the published wild type ChiB structure[20] as a template after simulated annealing runs at 2500 K to remove bias (Table 4). Model building was performed with O.[27]

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References and Notes

1. Gooday, G. W. *Chitin Chitosan Res*. **1997**, *3*, 233.
2. van Aalten, D. M. F.; Komander, D.; Synstad, B.; Gaseidnes, S.; Peter, M. G.; Eijsink, V. G. H. *Proc. Natl. Acad. Sci. U.S.A*. **2001**, in press.
3. Sakuda, S.; Isogai, A.; Masumoto, S.; Suzuki, A. *Agric. Biol. Chem*. **1987**, *51*, 3251.
4. Terwisscha van Scheltinga, A. C.; Armand, S.; Kalk, K. H.; Isogai, A.; Henrissat, B.; Dijkstra, B. W. *Biochemistry* **1995**, *34*, 15619.
5. Arai, N.; Shiomi, K.; Yamaguchi, Y.; Masuma, R.; Iway, Y.; Turberg, A.; Kolbl, H.; Omura, S. *Chem. Pharm. Bull*. **2000**, *48*, 1442.
6. Omura, S.; Arai, N.; Yamaguchi, Y.; Masuma, R.; Iway, Y.; Namikoshi, M.; Turberg, A.; Kolbl, H.; Shiomi, K. *J. Antibiot*. **2000**, *53*, 603.
7. Izumida, H.; Imamura, N.; Sano, H. *J. Antibiot*. **1996**, *49*, 76.
8. Kato, K.; Shizuri, Y.; Izumida, H.; Yokoyama, A. *Tetrahedron Lett*. **1995**, *36*, 2133.
9. Jaspars, M.; Rali, T.; Laney, M.; Schatzman, R. C.; Diaz, M. C.; Schmitz, F. J.; Pordesimo, E. O.; Crews, P. *Tetrahedron* **1994**, *50*, 7367.
10. Cimino, G.; de Rosa, S.; de Stefano, S.; Self, R.; Sodano, G. *Tetrahedron Lett*. **1983**, *24*, 3029.
11. Roll, D. M.; Chang, C. W. J.; Scheuer, P. J.; Gray, P. J.; Shoolery, J. N.; Matsumoto, G. K.; van Duyne, G. D.; Clardy, J. *J. Am. Chem. Soc*. **1985**, *107*, 2196.
12. Quinoa, E.; Crews, P. *Tetrahedron Lett*. **1987**, *28*, 3229.
13. Pham, N. B.; Butler, M. S.; Quinn, R. J. *J. Nat. Prod*. **2000**, *63*, 393.
14. Shin, J.; Lee, Hui-Seung; Seo, Youngwan; Rho, Jung-Rae; Cho, Ki Woong; Paul Valerie, J. *Tetrahedron* **2000**, *56*, 9071.
15. Arabshahi, L.; Schmitz, F. J. *J. Org. Chem*. **1987**, *52*, 3584.
16. Rodriguez, A. D.; Akee, R. K.; Scheuer, P. J. *Tetrahedron Lett*. **1987**, *28*, 4989.
17. Sampson, M. N.; Gooday, G. W. In *Chitin Enzymology*; Muzzarelli, R. A. A., Ed.; Atec Edizioni, 1996; Vol. 2, p 227.
18. Terwisscha van Scheltinga, A. C.; Kalk, K. H.; Beintema, J. J.; Dijkstra, B. W. *Structure* **1994**, *2*, 1181.
19. Perrakis, A. I.; Tews, I.; Dauter, Z.; Oppenheim, A. B.; Chet, I.; Wilson, K. S.; Vorgias, C. E. *Structure* **1994**, *2*, 1169.
20. van Aalten, D. M. F.; Synstad, B.; Brurberg, M. B.; Hough, E.; Riise, B. W.; Eijsink, V. G. H.; Wieringa, R. K. *Proc. Natl. Acad. Sci. U.S.A*. **2000**, *97*, 5842.
21. Hollis, T.; Mozingo, A. F.; Bortone, K.; Ernst, S.; Cox, R.; Robertus, J. D. *Protein Sci*. **2000**, *9*, 544.
22. Kim, D. Y.; Lee, I. S.; Jung, J. H.; Yang, S. I. *Arch. Pharmacol. Res*. **1999**, *22*, 25.
23. Kim, D.; Lee, I. S.; Jung, J. H.; Lee, C. O.; Choi, S. U. *Anticancer Res*. **1999**, *19*, 4085.
24. Ciminiello, A.; Carmela, D.; Fattorusso, E.; Magno, S.; Pansini, M. J. *J. Nat. Prod*. **1999**, *62*, 590.
25. Brurberg, M. B.; Nes, I. F.; Eijsink, V. G. H. *Microbiology* **1996**, *142*, 1581.
26. Brunger, A. T.; Adams, P. D.; Clore, G. M.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J.-S.; Kuszewski, J.; Nilges, M.; Pannu, N. S.; Read, R. J.; Rice, L. M.; Simonson, T.; Warren, G. L. *Acta Cryst*. **1998**, *D54*, 905.
27. Jones, T. A.; Zou, J. Y.; Cowan, S. W.; Kjeldgaard, M. *Acta Cryst*. **1991**, *A47*, 110.