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Discovery, chemistry, and chemical biology of microbial products*

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Abstract: Our long-standing and continual screening of microorganisms, especially for antiparasitic agents, has produced a wide variety of compounds of global importance, such as the avermectins. Recent discoveries include nafuredin, atpenins, argifin, and argadin. Nafuredin is a helminth-specific inhibitor of electron-transport enzyme, complex I, which exhibits anthelmintic activity against *Haemonchus contortus* in sheep. The atpenins are the most potent complex II inhibitors ever reported. Co-crystallization study of atpenin A5 and *E. coli* complex II indicated the binding mechanism of ubiquinone to complex II. Argifin and argadin are the first cyclic peptides to inhibit chitinase at low concentration. Though structurally similar, their chitinase inhibition mechanisms are quite different.

Keywords: antibiotics; microbial products; electron-transport enzymes; chitinase; enzyme inhibitors; natural products.

INTRODUCTION

Our long-term and extensive research work has encompassed the isolation of novel microorganisms, screening of biologically interesting products, structure elucidation, organic synthesis, biosynthesis, mode of action, and genomic analysis of producing strains [1]. As a result, we have discovered more than 340 new microbial products. Of these, 16 have contributed significantly to advances in human welfare and the life sciences through their use as medicines, animal health agents, agrochemical agents, and as reagents for biochemical research. For example, avermectins were isolated in the mid-1970s from the culture broth of *Streptomyces avermectinius* through cooperative research between our group and a research group at Merck Sharp & Dohme Research Laboratories. The avermectins possess potent anthelmintic and insecticidal activities [2]. Ivermectin, a mixture of 22,23-dihydro derivatives of avermectins B_{1a} and B_{1b}, is used worldwide for antiparasitic control of nematodes and arthropods in the veterinary field and for controlling some human nematode diseases, such as onchocerciasis, lymphatic filariasis, and strongyloidiasis. Ivermectin, an endectocide, is also used to kill mites which transmit human scabies. Among the compounds we have isolated, more than 60 have been targeted by synthetic organic chemists for total synthesis. A variety of natural products of complexity or interesting bioactivity have stimulated creation of new methods of synthetic production and evolution of the strategy and tactics for organic synthesis. After the discovery of avermectins, our continued extensive screening for microbial metabolites with antiparasitic activity has continually discovered promising candidates for antiparasitic drugs. Here we discuss four such new leads, nafuredin and atpenins (isolated as NADH-

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fumarate reductase inhibitors of anaerobic nematodes) and argifin and argadin (isolated as insect chitinase inhibitors).

NADH-FUMARATE REDUCTASE INHIBITORS

Differences in energy metabolisms of the human host and that of helminths are attractive targets for treatment of helminthiasis [3]. Mammals oxidize glucose to CO_2 completely through the enzymatic reaction of glycolysis and the tricarboxylic acid (TCA) cycle (Fig. 1). The electrons involved in glucose oxidation are transferred to NAD^+ and FAD and then pass into the electron-transport chain. The electrons are sequentially transported in mitochondria through complexes I–IV and finally accepted by oxygen. Protons are expelled from the matrix to intermembrane spaces in this process and generate a proton gradient, which generates ATP via ATP synthase (complex V). However, many adult helminths living in human hosts cannot use enough oxygen, and have developed a special energy-transducing system to adapt. Phosphoenolpyruvate generated from glucose by glycolysis does not enter into the TCA cycle, it is metabolized to oxaloacetate, malate, and fumarate sequentially. The terminal step of this pathway is the NADH-fumarate reductase system, which is found in many anaerobic organisms. The system is composed of complex I (NADH-rhodoquinone reductase) and complex II (rhodoquinol-fumarate reductase). Electrons from NADH are accepted by rhodoquinone through complex I, and then transferred to fumarate through complex II. Rhodoquinone has lower redox potential than ubiquinone and is suitable for this reverse reaction of complex II. The final electron acceptors are succinate, or similar volatile fatty acids. This anaerobic electron-transport system can provide ATP in the absence of oxygen.

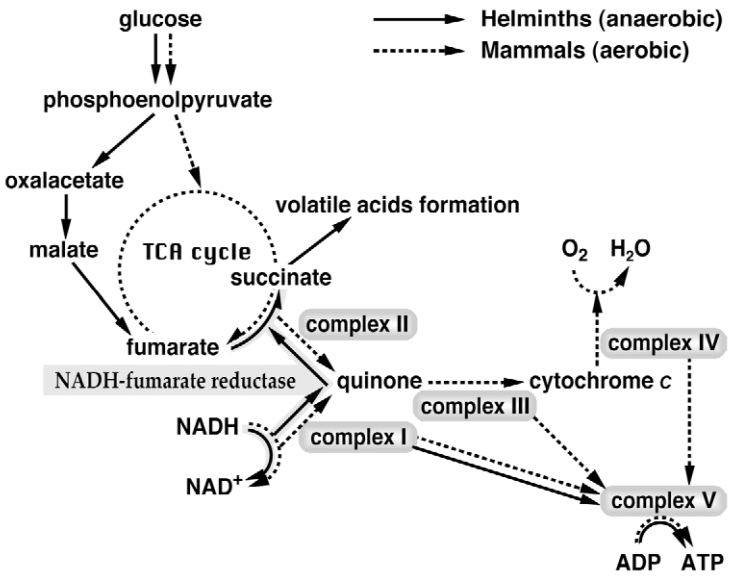
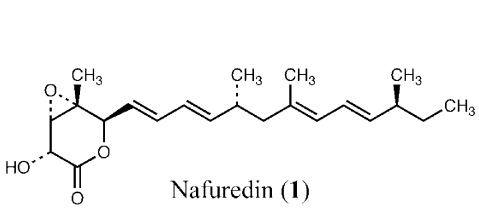


Fig. 1 Energy metabolism in helminths and mammals.

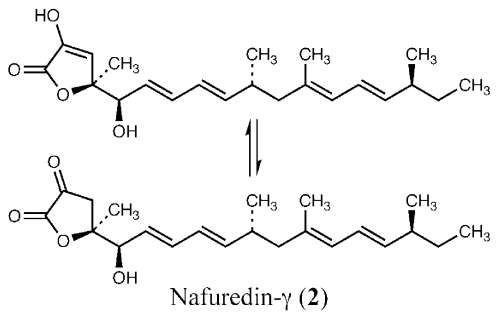
Our screening for inhibitors of NADH-fumarate reductase from microbial metabolites, using adult *Ascaris suum* (roundworm) mitochondria, has discovered nafuredin and atpenins.

Nafuredin

Nafuredin (**1**) was purified from the culture broth of *Aspergillus niger* FT-0554 isolated from a marine sponge [4,5]. The production of **1** was more than doubled by adding 50 % natural seawater. We later found several other nafuredin-producing strains, although all of the subsequent strains are *Fusarium* spp. The structure of **1** was elucidated by NMR studies as (6*E*,8*E*,12*E*,14*E*)-3,4-epoxy-2-hydroxy-4,10,12,16-tetramethyl-6,8,12,14-octadecatetraeno-5-lactone [5]. The relative configuration was shown by the nuclear Overhauser effect (NOE) study, and the absolute configuration was elucidated by comparison of the degradation products of **1** and the corresponding synthetic compounds [6].



Nafuredin (**1**)



Nafuredin-γ (**2**)

Compound **1** potently inhibited NADH-fumarate reductase (complexes I + II) and NADH-rhodoquinone reductase (complex I) of adult *A. suum* at the IC_{50} values of 12 and 24 nM, respectively (Table 1), while its inhibition against rhodoquinol-fumarate reductase (complex II) was very weak (IC_{50} = 80 μM) [4]. It also inhibited NADH-ubiquinone reductase (complex I) in L2 larvae of *A. suum*, which possess aerobic energy metabolism as in mammals, at the same concentration range. It means that **1** is effective against both adult (anaerobic) and larval (aerobic) stages of *A. suum*. As for mammalian complex I, the IC_{50} value for rat liver NADH-ubiquinone reductase was very weak (10 μM). Therefore, **1** is a potent complex I inhibitor of *A. suum*. Kinetic analysis of **1** showed the inhibition against *A. suum* complex I was uncompetitive with NADH and competitive with rhodoquinone. This indicated that the inhibition site of **1** is the quinone-binding domain in complex I.

Table 1 Effects of nafuredin (**1**) on electron-transport enzymes.

	Complex	IC_{50} (nM)			
		A. suum (adult)	*A. suum* (L2)	*H. contortus* (adult)	Rat liver
NADH-fumarate reductase	I+II	12	–	–	1 000
NADH-rhodoquinone reductase	I	24	9.0	195	>100 000
NADH-ubiquinone reductase	I	8	8.9	86	10 000
Rhodoquinol-fumarate reductase	II	80 000	–	–	–
Succinate-ubiquinone reductase	II	>100 000	–	–	>100 000

–: not tested.

The anthelmintic activity of **1** was studied using sheep infected by *Haemonchus contortus* (barber pole worm) [4]. As shown in Fig. 2, 2 mg/kg (p.o.) of **1** exerted anthelmintic activity against *H. contortus* by almost complete suppression of egg output of female worms within 11 days after treatment. A greater than 90 % egg reduction was observed at day 11. Complex I of *H. contortus* was also inhibited by **1**, although the inhibition was relatively weaker than against *A. suum* enzyme (Table 1). Therefore, the anthelmintic activity may be due to the effect against energy metabolism by **1**.

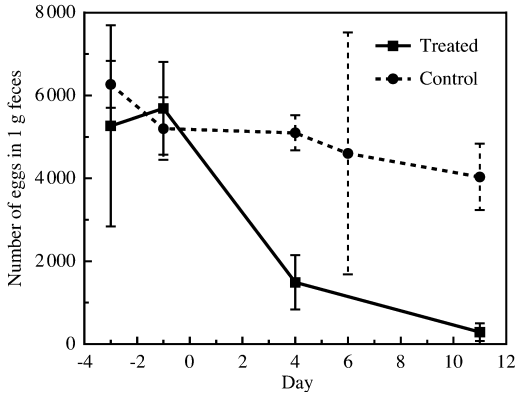


Fig. 2 Effects of treatment with nafuredin (**1**) on fecal egg counts in sheep infected with *H. contortus*. Eggs per gram of infected sheep feces on different days before and after oral treatment with 2 mg/kg of **1** were compared to untreated, infected sheep. Values are the means of two experiments (control) or three experiments (treated) ± S.D.

Compound **1** showed no side-effect and no loss of body weight at either 2 mg/kg p.o. treatment in sheep, 50 mg/kg p.o. treatment in mice, or 50 mg/kg i.p. treatment in mice.

Thus, **1** may be a selective inhibitor of helminth complex I. The specific inhibition by **1** suggested that the structure of the domain in helminth complex I differs somewhat from that of its mammalian counterparts. The study of **1** showed that complex I is a novel and promising target for anthelmintics.

Since **1** is a novel anthelmintic lead compound, it is important to establish the basic method for total synthesis. We accomplished total synthesis in 26 steps with an overall yield of 10 % [7]. Two fragments were derived from D-glucose and chiral 2-methylbutanol (Fig. 3).

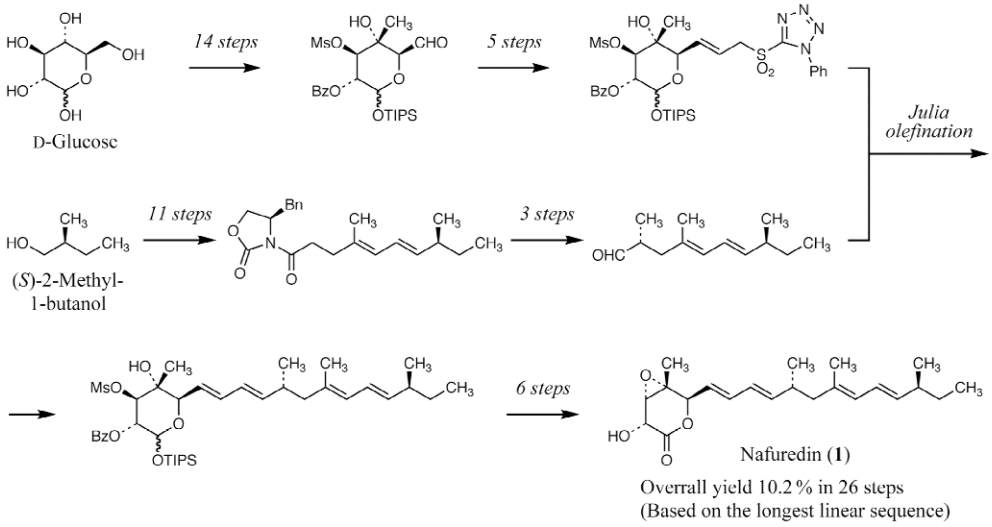


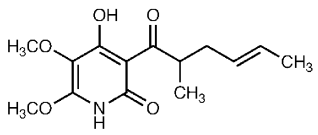
Fig. 3 Total synthesis of nafuredin (**1**).

During the course of the total synthetic studies of **1**, we found that **1** was converted to a novel γ-lactone derivative named nafuredin-γ (**2**) under mild basic conditions [8]. This is a keto-enol tautomer, and it may be formed via enolization, opening of the epoxide, and formation of the γ-lactone. The IC_{50} value of **2** against NADH-rhodoquinone reductase was 2.3 nM, while **2** did not inhibit bovine liver

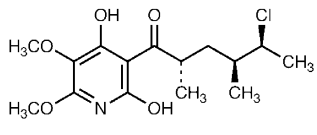
NADH-ubiquinone reductase at 10 μM. Therefore, **2** is, like **1**, a selective complex I inhibitor. Compound **2** also showed anthelmintic activity against *H. contortus* in sheep. Since the lactone moiety of **2** is simpler than that of nafuredin, **2** is useful for structure–activity relationship study. We have accomplished its total synthesis and are developing its analogs [9].

Atpenins

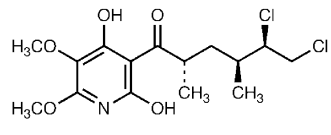
In further screening for NADH-fumarate reductase inhibitors, harzianopyridone (**3**) was isolated as a specific inhibitor of complex II. It was originally isolated from the culture broth of *Trichoderma harzianum* as an antifungal metabolite in 1989 [10]. We isolated atpenins A4 (**4**) and A5 (**5**) structurally closely related to **3** from the culture broth of *Penicillium* sp. as antifungal antibiotics in 1988 [11]. We consequently studied the electron-transport enzyme inhibition of **3** together with **4** and **5**. Their chromophore structures may be a tautomer of pyridone and pyridinol. The structures (below) are those depicted in the original papers. In contrast to **1**, **3–5** inhibited complex II (Table 2), and the inhibition was nonselective between helminths (rhodoquinol-fumarate reductase) and mammals (succinate-ubiquinone reductase, SQR) [12]. Among **3–5**, **5** showed the most potent inhibition against complex II. Although there are potent inhibitors of complexes I, III, and IV, no very potent inhibitors of complex II have been described. Carboxin and its derivatives, pesticides used for phytopathogenic fungi, are the most potent inhibitors of complex II [13]. Comparing **5** with carboxin, the IC_{50} value of **5** is 300-fold lower than that of carboxin (Table 2). Therefore, atpenins may be useful tools for clarifying the biochemical and structural properties of complex II.



Harzianopyridone (**3**)



Atpenin A4 (**4**)



Atpenin A5 (**5**)

Table 2 Effects of **3**–**5** and carboxin on electron-transport enzymes.

	Complex	IC_{50} (nM)			
		Harziano-pyridone (**3**)	Atpenin A4 (**4**)	Atpenin A5 (**5**)	Carboxin
NADH-fumarate reductase (adult *A. suum*)	I+II	1 600	110	14	–
NADH-rhodoquinone reductase (adult *A. suum*)	I	>100 000	>100 000	>100 000	–
Rhodoquinol-fumarate reductase (adult *A. suum*)	II	360	220	12	–
Succinate-ubiquinone reductase (bovine heart)	II	17	11	3.6	1 100

–: not tested.

Figure 4 shows the structure of complex II. Complex II consists of four subunits: flavoprotein (Fp), iron-sulfur protein (Ip), and two membrane-anchor proteins (CybL and CybS) [14]. Succinate dehydrogenase (SDH) activity requires only the Fp and Ip subunits, while SQR activity requires all subunits. We found that **5** potently inhibits both SQR and SDH activities of mitochondrial complex II. Therefore, **5** is suggested to bind to the central position of complex II that is the binding site of ubiquinone. Recently, the X-ray crystallographic structure of *Escherichia coli* complex II was eluci-

dated by Iwata and Cecchini's group [15]. We collaborated with them and reported the co-crystallization structure of *E. coli* complex II and **5** [16]. Compound **5** was located within the same hydrophobic pocket as ubiquinone but bound deeper (Q_2-site) than the site of ubiquinone (Q_1-site). The crystal structure of complex II with ubiquinone showed that ubiquinone only hydrogen-bonded to a tyrosine, while serine, histidine, and arginine also provided hydrogen-bonding to **5** in co-crystallization structure (Fig. 5). Thus, ubiquinone is suggested to bind at Q_1-site initially and then move to the Q_2-site to be reduced.

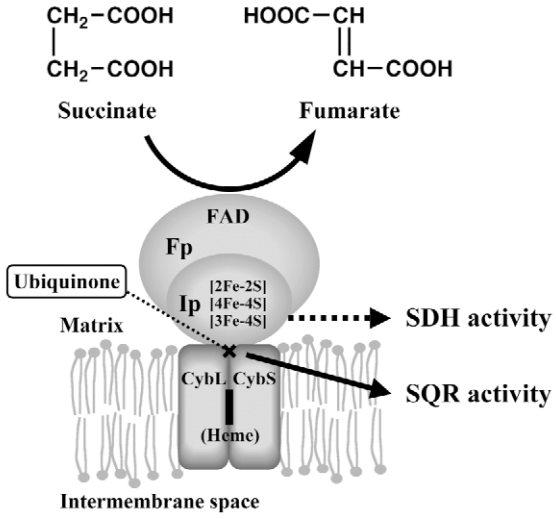


Fig. 4 Subunit structure and enzyme activities of complex II.

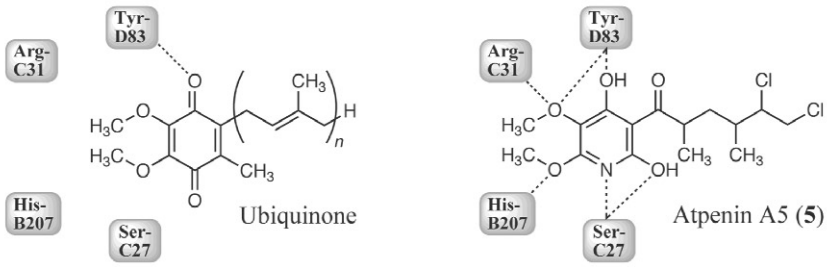


Fig. 5 Ubiquinone and atpenin A5 (**5**) bindings to *E. coli* complex II.

The structure of mammalian (porcine heart) complex II was elucidated last year [17], and that of helminths should be revealed soon. This information may clarify the interaction of **5** and complex II, and give a clue to the design of helminth-specific analogs of **5**.

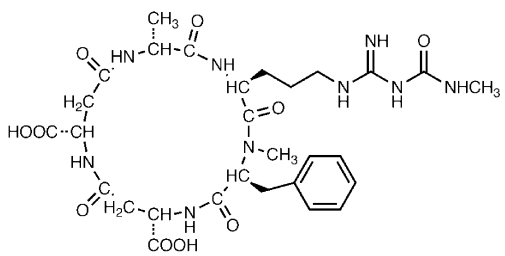
CHITINASE INHIBITORS

Chitin occurs in fungi, some algae, and many invertebrates including insects, but is not found in vertebrates. Thus, chitin synthesis and degradation processes might be specific targets for fungicides and insecticides [18]. Though chitin synthesis inhibitors, such as polyoxins active against phytopathogenic fungi and insecticidal benzoylphenylureas, are commercially available, chitin degradation inhibitors have not yet been commercialized.

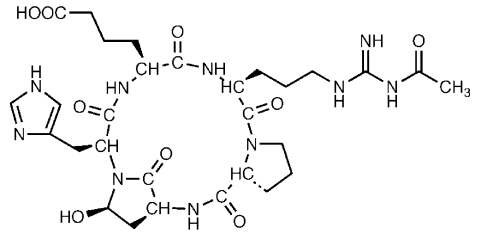
Chitinases degrade chitin by hydrolyzing it into oligomers of *N*-acetylglucosamine. Chitinases belong to either family 18 or 19 in the classification of glycosyl hydrolases. Family 18 chitinases are distributed widely from prokaryotes to eukaryotes, including vertebrates, while family 19 chitinases are mainly found in plants, where they function in defense against fungal and insect pathogens by destroying chitin-containing cell walls. Some mammalian chitinases have also recently been reported. Mammalian chitotriosidase and acid mammalian chitinase (AMCase) belong to family 18 chitinases. About 1 in 20 individuals are completely deficient in enzymatically active chitotriosidase, and markedly elevated chitotriosidase activity was observed in symptomatic Gaucher patients [19], which suggests chitotriosidase may not be essential for humans. AMCase is induced via a Th2-specific, IL-13-mediated pathway in epithelial cells and macrophages in an aeroallergen asthma model, and highly expressed in human asthma [20]. Inhibition of AMCase improves asthma symptoms, and AMCase may be an important target for asthma and other allergic diseases.

Only a few chitinase inhibitors have been reported, e.g., allosamidin, styloguanidine, and cyclo(L-arginyl-D-prolyl) [21]. Among them, the most potent inhibitor is a pseudotrisaccharide allosamidin isolated from *Streptomyces* sp. [22]. Allosamidin mimics the oxazolinium ion reaction intermediate of the substrate-assisted reaction mechanism. Inhibitors of chitinases would be expected to be insecticidal, fungicidal antibiotics or candidate drugs for allergic diseases.

In the course of searching for new insecticides of microbial origin, we screened inhibitors of chitinase derived from *Lucilia cuprina* (blowfly) [23]. We isolated a cyclic pentapeptide, argifin (**6**), from the culture broth of *Gliocladium* sp. [24,25]. It is the first cyclic peptide showing chitinase inhibition in a submicromolar range, and also the first chitinase inhibitor isolated from fungi. Subsequently, we isolated a second cyclic pentapeptide, argadin (**7**), from the culture broth of *Clonostachys* sp. [26]. Compounds **6** and **7** are both cyclic pentapeptides containing L-arginine, with modification at the guanidine residue, and each has one R-amino acid. An aldehyde residue of aspartic β-semialdehyde in **7** is bonded to an amino residue of adjacent histidine to form γ-lactam.



Argifin (**6**)



Argadin (**7**)

The inhibitory activities of **6** and **7** against six different chitinases were studied, in comparison with the known inhibitor allosamidin (Table 3) [25,27,28]. Compound **7** showed much more potent inhibition than **6** against all tested chitinases. Allosamidin was much more potent than **7** against chitinases from *L. cuprina*, *Streptomyces griseus*, and *Bacillus subtilis*, while **7** was the most potent against *Serratia marcescens* chitinase B, and human chitotriosidase. The kinetic study of **6** revealed that it inhibited *L. cuprina* chitinase by mixed inhibition. Compounds **6** and **7** inhibited normal molting of cockroaches when 20 μg/larva was injected.

Table 3 Chitinase inhibitory activities of argifin (**6**) and argadin (**7**).

	(μM)		
	Argifin (**6**)	Argadin (**7**)	Allosamidin
Lucilia cuprina chitinase (IC_{50})	0.103	0.003 4	0.000 4
Streptomyces griseus chitinase (IC_{50})	14.8	1.9	0.016
Bacillus subtilis chitinase (IC_{50})	19.0	1.9	–
Serratia marcescens chitinase B (K_i)	33	0.020	0.45
Aspergillus fumigatus chitinase B1 (IC_{50})	1.1	0.5	–
Human chitotriosidase (IC_{50})	4.5	0.013	0.04

–: not tested.

Since the inhibition of **7** against *Serratia* chitinase B was very strong and that of **6** was very weak, we compared crystal structures of *Serratia* chitinase B-complexed with **6** and **7** [27]. It is known that Asp142, Glu144, and Tyr214 are involved in the catalytic action at the active center of the *Serratia* chitinase B. Compound **6** bound at the active site of the enzyme, and its *N*-methylcarbamoylarginine residue interacted with Asp142, Glu144, and Tyr214 in the catalytic cavity of the enzyme (Fig. 6). The phenyl ring of the phenylalanine residue occupied a position equivalent to that of a pyranose ring of the substrate, permitting the formation of a triple sandwich with Trp97 and Trp220 of the enzyme. Compound **6** contained one intermolecular hydrogen bond. In the complex of **6** and the enzyme, six water molecules were observed that hydrogen-bonded to the inhibitor and enzyme, which indicated that **6** did not fit well with the catalytic cavity of enzyme. In contrast to the complex of **6** and the enzyme, **7** bound at the active site of the enzyme by a completely different manner (Fig. 6). It interacted with the three catalytic amino acids (Asp 142, Glu 144, and Tyr 214) at the histidine residue (imidazole and amide carbonyl). The acetylarginine and aminoadipic acid residues formed flat moiety through two hydrogen bonds, and the flat moiety occupied a position equivalent to that of a pyranose ring of the sub-

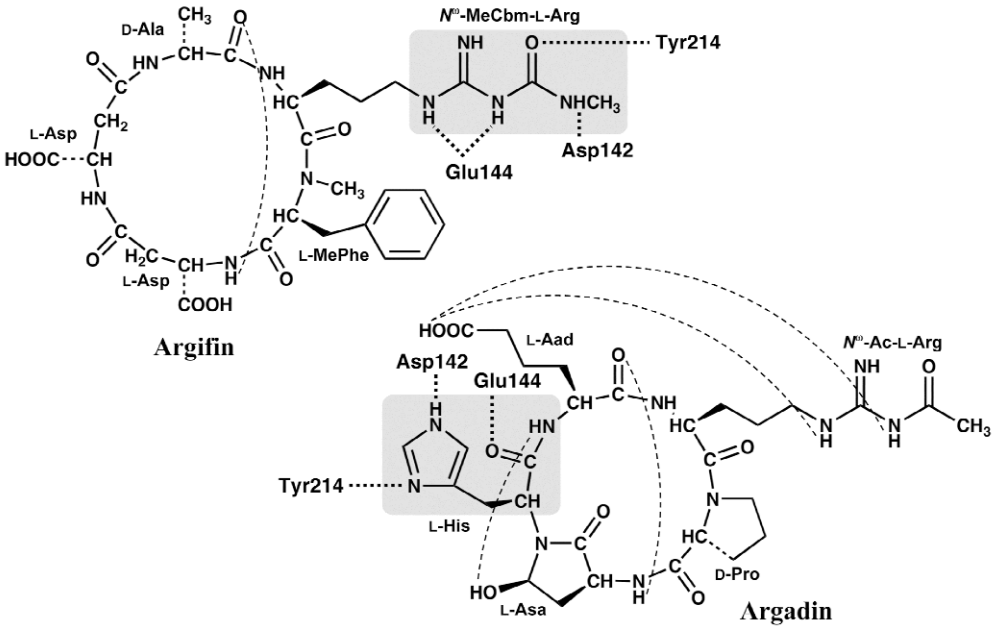


Fig. 6 Intramolecular hydrogen bonding (------) of argifin (**6**) and argadin (**7**) and their interaction (·····) with *Serratia* chitinase B.

strate and formed a triple sandwich with Trp97 and Trp220. Additionally, **7** contained two more intramolecular hydrogen bonds. Therefore, **7** occupied a more compact space and it was more conformationally restricted than **6**. In the complex of **7** and the enzyme, only one water molecule was observed in the cavity, which indicated that **7** bound more tightly to the enzyme catalytic cavity than **6**. It is interesting that **6** and **7** bound to the enzyme in quite different manners, although their structures are very similar. The crystal structures demonstrated why **7** showed highly potent inhibition compared to **6**.

The crystallographic studies of chitinases in complex with **6** and **7** were also done for *A. fumigatus* chitinase B1 and human chitotriosidase [28]. Compounds **6** and **7** bound to these enzymes in a similar fashion to that observed for *Serratia* chitinase B. However, subtle changes in the binding site dramatically affect affinity and selectivity.

Compounds **6** and **7** are cyclic peptides that mimic the carbohydrate substrate of the glycosyl hydrolase. It is very rare that peptides mimic carbohydrates, and it may be the first study that revealed the mechanism of interaction between the peptide-mimicking carbohydrates and the protein. The structures of chitinases in complex with **6** and **7** will provide opportunities for structure-based design and synthesis of derivatives, which may lead to more potent chitinase inhibitors with chemotherapeutic potential.

CONCLUSIONS

The biologically interesting microbial products discussed have provided important scientific information for use in the life sciences, including biochemistry as well as medicine. Our research encompasses study of microbial products as well as the producing strains. Avermectin has proved to be a globally important animal health agent as well as an indispensable human medication, and we have completed the genome-sequencing analysis of *Streptomyces avermectinius* [29]. This was the first genome analysis of any industrially important microorganism. With the information obtained, we are encouraged that it may become possible to engineer the production of new or more potent metabolites. More than 9 million base pairs exist in the linear chromosome of *S. avermectinius*, about twice the *E. coli* genome. The number of gene clusters of secondary metabolites was predicted to be more than 30, and 6.6 % of total genome DNA is used for gene clusters of secondary metabolites. We remain convinced that the isolation and screening of microorganisms, or genes of microorganisms, from a variety of bioenvironments, using a variety methods, together with the application of gene technology and the introduction of new screening methods, will provide numerous opportunities for the discovery of many more novel and greatly needed drugs, medicines, reagents, and other products.

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