VOL. 56 NO. 12, DEC. 2003 THE JOURNAL OF ANTIBIOTICS pp. 1033 — 1044

Novel α-Pyrones Produced by a Marine *Pseudomonas* sp. F92S91: Taxonomy and Biological Activities

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(Received for publication September 16, 2003)

Inhibitors of the enzymes involved in fatty acid biosynthesis (FAB) have been reported as antibacterial agents. These include thiolactomycin, cerulenin, triclosan, diazoborine, naphthyridinones, aminopyridines and pyridoindoles. Our search for new FAB inhibitors, using a lacZ reporter cell-based screen, led to several confirmed hits. Culture F92S91, later identified as a *Pseudomonas* sp. based on 16S profiling, was found to produce two α-pyrones (I and II) and three high molecular weight peptides. The pyrones were unstable under acidic conditions, and they were rearranged into a furanone derivative (III). Of these compounds, pyrone I was the most active with MICs (μg/ml) against *B. subtilis* (1~2), MRSA (2~4), *M. catarrhalis* (4) and VRE (2~64). Effects on macromolecular synthesis and membrane functions were tested in *B. subtilis*. Pyrone I nonspecifically inhibited incorporation of radiolabeled precursors into DNA, RNA and protein within 5 minutes of drug exposure, similar to that of triclosan. Both compounds also inhibited the cellular uptake of these precursors. Cerulenin did not have an effect until 30 minutes of drug treatment. Pyrone I and triclosan were membrane-active (BacLight test); however, pyrone I (at ≤128 μg/ml concentration) was not hemolytic to human RBCs in contrast to triclosan, which was hemolytic at 16 μg/ml. These data suggest that pyrone-I, unlike triclosan, selectively affects bacterial membrane function.

The increasing prevalence of multidrug-resistant bacteria and the appearance of *S. aureus* strains lacking susceptibility to even vancomycin have heightened the need for new antibiotics with novel modes-of-action[1,2]. In response, new bacterial targets are being identified and used for screening[3]. The ubiquitous type II fatty acid synthase (FAS-II), comprised of 7 distinct mono-functional peptides (Fig. 1), is essential for bacterial viability and lacks homology to the human FAS-I, which is a single multifunctional peptide[4]. Therefore, enzymes in FAS-II are considered to be important targets for antibiotic discovery and development. Inhibitors of the enoyl-ACP reductase (FabI), β-ketoacyl-ACP synthase (FabB) and acetoacyl-ACP synthase (FabH) have been known for some time (Fig. 1). Recently, several new classes of compounds active against FabI[4~6] and FabH[7] have been found (Fig. 2).

In our alliance with Millennium Pharmaceuticals, a *B. subtilis* strain harboring a lacZ reporter gene fusion to a cerulenin and triclosan-responsive promoter was constructed, and a high throughput screen for the detection of inhibitors of fatty acid biosynthesis was developed using this strain. Screening of the Wyeth Natural Products Library (NPL) led to several confirmed hits. One of the prioritized cultures, F92S91, which is a new species of *Pseudomonas* isolated from a marine sponge in Fiji, was found to produce novel α-pyrones (Fig. 3) and peptides (structures unresolved) that were active in the primary screen, and they exhibited antibacterial activity against Gram-positive pathogens. Coincidentally, the α-pyrone I (Fig. 3) was also identified from a culture broth of a *P. fluorescens* at the Schering-Plough Research Institute, NJ[8].

In this paper, we present the taxonomic characterization

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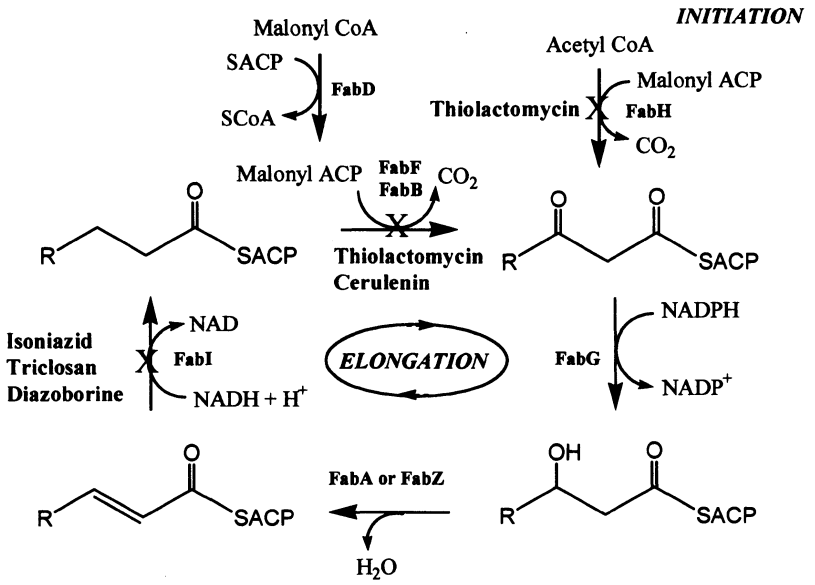
Fig. 1. Fatty Acid Biosynthesis in Bacteria.



Individual Fab enzymes are: D, malonyl-CoA ACP transacylase; H, β-ketoacyl-ACP synthase; B, β-ketoacyl synthase I; F, β-ketoacyl synthase II; G, β-ketoacyl-ACP reductase; A, β-ketoacyl-ACP dehydrase; and I, enoyl-ACP reductase. X indicates enzymes inhibited by known enzymes.

Fig. 2. Chemical structures of some known inhibitors of FabI and FabH.

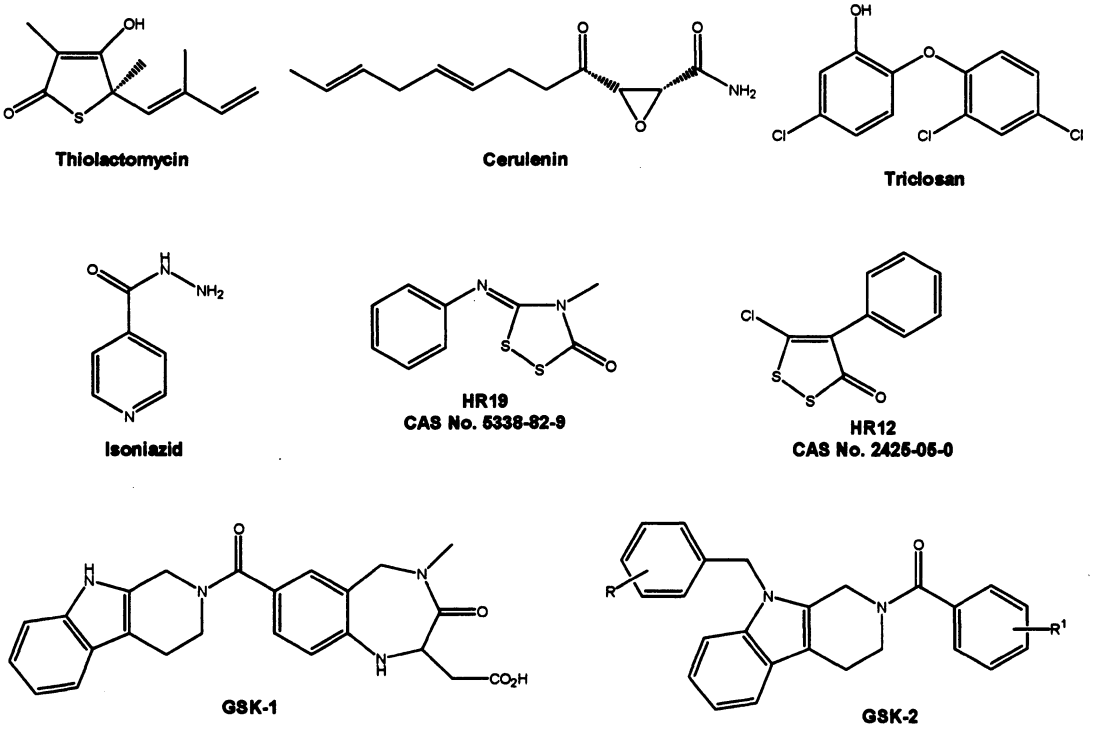
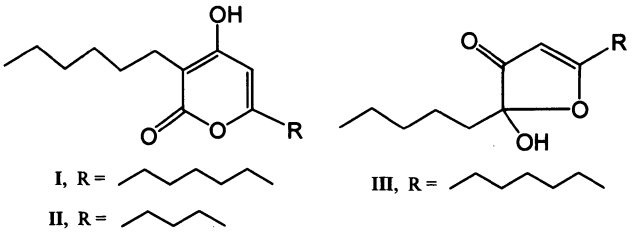


Fig. 3. Chemical structures of α-pyrones (I, II) and furanone (III).



of culture F92S91, *in vitro* antibacterial activities of the pyrones and peptides isolated from culture F92S91, and mechanistic studies of the α-pyrone I compared with triclosan. This work was presented at the 42nd ICAAC held in San Diego (M. P. SINGH, F. KONG, J. E. JANSO, D. A. ARIAS, P. SUAREZ, P. J. PETERSEN, V. S. BERNAN, G. CARTER & M. GREENSTEIN: Novel α-Pyrones and Peptides Produced by a Marine *Pseudomonas* sp. F92S91: Antimicrobial and Mechanistic Activities. Abstr. No. 1624 presented at the 42nd Interscience Conference of Antimicrobial Agents and Chemotherapy held in San Diego, CA, Sept. 27~30, 2002).

Materials and Methods

Media

Mueller Hinton (MH) medium was purchased from Becton Dickinson Microbiology Systems, Cockeysville, MD. Tryptic Soy Broth (TSB) and Heart Infusion Broth (HIB) were purchased from Difco Laboratories, Detroit, MI. ATCC Medium #21 (supplemented with 20 mg/liter of L-tryptophan) for *B. subtilis* was prepared as previously described[10].

Chemicals

[^{3}H]thymidine (Tdr, TRK.686), [^{3}H]uridine (Udr, TRK.410), and [^{3}H]amino acids (AA, TRK.550) were purchased from Amersham Corporation, Arlington Heights, IL. Galactan-Star® and Saphire II® were purchased from PE Biosystems, Bedford, MS. All reference antimicrobial agents and media components were purchased from Sigma Chemical Co., St. Louis, MO. Stock solutions (100× concentration, *e.g.* 12.8 mg/ml solution is needed for 128 μg/ml to be the highest concentration tested) of test compounds and reference antibiotics were prepared in suitable solvent (DMSO or water) and stored appropriately until needed. Two-fold serial dilutions of each compound were prepared in the same solvent, and 1 μl of the desired dilutions was transferred into wells of a 96-well microtiter plate. A similar volume of the solvent was used as an untreated control.

Bacterial Strains

B. subtilis PY79, the FAB assay organism, was provided by Millennium Pharmaceuticals, Inc, MA. Clinical isolates were collected from various medical centers in the United States, and QC strains were obtained from the ATCC. *B. subtilis* trpC2 BGSC1A1 (*B. subtilis* 327) and *E. coli imp* BAS849 were obtained from the Bacillus Genetic Stock Center, Columbus, Ohio, and S. A. BENSON[11], respectively.

Culture Conditions

A cryovial containing the frozen culture was thawed, and 10 μl of the bacterial suspension was inoculated into 20 ml of the appropriate medium in a 250 ml Erlenmeyer flask. The flask was incubated overnight at 37°C with shaking at 200 rpm. The absorbance (A_{600}) of the overnight culture diluted 1 : 10 in fresh medium ranged between 0.25~0.35. The overnight culture was diluted 1 : 200 (200 μl/40 ml in 250 ml flask) into fresh, prewarmed medium and was incubated as before until an A_{600} of 0.20~0.25 was attained (within 2~3 hours). This log-phase culture was immediately used for MIC/GIC determinations and a modified *Bac*Light membrane-damaging test.

FAB Induction Assay

The Wyeth Procedure No. 3988 was used to detect the FAB inhibitors in our natural products library (NPL). The assay organism *B. subtilis* PY79 (PyhfB::lacZ) was grown at 30°C and 200 rpm in the low-salt LB to an exponential phase (OD_{600}=0.60). Fifty microliters (50 μl) volumes of the culture were inoculateed into the wells of a 96-well white Optiplate containing 1 μl of the test drug at 50× concentration or water (or DMSO) for untreated control. The plate was incubated at 30°C for 5 hours with shaking. At the end of the incubation period, the plate was either frozen at −80°C or processed immediately. For immediate processing, 50 μl of the freshly prepared mixture of the substrate Galacton-Star (0.4 parts), Sapphire-II Enhancer (2 parts) in 7.6 parts of the lysis buffer (0.026% Sodium Deoxycholic acid, 0.053% Hexadecyltrimethyl-Ammonium Bromide, 265 mM NaCl, 395 mM HEPES, pH 7.5) was added to each well. The plate was incubated at room temperature for 1 hour and luminescence for each well was read using a Luminometer (Victor^2 V, Perkin Elmer, USA). The induction ratio (luminescence count for drug treated/untreated) for each well was calculated and

induction ratios of >5.0 were recorded as active.

Fermentation

Forty 250 ml erlenmeyer flasks, each containing 50 ml of fermentation production medium (dextrose 10.0 g/liter, yeast extract 5.0 g/liter, soluble starch 20 g/liter, $CaCO_3$ 1 g/liter, and NZ-amine 5 g/liter, pH 7.3), were inoculated with 2% v/v of a three day starter in TSB. The flasks were incubated at 22°C with aeration, and the culture was harvested after four days.

Isolation and Structure Elucidation

The above frozen culture (1 liter) was thawed and centrifuged. The supernatant was extracted with *n*-BuOH, and the mycelial pellet was extracted with MeOH. Both *n*-BuOH and MeOH extracts were combined, concentrated, and then loaded onto an HPLC column to give an active fraction L18748-fr17, which was further purified by semi-prep-HPLC to yield a novel pyrone compound **I**. The structure for **I** was elucidated by NMR and MS spectroscopic studies. The pyrone compound (**I**) was not stable under acidic conditions. It gradually decomposed to a furan derivative **III** in deuterated chloroform.

Metabolic Tests

F92S91 and five reference *Pseudomonas* cultures from the ATCC in Manassas, Virginia (*P. plecoglossicida* ATCC700383, *P. monteilii* ATCC700476, *P. putida* biovar A ATCC12633, *P. putida* biovar B ATCC17472, and *P. fluorescens* ATCC13525) were plated onto Tryptic Soy Agar (Difco) and incubated at 28°C for 24 hours. Identification was performed with the BBL Crystal™ enteric/nonfermentor ID kit (Becton Dickinson Labs) according to the manufacturer's instructions with a modification in the incubation temperature (28°C) and duration (40 hours).

Ribotyping

F92S91 and the five ATCC *Pseudomonas* sp. were typed using the RiboPrinter® Microbial Characterization System (Qualicon, Wilmington, DE). All samples were prepared and typed according to BRUCE[12]. In addition to *Eco*RI, all samples were cut with *Pvu*II in a separate batch run.

16S rDNA Analysis

Genomic DNA was isolated from F92S91 by a phenol/chloroform/isoamyl alcohol (Sigma) extraction. The 16S rDNA was amplified through PCR using partial primers of 8FPL and 1492RPL[13]. The PCR product was ligated into pCR®2.1 and transformed into InvF' using the Original TA Cloning® kit from Invitrogen Corporation Carlsbad, CA. Plasmids were isolated from successful transformants, and the DNA was sequenced on an ABI 3700 Sequencer from Applied Biosystems. The 16S rDNA sequences of the 11 *Pseudomonas* sp. and *E. coli* used in the analysis were obtained through the GenBank database.

Phylogenetic Analysis

The sequences were aligned in Clustal X 1.81[14] with default settings for the alignment parameters. A phylogenetic tree was generated in TREECON 1.3b[15] with the distances calculated according to the JUKES and CANTOR[16] method and with insertions and deletions taken into account. Bootstrap values were calculated using 1000 bootstrap samples, and neighbor-joining was used to infer tree topologies. The tree was rooted with *E. coli* (GenBank accession AX174877) as the outgroup.

In Vitro Antibacterial Activity

The *in vitro* antibacterial activities were determined by the microbroth dilution method as described earlier[10]. Briefly, microtiter plates containing 1 μl of two-fold serially diluted (range 12.80 to 0.006 mg/ml) antimicrobial agents in DMSO or sterilized water were inoculated with 100 μl of exponential phase culture adjusted to an inoculum density of $1\sim5\times10^5$ CFU/ml. The MICs were determined after 18 hours incubation at 37°C. The MIC was defined as the lowest concentration of the antimicrobial agent that completely inhibited visual growth of the organism.

Alternatively, microtiter plates containing 1 μl of serially diluted (range 12.80 to 0.006 mg/ml) test sample per well were inoculated with 100 μl of log-phase culture with an $A_{600}=0.20$. Plates were incubated at 37°C with agitation for 3 hour, and the absorbance at 490 nm of each well was recorded using the Molecular Devices microplate reader. The GIC was defined as the lowest concentration of the drug that prevented any increase in the absorbance during the 3 hour period.

Bactericidal activity against *B. subtilis* was determined by monitoring the change in absorbance at 490 nm using the Molecular Devices Thermomax plate reader setup to read in the negative kinetic mode every five minutes for 4 hours at 37°C. Decrease in the absorbance was used an the indication of loss of viability.

Macromolecular Synthesis

Uptake of appropriate radiolabeled precursors and their incorporation into TCA-precipitable material of *B. subtilis* were measured as previously described[10]. Cells (100 μl) treated with antibiotics or water (untreated control) were

pulse-labeled for 5 minutes by adding 2 μCi/ml (final conc.) of the radiolabeled precursors. To assess the effects on cellular uptake of precursors, the cells were harvested onto a glass filter using a 15-sec wash cycle with chilled saline. For incorporation of precursors into macromolecules, chilled TCA (10%, 100 μl) was added immediately to each well, and the plate was refrigerated for 1 hour. The precipitate was collected on a Wallac filtermat B and washed with chilled TCA (5%) using a Skatron 96-well harvester. Filter mats were dried in a microwave oven, solid scintillant (Pharmacia Meltilex B) was applied, and the isotope that was retained on the filter was quantitated in an LKB Betaplate scintillation counter. The levels of uptake and incorporation of [^{3}H]Tdr, [^{3}H]Udr, and [^{3}H]AA are expressed as the percent of that of the untreated control.

*Bac*Light Test

An exponential phase culture of *B. subtilis* PY79 (200 μl at A_{600} of 0.20) was treated with the test compound (2 μl of 100× conc. in DMSO) or the solvent for 15~30 minutes. Cells were pelleted by centrifugation (2,000 *g* for 2 minutes), washed once with saline (200 μl) and were then pelleted again. Supernatant (180 μl) was discarded, and 10 μl of the BacLight stain (1 : 10 fold diluted 1 : 1 mix of stain A and B from Molecular Probes Inc, Eugene, OR) was added to the cell suspension and vortexed for 10 seconds. Cells were allowed to stain in the dark for 15 minutes and were then examined by fluorescence microscopy using FTIC and Rhodamine filters for green (live) and red (dead) cells, respectively.

Hemolytic Activity

Saline washed RBCs (10^8 cells/ml) from freshly pooled human blood were treated with drug or water for 2 hours[17]. RBCs were pelleted by centrifugation and the A_{540} of the supernatant was measured. For 100% lysis, 25 μl of RBC suspension was added into 1 ml of water.

In Vivo Activity

The *in vivo* activities of compounds were assessed in mice infected intraperitoneally with *S. aureus* Smith (2.4×10^4 cells/mice). Antibiotic was administered intraperitoneally in single doses 1/2 hour after infection. Seven-day survival ratios with 5 dose levels and 5 animals per dose level were used for the determination of median effective doses (ED_{50}).

Results and Discussion

FAB Screen

In our collaboration with Millennium Pharmaceuticals, a *B. subtilis* strain harboring a lacZ reporter gene fusion to a cerulenin and triclosan-responsive promoter was constructed and was used for the detection of inhibitors of FAS II enzymes. Of 46,000 microbial extracts screened 280 extracts were found to be active. Further testing and bioprofile analysis (biological activities in our natural products database) reduced the number of confirmed hits to 25 cultures, which were fermented and prioritized for structure elucidation. Several known compounds were identified to be active in this screen, but they were not studied further.

Producing Organism

Bacterium F92S91 was isolated from a marine sponge from Fiji in the year 1992. It was fermented in four different media, and their extracts were deposited in the Wyeth natural products library for screening. Prior to the FAB screen, this culture was recorded as marginally active against Gram-positive bacteria (in our SSE screen), but was inactive in all other screens including those used for the detection of cytotoxic and DNA-damaging agents. Therefore, the FAB activity of this culture was considered as a unique activity, and the culture was prioritized for further evaluation.

Taxonomic Characterization

Culture F92S91 and five reference *Pseudomonas* species from the ATCC were plated onto TSA, and the morphological characteristics were compared after 24 hours incubation at 28°C. The cultures were then identified by the BBL Crystal™ enteric/nonfermentor ID kit. Culture F92S91 was metabolically similar to other genetically related *Pseudomonas* sp., but with the following distinct features: a) it grew well at 37°C similar to *P. monteilii* alone, b) it did not utilize any of the sugars or malonic acid in the BBL Crystal™ kit, similar only to *P. plecoglossicida*, and c) it did utilize lysine and GGL unlike the *P. plecoglossicida* (Table 1). Ribotyping of the F92S91 and other five reference strains was done using Qualicon's RiboPrinter® Microbial Characterization System with two distinct site-specific enzymes, *Eco*RI and *Pvu*II. RiboPatterns for the culture F92S91 were distinct from the closely related strains (Figs. 4 and 5). Additionally, isolation of the genomic DNA from F92S91, amplification

Table 1. Metabolic profile of culture F92S91 compared with reference strains.

	F92S91	*P. plecoglossicida* ATCC 700383	*P. monteilii* ATCC 700476	*P. putida* biotype a ATCC 12633	*P. putida* biotype b ATCC 17472	*P. fluorescens* ATCC 13525
Arabinose	-	-	-	-	-	-
Mannose	-	-	+	+	+	+
Sucrose	-	-	-	-	-	-
Melibiose	-	-	-	-	-	+
Rhamnose	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-
Galactose	-	-	-	+	-	+
Inositol	-	-	-	-	-	-
GGL	+	-	+	+	-	+
Esculin	-	-	-	-	-	-
Phenylalanine	+	-	+	+	+	+
Urea	+	+	+	+	+	+
Glycine	+	+	+	+	+	+
Citrate	+	+	+	+	+	+
Malonic acid	-	-	+	+	+	+
TTC	+	+	+	+	+	+
Arginine	+	+	+	+	+	+
Lysine	+	-	+	-	+	+
Growth at 37°C	+	-	+	-	+/-	-

Fig. 4. Riboprints generated with EcoRI.

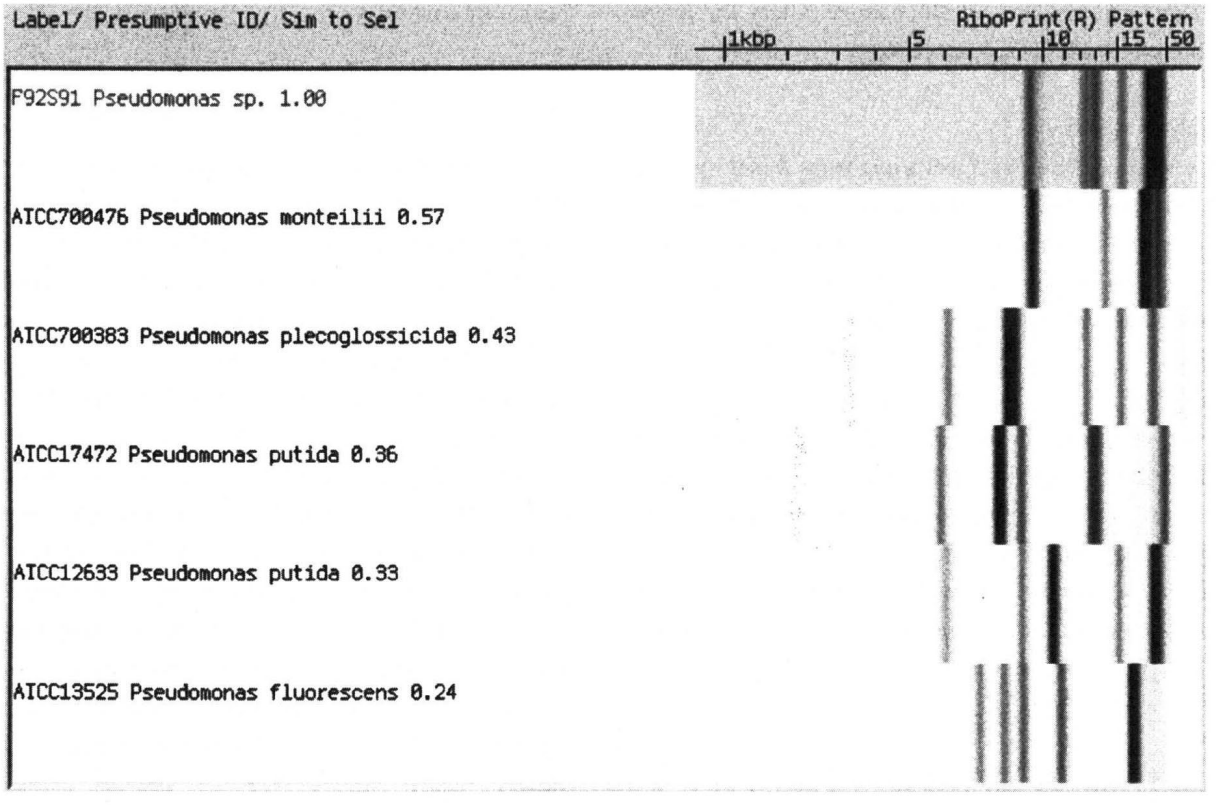


Fig. 5. Riboprints generated with PvuII.

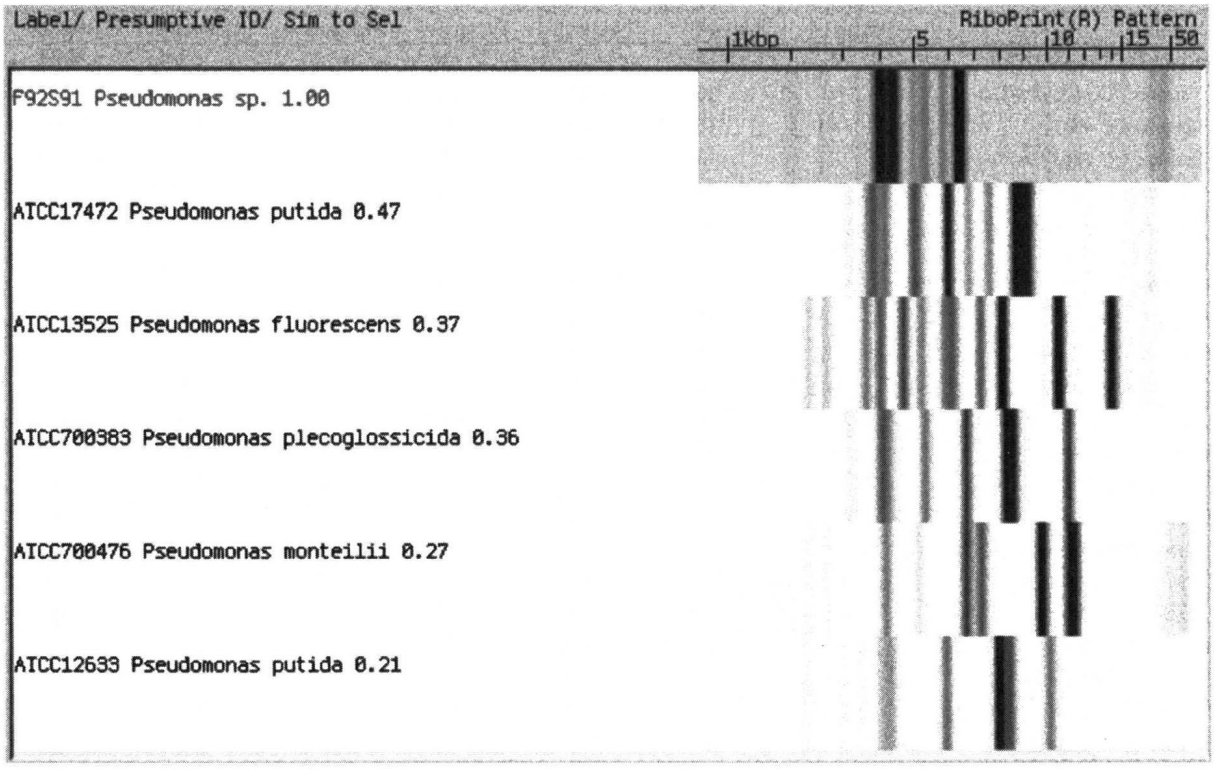


Fig. 6. 16S rDNA phylogenetic tree.

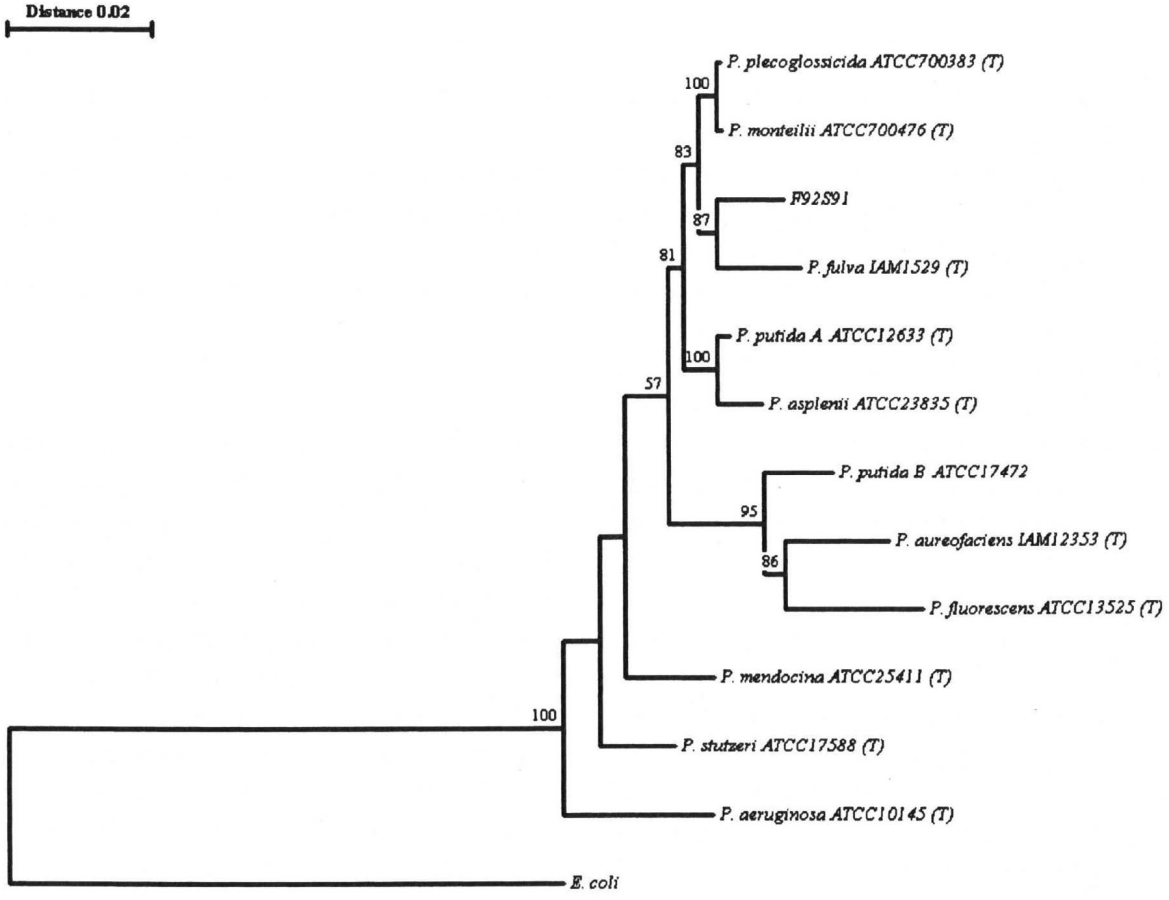


Table 2. Antimicrobial activities (MIC, μg/ml) of compounds isolated from F92S91.

Organism	Pyrone-I	Pyrone-II	Pyrone-III	Peptide-I	Cerulenin	Triclosan	Piperacillin
B. subtilis GC 6344 (168)	1	8	2	128	8	0.5	<0.25
S. aureus GC 1131 (MRSA)	4	16	16	64	64	<0.25	>128
S. aureus GC 4541	4	16	16	128	NT	NT	>128
S. aureus GC 4543 (Smith)	2	16	16	64	256	<0.25	0.50
S. aureus GC 2216 (ATCC)	2	16	32	64	64	<0.25	4
S. haemolyticus GC 4547	4	16	16	32	NT	NT	>128
MRCNS GC 4548	4	32	16	64	NT	NT	32
MRCNS GC 4549	64	32	32	32	NT	NT	64
MRCNS GC 6257	4	16	16	32	NT	NT	8
E. faecalis GC 6189	64	32	64	128	NT	NT	2
E. faecalis GC 4555	64	64	64	128	1	0.5	1
E. faecalis GC 2242 (VRE)	64	64	16	64	1	<0.25	4
E. faecium GC 4556	2	32	32	64	NT	NT	2
E. faecium GC 2243	64	32	64	64	NT	NT	128
E. faecium GC 4558	4	16	16	64	NT	NT	>128
S. pneumoniae GC 1894	16	32	32	16	128	8	2
S. pneumoniae GC 6242	64	128	64	16	NT	NT	<0.12
C. albicans GC 3066	>128	>128	>128	>128	1	8	>128
E. coli GC 4559 (wt)	>128	>128	>128	>128	256	<0.25	1
E. coli GC 4560 (imp)	128	32	128	32	2	<0.25	<0.12
E. coli GC 2203	>128	>128	>128	>128	NT	NT	1
M. catarrhalis GC 6219	4	8	4	32	64	<0.25	2
H. influenzae PT 7691	>128	8	128	>128	2	<0.25	<0.12
H. influenzae PT 7696	>128	8	16	>128	2	<0.25	<0.12

of the 16S rDNA by PCR, ligation of the PCR product into pCR2.1, transformation into InvF', and the isolation of the plasmid from the transformants provided material for DNA sequencing. Finally, the 16S rDNA sequences of the culture F92S91 were compared with DNA sequences (obtained from the GenBank) of eleven different *Pseudomonas* and an *E. coli* strain. Further analysis of 16S rDNA revealed that F92S91 was most closely related to *P. fulva* and was in the same clade as *P. plecoglossicida* and *P. monteilii*, but it branches away from them on the phylogenetic tree (Fig. 6). Metabolic characteristics, RiboPrints, and the genomic analyses of the culture F92S91 are suggestive of a new species of *Pseudomonas*.

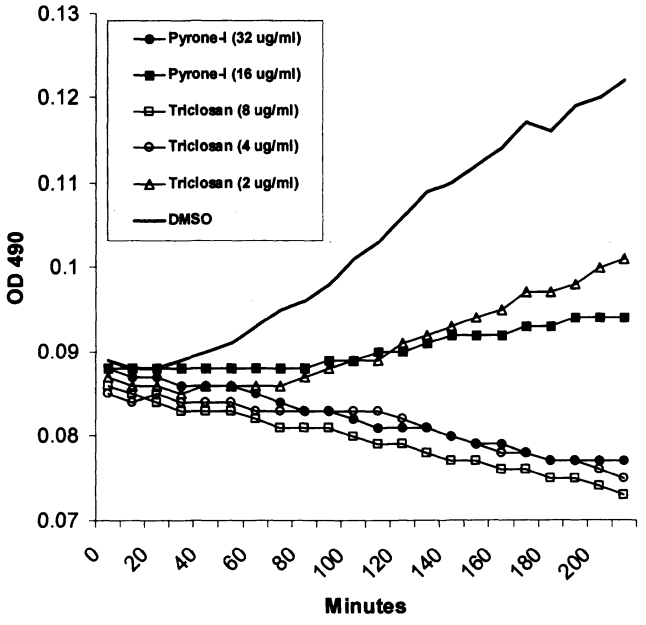
Novel Compounds Isolated

Culture F92S91 was found to produce two novel α-pyrones (Fig. 3) and two peptides (structures unresolved) that were active in the primary screen, and they exhibited antibacterial activity against Gram-positive pathogens. α-Pyrone **I** was the most active FAB and antibacterial component (Fig. 3). The furan derivative **III** was a degradation product of α-pyrone I. Coincidentally, the α-pyrone I was reported to be isolated from a culture broth of a *P. fluorescens*[8]. Chemical structures of the peptides were not completely resolved. Details of the isolation method and structure elucidation are being reported separately.

Biological Activities

The first two pyrones (Fig. 3) were found to be the compounds responsible for the original FAB activity, and their activities were detected at 1 to 2 μg/ml concentration. Pyrone I, the major component produced by the organism, demonstrated the greatest activity in the FAB assay, and it had the best antibacterial activity against the Gram-positive bacteria tested (Table 2). Minimum inhibitory concentrations (MICs) of pyrone I were 2 to 4 μg/ml against *S. aureus*, *M. catarrhalis* and *E. faecium*; 16~64 μg/ml against *E. faecalis*, *S. pneumoniae*; and >128 μg/ml against *E. coli* and *C. albicans*. It was also bactericidal against *B. subtilis* (Fig. 7).

Fig. 7. Bactericidal activity of pyrone and triclosan against *B. subtilis* PY79.



Log-phase culture at 0.20 of OD_{600} (100 μl/well) in a microtiter plate was treated with two-fold serially diluted drug, and change in the OD_{490} was recorded for 4 hours.

MOA Studies of α-Pyrone I

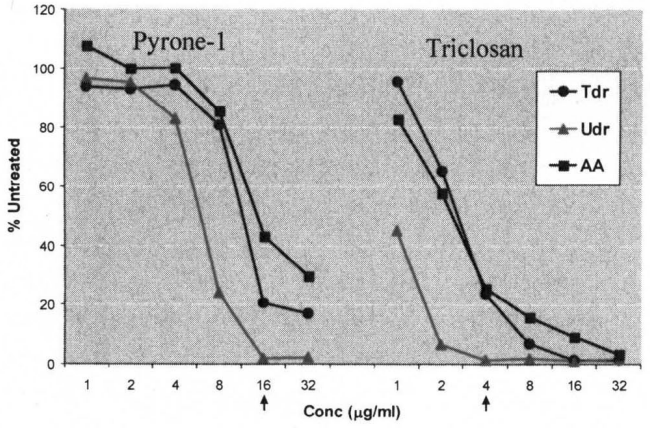
The cellular uptake and incorporation of appropriate radiolabeled precursors were measured in drug-treated log-phase *B. subtilis* cultures. Both pyrone I and triclosan rapidly affected the cellular uptake of thymidine, uridine, amino acids and acetates suggesting nonspecific membrane-damaging effects of these drugs on *B. subtilis*. Tracking of macromolecular synthesis also suggested rapid and nonspecific inhibition of cellular uptake of radiolabeled precursors for DNA, RNA and protein within 5 minutes of drug exposure. The most likely consequence was the nonspecific inhibition of all three precursors' incorporation into the macromolecules of drug-treated cells (Table 3). Under the same test conditions, ciprofloxacin, rifampin and chloramphenicol, predominantly inhibited DNA, RNA and protein synthesis, respectively. Cerulenin, a known inhibitor of the fatty acid biosynthesis enzyme FabB, exhibited only a weak inhibitory effect on the uptake and incorporation of uridine and amino acids. The nonspecificity of pyrone I and triclosan effects upon the cellular uptake of precursors appeared to be similar in the short term (Fig. 8), but a slightly different pattern emerged for prolonged drug treatments of 30 or 45 minutes (Fig. 9). Cells treated with 1×GIC of pyrone I appeared to have recovered from the

Table 3. Effects on cellular uptake of radiolabeled precursors and their incorporation into macromolecules of *B. subtilis* PY79.

Compound	Conc.	Thymidine		Uridine		Amino Acids	
	(μg/ml)	Uptake	Incorp.	Uptake	Incorp.	Uptake	Incorp.
Pyrone I	4	120	113	98	41	109	89
	8	70	88	90	19	62	48
	16	34	32	1	13	33	17
	32	6	51	3	16	7	21
Cerulenin	16	138	119	69	75	48	85
	32	140	124	50	58	50	92
Triclosan	2	31	18	6	4	9	14
	4	16	10	2	3	4	4
Ciprofloxacin	0.25	33	12	102	98	97	95
	0.5	31	9	104	103	97	95
Rifampin	0.25	96	83	96	7	67	25
	0.5	91	84	99	7	74	27
Chloramphenicol	4	96	91	92	105	64	30
	8	90	83	87	98	61	19

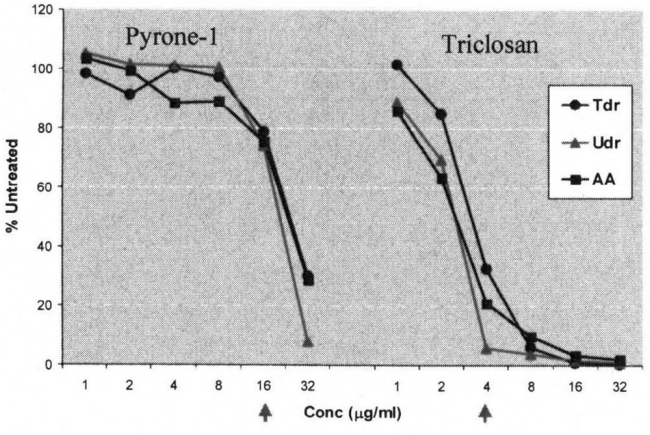
Data for 5 min drug treatment and 5 min pulse-labeling

Fig. 8. Concentration dependent effects of pyrone and triclosan on the cellular uptake of [^{3}H]thymidine (Tdr), [^{3}H]uridine (Udr) and [^{3}H]amino acids (AA) in a log-phase culture of *B. subtilis* 327 treated for 5 minutes.



The arrow indicates the GIC3h of the compounds tested.

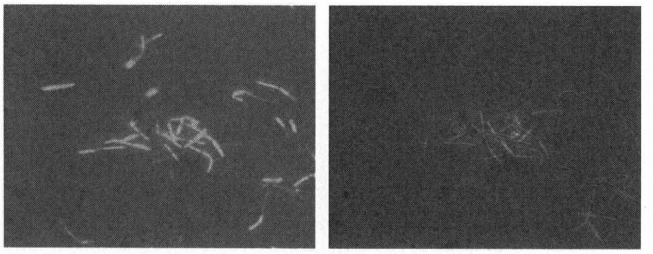
Fig. 9. Concentration dependent effects of pyrone and triclosan on the cellular uptake of [^{3}H]thymidine (Tdr), [^{3}H]uridine (Udr) and [^{3}H]amino acids (AA) in a log-phase culture of *B. subtilis* 327 treated for 45 minutes.



The arrow indicates the GIC3h of the compounds tested.

earlier nonspecific disturbances in their membrane function, whereas triclosan did not show such recovery. Triclosan is reported to be an inhibitor of the enzyme FabI, but it appears to have additional nonspecific effects in

Fig. 10. BacLight test of pyrone I.



Dead cells viewed with rhodamine and fluorescein filters

bacteria consistent with previously reported data[18,19].

Effects on Procaryotic and Eucaryotic Membranes

The membrane-damaging effects of pyrone I and triclosan on *B. subtilis* and human red blood cells were studied by using the BacLight viability test and hemolysis, respectively. Pyrone I and triclosan, both at 1~2 μg/ml, tested positive in the BacLight Live/Dead test. However, pyrone I did not cause significant hemolysis of hRBCs at 128 μg/ml after a 2-hour treatment. Triclosan and amphotericin B were more hemolytic than the pyrone I (Table 4).

In Vivo Efficacy and Toxicity

Pyrone I was tested at 2, 4, 8 and 16 mg/kg doses in mice with acute infection with the *S. aureus* Smith. This compound neither provided protection against the infection nor showed any toxicity in mice. The bioavailability of the drug through the intra-peritoneal route of administration was in question.

Conclusion

The FAB screen identified several extracts as active (0.6% hit rate), but only a few exhibited the desired antibacterial spectrum. Culture F92S91, producing the novel pyrones, was identified as a new species of *Pseudomonas*. Pyrone I was active against Gram-positive pathogens, and its primary mode-of-action appeared to be the disruption of membrane function. It lacked activity against Gram-negative organisms, and it did not provide *in vivo* protection against acute *S. aureus* infection in mice.

Table 4. Membrane-damaging effects in prokaryotic and eukaryotic cells.

Compound	Concentration (μg/ml)	BacLight Test[1]	% Hemolysis[2] 2h	24h
α-Pyrone-I	128	+	12	51
	64	+	7	8
	2	+	0	0
Triclosan	32	+	58	99
	16	+	13	31
	8	+	2	7
	2	+	0	0
Amphotericin B	4	-	87	99
	2	-	33	30

[1]Tested in *B. subtilis* 327 and PY79, treated for 30 min. +, red cells; -, green cells.

[2]Tested in human red blood cells, treated for 2 and 24 hours.

Activity against individual Fab enzymes may be tested to understand its precise target in the fatty acid biosynthetic pathway.

Acknowledgements

Authors would like to thank every one associated with the Wyeth-Millenium antibacterial collaboration. Special thanks to Drs. DAVID SHLAES, STEVE PROJAN, PHIL YOUNGMAN, CHRIS MURPHY and CHRISTIAN FRITZ for their support and guidance.

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