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Agrochelin, a New Cytotoxic Antibiotic from a Marine *Agrobacterium*

Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Biological Activity

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Agrochelin, a new alkaloid cytotoxic substance, was produced by the fermentation of *Agrobacterium* sp. The compound was obtained from the bacterial cells by solvent extraction and purified by silica gel chromatography. Agrochelin (**1**) and its acetyl derivative (**2**) exhibited cytotoxic activity.

During our screening for new antitumor compounds, we discovered a new substance named agrochelin (**1**) from the cells of marine *Agrobacterium* sp. The structure of agrochelin is closely related to the structures of yersiniabactin[1] and yersiniophore[2] produced by *Yersinia enterocolitica* and micacocidins[3,4] A, B, C isolated from *Pseudomonas* sp. In this paper, we report the taxonomy of the producing strain, fermentation and isolation of the antibiotic, as well as the physico-chemical properties and biological activity. Structural studies of agrochelin and its acetyl derivative will be described in a forthcoming paper.

Material and Methods

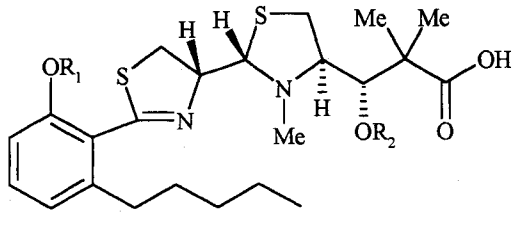
Producing Strain

Strain ALET-304 was isolated from a marine tunicate, *Ecteinascidia turbinata*, collected in Formentera Island (Spain). The tunicate was homogenized with sterile seawater, serially diluted and plated on Marine Agar 2216 (Difco). Plates were incubated at 28°C for 14 to 21 days. Colonies were picked and isolated to purity. Pure strains were fermented and cytotoxicity tests were carried out with the organic extracts from the fermentation broths. Strain ALET-304 was selected for further studies due to its cytotoxic activity.

Taxonomic Studies

Observation of growth on various media and tests for morphological and physiological characteristics were made on the basis of methods of COWAN[5] and COLWELL[6]. All media were supplemented with 2.7% of Instant Ocean Salts

Fig. 1. Structures of agrochelin (**1**) and acetyl derivative (**2**).



1 $R_1=R_2=H$
2 $R_1=R_2=Ac$

(Aquarium Systems, Inc., France).

Cellular fatty acid methyl esters were obtained by the method described by VAN DER AUWERA[7] and were separated by gas chromatography by using a Hewlett-Packard chromatograph, model HP 5890A. The chromatographic profile of strain ALET-304 was compared with the profile of our DataBase of reference strains.

ALET-304 DNA was obtained by boiling several colonies for 5 minutes in sterile water. 16S rDNA was amplified by PCR in a Eppendorf Mastercycler 53-30, following a standard PCR protocol. The primers used in the amplification corresponded to positions 8 to 29 and 710 to 689 in the *Escherichia coli* 16S rRNA sequence. PCR amplified DNA was purified by Ultra-free-MC 30000NMWL filter units (Millipore Inc.), and directly sequenced by using an automated DNA sequencer (373 XL Perkin Elmer).

The sequence was compared to the compilation of 16S rDNA genes available in the GenBank nucleotide library by BLAST searching, ALTSCHUL[8] through the U.S National Center for Biotechnology Information.

Fermentation

A seed culture was prepared by inoculating 50 ml of a medium consisting of Tryptone (Difco) 0.5%, yeast extract 0.2%, glucose 0.1% and Instant Ocean Salts 2.5%, with a frozen culture of the producing microorganism. After cultivation at 28°C for 30 hours with orbital agitation at 220 rpm, 40 ml culture was transferred to 2-liter Erlenmeyer flasks containing 500 ml of the same medium. The flasks were incubated at 28°C for 28 hours with orbital agitation at 220 rpm.

A fermentor of 75 liter capacity (Bioengineering LP-351) with 50 liter of the production medium consisting of dextrose 1%, Pharmamedia 1%, yeast extract 0.1%, $CaCO_3$ 0.4% and Instant Ocean Salts 1.8%, was inoculated with 2000 ml of the second stage seed culture. The fermentation was carried out at 28°C, 350 rpm and 35 liters air/minute. An overpressure of 0.5 bars is needed during the process. The production of the active compound in the fermentation broth was followed using an *in vitro* cytotoxic assay (against P-388 mouse-leukemia cells), using a modification of the Bergeron technique[9].

Analytical Procedure

Analytical TLC was done on pre-coated silica gel 60 F_{254} plates (0.2 mm thick, 20×20 cm, Merck) and the spots were detected under UV light (254 nm). Silica gel 60 (70~230 mesh, Merck) and Lichroprep RP-18 (40~63 μm, Merck) were used for column chromatography.

HPLC analysis was performed using an analytical pack cartridge Resolve C18 (10 μ, Waters Chromatography), using as a mobile phase methanol - 0.025 M phosphate buffer (pH=5.5) 8 : 2, at a flow rate of 2.0 ml/minute and with detection at 254 nm. The HPLC equipment was a Waters 991 with a photodiode-array detector and a Rheodyne injector.

IR spectra were recorded on a Perkin-Elmer 881 spectrophotometer. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. FAB-MS spectra were determined with a VG AutoSpec spectrometer.

Evaluation of Biological Activity

The cytotoxic activities of agrochelin and its acetyl derivative were determined *in vitro* in cell cultures of P-388 (ATCC CCL-46), mouse lymphoid neoplasm; A-549 (ATCC CCL-185), human lung carcinoma; HT-29 (ATCC HTB-38), human colon carcinoma and MEL-28 (ATCC HTB-72), human melanoma; following an adapted form of the method described by BERGERON[9].

Results and Discussion

Taxonomy of the Producing Strain

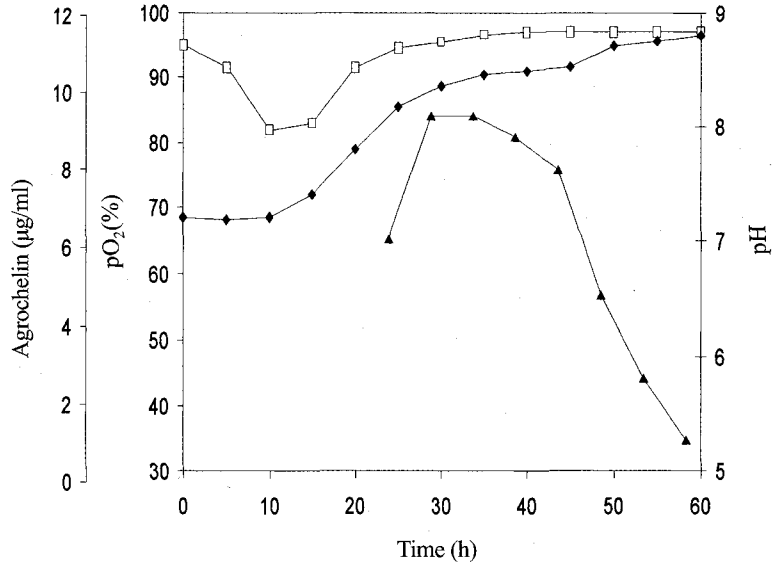
The producing strain is a non-sporulating, motile rod, occurring mostly single in young cultures which develops rosetta-shaped aggregates in older cultures. Colonies are low convex, circular, smooth, mucous, white to beige after 2 days incubation. Gram-negative, with an oxidase and catalase positive reaction and weakly for urease. Starch hydrolysis, gelatine liquefaction, skim milk coagulation, and indole production were negative. It can grow without NaCl in the media, but its growth was optimal with an addition of NaCl between 2 and 6%. Growth was observed up to 10% NaCl. Grows both at 4° and 37°C. It grows with glucose, glycerol, manitol or xylose as sole carbon source, not with maltose, sucrose or arabinose.

The fatty acid profile of strain ALET-304 was compared with the profiles of a DataBase composed of more than 500 reference strains. The profile of this strain correlated well with those of several "marine Agrobacterium" species. *Agrobacterium meteori* DSM 5824 was the most similar strain.

The fragment of the 16S rRNA gene sequence was aligned to its closest related sequences determined from the BLAST search. The closest-neighbouring species and strains were members of the genus *Agrobacterium* from the alpha subdivision of *Proteobacteria*. The closest relative was *Agrobacterium stellulatum* with a similarity of 93%.

Fig. 2. Time course production of agrochelin.

Agrochelin concentration (μg/ml) (▲), pO_2 (%) (□), pH (◆).



Based on the morphological[10,11], chemical and genetic characteristics of ALET-304, the strain was identified as a species of the genus *Agrobacterium*.

Fermentation

A typical time course profile of a 50 liter fermentation is shown in Fig. 2. The pH was increasing from 10 hours to the end of the process. The minimal partial pressure of oxygen was registered at 10 hours. The production of the active compound should start at 20 hours, and the maximum of the production was determined between 25 and 35 hours.

Isolation

The isolation procedure of agrochelin is summarized in Fig. 3. After completion of the cultivation, the whole broth (50 liter) was centrifuged to separate supernatant and cell mass. The cell mass was extracted twice with 3 liter of $CHCl_3$-CH_3OH-H_2O 2 : 1 : 1. The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuum to give a brown oil (4.8 g). The oily residue was dissolved in 500 ml of a mixture of 10% aqueous NaCl-MeOH 1 : 1 and defatted by partitioning twice with 500 ml of *n*-hexane. The aqueous alcohol fraction was extracted twice with 500 ml of $CHCl_3$, and the active $CHCl_3$ extracts were concentrated to yield a residue (2.3 g). The dissolved residue was applied to a silica gel VFC (vacuum flash chromatography) system and eluted with a stepwise gradient of EtOAc-MeOH. The eluate was analyzed by UV absorption at 254 nm on TLC, HPLC and by monitoring the cytotoxic activity against P-388 cells. The active fractions (1.2 g) were eluted with EtOAc-MeOH 1 : 1. MPLC on silica gel was repeated and the activity (570 mg) eluted with $CHCl_3$-MeOH 95 : 5. Further purification by C18 reversed phase chromatography gave 250 mg of pure agrochelin which eluted with MeOH : H_2O 85 : 15. The physico-chemical properties of agrochelin and its acetyl derivative are summarized in Table 1.

Biological Activity

The cytotoxic activity of agrochelin and its acetyl derivative against tumor cell lines is shown in Table 2. The IC_{50} value of agrochelin to P-388 cells was 0.053 μM, this compound showed only slightly lower activity against the rest of cell lines tested. The cytotoxic activities of the acetyl derivative were substancially reduced compared with those of agrochelin.

Fig. 3. Isolation procedure for agrochelin.

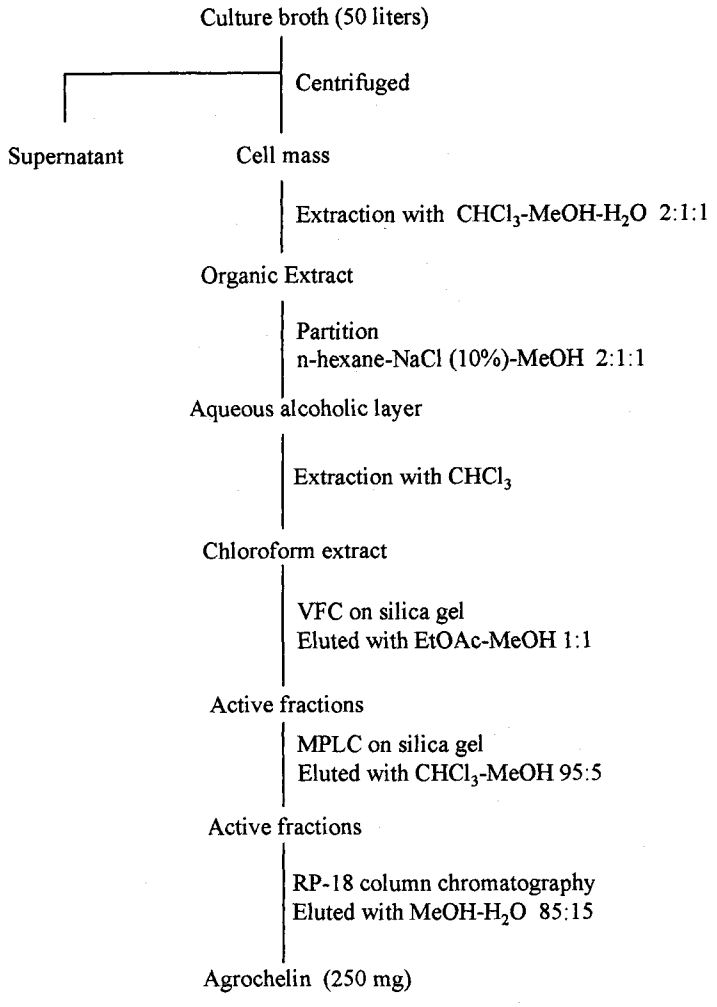


Table 1. Physico-chemical properties of agrochelin (**1**) and acetyl derivative (**2**).

	1	**2**
Appearance	Pale yellow oil	Pale yellow oil
Molecular formula	$C_{23}H_{34}N_2O_4S_2$	$C_{27}H_{38}N_2O_6S_2$
FAB-MS $(M+H)^+$	467	551
HRFAB-MS $(M+H)^+$	467.2042 (calcd. 467.2038)	551.2229 (calcd. 551.2249)
$[\alpha]_D^{25}$	- 20.5° (c 0.2, $CHCl_3$)	
$UV\lambda_{max}^{MeOH}$ nm	210, 264	210, 265
IR υ_{max}(KBr) cm^{-1}	2934, 1702, 1582	2933, 1733, 1459
	1448,1208, 1162	1367, 1200
TLC[a] (Rf value)[b]	0.52	0.60
HPLC (Rt, minutes)[c]	3.15	2.32

[a] Silica gel 60 F_{254}, Merck.
[b] Solvent: $CHCl_3$ - MeOH (9 : 1).
[c] Resolve C18 redial pack cartridge (10 μ); mobile phase: MeOH - 0.025 M buffer phosphate (pH=5.5) (4 : 1); flow rate: 2 ml/minute; detection: 254 nm.

Table 2. Cytotoxic activity of agrochelin and acetyl derivative (IC_{50}, μM).

Compound	P-388	A-549	HT-29	MEL-28
Agrochelin	0.053	0.107	0.268	0.268
Acetyl derivative	3.630	7.270	7.270	7.270

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