Karalicin, a New Biologically Active Compound from *Pseudomonas fluorescens/putida*

I. Production, Isolation, Physico-chemical Properties and Structure Elucidation

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An original compound, named karalicin, was isolated from a fermentation broth of the *Pseudomonas fluorescens/putida* strain SS-3 (CCM 4430). Production, physico-chemical properties and structure elucidation are described.

Several academic groups and private companies have to date a common goal in the finding of antiviral agents, since there is still a need for selective and efficient drugs to treat respiratory viral diseases, herpetic diseases and immunodeficiency-causing agents[1].

Researchers in this field mainly follow two methods; one is the screening of natural compounds; while the second is the synthesis of more efficacious derivatives starting from active parental molecules[2].

The first method has proven to be efficient when large number of natural compounds can be screened. This is quite a long and expensive job, since screenings for antiviral activity require cell cultures and more sophisticated equipment than that needed in the search for antibacterial drugs.

During a screening program for the detection of antiviral drugs using an original and rapid method, we isolated a strain of *Pseudomonas fluorescens/putida*, which produced a new compound, named karalicin (Fig. 1).

In this paper we describe the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties and structure elucidation. The biological properties will be presented in the accompanying paper[3].

Taxonomy of the Producing Strain SS-3

The producing strain was isolated from a sewer channel in the city of Cagliari (once known as Karalis, Sardinia, Italy). It was identified as a *Pseudomonas fluorescens/putida* biotype 5 and deposited in the Czech Collection of Microorganisms (CCM) with the accession number CCM 4430. The rapidly growing colonies produced a light yellow pigment. The ability of the strain to utilize several substrates is indicated in Table 1.

The biologically active colonies were selected after several passages on the medium AR, which had the following composition: yeast extract 2 g, casaminoacids 10 g, soluble starch 4 g, agar purified 11 g, NaCl 6 g, KCl 0.4 g, $CaCl_2 \cdot 2H_2O$ 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.1 g in 1 liter

Fig. 1. Structure of karalicin.

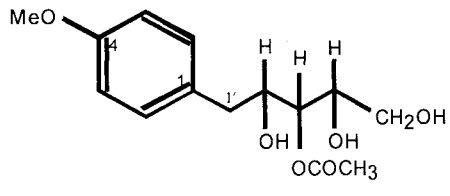

Table 1. Utilization of subtrates by strain *Pseudomonas fluorescens/putida* SS-3.

Substrate	utilization	Substrate	utilization
Rhamnose	-	Ribose	-
Inositol	+	Sucrose	+
D-Maltose	-	Lactose	+
D-Mannitol	+	D-Glucose	+
D-Sorbitol	-	D-Arabinose	-
Sodium citrate	+	3-Hydroxybenzoate	-
L-Fucose	-	Propionate	-
Malonate	+	Salicin	-
Melezitose	-	Itaconate	-
Acetate	+	L-Alanine	+
D-Melebiose	-	Caprate	+
Valerate	+	Citrate	+
Histidine	+	5-Ketogluconate	-
Glycogen	-	2-Ketogluconate	-
3-Hydroxybutyrate	+	N-Acetylglucosamine	+
L-Serine	+		

Table 2. Physico-chemical properties of karalicin.

Appearance	Brown powder
mp (C°)	134-136
Molecular formula	$C_{14}H_{20}O_6$
Optical rotation	$[\alpha]_D$ - 44.5 (*c* 0.1, $CHCl_3$)
EI-MS (m/z) (rel.int.)	284 (3), 266 (15), 222 (7), 206 (6), 162 (10), 144 (70), 134 (12), 122 (22), 121 (40), 108 (10), 84 (100)
Anal.	calcd for $C_{14}H_{20}O_6$: 284.1260
	found : 284.1265
Solubility	
Soluble:	MeOH, PrOH, EtOAc, $CHCl_3$, Me_2CO, DMSO
Sparingly soluble:	Water
Insoluble:	Hexane

of deionized water. After this treatment, stable hyper-producing colonies were obtained.

Fermentation

Inoculum for fermentation was prepared growing the strain in the AR broth (50 ml) in 250 ml Erlenmeyer flasks, for 18 hours at 28°C under shaking conditions (250 rpm) in a dry incubator (New Brunswick, model G-25). The microbial suspension was then seeded in the surface of purified agar-solidified AR medium plates of 18 cm of diameter and grown in static conditions for 5 days at 28°C.

Subsequently, the plates underwent a cycle of freezing and thawing, the fluid obtained was collected, pooled, centrifuged at 8,000 rpm for 15 minutes and extracted twice with 20% $CHCl_3$ (v/v). After evaporation of $CHCl_3$ in a vacuum distillator, a brown powder was obtained, which was used for purification of the active principles.

Isolation of Karalicin

A total of about 500 litres of culture of *Ps. fluorescens/putida* SS-3 was used for producing and extracting the active compound. A total of 12 g of a chloroform extract was obtained from about 250 litres of fluid extract. It was a brown powder which could easily be dissolved in DMSO and the activity was stable for several months when kept at 4~8°C.

The raw extract was dissolved in $CHCl_3$ - PrOH 1 : 1 (v/v) and passed through a Sephadex LH-20 column (Pharmacia, Uppsala, Sweden). The eluent was $CHCl_3$ - PrOH 1 : 1. Thin-layer chromatography (TLC) was used for screening the eluted fractions. In TLC the eluent $CHCl_3$ - PrOH 95 : 5 was used; 8 fractions were collected; the antiviral activity was mainly concentrated in the 3rd fraction. This fraction was again dissolved in Me_2CO - dichloromethane 7 : 3 (v/v) and eluted in a Sephadex LH-20 column. Six fractions were obtained; among these biological activity was concentrated in the 4th fraction.

2.5 g of a raw active fraction were obtained by this procedure. It was then chromatographed on a silica-gel column eluted with increasing amounts of MeOH in $CHCl_3$, affording the fractions S1 (720 mg; $CHCl_3$ - MeOH 19 : 1), S2 (347 mg; 9 : 1), S3 (165 mg; 4 : 1), S4 (100 mg; MeOH). Further purification on silica-gel with $CHCl_3$ - MeOH 9 : 1 of the active fraction S3 gave pure karalicin (35 mg). This substance underwent chemical-physical characterization for elucidating the structure formula (Italian Patent N. RM 94A.000688 24/10/94). Fig. 1 reports karalicin's structure.

Physico-chemical Properties

The physico-chemical properties of karalicin are summarized in Table 2. The molecular formula was determined as being $C_{14}H_{20}O_6$ on the basis of mass spectra run on a AEI-12 instrument at 70 eV. Optical rotation was determined on a Perkin Elmer 243 polarimeter.

Structure Elucidation of Karalicin

NMR spectra were obtained on a Varian Gemini-300 spectrometer in $CDCl_3$ (TMS as reference). NMR

Table 3. NMR data for karalicin.

Position	δ_C	δ_H	Long range H/C connectivities ^2J	^3J
1	130.5	-		
2, 6	129.6	7.10	113.9	158.1
				34.1
3, 5	113.9	6.82	129.6	130.5
			158.1	
4	158.1	-		
1'	34.1	2.80+	130.5	129.6
2'	61.4	3.56		
3'	81.4	4.75		171.1
4'	76.7	4.15		
5'	52.3	3.40		
		2.80+		
6'	171.1	-		
7'	21.0	2.13	171.1	
8'	55.2	3.77		158.1
OH	-	3.20		

+ Overlapped

parameters of karalicin, $C_{14}H_{20}O_6$, M^+ 284 are represented in Table 3. Direct ^1H and ^{13}C connectivities were obtained by HETCOR[4] experiments, while the assignment of the quaternary carbons followed INEPT[5] measurements. The spectroscopic data suggested a compound with the gross structure of a methoxy-benzene *para*-substituted by a C_5 chain containing an acetyl group. In accordance, the mass spectrum displayed the tropilium ion at m/z 122~121 and fragments derived by the losses of 122 and 60 a.m.u. from the molecular and $(M-18)^+$ ions.

Inspection of the carbon signals attributed to the chain revealed signals for two methylenes, (one of which oxygenated (δ_C 52.3)) and for three oxymethines. In the INEPT experiment, one of the oxymethines (δ_C 81.4; δ_H 4.75) showed long-range connectivity with the signal (δ_C 171.1) attributed to the carbonyl of the acetyl group. Moreover, in the COSY[6] spectrum the relative proton signal (δ_H 4.75) showed cross peaks to both CH signals at 4.15 and 3.56, while no cross peak to the methylene signals was observed. These data located the acetyl-bearing oxymethine at the 3′ position, and allowed the structure in Fig. 1 to be assigned to karalicin.

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