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Cystocin, a Novel Antibiotic, Produced by *Streptomyces* sp. GCA0001: Production and Characterization of Cystocin

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Abstract 3'-[S-Methyl-cysteinyl]-3'-amino-3'-deoxy-N,N-dimethyl adenosine, cystocin, is a biosynthesized antibiotic material newly identified from *Streptomyces* sp. GCA0001. Its structure was found to be similar to puromycin, where the terminal tyrosine is replaced by a methyl cysteine. NMR data prove that the 3-amino ribose is connected to dimethylamino-purine through the anomeric carbon at 1'-carbon. The methyl cysteinyl unit is connected to the amino unit of ribose by peptide bond. The verification of the structure was performed by comparing the puromycin nucleosides resulted from the hydrolysis of cystocin and puromycin, respectively. Antibiotic activity of cystocin against *Streptococcus* was found to be two times more potent than that of puromycin.

Key words: Cystocin, 3'-[S-methyl-cysteinyl]-3'-amino-3'-deoxy-N,N-dimethyl adenosine, puromycin, antibiotic activity, *Streptomyces*

Peptidyl transferase catalyzes peptide bond formation during protein synthesis on the ribosome by a process involving the transfer of a peptide chain from peptidyl-tRNA to aminoacyl-tRNA. Puromycin, which was isolated from *Streptomyces alboniger* in 1952 [12], is a well-known antibiotic [1, 2], and interferes with protein synthesis at the stage of chain growth by competing with the aminoacyl-tRNA [13, 14], since it is structurally similar to the aminoacyl-adenosyl terminal of aminoacyl-tRNA [5]. It has, however, been proved that the aromatic aminoacyl residue is not necessary for the biological activities [3].

During the course of screening fermentation broths for antibacterial activity, a derivative of puromycin was isolated from *Streptomyces* sp. GCA0001. 3'-[S-Methyl-cysteinyl]-3'-amino-3'-deoxy-N,N-dimethyl adenosine, cystocin in Fig. 1, is a newly biosynthesized derivative of puromycin. This article describes the isolation, physicochemical properties, and structure of cystocin.

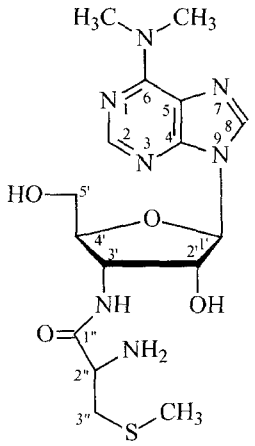

Fig. 1. Structure of cystocin.

Materials and Methods

Microorganism and Phylogenetic Study

Streptomyces sp. GCA0001 was isolated from several soil samples around Sunmoon University, Korea, and maintained on R2YE agar after cultivation [8]. *Streptomyces* sp. GCA0001 was deposited in the Korean Collection for Type Cultures (KCTC, Tajeon, Korea) with an accession number of KCTC0930BP.

Since *Streptomyces* sp. GCA0001 showed the color and shape of actinomycetes, phylogenetic study using 16S rRNA sequence was performed according to the Distance Model of Jukes and Cantor [6].

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Fermentation and Isolation

Spores of *Streptomyces* sp. GCA0001 were inoculated into three 500-ml flasks containing 150 ml of seed medium (yeast extract 0.75 g, beef extract 0.45 g, tryptose 0.75 g, soluble starch 3.6 g, dextrose 0.75 g, and calcium carbonate 0.6 g were added to 100 ml of water), and cultured on the rotary shaker with an agitation speed of 250 rpm for 3 days at 29°C. To obtain enough material for analysis, fermentation was performed with 450 ml of the above solution as an inoculum in a 14-l fermenter at 29°C for 4 days with an aeration rate of 3.0 v/v/m and an agitation speed of 300 rpm. The fermentation medium consisted of bacto-soyton 315 g, dextrin 450 g, calcium carbonate 63 g, cobalt chloride 0.216 g, and water to make a 9 l solution. The pH was adjusted to 7.4 with 1.0 M HCl solution prior to sterilization.

The fermentation broth was centrifuged, and supernatant solution containing cystocin was extracted with chloroform repeatedly. This solution was dried to solid in vacuum, and the resulting residue dissolved in $CHCl_3$:CH_3OH (9:1, v/v) was applied to silica gel column (silica 60, Merck) using the same $CHCl_3$-CH_3OH mixture as an eluent. To obtain the pure material, several applications to silica gel column were performed by changing the ratio of $CHCl_3$:CH_3OH mixture.

General Procedures

Elemental analysis was performed at Korea Basic Science Institute (KBSI, Seoul). UV absorption spectra were obtained by spectrophotometer (Shimazu UV-106A) using methanol solution of the antibiotic. FT-IR spectra were recorded with a Shimazu FTIR-8101 spectrometer. TLC was performed using aluminum plate coated with Silica gel 60 F_{254} (Merck), and chloroform-methanol (5:1) mixture was used as a developing solution. All mass spectrometric analyses were performed using a tandem mass spectrophotometer (JEOL, JMS-HX110/110A), a four-sector instrument of E1B1E2B2 configuration. The ion source was operated at 10 KeV accelerating voltage in positive-ion mode with a mass resolution of 1000 (10% valley). NMR spectra, including homo(^{1}H-^{1}H) and hetero(^{1}H-^{13}C) COSY, were obtained on VARIAN UNITY-300 INOVA spectrometers with $CDCl_3$ solutions of antibiotic at ambient temperature (^{1}H-NMR at 300 MHz and ^{13}C-NMR at 75 MHz). Chemical shifts are given in ppm using tetramethyl silane as an internal standard.

Hydrolysis of Cystocin and Puromycin

Puromycin, which was used for the identification of nucleoside derivatives resulting from the hydrolysis of cystocin by protease K (4.3 units/mg, Aldrich), was purchased from Aldrich. One hundred mg of cystocin, which is partially insoluble in water, was dissolved in 0.041 M KH_2PO_4 (pH=7.5) by adding methyl alcohol drop by drop up to 3 ml. After adding 10 mg of protease, the reaction mixture was kept at 38°C overnight. For puromycin, 23 mg of the sample and 4 mg of protease were used under the same reaction condition. The resulting hydrolyzed mixtures were freeze-dried, and the extracted methanol solution of the dried products was column chromatographed on silica gel 60 (Merck). The CH_3Cl:CH_3OH (3:1) mixture was used as an eluting solvent. After checking with TLC, solutions of resulting nucleoside derivatives were collected and dried for the identification using TLC and NMR.

Results and Discussion

Microorganism and Phylogenetic Study

The microscopic observation revealed that the spore mass was white in color and straight chain, showing resistance to antibiotics such as apramycin and neomycin.

The 16S rRNA from *Streptomyces* sp. GCA0001 has 582 bases as shown in Fig. 2. The 16S rRNA from *Streptomyces* sp. GCA0001 was found to have more than 97% similarity to *Streptomyces lydicus* ATCC 25470T (98.62%), *Streptomyces mashuense* DSM 40221T (98.24%), *Streptomyces albus* subsp. Albus DSM 40313T (97.95%), *Streptomyces albulus* ISP 5492T (97.41%), and *Streptomyces thermocoprophilus* DSM 41700T (97.06%), but the exact phylogenetic determination was not performed, since most of 16S rRNA sequences of *Streptomyces* have not yet been determined so far. Thus, the *Streptomyces* sp. GCA0001 can not be assigned any of the species determined up to now, and it has been deposited in KCTC as a new strain.

Fermentation and Isolation

During the fermentation process, 20 ml of antifoam aqueous solution (Antifoam 204, Sigma: 10% v/v) was added to prevent excess foaming. Also, $MgSO_4$ was fed according to the schedule into the final concentration of 0.5 M to increase the yield of cystocin [4, 9]. During the process of adding $MgSO_4$, 10 g of dextrin and 5 g of yeast extract were also added into the fermenter as a carbon source and a nitrogen source, respectively. A detailed optimization of the fermentation process had been published elsewhere [7].

The fermentation broth of *Streptomyces* sp. GCA0001 (9 liters) was centrifuged at 4,000 rpm for ten minutes, and

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GCGGC GTGCT TAACA CATGC AAGTC GAACG ATGAA CCTCC TTCGG GAGGG   50
GATTM GTGGC GAACG GGTGA GTAAC ACGTG GGCAA TCTGC CCTGC ACTCT  100
GGGAC AAGCC CTGGA AACGG GGTCT AATAC CGGAT ACGAC ACGGG ATCGC  150
ATGAT CTCCG TGTGG AAAGC TCCGG CGGTG CAGGA TGAGC CCGCG GCCTA  200
TCAGC TTGTT GGTGG GGTAA TGGCC TACCA AGGCG ACGAC GGGTA GCCGG  250
CCTGA GAGGG CGACC GGCCA CACTG GGACT GAGAC ACGGC CCAGA CTCCT  300
ACGGG AGGCA GCAGT GGGGA ATATT GCACA ATGGG CGMAA GCCTG ATGCA  350
GCGAC GCCGC GTGAG GGATG ACGGC CTTCG GGTTG TAAAC CTCTT TCAGC  400
AGGGA AGAAG CGCAA GTGAC GGTAC CTGCA GAAGA AGCGC CGGCT AACTA  450
CGTGC CAGCA GCCGC GGTAA TACGT AGGGC GCAAG CGTTG TCCGG AATTA  500
TTGGG CGTAA AGAGC TCGTA GGCGG CTTGT CACGT CGGAT GTGAA AGCCC  550
GGGGC TTAAC CCCGG GTCTG CATTC GATAC GG                       582
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Fig. 2. The sequence of 16S rRNA from *Streptomyces* sp. GCA0001.

Table 1. Physicochemical properties of cystocin.

Appearance	White powder
Molecular formula	$C_{16}H_{25}N_7O_4S$
FAB-MS(positive)	*m/z*=412.2 $(M+H)^+$
Elemental Analysis	
Calculated:	C 46.7, H 6.17, N 23.8, S 7.76%
Found:	C 46.7, H 6.13, N 23.8, S 7.78%
Melting point	156- 158°C
UV-λ_{max} (in methanol)	274 nm
FT-IR (in KBr pellet)	ca. 3440, 3395, 3120, 2900, 1650, 1600, 1510, 1430, 1220, and 1070 cm^{-1}

the supernatant solution was extracted three times with an equal volume of chloroform. The chloroform solution was dried at reduced pressure to produce approximately 5 g of the solid material. This material was subjected to silica gel chromatography (100 g of silica 60, Merck) using $CHCl_3$-CH_3OH (9:1 v/v) as the developing solvent, and 600 mg of crude cystocin product was obtained after vacuum drying. The above crude cystocin (600 mg) was again chromatographed on 20 g of silica gel using the same developing solvent to produce 220 mg of the cystocin, which contained a small amount of impurity as determined by TLC ($CHCl_3$:CH_3OH, 5:1). Thus, this material was dissolved again in 5 ml of chloroform, and re-precipitated by adding a small volume of hexane slowly to give 200 mg of pure cystocin. This final material was verified to be pure by TLC ($CHCl_3$:CH_3OH, 5:1), having an R_f value of 0.5.

Characterization of Cystocin

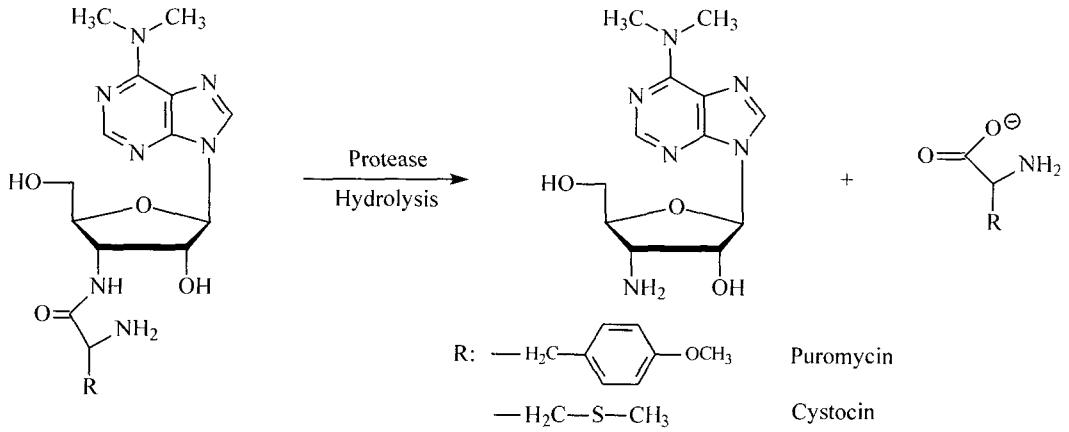
Cystocin(1) was obtained as a white solid with a melting point of 156- 158°C, and maximum UV absorption was at 274 nm, when obtained from methanol solution. All the physicochemical properties including FT-IR data are summarized in Table 1.

The positive FAB-MS spectra suggested that the molecular weight of cystocin was 411 amu, and the molecular formula could be assigned to $C_{16}H_{25}N_7O_4S$, when considering the result of the elemental analysis. Analysis of the ^{1}H-NMR, ^{13}C-NMR, and ^{1}H-^{1}H and ^{1}H-^{13}C COSY NMR spectra revealed the presence of four quaternary carbons (three olefinic carbons and one carbonyl carbon) and twelve proton-bearing carbons. The 2 and 8 carbon of the purine ring could be easily assigned from ^{1}H-NMR, since two singlets at 8.16 and 8.08 ppm are the typical peaks of the purine system. The ^{1}H-^{1}H COSY showed the couplings among 1' and 2', 2' and 3', 3' and 4', 4' and 5', respectively, which was indicative of an amino ribose unit. Further analysis of NMR spectra (NOESY) showed that these two ring systems were connected through the anomeric carbon at 1' (d=5.92, *J*=3.9 Hz). The complete assignment of NMR peaks is tabulated in Table 2.

Further proof of the structure was obtained by comparison of the puromycin nucleosides produced by hydrolyses of cystocin and puromycin (see Scheme 1) [10]. Since cystocin has a closely related structure of puromycin (the terminal tyrosine is replaced by a methyl cysteinyl unit at cystocin), as shown in Scheme 1, the nucleoside from the hydrolysis of cystocin is expected to be the same as that of puromycin. Hydrolyses of each material as described above produced 53 and 13 mg of puromycin nucleosides, respectively. These two materials showed the same R_f values of 0.3 when developed using CH_3Cl:CH_3OH (3:1) mixture as eluting solvent. Also, NMR spectra of

Table 2. ^{1}H and ^{13}C NMR assignments for cystocin (in $CDCl_3$).

Carbon No.	Carbon shift (δ)	Proton shift (δ) and multiplicity
1(N)	-	-
2	152.3(CH)	8.16 (s, 1H)
3(N)	-	-
4	120.2(Q)	-
5	154.8(Q)	-
6	150.1(Q)	-
7(N)	-	-
8	138.4(CH)	8.08 (s, 1H)
9(N)	-	-
1	90.0(CH)	5.92 (d, 1H, *J*=3.9 Hz)
2	73.79(CH)	4.71 (dd, 1H, *J*=6.3, 3.9 Hz))
3	50.6(CH)	4.47 (dd, 1H, *J*=12.9, 6.3 Hz)
4	84.1(CH)	4.24 (m, 1H)
5	61.5(CH_2)	4.09 (d, 1H, *J*=13.8 Hz), 3.83 (d, 1H, *J*=13.5 Hz)
1"	174.5(Q)	
2"	54.1(CH)	-
3"	39.5(CH_2)	3.60 (dd, 1H, *J*=8.4, 3.9 Hz)
3"-S-CH_3	15.5(CH_3)	3.00 (dd, 1H, *J*=13.8, 3.9 Hz), 2.72 (dd, 1H, *J*=13.5, 8.4 Hz)
6-N-CH_3	38.5(CH_3)	2.11 (s, 3H)
		3.5 (bs, 6H)



Scheme 1. Hydrolysis of cystocin and puromycin.

these two products could not be distinguished from each other [11]. Thus, the structure of cystocin could be concluded, as shown in Fig. 1.

To evaluate the pharmaceutical potential of cystocin, this material was tested at KRICT, Korea. The initial result showed that cystocin was two times more potent than puromycin in antibacterial activity against *Streptococcus*, with the typical MIC around 3-6 mg. The detailed biological activity is under further study, and will be published elsewhere.

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