γ-Pyrone Compounds with Selective and Potent Anti-*Helicobacter pylori* Activity

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Many recent studies have shown that peptic ulcer diseases are mainly caused by *Helicobacter pylori* (*H. pylori*) infection[1,2]. Eradication of this bacterium dramatically decreases the recurrence rate in peptic ulcer patients. Treatment regimens including a proton pump inhibitor and antimicrobial agents such as amoxicillin and clarithromycin are now recommended[3]. However, these therapies have problems including side effects (*e.g.* diarrhea), build-up of drug resistance, and poor compliance[4,5]. Therefore the development of a new class of anti-*H. pylori* agents is needed.

In the course of our screening for anti-*H. pylori* agents, *N*-acetyl aureothamine (**1**) was found from the culture broth of *Streptomyces netropsis* JCM 4544. In this paper, we describe the fermentation, isolation, structure elucidation and biological properties of **1**. In addition, anti-*H. pylori* activities of other γ-pyrone compounds are reported.

A slant culture of the strain JCM 4544 grown on Bennett's agar was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 1%, potato starch 2%, Polypeptone (Nihon Pharmaceutical Co., Ltd.) 0.5%, yeast extract 0.5% and $CaCO_3$ 0.4%. After incubation at 28°C for three days on a rotary shaker at 220 rpm, the seed culture was inoculated into a 500-ml Erlenmeyer flask containing 90 ml of the production medium consisting of brown rice 33%, Polypeptone 0.5%, yeast extract 0.5%, meat extract 0.3%, brain heart infusion 0.6% and K_2HPO_4 0.3%. The fermentation was carried out under static condition at 28°C for seven days.

The fermentation broth from 30 flasks was extracted with acetone/H_2O (8 : 2). After removal of the organic solvent, the extract was applied to a Diaion HP-20 column. The column was washed with MeOH - H_2O (8 : 2) and MeOH, and eluted with acetone. The acetone eluate was subjected to silica gel column chromatography using $CHCl_3$/MeOH (20 : 1) as an eluent. The active fraction was further applied to a silica gel column and developed with Hexane/EtOAc (1/9). The combined active fraction was finally purified by ODS HPLC on Cosmosil $5C_{18}$-AR with MeOH/H_2O (75 : 25) to yield 4.6 mg of **1**.

The physico-chemical properties of **1** are listed in Table 1. The molecular formula of **1** was determined to be $C_{24}H_{27}NO_5$ by high-resolution matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS[6,7] and NMR data that indicated twelve degrees of unsaturation. The IR spectrum suggested the presence of an NH group (3260 cm^{-1}) and conjugated and/or amide carbonyl groups (1660 cm^{-1}). The ^1H NMR and DEPT spectra indicated the presence of four methyls, a methoxy, a methylene, an oxygenated methylene, an oxygenated methine, two olefinic methines, six olefinic quaternary carbons, two carbonyls, and a 1,4-disubstituted benzene ring, which accounted for ten of these twelve degrees of unsaturation. The ^1H and ^{13}C NMR chemical shifts are shown in Table 2.

Analysis of one- and two-dimensional NMR spectra including COSY, HMQC and HMBC led to the assignments of three partial structures **a**~**c** as shown in Fig. 2. Partial structure **a** was deduced through analysis of HMBC correlations for H-23, H-24 and H-25. Singlet methyl protons H-23 (δ_H 2.03) gave cross peaks C-4, C-5 and C-6. Other singlet methyl protons H-24 (δ_H 1.85) were correlated to C-2, C-3 and C-4, and methoxyl protons H-25

Table 1. Physico-chemical properties of *N*-acetyl aureothamine (**1**).

Appearance	Colorless gum
Molecular weight	409
Molecular formula	$C_{24}H_{27}NO_5$
HRMALDI-TOFMS (m/z)	
Found:	410.1967 $(M+H)^+$
Calcd:	410.1968
$[\alpha]^{25}_D$	+ 24.0° (c 0.10, $CHCl_3$)
UV (MeOH) λ_{max} nm(ε)	295 (31900)
IR νmax (film) cm^{-1}	3260, 2930, 1660, 1580, 1530, 1520 1460, 1410, 1370, 1320, 1260, 1160

(δ_H 3.93) were correlated to C-2. The presence of a dienone moiety was supported by the ^{13}C chemical shift of C-4 (δ_C 180.7). In partial structure **b**, a tetrahydrofuran ring moiety was established by interpretation of the COSY and HMBC spectra. A methine proton H-7 (δ_H 5.13) was coupled to methylene protons H-8 (δ_H 3.04, 2.91) in the COSY spectrum. The C^8-C^9-C^{10} portion was disclosed by HMBC correlations (H-7/C-9; H-8/C-9, C-10; H-10/C-9). The chemical shifts of the C-7 methine (δ_C 73.2 and δ_H 5.13) and the C-10 methylene (δ_C 70.2 and δ_H 4.86, 4.74) and HMBC correlations (H-7/C-10; H10/C-7) indicated the presence of an ether linkage between C-7 and C-10. The $C^9{=}C^{11}{-}C^{12}(C^{22}){=}C^{13}$ portion was deduced from correlations in the HMBC spectrum (H-11/C-8, C-10, C-13, C-22; H13/C-11, C-22; H22/C-12, C13). In partial structure **c**, a 1,4-disubstituted benzene ring moiety was easily assigned by interpretation of the COSY and HMBC spectra. An NH proton (δ_H 7.26), which showed HMBC correlations with C-16 and C-17, could be attached to C-17.

Connection of partial structures **a**~**c** was accomplished by interpretation of the HMBC spectrum. HMBC correlations from H-7 and H-8 to C-6 showed connection between partial structures **a** and **b**. An olefinic methine proton H-13 was correlated to C-15 and C-19, indicating connection between partial structures **b** and **c**. An HMBC correlation from singlet methyl protons H-21 (δ_H 2.19) to a carbonyl (δ_C 168.2) and an NOESY cross peak between H-21 and an NH proton (δ_H 7.26) revealed the presence of an acetamide moiety. An ether linkage between C-2 and C-6 was deduced from the degrees of unsaturation and the low-field ^{13}C chemical shifts of C-2 (δ_C 162.1) and C-6 (δ_C 155.2). The (9*Z*, 12*E*)-geometry of the diene system was suggested by NOESY cross peaks (H-8/H-11; H-10/H-22; H-22/H-15) and the high-field ^{13}C chemical shift of C-22 (δ_C 17.5). Thus, the structure of **1** was determined to be that shown in Fig 1. A literature search suggested that **1** was identical to *N*-acetyl aureothamine, which has been previously synthesized from aureothin[8]. However, neither the physico-chemical properties nor the biological activities of **1** have been repoted in the literature. This paper describes the first isolation of **1** from the nature and the assignments of ^1H and ^{13}C chemical shifts.

Antimicrobial activities of *N*-acetyl aureothamine (**1**) are shown in Table 3. **1** exhibited potent anti-*H. pylori* activity with MIC value of 0.003 μg/ml. We also evaluated the anti-*H. pylori* activities of other γ-pyrone compounds, aureothin[8] (**2**) and actinopyrone A[9,10] (**3**). Of these compounds, **3** displayed the most potent anti-*H. pylori* activity, which was 250-fold higher than that of amoxicillin

Table 2. ^1H and ^{13}C NMR data of *N*-acetyl aureothamine (**1**) in $CDCl_3$.

No.	^{13}C	^1H
2	162.1	
3	100.0	
4	180.7	
5	120.0	
6	155.2	
7	73.2	5.13 (dd, 7.3, 6.1)
8	38.3	3.04 (dd, 15.3, 7.3)
		2.91 (dd, 15.3, 6.1)
9	137.8	
10	70.2	4.86 (d, 12.2)
		4.74 (d, 12.2)
11	126.7	6.16 (s)
12	134.4	
13	130.1	6.29 (s)
14	133.5	
15, 19	129.7	7.22 (d, 8.5)
16, 18	119.5	7.49 (d, 8.5)
17	136.5	
20	168.2	
21	24.6	2.19 (s)
22	17.5	2.01 (s)
23	9.4	2.03 (s)
24	6.9	1.85 (s)
25	55.3	3.93 (s)
20-NH		7.26 (s)

Fig. 1. Structures of *N*-acetyl aureothamine (**1**), aureothin (**2**) and actinopyrone A (**3**).

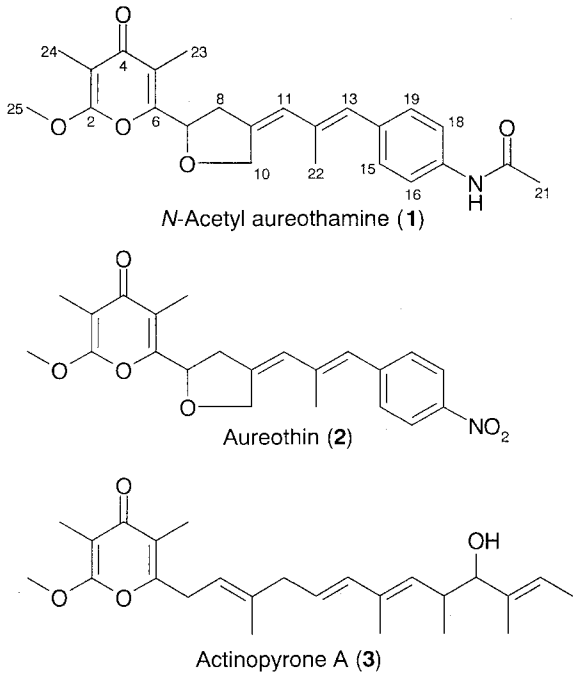

Fig. 2. Partial structures of *N*-acetyl aureothamine (**1**).

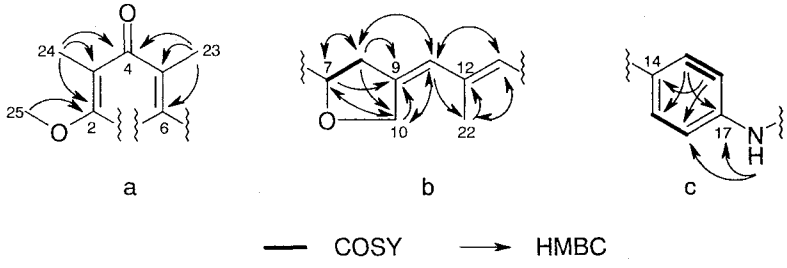

Table 3. Antimicrobial activities of *N*-acetyl aureothamine (**1**), aureothin (**2**), actinopyrone A (**3**) and the reference compounds.

Test organisms	MIC(μg/ml)				
	1	**2**	**3**	amoxicillin	clarithromycin
Helicobacter pylori ATCC 43504	0.003	0.00078	0.0001	0.025	0.013
Staphylococcus aureus FDA209P JC-1	>50	>50	>100	0.39	1.56
Bacillus subtilis ATCC 6633	>50	>50	>100	0.025	0.025
Peptostreptococcus productus CAYA 12-2	>50	>50	>100	0.05	0.025
Bifidobacterium bifidum CAYA21-1	>50	>50	>100	0.39	0.1
Clostridium perfringens CAYA 39-1	>50	>50	>100	0.05	0.39
Escherichia coli O-1	>50	>50	>100	0.78	50
Klebsiella pneumoniae ATCC 10031	>50	>50	>100	100	6.25
Pseudomonas aeruginosa ATCC 10490	>50	>50	>100	>100	>100
Bacteroides fragilis GAI 5562	>50	>50	>100	12.5	1.56

and 130-fold higher than that of clarithromycin. Furthermore, these compounds were inactive against other Gram-positive and Gram-negative bacteria tested, unlike amoxicillin and clarithromycin, which are active against a variety of microorganisms and therefore cause diarrhea as a side effect. *In vitro* cytotoxic activities of these compounds were examined against HeLa S3 cells. The IC_{50} values of **1**, **2** and **3** were 5.0, 10 and >10 μg/ml, respectively. These results suggest that γ-pyrone compounds are selective and potent anti-*H. pylori* agents and have low potential for diarrhea caused by the disturbance of intestinal microbial flora.

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