Notes

CYSFLUORETIN, A NEW INHIBITOR OF GLUTATHIONE *S*-TRANSFERASE, PRODUCED BY *Streptomyces* SP. MI384-DF12

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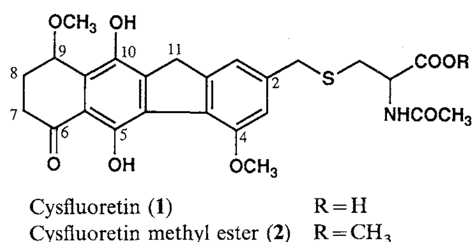
We have previously reported that benastatins A, B and C, and bequinostatins A, B, C and D were isolated from the culture broth of *Streptomyces* sp. MI384-DF12 and are novel inhibitors of glutathione *S*-transferase (GST, EC 2.5.1.18)[1~5]. In our continuing study of the strain, we have found that this strain produces another inhibitor designated cysfluoretin (**1**) which does not contain a benzo[*a*]-naphthacene skeleton and differs from benastatins and bequinostatins as shown in Fig. 1. Here we wish to describe the isolation, physico-chemical properties, structure and biological activities of **1**.

Streptomyces sp. MI384-DF12 (FERM P-11270) was cultured as described previously[1].

The isolation procedure is shown in Fig. 2. The culture broth (66 liters) was filtered at pH 4 and separated into the mycelial cake and the culture filtrate. The mycelial cake was extracted with 75% aq Me_2CO; the extract was filtered and concentrated *in vacuo* to an aqueous solution. The solution was extracted with EtOAc and the extract was concentrated to dryness under reduced pressure. The dried material was chromatographed on a column of silanised silica gel with a linear gradient of 40 to 100% aq MeOH. The eluate was evaporated to dryness and applied to a silica gel column. After washing the column with $CHCl_3$ - MeOH - AcOH (95 : 5 : 1), the active substance was eluted with $CHCl_3$ - MeOH - AcOH (90 : 10 : 1). The eluate was evaporated to dryness. This powder was dissolved in a small volume of MeOH - AcOH (100 : 1) and the solution was subjected to Sephadex LH-20 column chromatography developed with MeOH - AcOH (100 : 1). The eluate was concentrated under reduced pressure to give a reddish brown powder. The crude powder was further purified by a reversed phase HPLC using a Capcell Pak C_{18} column (2.0 × 25 cm, flow rate 8 ml/minute) with a solvent mixture of CH_3CN - H_2O - AcOH (35 : 65 : 1). The fractions containing **1** were collected and evapo-

Fig. 1. Structures of cysfluoretin and its methyl ester.



Cysfluoretin (**1**) R = H
Cysfluoretin methyl ester (**2**) R = CH_3

Fig. 2. Isolation procedure of cysfluoretin.

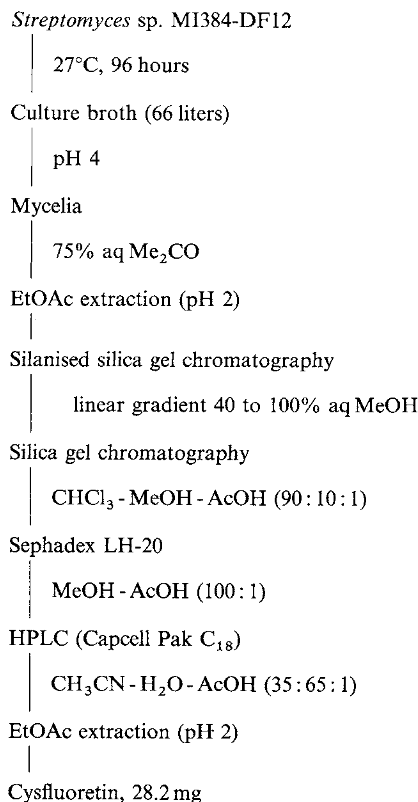


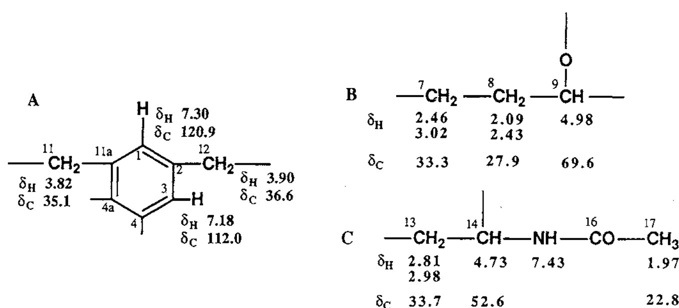
Table 1. Physico-chemical properties of cysfluoretin.

Appearance	Yellow powder
MP	114～116°C (dec)
$[\alpha]_D^{24}$	−24.0° (*c* 0.5, MeOH)
Molecular formula	$C_{25}H_{27}O_8NS$
FAB-MS (*m/z*, Negative)	500 $(M-H)^-$
HRFAB-MS (*m/z*, Negative)	
Found:	500.1394 $(M-H)^-$
Calcd:	500.1379 for $C_{25}H_{26}O_8NS$
UV λ_{max} nm (log ε)	
in H_2O - MeOH (1:9)	217 (4.66), 238 (sh, 4.37), 309 (sh, 4.19), 335 (4.34), 397 (4.10)
in 1 N HCl - MeOH (1:9)	218 (4.63), 237 (sh, 4.33), 309 (sh, 4.19), 333 (4.32), 396 (4.07)
in 1 N NaOH - MeOH (1:9)	222 (4.52), 240 (sh, 4.30), 307 (sh, 4.19), 330 (4.32), 404 (3.97), 454 (sh, 3.56)
IR ν_{max}^{KBr} cm^{-1}	3330, 2980, 1745, 1658, 1582, 1440, 1373, 1322, 1270, 1098, 1006, 960, 890
Rf value on TLC	0.20 ($CHCl_3$ - EtOAc - AcOH, 60:35:5, silica gel)
Color reaction	Phosphomolybdate-H_2SO_4, $FeCl_3$, Pauly, 2,4-Dinitrophenylhydrazine, Potassium hexachloroplatinate (IV)
Solubility	Soluble: DMSO, MeOH, Me_2CO, EtOAc Insoluble: H_2O

Table 2. ^{13}C and ^1H NMR data of cysfluoretin and its methyl ester in acetone-d_6.

Carbon	Cysfluoretin (**1**)		Cysfluoretin methyl ester (**2**)	
	δ_C ppm (100 MHz)	δ_H ppm (*J* in Hz, 400 MHz)	δ_C ppm (100 MHz)	δ_H ppm (*J* in Hz, 400 MHz)
1	120.9 (d)	7.30 (1H, br s)	121.0 (d)	7.35 (1H, br s)
2	141.9 (s)		141.8 (s)	
3	112.0 (d)	7.18 (1H, br s)	112.0 (d)	7.22 (1H, br s)
4	153.1 (s)		153.2 (s)	
4a	127.5 (s)		127.5 (s)	
4b	131.1 (s)		131.2 (s)	
5	152.5 (s)	12.17 (5-OH, s)	152.5 (s)	12.21 (5-OH, s)
5a	115.4 (s)		115.4 (s)	
6	206.3 (s)		206.6 (s)	
7	33.3 (t)	2.46 (1H, m)	33.3 (t)	2.49 (1H, m)
		3.02 (1H, m)		3.02 (1H, m)
8	27.9 (t)	2.09 (1H, m)	27.9 (t)	2.10 (1H, m)
		2.43 (1H, m)		2.45 (1H, m)
9	69.6 (d)	4.98 (1H, br t, 2.8)	69.5 (d)	5.01 (1H, br t, 2.7)
9a	127.4 (s)		127.4 (s)	
10	142.6 (s)	9.52 (10-OH, br s)	142.7 (s)	9.57 (10-OH, br s)
10a	135.8 (s)		135.8 (s)	
11	35.1 (t)	3.82 (2H, br s)	35.1 (t)	3.89 (1H, br s)
11a	148.0 (s)		148.1 (s)	
12	36.6 (t)	3.90 (2H, s)	36.5 (t)	3.92 (2H, s)
13	33.7 (t)	2.81 (1H, dd, 14.4, 7.8)	33.7 (t)	2.78 (1H, dd, 14.0, 7.6)
		2.98 (1H, dd, 14.4, 5.4)		2.93 (1H, dd, 14.0, 5.8)
14	52.6 (d)	4.73 (1H, ddd, 7.8, 7.8, 5.4)	52.6 (d)	4.73 (1H, m)
		7.43 (14-NH, br d, 7.8)		7.49 (14-NH, br d, 8.0)
15	172.4 (s)		172.1 (s)	
15-OCH_3			52.5 (q)	3.69 (3H, s)
16	170.4 (s)		170.1 (s)	
17	22.8 (q)	1.97 (3H, s)	22.6 (q)	1.95 (3H, s)
18	57.5 (q)	4.20 (3H, s)	57.5 (q)	4.24 (3H, s)
19	56.8 (q)	3.38 (3H, s)	56.8 (q)	3.40 (3H, s)

Fig. 3. Partial structures of cysfluoretin.

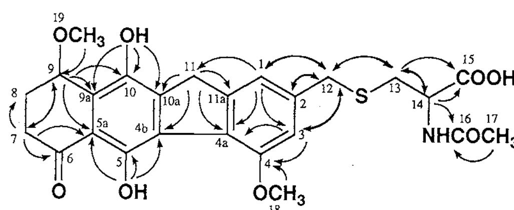


rated to dryness to give a yellowish powder. The powder was suspended in water and extracted with an equal volume of EtOAc. The extract was concentrated to dryness under reduced pressure to give pure **1**. The total yield of **1** was 28.2 mg.

The physico-chemical properties of **1** are summarized in Table 1. **1** is soluble in DMSO, MeOH, Me_2CO and EtOAc, but insoluble in water. The molecular weight and formula of **1** were elucidated as $C_{25}H_{27}O_8NS$ (MW 501) from the FAB-MS peak at m/z 500 $(M-H)^-$, HRFAB-MS [found: m/z 500.1394 $(M-H)^-$, calcd: m/z 500.1379 for $C_{25}H_{26}O_8NS$] and ^1H and ^{13}C NMR spectra of **1** (Table 2). The result of an X-ray micro analyser supported the fact that **1** contained sulfur. In the UV spectrum of **1**, the absorption band in alkaline solution exhibited bathochromic shifts from 396 nm in 1 N HCl-MeOH (1 : 9) to 404 and 454 nm in 1 N NaOH-MeOH (1 : 9), suggesting the presence of a phenolic hydroxyl group. The IR spectrum of **1** showed the presence of a hydroxyl group (3330 cm^{-1}), and the presence of a carboxylic acid (1745 cm^{-1}), an amide bond (1658, 1582 cm^{-1}) and an aromatic ketone (1658 cm^{-1}) group which were supported by ^{13}C NMR signals at δ_C 172.4 (C-15), 170.4 (C-16) and 206.3 (C-6) ppm. Futhermore, the ^{13}C NMR spectrum of **1** revealed twelve sp^2 carbon signals excluding those of the three carbonyl carbons and ten sp^3 carbon signals. Three of these signlas (C-4, C-5 and C-9) appeared at lower field (δ_C 153.1, 152.5 and 69.6 ppm) indicating oxygen-bearing carbons, and also the signal (C-14) at δ_C 52.6 ppm which indicated a nitrogen-bearing carbon. In the ^1H NMR spectrum of **1**, two methyl protons at δ_H 4.20 ($18\text{-}H_3$) and 3.38 ($19\text{-}H_3$) ppm suggested the presence of methoxyl groups from their chemical shifts.

The ^1H-^1H COSY spectrum and spin decoupling experiments of **1** showed the following results, a *meta* spin-spin coupling between aromatic protons

Fig. 4. ^1H-^{13}C correlation for **1** by the HMBC experiments.



at δ_H 7.30 (1-H) and 7.18 (3-H) ppm, long-range spin couplings between the signal at δ_H 7.30 (1-H) ppm and two methylenes at δ_H 3.82 ($11\text{-}H_2$) and 3.90 ($12\text{-}H_2$) ppm, and linkages from C-7 to C-9 and C-13 to 14-NH. The methyl protons at δ_H 1.97 ($17\text{-}H_3$) ppm suggested the presence of an acetyl group from its chemical shift. From the above results, the presence of three partial structures (Fig. 3. A, B and C) were revealed.

As shown in Fig. 4, in the HMBC (heteronuclear multiple bond connectivity) spectrum of **1**, the phenolic hydroxyl proton at δ_H 12.17 (5-OH) ppm correlated with three carbons at δ_C 131.1 (C-4b), 152.5 (C-5) and 115.4 (C-5a) ppm, and also the other phenolic hydroxyl proton at δ_H 9.52 (10-OH) ppm correlated with three carbons at δ_C 127.4 (C-9a), 142.6 (C-10) and 135.8 (C-10a) ppm, suggesting the presence of a hydroquinone moiety. The partial structures A and B could be connected through this hydroquinone moiety.

The methine proton at δ_H 4.73 (14-H) ppm coupled to the carbonyl carbon at δ_C 172.4 (C-15) ppm, and the presence of a carboxyl group was confirmed by the preparation of **2** [FAB-MS peak at m/z 514 $(M-H)^-$]. In the HMBC spectrum of **2**, the methyl protons at δ_H 3.69 ($15\text{-}OCH_3$) ppm correlated with the carbon at δ_C 172.1 (C-15).

The methylene protons at δ_H 3.90 ($12\text{-}H_2$) ppm showed cross peak with the carbon signal at δ_C 33.7

(C-13) ppm, and also the methylene protons at δ_H 2.81, 2.98 (13-H_2) ppm showed cross peak with the carbon signal at δ_C 36.6 (C-12) ppm in the HMBC spectrum of **1**. Therefore, the partial structures A and C could be connected through the remaining sulfur. From the above results, the structure of **1** was determined to be 7,8,9,11-tetrahydro-5,10-dihydroxy-4,9-dimethoxy-2-(4-acetamido-4-carboxy-2-thia-butyl)-6*H*-benzo[*b*]fluoren-6-one. The absolute configuration remains to be determined.

The inhibitory activity of **1** against partially purified GST from rat liver was measured as described previously[3]. Its IC_{50} value was 9.4 μg/ml. **1** had no significant antimicrobial activity at 100 μg/ml. **1** has no toxic indications after ip injection in mice at a dose of 100 mg/kg.

S-Conjugates of glutathione are formed non-enzymatically and enzymatically in biological systems during the metabolism of drugs and environmental chemicals. Enzymatic formation involves GST which catalyzes the conjugation of a wide range of aromatic halides with reduced glutathione. The formation of *S*-conjugates of glutathione is the initial step in the mercapturic acid pathway, leading to the ultimate excretion of mercapturic acid derivatives of glutathine, CH_3CO-$NHCH(COOH)CH_2$-S-R[6]. Thus, cysfluoretin which has a mercapturic acid moiety is structurally interesting.

References

1) Aoyagi, T.; T. Aoyama, F. Kojima, N. Matsuda, M. Maruyama, M. Hamada & T. Takeuchi: Benastatins A and B, new inhibitors of glutathione *S*-transferase, produced by *Streptomyces* sp. MI384-DF12. I. Taxonomy, production, isolation, physico-chemical properties and biological activities. J. Antibiotics 45: 1385～1390, 1992

2) Aoyama, T.; H. Naganawa, Y. Muraoka, H. Nakamura, T. Aoyagi, T. Takeuchi & Y. Iitaka: Benastatins A and B, new inhibitors of glutathione *S*-transferase, produced by *Streptomyces* sp. MI384-DF12. II. Structure determination of benastatins A and B. J. Antibiotics 45: 1391～1396, 1992

3) Aoyama, T.; F. Kojima, T. Yamazaki, T. Tatee, F. Abe, Y. Muraoka, H. Naganawa, T. Aoyagi & T. Takeuchi: Benastatins C and D, new inhibitors of glutathione *S*-transferase, produced by *Streptomyces* sp. MI384-DF12. Production, isolation, structure determination and biological activities. J. Antibiotics 46: 712～718, 1993

4) Aoyama, T.; F. Kojima, F. Abe, Y. Muraoka, H. Naganawa, T. Takeuchi & T. Aoyagi: Bequinostatins A and B, new inhibitors of glutathione *S*-transferase, produced by *Streptomyces* sp. MI384-DF12. Production, isolation, structure determination and biological activities. J. Antibiotics, 46: 914～920, 1993

5) Yamazaki, T.; T. Tatee, T. Aoyama, F. Kojima, T. Takeuchi & T. Aoyagi: Bequinostatins C and D, new inhibitors of glutathione *S*-transferase, produced by *Streptomyces* sp. MI384-DF12. J. Antibiotics, 46: 1309～1311, 1993

6) Boyland, E. & L. F. Chasseaud: The role of glutathione and glutathione *S*-transferases in mercapturic acid biosynthesis. Adv. Enzymol. 32: 173～219, 1969