Salfredins, New Aldose Reductase Inhibitors Produced by *Crucibulum* sp. RF-3817

I. Fermentation, Isolation and Structures of Salfredins

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New inhibitors of aldose reductase, designated salfredins A_3, A_4, A_7, C_1, C_2, C_3 and B_{11}, were isolated from the fermentation broth of *Crucibulum* sp. RF-3817 by successive purification procedures of solvent extraction, silica gel column chromatographies and reverse-phase HPLC. Their structures were established by spectroscopic methods, including UV, SI-MS and NMR. The structures of salfredins A_4 and B_{11} were confirmed by X-ray crystallographic analysis.

Aldose reductase (Alditol: NADP+oxidoreductase, EC1.1.1.21) plays a key role in the "polyol pathway", and inhibition of this enzyme represents a new pharmacological approach towards the treatment of certain chronic complications of diabetes mellitus[1~3].

In a screening program for unique microbial products with pharmacological activity, new aldose reductase inhibitors[4,5], designated as salfredins A_3 (**1a**), A_4 (**2a**), A_7 (**3a**), C_1 (**4a**), C_2 (**5a**), C_3 (**6a**) and B_{11} (**7a**), were isolated from the fermentation broth of *Crucibulum* sp. RF-3817. The taxonomy, fermentation, isolation, physico-chemical properties and structures of salfredin congeners are described in this paper. Their biological properties will be reported in a following paper (in preparation).

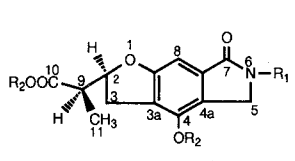
Taxonomy

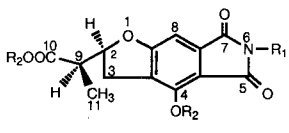
The strain RF-3817 was isolated as follows. First, peridioles within the peridium formed on a rotten wood block were immersed in a solution of sodium hypochlorite for one minute to sterilize the surface of the peridioles. Next, the peridioles were washed with sterile water and the basidiospores formed within the peridioles were placed on an appropriate medium to isolate the strain. On the hyphae of the strain grown on the medium, clamp connections were observed, the width of each hypha being 1 to 5 μm.

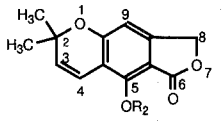
The peridium of the strain formed on the rotten wood block is pyriform, 5 to 8 mm in height, 4 to 6 mm in diameter, and has an ocher color. In the upper portion of the peridium, there is an epiphragm having a pale yellowish-white color, which will dehisce when matured. Within the peridium are lenticular peridioles. The peridioles, 1 to 1.5 mm in size, are attached by funiculi to the inner wall of the peridium. Within the peridioles, basidiospores, 3 to 5 by 7 to 10 μm, are formed.

These properties were compared with the characteristics of other genera belonging to the order Nidulariales,

Fig. 1. Structures of salfredins.







		R_1	R_2
A_3	(1a)	$-CH(CH_2CH_2COOH)COOH$	H
	(1b)	$-CH(CH_2CH_2COOCH_3)COOCH_3$	CH_3
A_4	(2a)	$-CH_2COOH$	H
	(2b)	$-CH_2COOCH_3$	CH_3
	(2c)	$-CH_2COOCH_2CH_3$	CH_3
A_7	(3a)	$-CH(CH_3)COOH$	H
	(3b)	$-CH(CH_3)COOCH_3$	CH_3
C_1	(4a)	H	H
	(4b)	CH_3	CH_3
C_2	(5a)	$-CH_2COOH$	H
	(5b)	$-CH_2COOCH_3$	CH_3
	(5c)	$-CH_2COOCH_2CH_3$	CH_3
C_3	(6a)	$-CH(CH_3)COOH$	H
	(6b)	$-CH(CH_3)COOCH_3$	CH_3
B_{11}	(7a)	-----	H
	(7b)	-----	CH_3

the family Nidulariaceae described by BRODIE[6] and, by IMAZEKI and HONGO[7]. According to the comparison, the strain was identified as a strain of the genus *Crucibulum* and named *Crucibulum* sp. RF-3817. The strain RF-3817 was deposited with the Agency of Industrial Science and Technology, Japan, under the accession No. FERM BP-2888.

Fermentation

Fermentation for the production of salfredins (A_3, A_4, A_7, C_1, C_2, C_3 and B_{11}) was carried out as follows. The culture of *Crucibulum* sp. RF-3817 on an agar slant (20% potato decoction, glucose 1.0%) was inoculated into 500-ml Erlenmyer flasks containing 100 ml of PsSY seed medium consisting of potato starch 2.0%, sucrose 2.0%, and yeast extract (Difco) 0.5% in tap water, without adjusting pH, and cultured on a rotary shaker (180 rpm) at 28°C for 5 days. For the production of salfredins, a 4-ml aliquot of each culture was transferred into one hundred 500-ml Erlenmyer flasks, each containing 100 ml of SSY medium consisting of soluble starch 2.0%, sucrose 2.0%, yeast extract (Difco) 0.5%, without adjusting pH, and incubated on a rotary shaker (180 rpm) at 28°C for 10~15 days.

Among the salfredins, the most potent compounds according to the aldose reductase inhibitor assay were salfredins A_4 and C_2, which have a glycine residue in the molecule. Rat lens aldose reductase inhibitory activity was measured as follows[8]. Rat lenses were homogenized in 5 mM mercaptoethanol and then centrifuged at 10,000 rpm for 20 minutes. The obtained supernatants were used as the enzyme source. Aldose reductase activities were assayed spectrophotometrically by determining the decrease in NADPH concentration at 340 nm. The reaction mixture contained 0.1 M phosphate buffer (pH 6.2), 0.2 M lithium sulfate, 0.1 M NADPH, 10 mM DL-glyceraldehyde and the enzyme supernatant. The reaction was initiated by the addition of NADPH and monitored spectrophotometrically for the first 2 minutes. Enzyme activity was adjusted so that the average reaction rate of the control sample gave 0.020 absorbance unit/minute. The percent inhibition of each compound was calculated by comparison with the control solutions, and IC_{50} values were obtained from least square analysis of the log dose response curves.

To achieve their efficient production, the fermentation conditions were examined for salfredin A_4 production in the presence of various amounts of glycine in SSY medium (Fig. 2). As expected, the addition of glycine markedly enhanced the productivity of salfredin A_4 and the highest production was observed in the presence of 1.0% glycine, a level which corresponded to as much as 25 times that of the glycine-free medium. Further addition of glycine (up to 2.0%) lowered its production level as shown in Fig. 2. The presence of 1.0% glycine also markedly stimulated salfredin C_2 production as shown in Table 1.

Fig. 2. Effect of addition of glycine on salfredin A_4 production in SSY medium.

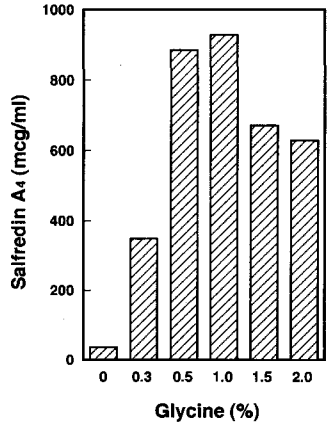


Table 1. Effect of glycine on salfredin A_4, C_2 and A_3 production in SSY and CCY media.

Medium	pH	PCV %	HPLC (mcg/ml) Salfredin		
			A_4	C_2	A_3
SSY	4.6	8	27	8	83
SSY + glycine 1.0%	4.1	10	1270	120	105
CCY	4.9	18	120	23	65
CCY + glycine 1.0%	4.3	20	2579	798	166

PCV: % packed cell volume.

Even when a more productive CCY medium (corn starch 3.0%, glucose 2.0%, corn steep liquor 3.0%, peanut meal 1.0%, dried yeast (Iwaki Co.) 0.5%, $CaCO_3$ 0.2%, without adjustment pH) was used in place of SSY medium, over 20-fold stimulation by addition of 1.0% glycine was observed for the production of salfredins A_4 and C_2. On the other hand, the production of salfredin A_3 containing a glutamic acid residue instead of a glycine residue was not significantly affected by addition of glycine as shown in Table 1.

The amount of salfredins A_4, C_2 and A_3 in the cultured broth was determined by analytical HPLC (column: Ultron VX-ODS 4.6 × 150 mm; A_4 and A_3, solvent: CH_3CN-0.1% TFA aq. (21:88), C_2, solvent: (18:82), flow rate: 1.0 ml/minute; detection: 220 nm).

The findings strongly suggested that glycine is the biosynthetic precursor of salfredins A_4 and C_2.

In order to obtain a large quantity of salfredin A_4 to

conduct chemical modifications of salfredins, which are described in the accompanying paper[9], large-scale fermentation using a 500-liter fermentor was carried out with addition of 1.0% glycine to the SSY medium. A 800-ml portion of the seed culture in a 2000-ml flask, grown in PsSY medium on a rotary shaker (180 rpm) at 28°C for 5 days, was transferred to a 30-liter jar fermentor containing 18 liters of the PsPY seed medium. The culture was incubated at 28°C for 5 days under agitation at 250 rpm and aeration of 10.8 liters per minute.

To produce sulfredin A_4, 12 liters of the resulting culture fluid was transferred into a 500-liter fermentor containing 300 liters of the SSY medium supplemented with 1.0% glycine. The fermentation was carried out at 28°C for 14 days under agitation at 220 rpm and aeration of 180 liters per minute. The back pressure of the fermentor was set at 0.35 kg/cm^2.

The time course of salfredin A_4 production is shown in Fig. 3. Salfredin A_4 production reached maximum (731 μg/ml) on the 13th day after incubation in a 500-liter fermentor. The fermentation broth thus obtained was processed on a large scale essentially according to the protocol described in Fig. 4. The overall yield of salfredin A_4 was approximately 62.0 g which was used for chemical modification described in the ensuing paper[9].

In a similar way, about 1.5 g of salfredin C_2 was obtained by the same large-scale fermentation.

Isolation

Production from SSY medium: The isolation and purification procedures of the salfredins are outlined in Fig 4. The mycelium was removed from the fermentation broth (SSY medium, 8 liters, pH 4.6) by filtration. The filtrate was extracted twice with ethyl acetate (3 liters) after adjusting it with 2 N HCl to pH 2.0. The organic layers were washed with saturated NaCl solution, dried over anhydrous Na_2SO_4, and evaporated to dryness *in vacuo*, giving an oily extract (4.422 g). This extract was applied to a column of MCI GEL CHP-20P (75~150 μ, 200 ml). The column was developed with gradient mixtures of CH_3CN - 0.1% TFA aq. [(20 : 80) ~ (80 : 20)]. The active fractions were primarily separated into six portions, which were concentrated to dryness, giving fraction 1 (containing mainly salfredin A_3, 222 mg), fraction 2 (containing salfredins A_3 and A_4, 954 mg), fraction 3 (containing salfredin A_7, 314 mg), fraction 4

Fig. 3. Time course of salfredin A_4 production in SSY + glycine 1.0% medium in a 500-liter fermentor.

□ Salfredin A_4, ○ % packed cell volume (PCV), ▲ pH.

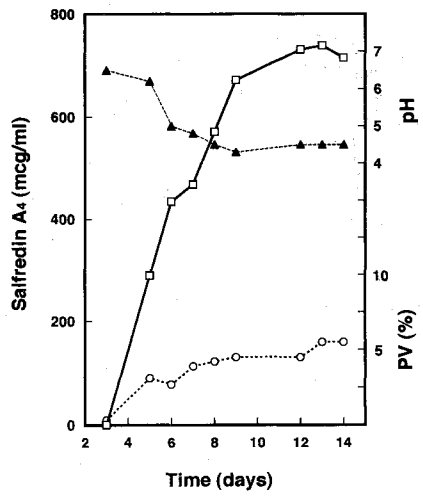
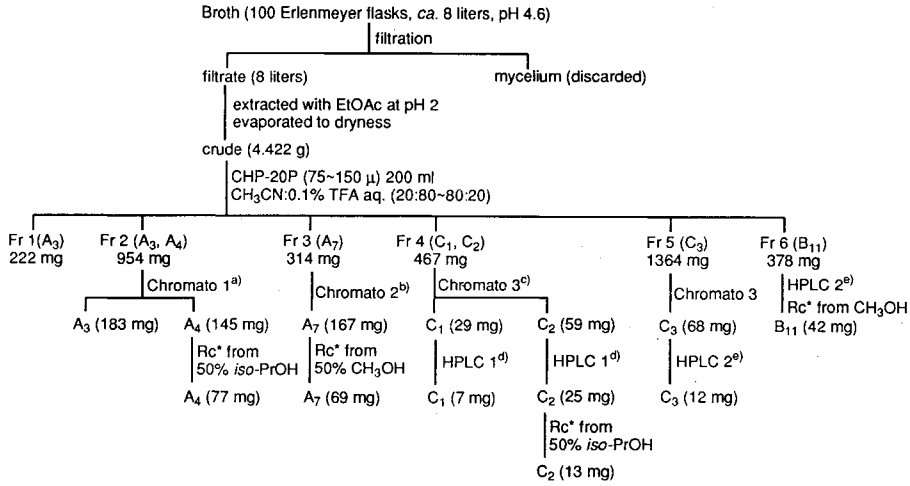


Fig. 4. Isolation and purification of salfredins.



a) Chromato 1 (LiChroprep PR-18 (25~40 μ) 20 i.d. x 500 mm, CH_3CN:0.1% TFA aq. (13:87). b) Chromato 2 [CH_3CN:THF (8:2)]:0.1% TFA aq. (13:87). c) Chromato 3 CH_3CN:0.1% TFA aq. (20:80). d) HPLC 1 (Cosmosil-5C18, 20 i.d. x 150 mm, CH_3CN:0.1% TFA aq. (20:80). e) HPLC 2 [CH_3CN:THF (8:2)]:0.1% TFA aq. (23:77)
Rc* = Recrystallization

(containing salfredins C_1 and C_2, 467 mg), fraction 5 (containing salfredin C_3, 1,364 mg) and fraction 6 (containing B_{11}, 378 mg). These fractions were purified as described below.

a) Fraction 2 was chromatographed on a column of LiChroprep PR-18 (25~40 μ, 20 i.d. × 500 mm) with a mixture of CH_3CN - 0.1% TFA aq. (13 : 87), giving both salfredin A_3 (183 mg, colorless amorphous powder) and A_4 (145 mg), which was recrystallized with 50% *iso*-PrOH to yield salfredin A_4 (77 mg) as colorless fine needles.

b) Fraction 3 was applied to a column of LiChroprep PR-18 (25~40 μ, 20 i.d. × 500 mm) with a mixture of [CH_3CN - THF (8 : 2)]: 0.1% TFA (13 : 87), giving salfredin A_7, which was recrystallized with a mixture of CH_3OH - H_2O (1 : 1) to yield pure salfredin A_7 (69 mg) as colorless fine needles.

c) Fraction 4 was chromatographed on the same column with a mixture of CH_3CN - 0.1% TFA aq. (20 : 80) to separate salfredins C_1 (29 mg) and C_2 (59 mg).

c-1) The C_1 component was further purified by preparative reverse phase HPLC [column: Cosmosil-5-C18, 20 i.d. × 150 mm; solvent: CH_3CN - 0.1% TFA aq. (20 : 80)] to afford pure salfredin C_1 (7 mg) as a pale yellow amorphous powder.

c-2) The C_2 component was purified by the same HPLC procedure to give salfredin C_2 (25 mg), which was recrystallized with 50% *iso*-PrOH to yield pure C_2 (13 mg) as colorless fine needles.

d) Fraction 5 was subjected to column chromatography [column: LiChroprep PR-18, 25~40 μ, 20 i.d. × 500 mm; solvent: CH_3CN - 0.1% TFA aq. (20 : 80)] giving salfredin C_3 (68 mg), which was further purified by preparative reverse phase HPLC [column: Cosmosil-5-C18, 20 i.d. × 150 mm; solvent: [CH_3CN - THF (8 : 2)]: 0.1% TFA aq. (23 : 77) to yield salfredin C_3 (12 mg) as a pale yellow oil.

e) Fraction 6 was purified by the same HPLC procedure to give an eluate, which was recrystallized with MeOH to afford salfredin B_{11} (42 mg) as colorless fine needles.

Structures of Salfredins A_3 (**1a**), A_4 (**2a**), A_7 (**3a**), C_1 (**4a**), C_2 (**5a**), C_3 (**6a**), B_{11} (**7a**)

Salfredin congeners were acidic in nature and were soluble in methanol, ethanol, acetone, and chloroform but insoluble in water. Their physico-chemical properties are summarized in Table 2.

Salfredins were classified into three groups, A, B and C, by the modes of UV absorption spectra, which showed bathochromic shifts in alkaline solutions (Fig. 5). These

Fig. 5. UV spectra of salfredins.

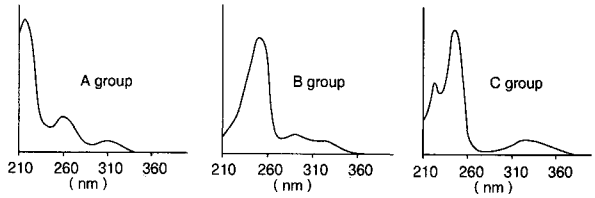


Table 2. Physico-chemical properties of salfredins.

	A_4 (**2a**)	A_3 (**1a**)	A_7 (**3a**)
Appearance	prisms (acidic)	powder (acidic)	prisms (acidic)
MP °C	>300°C	-	293~296 (dec)
Formula	$C_{15}H_{15}NO_7 \cdot 1/10H_2O$	$C_{18}H_{19}NO_9 \cdot 7/10H_2O$	$C_{16}H_{17}NO_7 \cdot 3/10H_2O$
Calcd	C 55.76, H 4.74, N 4.34	C 53.26, H 5.06, N 3.45	C 56.40, H 5.21, N 4.11
Found	C 55.61, H 4.75, N 4.61	C 53.11, H 5.19, N 3.79	C 56.34, H 5.19, N 4.33
SI-MS (*m/z*)	322 $[M+H]^+$	394 $[M+H]^+$	336 $[M+H]^+$
$\lambda_{max}^{MeOH}(E_{1\%}^{1\,cm})$ nm			
MeOH	215(810), 258(190), 303(60)	same as A_4	same as A_4
MeOH:0.1N HCl (9:1)	215(710), 258(190), 303(60)		
MeOH:0.1N NaOH (9:1)	220sh(630), 275(150), 316(60)		
λ_{max}^{KBr} cm^{-1}	3466, 1725, 1691, 1634.	3400, 1714, 1653, 1605	-
HPLC (min)	13.5[a]	12.1[a]	6.8[b]

	C_1 (**4a**)	C_2 (**5a**)	C_3 (**6a**)	B_{11} (**7a**)
Appearance	pale yellow powder (acidic)	prisms (acidic)	pale yellow oil (acidic)	prisms (acidic)
MP °C	-	246~247	-	179~180
Formula	$C_{13}H_{11}NO_6$	$C_{15}H_{13}NO_8 \cdot 2/10H_2O$	$C_{16}H_{15}NO_8$	$C_{13}H_{12}O_4$
Calcd		C 53.17, H 3.99, N 4.13		C 67.24, H 5.21
Found		C 53.08, H 4.08, N 4.37		C 67.10, H 5.14
SI-MS (*m/z*)	278 $[M+H]^+$	336 $[M+H]^+$	350 $[M+H]^+$	233 $[M+H]^+$
$\lambda_{max}^{MeOH}(E_{1\%}^{1\,cm})$ nm				
MeOH	223(670), 348(840), 335(110)	same as C_1	same as C_1	250(1500), 295(170), 325(120)
MeOH:0.1N HCl (9:1)	223(580), 348(770), 335(110)			250(1500), 295(170), 325(120)
MeOH:0.1N NaOH (9:1)	234(780), 247sh(580), 393(150)			245(1500), 295(170), 325(120)
λ_{max}^{KBr} cm^{-1}	-	3100, 1696, 1613	-	3392, 1744, 1626, 1595
HPLC (min)	8.7[c]	12.6[c]	16.0[d]	8.7[e]

[a] Column, Cosmosil 5C18 (4.6 i.d. x150 mm); Flow rate, 1 ml/min; detection, UV at 220 nm; solvent, CH_3CN:0.1% TFA = 13:87. [b] Solvent, CH_3CN:0.1% TFA = 20:80. [c] Solvent, (CH_3CN:THF = 8:2):0.1% TFA = 20:80. [d] Solvent, (CH_3CN:THF=8:2):0.1% TFA = 22:78. [e] Solvent, (CH_3CN:THF = 8:2):0.1% TFA = 40:60.

Table 3. ^{1}H NMR chemical shifts of salfredins.

A_4 (**2a**)	A_3 (**1a**)	A_7 (**3a**)
1.15 (3H, d, 7.0, 11-CH_3)	1.15 (3H, d, 7.0, 11-CH_3)	1.14 (3H, d, 7.0, 11-CH_3)
2.72 (1H, dq, 7.0, 7.0, 9-CH)	1.8~2.4 (4H, m, 13- and 14-CH_2)	1.47 (3H, d, 7.4, 12-CH_3)
2.94 (1H, dd, 7.2, 16.6, 3-CH_2)	2.73 (1H, dq, 7.0, 7.0, 9-CH)	2.71 (1H, dq, 7.0, 6.8, 9-CH)
3.31 (1H, dd, 9.0, 16.6, 3-CH_2)	2.98 (1H, dd, 7.5, 16.5, 3-CH_2)	2.97 (1H, dd, 7.4, 16.6, 3-CH_2)
4.20 (2H, s, 5-CH_2)	3.2~3.4 (1H, m, 3-CH_2)	3.2~3.4 (1H, m, 3-CH_2)
4.30 (2H, s, 12-CH_2)	4.25 (2H, s, 5-CH_2)	4.24 (2H, s, 5-CH_2)
5.00 (1H, ddd, 7.0, 7.2, 9.0, 2-CH)	4.75 (1H, m, 12-CH)	4.77 (1H, q, 7.4, 12-CH)
6.49 (1H, s, 8-CH)	5.03 (1H, m, 2-CH)	5.00 (1H, m, 2-CH)
	6.49 (1H, s, 8-CH)	6.49 (1H, s, 8-CH)

C_2 (**5a**)	C_3 (**6a**)	B_{11} (**7a**)
1.15 (3H, d, 7.0, 11-CH_3)	1.14 (3H, d, 7.0, 11-CH_3)	1.46 (6H, s, 2-CH_3 x2)
2.80 (1H, dq, 7.0, 7.0, 9-CH)	1.49 (3H, d, 7.3, 12a-CH_3)	5.21 (2H, d, J = 0.8, 8-CH_2)
3.02 (1H, dd, 7.4, 16.8, 3-CH_2)	2.79 (1H, dq, 7.0, 7.0, 9-CH)	5.64 (1H, d, J = 10.0, 3-CH)
3.2~3.4 (1H, m, 3-CH_2)	3.00 (1H, dd, 7.3, 16.6, 3-CH_2)	6.39 (1H, d, J = 0.8, 9-CH)
4.19 (2H, s, 12-CH_2)	3.2~3.4 (1H, m, 3-CH_2)	6.67 (1H, d, J = 10.0, 4-CH)
5.17 (1H, m, 2-CH)	4.74 (1H, q, 7.3, 12-CH_2)	7.77 (1H, s, OH)
6.78 (1H, s, 8-CH)	5.17 (1H, m, 2-CH)	
	6.75 (1H, s, 8-CH)	

Solvent: **1a**, **2a**, **3a**, **5a**, **6a** in d_6-DMSO, **7a** in $CDCl_3$.

salfredins were then converted into the corresponding methyl ethers [δ_H 3.97~4.21(s)] by diazomethane treatment. The findings suggest that salfredins possess phenol moieties on these molecules.

IR spectra of salfredins A and C showed absorption bands at *ca.* 1700~1745 cm^{-1}, indicating the presence of carboxylic acid moieties, which were ascertained by ^{13}C NMR [δ_C *ca.* 171~175(s)]. Similar structural moieties were present in all members of the A and C groups, indicating their close structural and biosynthetic relationship.

The relative stereostructure of salfredin A_4 (**2a**) was confirmed by X-ray crystallographyic analysis and ^{1}H and ^{13}C NMR chemical shifts of **2a** could be reasonably assigned, and the mass spectra were consistent with its structure. Therefore, the structures of other components were subsequently determined by comparing these MS and NMR spectroscopic data with those of **2a**. Salfredins A and C had the same furoisoindol nucleus but differed from each other in the *N*-alkyl carboxylic acid side chain moiety, except for B_{11}.

The ^{1}H and ^{13}C NMR chemical shifts of salfredins are displayed in Tables 3 and 4.

1. Structure of Salfredin A Group

The molecular formula of **2a** was established as $C_{15}H_{15}NO_7$ from the elemental analysis, SI-MS and ^{13}C NMR spectra. As methylation of **2a** with diazomethane gave trimethyl compound **2b**, **2a** was assumed to have two carboxylic acids and one phenol moiety, and this was confirmed by ^{1}H and ^{13}C NMR spectra of **2b** [δ_H 3.71 and 3.76 ($COOCH_3$), 3.97 (OCH_3)].

Table 4. ^{13}C NMR chemical shifts of salfredins.

	A_4 (**2a**)	A_3 (**1a**)	A_7 (**3a**)	C_2 (**5a**)	C_3 (**6a**)	B_{11} (**7a**)
1	12.17 q[a]	11.90 q	12.56 q	11.86 q	11.81 q	28.11 q
2	30.85 t	24.13 t	15.75 q	30.44 t	14.90 q	28.11 q
3	43.67 t	30.18 t	31.24 t	38 t[b]	30.46 t	70.45 t
4	44.08 d	30.64 t	44.42 d	43.69 d	43.79 d	77.87 s
5	47.95 t	43.86 d	44.92 t	85.44 d	46.46 d	102.27 d
6	84.28 d	44.72 t	49.55 d	97.49 d	85.61 d	103.80 s
7	94.64 d	53.08 d	84.51 d	109.10 s	97.63 d	108.78 s
8	117.05 s	84.22 d	94.79 d	119.76 s	109.22 s	115.22 d
9	121.88 s	94.76 d	117.20 s	134.78 s	120.02 s	129.03 d
10	133.43 s	117.22 s	122.11 s	152.12 s	135.03 s	147.04 s
11	148.93 s	121.88 s	133.71 s	165.57 s	152.47 s	152.09 s
12	161.13 s	133.18 s	149.08 s	165.69 s	165.98 s	160.73 s
13	168.14 s	148.80 s	161.24 s	166.60 s	166.08 s	172.59 s
14	171.09 s	161.22 s	168.06 s	169.05 s	166.91 s	
15	174.98 s	168.83 s	173.52 s	174.21 s	171.65 s	
16		172.44 s	174.98 s		174.66 s	
17		173.87 s				
18		174.90 s				

Solvent: **1a**, **2a**, **3a**, **5a**, **6a** in d_6-DMSO, **7a** in $CDCl_3$.

[a] Multiplicity determined by single-frequency off-resonance decoupling (SFORD) experiments.

[b] The value is obscure because of solvent interference.

The relative stereostructure of **2a**, which had been postulated by NMR studies of **2a** and **2b**, was confirmed by X-ray analysis to be 2-[6-carboxymethyl-4-hydroxy-7-oxo-2,3,5,6-tetrahydrofuro(2,3-f)isoindol-2-yl]-propionic acid having an *N*-ethanoic acid (N–CH_2COOH) produced from glycine in the molecule.

The molecular formulae of salfredins A_3 (**1a**) and A_7 (**3a**) were determined as $C_{18}H_{19}NO_9$ and $C_{16}H_{17}NO_7$ by SI-MS and ^{13}C NMR, respectively. Methylation of **1a** with diazomethane gave the tetramethyl compound (**1b**). Based on detailed NMR studies of these com-

Fig. 6. A perspective view of salfredin A_4 (**2a**) drawn by PLUTO.

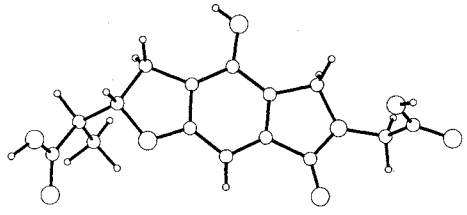
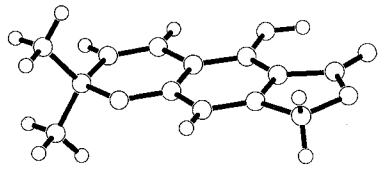


Fig. 7. A perspective view of salfredin B_{11} (**7a**) drawn by PLUTO.



pounds, it was concluded that **1a** possessed a glutamic acid residue [(N)–CH(COOH)–CH_2CH_2– COOH] [12-CH: δ_H 4.75 (1H, m), δ_C 53.08 (d); 13 and 14-CH_2: δ_H 1.8~2.4 (2H) and 2.24 (2H), δ_C 24.13 (t) and 30.18 (t); 12a and 15-CO: δ_C 172.44 (s) and 173.87 (s)] and **3a** had an alanine one [(N)–CH(CH_3)–COOH] [12-CH: δ_H 4.77 (1H, q), δ_C 49.55 (d); 12a-CH_3: δ_H 1.47 (3H, d), δ_C 15.2 (q); 13-CO: δ_C 172.9 (s)] in place of a glycine one [(N)–CH_2–COOH] [12-CH_2: δ_H 4.20 (2H, s), δ_C 43.67 (t); 13-CO: δ_C 171.09 (s)] on the same basic furoisoindol propionic acid nucleus of **2a**. The ^1H and ^{13}C NMR chemical shifts of furoisoindol propionic acid of both **1a** and **3a** agreed reasonably with **2a**. Therefore, the relative stereostructures of **1a** and **3a** were determined to be 2-[6-(1,3-dicarboxy)propyl-4-hydroxy-7-oxo-2,3,5,6-tetrahydrofuro(2,3-f)isoindol-2-yl]-propionic acid and 2-[6-(1-carboxy)ethyl-4-hydroxy-7-oxo-2,3,5,6-tetrahydrofuro(2,3-f)isoindol-2-yl]-propionic acid as shown in Fig. 1.

2. Structure of Salfredin C Group

The molecular formula of **5a** was determined as $C_{15}H_{13}NO_8$ by SI-MS and ^{13}C NMR, and the mass number of **5a** [m/z 336 $(M+H)^+$] was 14 mass units more than that of **2a** [m/z 322 $(M+H)^+$]. By comparision with the protons and carbons chemical shifts of **2a** and **5a** in NMR spectra, the structure of **5a** was similar to that of **2a** with respect to having the same dihydrofuran ring side chain and glycine residue. On the other hand, there was one less methylene group (–CH_2–) of **5a** [δ_H 3.02 and 3.2~3.4 (3-CH_2), and 4.19 (12-CH_2); δ_C 30.44 (t) and 38 (t)†] than that of **2a** [δ_H 2.94 and 3.31 (3-CH_2), 4.20 and 4.30; δ_C 30.85 (t), 43.67 (t) and 47.95 (t)], and there was one more quaternary carbon of **5a** [δ_C 109.10, 119.76, 134.78, 152.12, 165.57, 165.69, 166.60, 169.05 and 174.21] than that of **2a** [δ_C 1170.05, 121.88, 133.43, 148.98, 161.13, 168.14, 171.09 and 174.98]. In the UV spectra, the absorption band of **5a** shifted to a longer wave length than that of **2a**. These data was suggested that **5a** was oxidated derivative of **2a** at C-5 position. The conversion of **2a** into **5a** was achieved by chemical method. The oxidated derivatives of **2b** by Jones oxidation was identified with **5b** which was derived from **5a** by methylation. From these results, the relative stereostructure of **5a** was determined to be 2-[6-carboxymethyl-4-hydroxy-5,7-dioxo-2,3,5,6-tetrahydrofuro(2,3-f)isoindol-2-yl]-propionic acid having a furophthalimide skeleton. The molecular formulae of **6a** and **4a** were determined to be $C_{16}H_{15}NO_8$ and $C_{13}H_{11}NO_6$ by SI-MS, respectively. From ^1H and ^{13}C NMR spectra, both **4a** and **6a** showed the corresponding signals to the same furophthalimide moiety of **5a**, but they displayed no signals of a carboxymethyl (–CH_2COOH) residue, and **6a** showed the signals of the –CH(CH_3)COOH residue. Moreover, **4a** was converted into the *N*-methyl compound (**4b**) by treatment with diazomethane. From these results, the relative stereostructures of **4a** and **6a** were determined to be 2-[4-hydroxy-5,7-dioxo-2,3,5,6-tetrahydrofuro(2,3-f)-isoindol-2-yl]-propionic acid and 2-[6-carboxymethyl-4-hydroxy-5,7-dioxo-2,3,5,6-tetrahydrofuro(2,3-f)-isoindol-2-yl]-propionic acid, respectively. These structures were supported by SI-MS and NMR data of the corresponding methyl compounds (**4b** and **5b**).

3. Structure of Salfredin B

The molecular formula of **7a** was established as $C_{13}H_{12}O_4$ from the elemental analysis, SI-MS and ^{13}C NMR spectra. Compound **7a** did not have a nitrogen atom and propionic acid residue. IR spectra of **7a** displayed an absorption band at 1744 cm^{-1}, indicating the presence of a lactone moiety. Since methylation of **7a** with diazomethane gave methyl compound **7b**, **7a** was assumed to have one phenol moiety.

The structure of **7a** was postulated by NMR studies of

† The value is obscure because of solvent interference.

7a and finally confirmed by X-ray crystallographic analysis to be 5-hydroxy-2,2-dimethyl-1,7-dioxa-cyclopenta(g)coumarin-6-one.

Experimental

NMR spectra were measured with a Varian VXR-200 spectrometer in $CDCl_3$ or DMSO-d_6 solution with the internal standard TMS. Mass spectra were obtained with a Hitachi M-90 spectrometer, IR spectra with a Jasco IR-700 infrared spectrometer, and UV spectra with a Hitachi 320 spectrophotometer. Preparative TLC was performed with Merck SiO_2 F60.

1. Methylation of Salfredins

a) A mixture of salfredin A_4 (**2a**, 1.0 g), K_2CO_3 powder (1.94 g) and CH_3I (1.8 ml) in dry DMF (10 ml) was stirred at 50°C on an oil bath for 1 hour. After cooling of the reaction mixture, toluene (30 ml) was added to this and then the mixture was made acidic with 2 N HCl. The toluene layer was washed three times with H_2O, dried over Na_2SO_4, and evaporated *in vacuo* giving a pale yellow oily residue (**2b**, 1.1 g, *ca.* 100% yield).

b) To a solution of **2a** (6 mg) in MeOH (0.5 ml) was added excess trimethylsilyldiazomethane (10% *n*-hexane solution) at room temperature. After the reaction mixture was left standing overnight, the organic solvent was removed *in vacuo* and the resulting residue was purified by TLC (KGF, toluene - EtOH 1 : 1, Rf 0.3), giving **2b** (3.5 mg). **2b**: LSI-MS *m/z* 364 $[M+H]^+$, M = $C_{18}H_{21}NO_7$ (363). ^1H NMR ($CDCl_3$) δ 1.34 (3H, d, J=7.2 Hz, 11-CH_3), 2.82 (1H, dq, J=7.2, 7.2 Hz, 9-CH), 3.14 (1H, dd, J=7.2, 16.2 Hz, 3-CH_2), 3.49 (1H, dd, J=9.2, 16.2 Hz, 3-CH_2), 3.71 (3H, s, $COOCH_3$), 3.76 (3H, s, $COOCH_3$), 3.97 (3H, s, 4-OCH_3), 4.38 (2H, s, 5-CH_2), 4.47 (2H, s, 12-CH_2), 5.00 (1H, ddd, J=7.2, 7.2, 9.2 Hz, 2-CH), 6.93 (1H, s, 8-CH). ^{13}C NMR ($CDCl_3$) δ 13.76 q, 32.86 t, 44.29 t, 45.32 d, 49.19 t, 52.46 q, 52.79 q, 58.99 q, 84.61 d, 99.35 d, 119.46 s, 123.74 s, 134.37 s, 151.51 s, 162.16 s, 168.90 s, 169.98 s, 174.49 s.

c) Tetramethyl salfredin A_3 (**1b**) (TLC: toluene - EtOH 1 : 1, Rf 0.4, 85% yield) was obtained from **1a** by trimethylsilidyldiazomethane method. **1b**: LSI-MS *m/z* 450 $[M+H]^+$, M = $C_{22}H_{27}NO_9$ (449). ^1H NMR ($CDCl_3$) δ 1.34 (3H, d, J=7.2 Hz, 11-CH_3), 2.1 ~ 2.5 (4H, m, 13- and 14-CH_2), 2.81 (1H, dq, J=7.2, 7.2 Hz, 9-CH), 3.15 (1H, dd, J=7.4, 16.0 Hz, 3-CH_2), 3.50 (1H, dd, J=9.3, 16.0 Hz, 3-CH_2), 3.62 (3H, s, 10-$COOCH_3$), 3.72 (3H, s, 12-$COOCH_3$), 3.73 (3H, s, 15-$COOCH_3$), 3.98 (3H, s, 4-OCH_3), 4.32 (1H, d, J=16.2 Hz, 5-CH_2), 4.50 (1H, d, J=16.2 Hz, 5-CH_2), 4.9 ~ 5.1 (2H, m, 2- and 12-CH), 6.91 (1H, s, 8-CH). ^{13}C NMR ($CDCl_3$) δ 13.28 q, 25.04 t, 30.68 t, 32.49 t, 44.89 d, 45.11 t, 51.80 q, 51.99 q, 52.45 q, 53.29 d, 58.59 q, 84.16 d, 98.82 d, 119.00 s, 123.56 s, 133.81 s, 151.16 s, 161.79 s, 168.74 s, 171.12 s, 172.81 s, 173.99 s.

d) Trimethyl salfredin C_1 (**4b**) (TLC: Rf 0.6, 85% yield) was afforded from **4a** by the trimethylsilyldiazomethane method. **4b**: LSI-MS *m/z* 320 $[M+H]^+$, M = $C_{16}H_{17}NO_6$ (319). ^1H NMR ($CDCl_3$) δ 1.33 (3H, d, J=7.2 Hz), 2.83 (1H, dq, J=7.2, 7.2 Hz), 3.07 (1H, dd, J=7.6, 16.8 Hz), 3.11 (3H, s), 3.42 (1H, dd, J=9.6, 16.8 Hz), 3.72 (3H, s), 4.20 (3H, s), 5.10 (1H, ddd, J=7.2, 7.6, 9.6 Hz), 6.93 (1H, s). ^{13}C NMR δ ($CDCl_3$) 13.04 q, 23.91 q, 31.70 t, 44.83 d, 52.11 q, 61.65 q, 85.51 d, 100.63 d, 112.44 s, 122.33 s, 136.66 s, 154.64 s, 165.70 s, 166.74 s, 167.72 s, 173.60 s.

e) Trimethyl salfredin C_2 (**5b**) (TLC: Rf 0.6, 85% yield) was prepared from **5a** by the trimethylsilyldiazomethane method. **5b**: LSI-MS *m/z* 378 $[M+H]^+$, M = $C_{18}H_{19}NO_8$ (377). ^1H NMR ($CDCl_3$) δ 1.34 (3H, d, J=7.0 Hz, 11-CH_3), 2.84 (1H, dq, J=7.0, 7.0 Hz, 9-CH), 3.09 (1H, dd, J=7.8, 16.8 Hz, 3-CH_2), 3.45 (1H, dd, J=9.8, 16.8 Hz, 3-CH_2), 3.73 (3H, s, $COOCH_3$), 3.76 (3H, s, $COOCH_3$), 4.21 (3H, s, 4-OCH_3), 4.38 (2H, s, 12-H), 5.13 (1H, ddd, J=7.0, 7.8, 9.8 Hz, 2-CH), 6.97 (1H, s, 8-CH). ^{13}C NMR ($CDCl_3$) δ 13.45 q, 32.17 t, 39.20 t, 45.26 d, 52.58 q, 53.07 q, 62.12 q, 86.05 d, 101.43 d, 112.62 s, 123.15 s, 136.82 s, 155.45 s, 166.11 s, 166.46 s, 167.21 s, 168.43 s, 174.01 s.

f) Methyl salfredin B_{11} (**7b**): A mixture of salfredin B_{11} (1.4 mg), CH_3I (0.5 ml) and Ag_2O (10 mg) in acetone (1 ml) was stirred and gently refluxed on an oil bath for 30 minutes. The reaction mixture was filtered and the filtrate was evaporated *in vacuo*. $CHCl_3$ (1 ml) was added to the residue, and the insoluble substance was filtered off. The solvent was evaporated *in vacuo* giving **7b** (1 mg).

Salfredin B_{11} (**7a**): ^1H NMR ($CDCl_3$) δ 1.46 (6H, s, 2-CH_3 × 2), 5.21 (2H, d, J=0.8 Hz, 8-CH_2), 5.64 (1H, d, J=10.0 Hz, 3-CH), 6.39 (1H, d, J=0.8 Hz, 9-CH), 6.67 (1H, d, J=10.0 Hz, 4-CH), 7.77 (1H, s, OH).

Methyl salfredin B_{11} (**7b**): ^1H NMR ($CDCl_3$) δ 1.52 and 1.56 (each 3H, s, 2-CH_3 × 2), 4.13 (3H, s, 5-OCH_3), 5.16 (2H, s like, 8-CH_2), 5.68 (1H, d, J=10.2 Hz, 3-CH), 6.54 (1H, s like, 9-CH), 6.71 (1H, d, J=10.2, 4-CH).

2. Conversion of Salfredin A_4 into Salfredin C_2

To a mixture of 4-methoxy salfredin A_4 diethylester (**2c**) (2.088 g) in acetone (53 ml) and $MgSO_4$ (5.34 g) was added dropwise 8 N Jones reagent (5.6 ml) over 55 minutes with stirring at room temperature. After stirring for 2 hours, *iso*-PrOH (0.1 ml) was added to this reaction mixture and then the color of this mixture changed from red to green. The green precipitate was removed from this mixture by filtration, then the filtrate was mixed with 1% $NaHCO_3$ (25 ml) and concentrated *in vacuo* for removing acetone. The resulting mixture was extracted twice with EtOAc (75 ml) and the extracts were washed with saturated NaCl solution and evaporated *in vacuo* giving the crude residue, which was purified by column chromatography (column: Merck, silica gel 60, 40 ~ 63 nm, 90 g; solvent: toluene - EtOAc 9 : 1) to afford 4-methoxy salfredin C_2 diethylester **5c** (colorless amorphous powder, 826 mg, 38% yield).

4-Methoxy salfredin C_2 diethylester **5c** was identified

with the authentic sample derived from salfredin C_2, which was methylated with diazomethane, followed by alkaline hydrolysis and ethyl esterification with EtOH and HCl.

5c: ^{1}H NMR ($CDCl_3$) δ (1.26 (3H, t, J=7.2 Hz), 1.28 (3H, t, J=7.0 Hz), 1.33 (3H, d, J=7.0 Hz), 2.82 (1H, dq, J=7.0, 7.0 Hz), 3.10 (1H, dd, J=7.5, 16.8 Hz), 3.43 (1H, dd, J=9.7, 16.8 Hz), 4.18 (2H, q, J=7.0 Hz), 4.21 (3H, s), 4.22 (2H, q, J=7.2 Hz), 4.36 (2H, s), 5.11 (1H, ddd, J=7.0, 7.5, 9.7 Hz), 6.97 (1H, s). ^{13}C NMR ($CDCl_3$) δ 13.09 q, 14.13 q, 14.16 q, 31.66 t, 38.93 t, 44.94 d, 60.97 t, 61.66 q, 61.79 t, 85.71 d, 100.95 d, 112.17 s, 122.73 s, 136.38 s, 154.97 s, 165.73 s, 166.03 s, 166.82 s, 167.51 s, 173.11 s.

3. X-Ray Crystallographic Analysis of Salfredin A_4

The structure of A_4 was determined by X-ray analysis. Colorless plate crystals were obtained from 50% *iso*-PrOH solution. Crystal data: $C_{15}H_{15}NO_7$, Mr=321.3, monoclinic, $P2_1$, a=5.615(1), b=23.408(4), c=5.597(1) Å, β=94.80(2)°, V=733.0(2) Å^3, Z=2, Dx=1.456 g/cm^3, CuKα radiation, λ=1.54178 Å, μ=1.01 mm^{-1}, F(000)=336.

A crystal with dimensions 0.20×0.15×0.25 mm was used for X-ray measurements at 295 K on a Rigaku AFC-5R diffractometer equipped with a graphite monochromator. Cell constants were determined from 24-well centered reflections in the range $45<2\theta<55°$. Intensity data were collected to a maximum of 2θ-value of 130° by the $\omega/2\theta$ scan technique. The total number of independent reflections measured was 1286, of which 1258 were considered to be observed [$F>=4\sigma(F)$]. No absorption correction was applied. The structure was solved by direct methods and all H atoms were located in difference Fourier maps. The structure was refined by full-matrix least-squares, with anisotropic temperature factors for non-H atoms and isotropic temperature factors for H atoms. The weighting scheme employed was $\omega=1/\sigma$ (F). The refinement converged to R=0.038, ωR=0.036. The residual densities are in the range $-0.22\sim0.23$ e/Å^3. All crystallographic calculations were done on a VAX3100 workstation using the program system Xtal3.2[10] with the scattering factors included in the program.

4. X-Ray Crystallographic Analysis of Salfredin B_{11}

The structure of B_{11} was determined by X-ray analysis. Colorless prismatic crystals were obtained from *iso*-PrOH solution.

Crystal data: monoclinic; space group C2/m, a=15.172(5), b=6.887(2), c=10.658(2) Å, β=104.71(1)°, V=1077.2(5) Å^3, Z=4, Dx=1.43 g/cm^3. A crystal with dimensions 0.05×0.10×0.30 mm was mounted on a Rigaku AFC-5R diffractometer with Ni-filtered Cu-Kα radiation. The data was measured using ω-2θ scan technique to a maximum 2θ-value of 140°. A total of 997 unique reflections were measured. An empirical absorption correction using the program DIFABS[11] was applied which resulted in transmission factors ranging from 0.71 to 1.59. The data were corrected for Lorenz and polarization effects. The structure was solved by direct methods[12]. Non-hydrogen atoms were refined anisotropically. Hydrogen atoms were refined isotropically. The final cycle of full-matrix least-squares refinement was based on 852 observed reflections [$I>1.0\sigma(I)$] and 129 variable parameters and converged (The largest parameter shift was 0.04 times its esd) with an agreement factor of $R=\sum||Fo|-|Fc||/\sum|Fo|=0.039$. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.16 and -0.19 e/Å^3, respectively. Neutral atom scattering factors were taken from CROMER and WABER. All calculations were performed using the teXsan crystallographic software package of Molecular Structure Corporation.

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