FR901228, A NOVEL ANTITUMOR BICYCLIC DEPSIPEPTIDE PRODUCED BY *Chromobacterium violaceum* No. 968

II. STRUCTURE DETERMINATION

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The structure of FR901228 ($C_{24}H_{36}N_4O_6S_2$), including stereochemical details, was determined by a combination of spectroscopic, chemical evidence and single-crystal X-ray crystallographic analysis using anomalous dispersion from the S atoms.

In the course of screening soil samples for new antitumor compounds, FR901228 (**1**) was discovered from the fermentation of a bacterial culture. The taxonomy, fermentation, isolation, physico-chemical characterization and biological properties of **1** are the subjects of the preceding paper[1]. We report here on the full account of structure determination of **1**.

Plane Structure Elucidation for 1

From an ethyl acetate extract of fermentation broth of *Chromobacterium violaceum* No. 968 was obtained FR901228 (**1**) as colorless prisms: mp 235~245°C (dec), $[\alpha]_D^{23}$ +39° (*c* 1.0, $CHCl_3$). The molecular formula, $C_{24}H_{36}N_4O_6S_2$, was determined by the interpretation of elementary analysis[1], ^1H and ^{13}C NMR data (Table 1) and high-resolution FAB-MS (matrix: 3-nitrobenzyl alcohol, obsd 541.2164; $C_{24}H_{36}N_4O_6S_2+H$ requires 541.2154). The nature of the two sulfur atoms was assigned as a disulfide functionality from the FAB-MS data (vide infra). Low-resolution FAB-MS spectra in a matrix of dithiothreitol-dithioerythritol (magic bullet)[2] showed a *quasi* molecular ion at *m/z* 543, while in 3-nitrobenzyl alcohol a protonated molecular ion at *m/z* 541 was observed. The difference of 2 mass units is ascribable to reduction of the disulfide to a dithiol by the reducing action of dithiothreitol[3].

The ^{13}C NMR spectrum revealed signals for all 24 carbons, including 5 CH_3, 4 CH_2, 9 CH and 6 quaternary carbons. Since five amide or ester carbonyls (δ_C 172.5, 172.1, 169.9, 168.8, 165.6) and four olefinic carbons (δ_C 131.6 (s), 131.5 (d), 130.4 (d), 127.8 (d)) account for 7 of the 9 degrees of unsaturation required by the molecular formula, **1** must be a bicyclic compound. Strong absorptions at

Fig. 1. Structure of FR901228 (**1**).

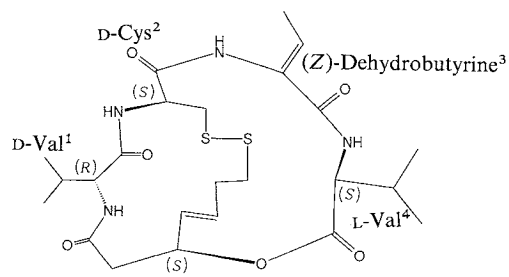
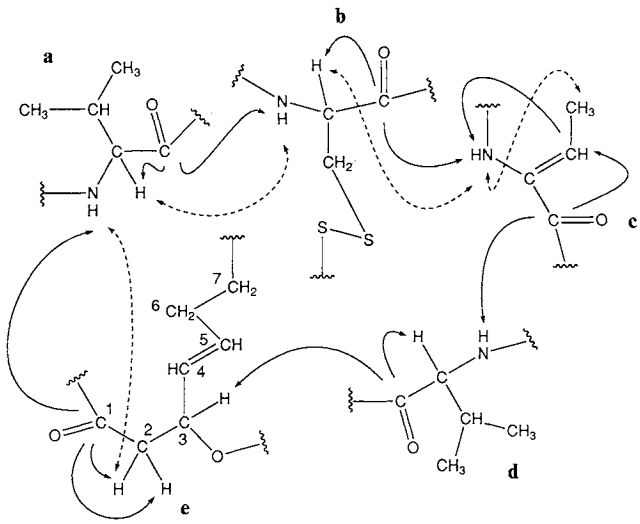


Fig. 2. Partial structures **a**, **b**, **c**, **d**, and **e** for **1**.



Selected long-range ^{13}C-^{1}H coupling patterns are indicated by arrows and critical NOE correlations by double-headed dotted arrows.

1660, 1520 cm^{-1} together with a band at 1740 cm^{-1}, in the IR spectrum, suggested that **1** is a peptide with an ester or lactone functionality. Total acid hydrolysis of **1** yielded valine and ammonia in a 2:1 molar ratio. In the ^{1}H NMR spectrum of **1**, the two Val residues, the ^{1}H spin system –NHCH(CH_2–)CO– (δ_H 7.73 (d, 7, exchangeable), 4.68 (ddd, 5, 7, 10.5), 3.25 (dd, 5, 16), 3.21 (dd, 10.5, 16)) and an ethylidene group (δ_H 6.21 (q, 7), 1.66 (3H, d, 7)) were characterized with the aid of ^{1}H-^{1}H COSY. The presence of a cysteine residue in partial structure **b** was first inferred from the ^{1}H spin system –NHCH(CH_2–)CO– in conjunction with the disulfide functionality, and eventually substantiated by the detection of cysteic acid in the acid hydrolysate of the performic acid oxidation product of **1** (see Experimental section). The ethylidene group, a singlet amide ^{1}H signal (δ_H 8.46) and high-field shifted amide carbonyl (δ_C 165.6) implied the presence of dehydrobutyrine[4] (partial structure **c**), a well-known dehydro amino acid found in microbial peptides, and the existence of **c** was supported by the long-range ^{13}C-^{1}H coupling between the carbonyl and the ethylidene olefinic proton (δ_H 6.21). Extensive analysis of ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY and COLOC led to the assignment of a partial structure **e** as 3-oxy-4-heptenoyl-7-yl. The sum of the partial structures **a**~**e** satisfies the molecular formula of **1**. The plane structure for **1** was readily assembled by connecting **a**, **b**, **c**, **d**, **e** units on the basis of long-range ^{13}C-^{1}H coupling patterns observed in COLOC[5] spectra as shown in Fig. 2. The long-range coupling of Val carbonyl carbon in **d** to oxymethine proton 3-H in **e** indicated that **1** possessed a 16-membered lactone. The sequence of the acyl tetrapeptide, acyl-Val^1-Cys^2-dehydrobutyrine^3-Val^4, was further supported by NOEs, detected in NOESY spectra, between NH and CαH pairs from neighboring amino acid residues. In addition, the (*Z*) configuration of the double-bond in the dehydro amino acid (**c**) was assigned by an NOE between the ethylidene methyl protons and the amide proton as shown in Fig. 2 by the dotted arrow. The assumption of a bond between the disulfide in **b** and C-7 in **e** would give the plane structure for **1**. The ^{1}H and ^{13}C NMR spectral assignment are summarized in Table 1.

Table 1. ^{1}H and ^{13}C NMR signal assignments of FR901228 (**1**).

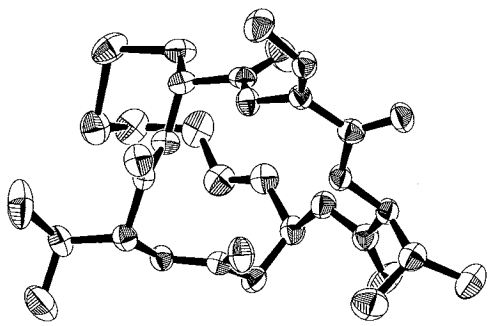
Assignment		δ_H[a] (mult., J (Hz))	δ_C[b] (mult.)	Assignment		δ_H[a] (mult., J (Hz))	δ_C[b] (mult.)
Acyl	1		172.5 (s)	D-Cys[2]	NH	7.73 (d, 7)	
	2	2.67 (dd, 13.5, 6.5),	38.4 (t)		α	4.68 (ddd, 10.5, 7, 5)	57.7 (d)
		3.14 (dd, 13.5, 2)			β	3.21 (dd, 16, 10.5),	36.1 (t)
	3	5.70 (m)	71.5 (d)			3.25 (dd, 16, 5)	
	4	5.80 (br d, 16)	131.5 (d)		CO		168.8 (s)
	5	5.95 (dm, 16)	130.4 (d)	Dehb[3c]	NH	8.46 (s)	
	6	2.70~2.59 (2H, m)	31.0 (t)		α		131.6 (s)
	7	3.00 (m)	38.8 (t)		β	6.21 (q, 7)	127.8 (d)
		3.14 (m)			γ	1.66 (3H, d, 7)	13.4 (q)
D-Val[1]	NH	8.98 (d, 4)			CO		165.6 (s)
	α	3.96 (dd, 6, 4)	63.0 (d)	L-Val[4]	NH	7.81 (d, 7)	
	β	2.27 (m)	29.6 (d)		α	4.40 (dd, 7, 5)	59.1 (d)
	γ	1.07 (3H, d, 7),	19.4 (q)		β	2.33 (m)	32.0 (d)
		1.10 (3H, d, 7)	19.5 (q)		γ	0.98 (3H, d, 7),	18.8 (q)
	CO		172.1 (s)			1.02 (3H, d, 7)	18.9 (q)
					CO		169.9 (s)

[a] 400 MHz in DMF-d_7.
[b] 100 MHz in DMF-d_7.
[c] Dehydrobutyrine.

Structure of **1**

Reverse-phase chiral HPLC analyses of the acid hydrolysate of **1** allowed us to assign the D configuration for the one of the Val residues and the L configuration for the another Val. The (*E*) geometry of the double bond in the acyl component (**e**) was evident form the vicinal coupling constant of 16 Hz. The remaining structural problems were as follows: (1) The differentiation of the positions of D-Val and L-Val; (2) stereochemical assignment of cysteine residue in **b**; (3) absolute stereochemistry on C-3 in the acyl component (**e**). These were solved by single-crystal X-ray crystallographic analysis of **1**. From methanol solution, **1** crystallized in prisms. The structure was solved by direct methods (MULTAN 84) and the perspective view is shown in Fig. 3. The absolute configurations (3*S*, D-Val[1], D-Cys[2], L-Val[4]) were assigned by the Bijvoet rule[6] using the anomalous dispersion effect from the sulfur atoms. From the above information, the absolute structure of FR901228 (**1**) was determined as depicted in Fig. 1.

Fig. 3. ORTEP drawing of **1**.



FR901228 is an uncommon cage-shaped bicyclic depsipeptide, that is, a 16-membered macrocyclic lactone with a 15-membered ring encompassing the disulfide bond formed from the D-cysteine and the thiol group of a novel acid, 3-hydroxy-7-mercapto-4-heptenoic acid.

Experimental

Low-resolution and high-resolution FAB-MS spectra were measured with a VG ZAB-SE mass spectrometer. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AM400wb spectrometer. All 2D NMR experiments were carried out employing the standard Bruker software library.

Chiral Assignment of D-Val and L-Val

One mg of **1** was hydrolyzed with 6 N HCl (1 ml) at 110°C for 18 hours in a sealed tube. The mixture

was evaporated to dryness under reduced pressure and the residue was dissolved in aq 0.25 mM $CuSO_4$ and the solution was subjected to HPLC analysis. The analytical conditions were as follows: column Chiralpack WE (4.6×250 mm); mobile phase aq 0.25 mM $CuSO_4$; flow rate 1 ml/minute; detection 254 nm; temperature 40°C. Retention time of D-Val (16.5 minutes), L-Val (23.1 minutes).

Detection of Cysteic Acid

Thirty percent hydrogen peroxide (0.25 ml) was added to formic acid (4.75 ml) and the solution was kept at room temperature for 2 hours. Five mg of **1** was dissolved in formic acid (1 ml)-MeOH (0.2 ml). To the mixture was added the perfomic acid at −10°C and the mixture was allowed to stand at the same temperature for 3 hours. Twenty ml of ice water was added to the mixture and lyophilized. The residue was hydrolyzed at 110°C with 6 N HCl for 18 hours and evaporated to dryness. The residue was dissolved in 0.01 N HCl and the solution was analyzed with a Hitachi 835 automatic amino acid analyzer. Rt of cysteic acid, Val and ammonia were 7.7, 25.2, 42.9 minutes, respectively. The peak of retention time 7.7 minutes was coeluted with authentic DL-cysteic acid.

X-Ray Structure Analysis of **1**

A prismatic crystal of size 0.3×0.2×0.2 mm (density not measured) was used for data collection on a Rigaku AFC5R diffractometer with graphite-monochromated CuK_α radiation ($\lambda=1.54178$ Å). Cell parameters were obtained from a least-squares refinement using the setting angles of 25 reflections with $53<2\theta<61°$. The crystal data are as follows; chemical formula $C_{24}H_{36}N_4O_6S_2\cdot CH_3OH$ ($C_{25}H_{40}N_4O_7S_2$), orthorhombic, space group $P2_12_12_1$, $a=21.364$ (1), $b=15.451$ (1), $c=8.769$ (1) Å, $Z=4$, Dcalc=1.31 gcm^{-3}.

Intensity data were collected at room temperature in the $\omega/2\theta$ scan mode up to $2q=130°$ with a scan rate of 4°/minute. The ω scan range was $(1.0+0.3\tan\theta)°$. Background measurement were counted for 6 seconds on either side of the peak. Three reference reflections monitored every 100 reflections showed no appreciable intensity decrease. Of 2803 unique observed reflections, 2568 reflections with $|Fo|\geq 3\sigma$ (Fo) were used for structure determination. Corrections were made for Lorentz and polarization effects, but not for absorption effect ($\mu(CuK_\alpha)=20.8\,cm^{-1}$).

The structure was solved by direct methods (MULTAN84) and refined by full-matrix least squares. The quantity minimized was $\sum w(|Fo|-|Fc|)^2$, where $w=[\sigma 2(Fo)]^{-1}$. The refinements were made with anisotropic thermal parameters for non-H atoms. H atoms located on difference maps and calculated geometrically were refined isotropically. The absolute configuration was determined by comparing 53 Bijvoet pairs. The final R values were 0.038 for the structure of (3*S*, D-Val[1], D-Cys[2], L-Val[4])-configurations and 0.049 for that of the opposite configurations. The significance of this R-value difference was also justified on a basis of HAMILTON's R-factor-ratio test at a significance level $\gg 0.995$. Final wR value was 0.034. The ratio of maximum least-squares shifts to error in the refinement cycle for non-H atoms is 0.2. Atomic scattering factors used were taken from International Tables for X-ray Crystallography Vol. IV (Kynoch Press, Birmingham, England, 1974). Crystal data and atomic coordinates are available as supplementary material.

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