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Fredericamycin B and C from *Streptomyces griseus*: Structure Elucidation after 23 Years

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It is now more than 23 years since the first two articles about the new antitumor antibiotic fredericamycin A (FM-A, NSC-305263) with the fascinating unique spiro-structure were published in this journal[1,2]. Shortly after the structure of fredericamycin A (**1**) was elucidated by X-ray analysis[3] and later on confirmed by NMR experiments[4,5]. Up to now, there are many publications concerning the biology and chemistry of FM-A and also many patents were filed. But there are no reports in the literature concerning the structures of the two by-products described by PANDEY *et al.* in the first publication[1], called fredericamycins B (**2**) and C (**3**).

During our ongoing search for new antitumor and antiinfective compounds from natural sources, we examined the fermentation products of *Streptomyces griseus* ATCC 49344. Beside the main component fredericamycin A, we isolated also two intensively coloured violet pigments from this strain, which turned out to be identical in their physico-chemical properties with the formerly described fredericamycins B (**2**) and C (**3**)[1].

Materials and Methods

General

Nuclear magnetic resonance (NMR) spectra were measured on a Bruker Avance DRX 500 spectrometer with the solvent signals as internal standard. Analytical HPLC examinations were run on a Waters Millennium system with two independent pumps (Model 590) and a PDA 996 photodiode array detector, using a acetonitrile - water gradient on a Chromolith SpeedROD C-18e column (Merck, Darmstadt). Ultraviolet-visible (UV-VIS) spectra were taken directly from the analytical HPLC-PDA runs and show relative intensities. Size exclusion chromatography was conducted on Kronlab glass columns (2.5×100 cm) with Sephadex® LH-20 (Pharmacia) as column material and methanol or CH_2Cl_2/TFA as eluent. High resolution mass spectra were run on a Micromass LCT with a TOF detector, combined with a Hewlett-Packard 1100 analytical HPLC. Analytical thin layer chromatography (TLC) was done on silica gel plates (ALUGRAM® SIL G/UV_{254}, 0.2 mm, Macherey-Nagel, Düren).

Microorganism

Streptomyces griseus ATCC 49344 was purchased at the American Type Culture Collection (ATCC) and cultivated on agar plates (YMG medium).

Fermentation

Fredericamycins A, B and C were produced in a 150-liter stainless steel fermenter, containing 100 liters of YMG-medium (10 g malt extract, 4 g glucose and 4 g yeast extract per liter, pH=7.5), with a growth time of 7 days at 27°C under aeration of 0.5 vvm and agitation of 150 rpm.

Results and Discussion

Isolation

The whole fermentation broth (~100 liters) was brought to pH=1 with TFA and then frozen at −20°C for a complete precipitation of insoluble components. After warming up to 4°C, the broth was centrifuged at 5000 *g*, the supernatant was discarded and the mycelial part together with the precipitate (~11 liters) was lyophilised to give 2.7 kg of dry material.

This dry material (550 g) was extracted three times with three liter of dichloromethane containing 0.1% TFA. After filtration and removal of the solvent, the resulting black oily gum (32 g) was triturated three times with 600 ml dichloromethane/cyclohexane (1 : 9 v/v) to remove fatty components, then extracted three times with 300 ml methanol. The insoluble part (black solid, containing the main amount of fredericamycin A and B) was separated by centrifugation and the solvents were removed to result in 3 g of a dark black solid. One gram of this solid was triturated with 10 ml methanol and the supernatant was separated by size exclusion chromatography on Sephadex

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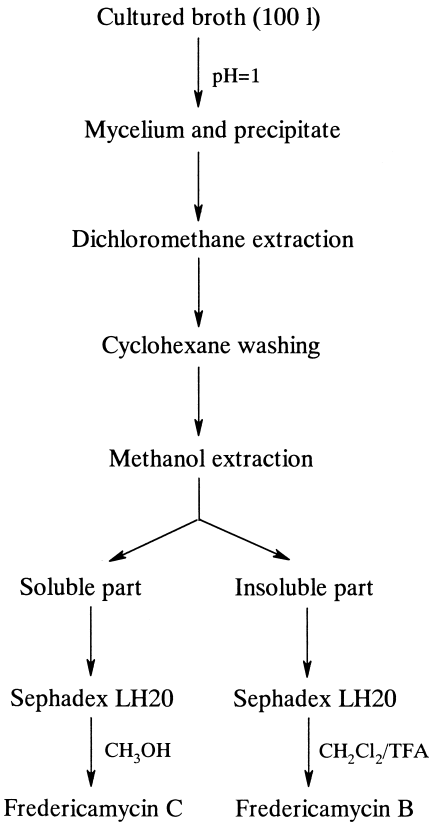
LH-20 with methanol as eluent (column 2.5×100 cm, flow 1 ml/minute). The slowly moving, intensively coloured violet band was collected and resulted after removal of the solvent in 2.6 mg of a black solid, which turned out to be identical in its physico-chemical properties[1] with fredericamycin C (**3**), with a molar mass of m/z=572 (negative ESI-MS).

Compound **3** is quite instable and reacts after addition of acids quickly under elimination of one molecule of water to a more unpolar substance with m/z=554, which we called fredericamycin C_1 (**4**). This substance is an artifact which occurs during the work-up using acids, but its structure is closely related to fredericamycin B (**2**) and very useful for the structure elucidation of the latter.

For the isolation of **2**, 11 mg of the black solid which contains the main part of fredericamycin A (see above) was dissolved in 7 ml of dichloromethane (containing 0.1% of TFA) and then separated on a Sephadex LH-20 column eluted with the same solvent. The slowly moving, intensively coloured violet band was collected and resulted in 0.7 mg of a black solid, which turned out to be identical with fredericamycin B (**2**)[1], with a molecular mass of m/z=553 (negative ESI-MS).

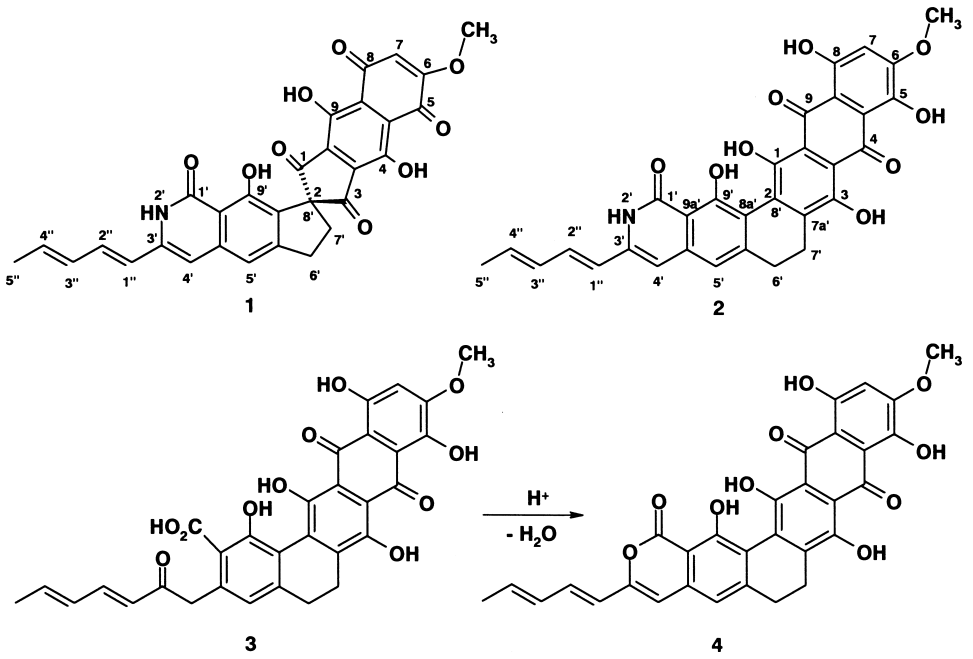
Fig. 1. Isolation procedure for fredericamycins.



Structure Elucidation

The physicochemical properties of the fredericamycins

Fig. 2. Structures of fredericamycins A (**1**), B (**2**), C (**3**) and C_1 (**4**).

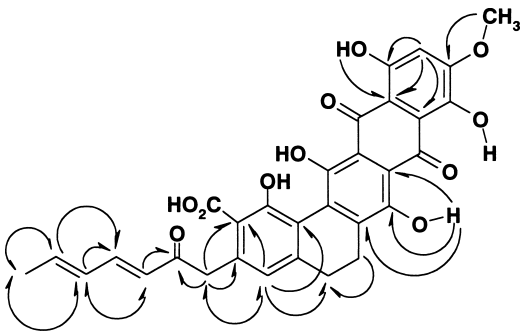


1～**4** are summarised in Table 1. The NMR-data of **1**, **3** and **4** are shown in Tables 2 and 3.

Knowing the physicochemical properties[1] of fredericamycin A (**1**) and its NMR characteristics[4,5], similarities and differences towards **2**, **3** and **4** are evident. The molecular masses are all in the same range, as well as the NMR shifts for $C_{1''}$ to $C_{5''}$ and for $C_{1'}$ to $C_{9'}$ of **1**, **3** and **4** (Tables 2 and 3). A methoxy group exists in all four compounds. In contrary, the UV-VIS spectra of **2**～**4** are very similar in the region of 400～600 nm (Fig. 4), but distinctly different to **1** and show a bathochromic shift of their λ_{max} in comparison to **1**, suggesting an extended conjugated system. In the ^{13}C-NMR of **2**～**4** the characteristic signal of the spiro-center in **1** is missing, as well as the two keto-signals of the spiro-ring.

Fredericamycin C (**3**) is the most unstable compound. Its molecular mass is *m/z*=572, corresponding to $C_{31}H_{24}O_{11}$. The UV-VIS spectrum in the region of 300～500 nm is less structured than those of **1**, **2** and **4** (Fig. 4), but it shows almost the same λ_{max} like **2** and **4**. This indicates an interruption of the conjugated system in the benzolactam-diene part ($C_{1'}$～$C_{9'}$ and $C_{1''}$～$C_{5''}$) in comparison to **1**, **2** and **4**.

Fig. 3. Selected HMBC correlations of **3**.



Taking all this information together and including also the information from extensive 2D-NMR measurements (Fig. 3), we were able to propose the structure for fredericamycin C (**3**). Additionally, the structure was confirmed by comparison of the ^{1}H, ^{13}C and UV-spectra of the structurally related quinoid compounds austrocortinin[6], obelmycin H[7], cynodontin[8] and other polyhydroxy-anthraquinones[9,10]. The structures of **3** and **2** are also supported by considerations about the biosynthesis, which can be easily deduced from a polyketide-pathway, using the same precursor polyketide suggested for fredericamycin A[11].

As mentioned above, **4** is derived from **3** by elimination of one molecule of water (−18) in the presence of acids, *e.g.* TFA. Its molecular mass is *m/z*=554, corresponding to $C_{31}H_{22}O_{10}$. Looking at the NMR data of **4**, one recognizes the absence of the carbonyl-signal at position 3′ of **3**, as well as the absence of the carboxy-group at position 2′. The UV spectrum of **4** is quite similar to **3**, but shows a slight bathochromic shift from 574 to 582 nm. This leads to the structure **4** given in Fig. 1, additionally confirmed by 2D-NMR data. Closely related to this structure are benaphthamycin[12] and WS79089A[13], also showing a 2-oxahexaphene moiety and a very similar skeleton.

At last we looked at fredericamycin B (**2**). Unfortunately, the obtainable quality of **2** was not good enough for a complete structure elucidation by NMR due to its instability, therefore the structure was deduced from the

Fig. 4. UV-spectra of fredericamycins A (**1**), B (**2**), C (**3**) and C_1 (**4**) in acetonitrile/water.

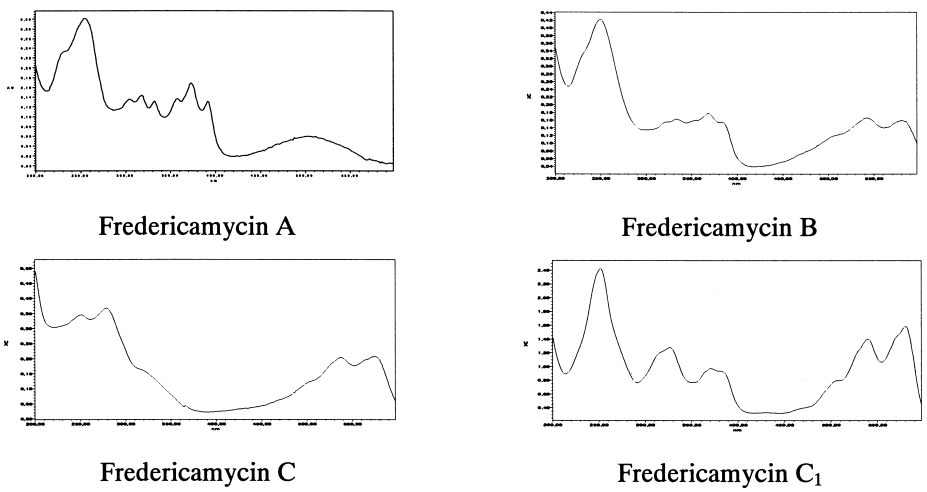


Table 1. Physico-chemical properties of the fredericamycins A (**1**), B (**2**), C (**3**) and C_1 (**4**).

	1	**2**	**3**	**4**
Appearance	Red powder	Black powder	Black powder	Black powder
Molecular formula	$C_{30}H_{21}NO_9$	$C_{31}H_{23}NO_9$	$C_{31}H_{24}O_{11}$	$C_{31}H_{22}O_{10}$
Molecular weight (*m/z*)	539	553	572	554
HR-ESI-MS				
Found	---	553.1402	572.1334	554.1243
Calculated	---	553.1373	572.1319	554.1213
UV λ nm (% A_{rel}) in CH_3CN/H_2O pH=6	255 (100), 304 (47), 317 (49), 332 (45), 358 (48), 372 (58), 392 (45), 504 (23)	260 (100), 328 (51) 348 (55), 370 (61), 387 (70), 510 (46), 548 (65), 586 (68)	255 (96), 276 (100), 315 (46), 537 (43), 560 (38), 574 (41)	256 (100), 339 (55), 386 (55), 407 (40), 505 (30), 542 (59), 582 (67)
TLC (R_f)[a] Lit.[1)]	0.76	0.89	0.64	---
(R_f)[b]	0.66	0.82	0.61	0.93
(R_f)[c]	0.90	0.95	0.72	0.97

[a] Silica Gel (F_{254}, E. Merck, Darmstadt)[1)]: $CHCl_3/CH_3OH$/HOAc 87:3:3

[b] Silica Gel (SIL G/UV_{254}, Macherey-Nagel, Düren): $CHCl_3/CH_3OH$/HOAc 87:3:3

[c] Silica Gel (SIL G/UV_{254}, Macherey-Nagel, Düren): $CHCl_3/CH_3OH$ 9:1 (0.1 % TFA)

Table 2. ^{1}H-NMR data of **1**[5)] (DMSO-d_6, 300 MHz), **3** (DMSO-d_6, 500 MHz) and **4** ($CDCl_3$, 500 MHz); *J* (Hz).

Position	**1**[5)]	**3**	**4**
1-OH	---	---[b]	12.95 (1H, s)
3-OH	---	13.21 (1H, s)	13.31 (1H, s)
4-OH	12.60[a] (1H, s)	---	---
5-OH	---	---[b]	12.93 (1H, s)
6-OCH_3	3.97[a] (3H, s)	3.92 (3H, s)	3.98 (3H, s)
7	6.58[a] (1H, s)	6.88 (1H, s)	6.66 (1H, s)
8-OH	---	12.72 (1H, s)	12.92 (1H, s)
9-OH	12.60[a] (1H, s)	---	---
1'-COOH	---	14.30 (1H, s br.)	---
2'-NH	11.60 (1H, d, 0.9)	---	---
4'	6.71 (1H, d, 0.9)	4.23 (2H, s)	6.29 (1H, s)
5'	7.03 (1H, s)	6.50 (1H, s)	6.79 (1H, s)
6'	3.20 (2H, t, 7.5)	2.61 (2H, t br.)	2.80 (2H, t br)
7'	2.46 (2H, t, 7.5)	2.77 (2H, t br.)	2.80 (2H, t br)
9'-OH	13.15 (1H, s)	---[b]	11.91 (1H, s)
1''	6.26 (1H, d, 15.9)	6.14 (1H, d, 15.4)	6.02 (1H, d, 15.0)
2''	7.15 (1H, dd, 15.9, 10.5)	7.16 (1H, dd, 15.4, 10.0)	7.05 (1H, dd, 15.0, 11.1)
3''	6.21 (1H, ddq, 15.0, 10.5, 1.2)	6.26 (1H, m)	6.18 (1H, m)
4''	5.93 (1H, dq, 15.0, 6.0)	6.26 (1H, m)	6.05 (1H, m)
5''	1.83 (3H, dd, 6.3, 1.2)	1.82 (3H, d, 4.9)	1.85 (3H, d, 6.5)

[a] Signals observed only on addition of traces of TFA-d

[b] Signal not observed

Table 3. ^{13}C-NMR data (125 MHz) of **1** (DMSO-d_6+TFA-*d*), **3** (DMSO-d_6) and **4** ($CDCl_3$).

Position	1[a]	3	4
1	198.4 (s)	156.4 (s)	153.9 (s)
2 = 8'	64.2 (s)	137.1 (s)	140.8 (s)
3	198.4 (s)	152.6 (s)	156.5 (s)
3a	137.9 (s)	112.4 (s)	111.5 (s)
4	151.7 (s)	187.3 (s)	189.7 (s)
4a	118.5 (s)	113.4 (s)	111.6 (s)
5	183.0 (s)	149.0 (s)	149.5 (s)
6	161.6 (s)	157.7 (s)	157.4 (s)
6-OCH_3	57.6 (q)	56.8 (q)	56.7 (q)
7	111.9 (d)	106.0 (d)	106.7 (d)
8	189.6 (s)	158.6 (s)	160.2 (s)
8a	119.1 (s)	105.9 (s)	106.2 (s)
9	151.2 (s)	186.8 (s)	187.1 (s)
9a	135.6 (s)	114.6 (s)	124.8 (s)
1'	166.8 (s)	169.6 (s)	166.3 (s)
3'	134.4 (s)	197.2 (s)	153.3 (s)
4'	106.2 (d)	47.4 (t)	105.3 (d)
4a'	139.9 (s)	140.8 (s)	138.7 (s)
5'	108.8 (d)	120.9 (d)	114.5 (d)
5a'	154.2 (s)	144.2 (s)	150.5 (s)
6'	32.3 (t)	28.8 (t)	30.0 (t)
7'	34.5 (t)	21.4 (t)	21.8 (t)
7a'	---	137.5 (s)	131.5 (s)
8' = 2	64.2 (s)	137.1 (s)	140.8 (s)
8a'	123.0 (s)	121.1 (s)	117.3 (s)
9'	155.9 (s)	164.7 (s)	159.4 (s)
9a'	110.7 (s)	116.1 (s)	105.1 (s)
1''	122.2 (d)	128.1 (d)	119.7 (d)
2''	132.9 (d)	141.8 (d)	134.7 (d)
3''	131.3 (d)	130.5 (d)	130.6 (d)
4''	133.9 (d)	139.7 (d)	136.1 (d)
5''	18.3 (q)	18.6 (q)	18.2 (q)

[a] assignment in accordance to Lit. [5]

UV-spectra and the HR-ESI-MS data in analogy to **3** and **4**. Compound **2** shows a molecular mass of m/z=553 ($C_{31}H_{23}NO_9$), indicating at least one nitrogen and suggesting the exchange of the lacton-group in **4** by a lactam-function.

The UV-spectrum of **2** is very close to **4** in its shape and λ-values, as well as the retention times on the HPLC and the Rf-values on TLC. All these results lead to the tentative structure **2** for fredericamycin B. Closely related to this structure are ericamycin[14] and BE-19412A[15], also showing a 2-azahexaphene moiety and a very similar skeleton.

Discussion

Fredericamycin A (**1**) is an antitumor antibiotic with a unique spiro-structure and strong physiological effects, which is known now since 23 years. After the isolation of fredericamycins B (**2**) and C (**3**), we were able to determine the structure of those minor compounds using extensive NMR measurements and by comparison of the physico-chemical properties. Considerations about the biogenesis of all compounds **1**～**3** lead to the same precursor polyketide[11], underlining the close biosynthetic relationship. Nevertheless, the minor compounds **2**～**4** show only very weak cytotoxicity and antimicrobial activity, as described in the first publications[1,2], which is consistent with our own *in vitro* data.

With these results, the long way to the structure elucidation of the fredericamycins has finally been completed.

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