NEW POLYENIC ANTIBIOTICS ACTIVE AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

IV. STRUCTURAL ELUCIDATION OF ENACYLOXIN IIa

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The chemical structure of a unique polyenic antibiotic enacyloxin IIa (former name: fr. 2) produced by *Frateuria* (formerly *Gluconobacter*) sp. W-315 has been determined by extensive spectroscopic studies, in particular by NMR spectral analysis. It has a novel non-lactonic structure involving 3,4-dihydroxycyclohexanecarboxylic acid with a chlorine-containing polyenic and polyhydroxy acyl side chain attached as an ester to the 3-hydroxyl substituent of the acid.

Frateuria sp. W-315 produces a series of antibiotics named enacyloxins (ENXs) which are active against Gram-positive and Gram-negative bacteria, slightly active against fungi, but inactive against yeasts[1,2]. In an earlier paper[3], we first reported the structure of enacyloxin IIa (ENX IIa, **1**)†† as shown in Fig. 1.

This paper presents the detailed structural elucidation of ENX IIa. As already reported in ref 1, the UV spectrum of **1** showed a broad absorption maximum at 365 nm indicating the presence of a conjugated polyenic chromophore in the molecule. Scanning the UV spectrum in liquid nitrogen clearly revealed the presence of carbonyl-conjugated pentaene chromophore[4] as shown in Fig. 2. The UV spectra of **1** in methanol exhibited little change under acidic and alkaline conditions suggesting a

Fig. 1. Enacyloxin IIa (**1**, R=H) and its methyl ester (**2**, R=CH_3).

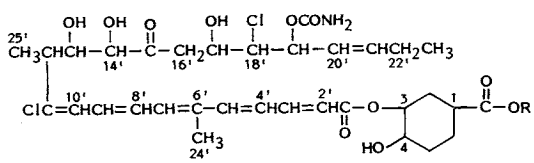
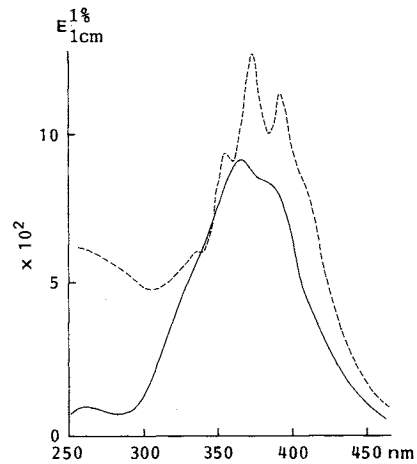


Fig. 2. UV spectra of ENX IIa (MeOH-glycerol=1:4).

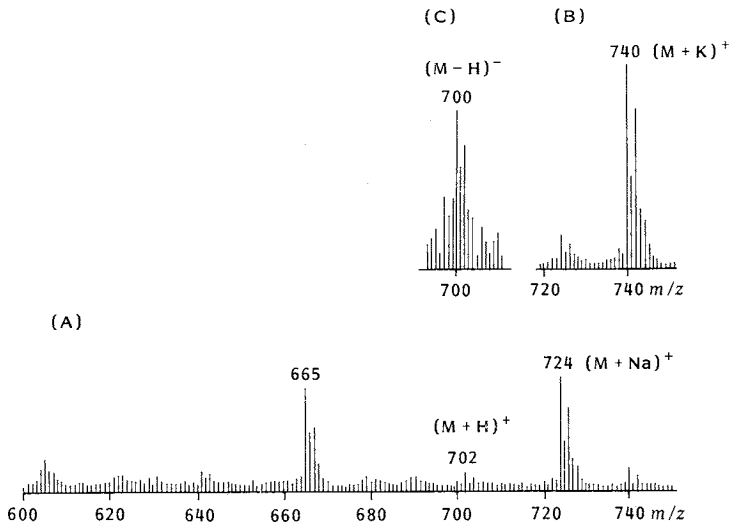
——: Scanned at room temperature, ——: scanned in liquid nitrogen.



†† In ref 3, the name of the antibiotic had been reported as enacyloxin II, which has now been renamed as enacyloxin IIa.

Fig. 3. FAB mass spectra of ENX IIa.

A: Positive FAB mass spectrum, B: positive FAB mass spectrum added with KCl, C: negative FAB mass spectrum.



non-ionizable functionality as part of the chromophore.

The IR spectrum of **1** exhibits the presence of hydroxyl (3400 cm^{-1}), carboxylic (~2500 and 1710 cm^{-1}) and polyenic (1620, 1575 and 975 cm^{-1}) groups.

FAB-MS and BEILSTEINS's test revealed the presence of two chlorine atoms in **1** (Fig. 3). The splitting pattern of $(M+Na)^+$ ion closely approximated the theoretical distribution of isotope abundances. Based on HRFAB-MS data (724.2226 ($(M+Na)^+$), calcd for $C_{33}H_{45}O_{11}NCl_2Na=724.2267$), the molecular formula of **1** was determined to be $C_{33}H_{45}O_{11}NCl_2$.

The carbon framework of **1** was elucidated by detailed analysis of the NMR spectra measured in CD_3OD and the results are summarized in Table 1. The ^{13}C NMR spectra showed 33 signals ($-CH_3\times3$, $>CH_2\times5$, $>CH-\times2$, $>CH-X$ (X=O or Cl)$\times7$, $=CH-\times10$, $>C=\times2$, $>C=O\times1$, $-COOR\times1$, $-COOH\times1$ and $-OCONH_2\times1$). The presence of a carbamate group was demonstrated by its typical chemical shift (158.6 ppm), a similar δ value to that of irumamycin (158.1 ppm)[5], a factor of nebramycin (159.8 ppm)[6], kijanimicin (157.4 ppm)[7] or kabiramide C (157.3 ppm)[8]. The ^1H NMR spectra of **1** and its methyl ester (**2**) are presented in Fig. 4. COSY, phase-sensitive double quantum filter COSY and HOHAHA spectra revealed the presence of $-OCO-CH=CH-CH=CH-$, $-C(CH_3)=CH-CH=CH-CH=$, $-CH(CH_3)-CHX-CHX-$, $CHX-CHX-CHX-CH=CH-CH_2-CH_3$ and $-CH-CH_2-CHX-CHX-CH_2-CH_2-$ (X=O or Cl). These fragments separated by quaternary carbons or hetero atoms were connected on the basis of cross peaks in the HMBC spectra as follows: C-1′→(2′-H, 3′-H), C-5′→(3′-H, 7′-H, 24′-H), C-6′→(4′-H, 5′-H, 24′-H), C-7′→(9′-H, 24′-H), C-10′→(8′-H, 9′-H, 12′-H), C-11′→(9′-H, 10′-H, 13′-H, 25′-H), C-15′→(14′-H, 16′-H), C-21′→(19′-H, 22′-H), C(carbamate)→(19′-H) and C_{COOH}→(1-H, 6-H). The above results revealed that **1** consists of two fragments, a 3,4-dihydroxycyclohexanecarboxylic acid moiety and a linear polyoxygenated polyene carboxy residue with the carbamate group at C-19′ as shown in Fig. 5. The signals of methylene protons (16′-H_2) of **1** gradually disappeared on standing in CD_3OD due to the hydrogen-deuterium exchange *via* enolization of the adjacent carbonyl function. The free

Table 1. ^{13}C- and ^{1}H-resonances of enacyloxin IIa.

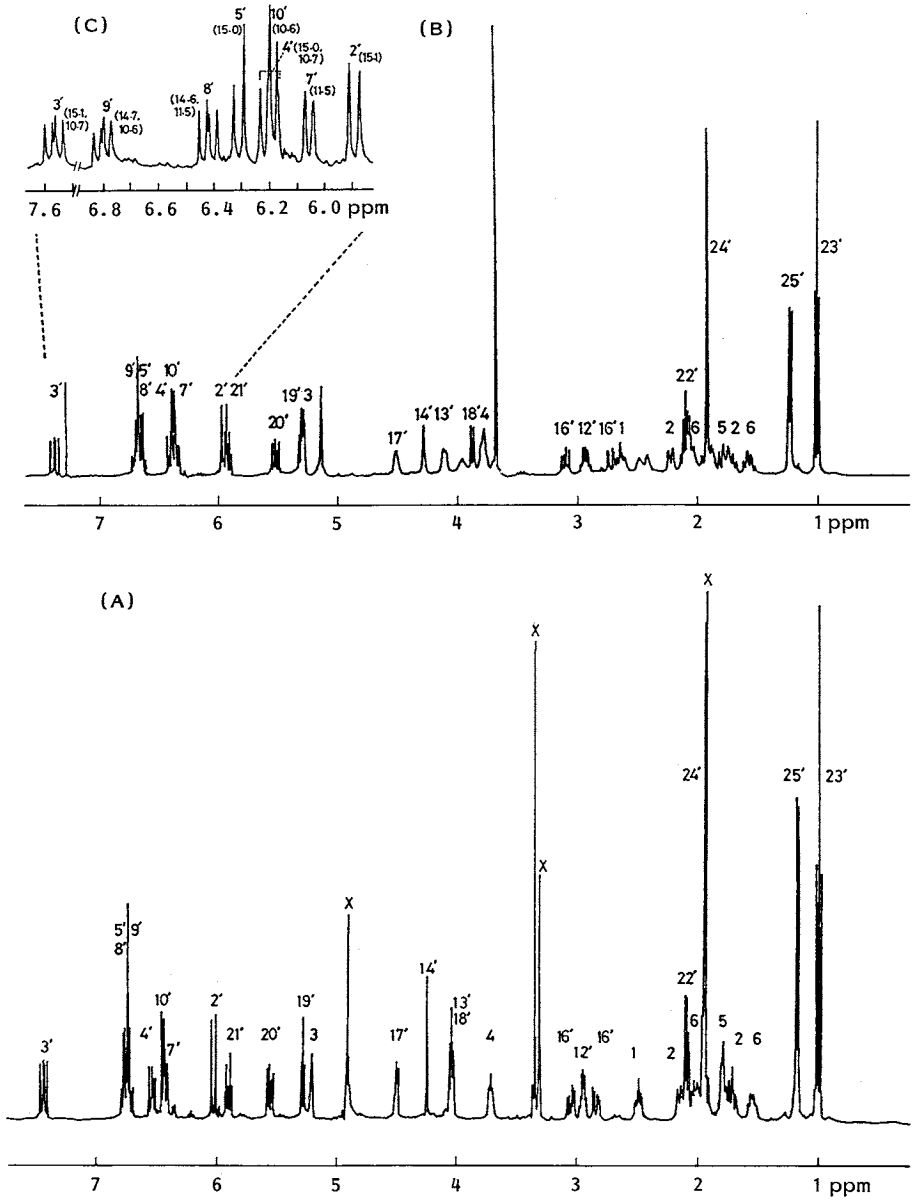
Position	^{13}C	J_{C-H} (Hz)	^{1}H (J/Hz)
1-COOH	181.5		
1	40.1		2.49 (dddd, J=11.6, 11.6, 3.5, 3.5)
2	32.9		1.72 (ddd, J=14.1, 12, 3.0)
			2.15 (dm, J=14.2)
3	73.5	150.7	5.21 (dm, J=4.3)
4	70.8	139.6	3.72 (ddd, J=9.4, 6.4, 2.9)
5	29.7		1.80 (m)
6	28.2		1.55 (m), 2.00 (m)
1′	168.6		
2′	121.5		6.02 (d, J=15.1)
3′	146.7		7.42 (dd, J=15.0, 11.2)
4′	127.2		6.52 (dd, J=15.0, 11.2)
5′	146.5		6.75 (d, J=15.0)
6′	137.4		
7′	136.9		6.41 (br d, J=10.1)
8′	131.5		6.76 (dd, J=14.9, 10.1)
9′	131.6		6.71 (dd, J=14.9, 9.8)
10′	128.4		6.44 (d, J=9.8)
11′	140.6		
12′	47.5		2.94 (dq, J=9.5, 6.7)
13′	74.0	144.3	4.05 (br d, J=9.3)
14′	78.8	141.5	4.25 (br s)
15′	211.7		
16′	44.6		2.84 (dd, J=17.0, 4.6)
			3.05 (dd, J=17.0, 7.9)
17′	66.9	146.1	4.50 (m)
18′	67.9	149.8	4.05 (dd, J=7.9, 2.5)
19′	75.5	149.8	5.28 (dd, J=7.8, 7.3)
19′-$OCONH_2$	158.6		
20′	126.1		5.55 (ddt, J=15.3, 7.3, 1.2)
21′	139.2		5.89 (dt, J=15.3, 6.4)
22′	26.3		2.10 (qdd, J=7.4, 6.4, 1.2)
23′	13.5		1.01 (t, J=7.4)
24′	12.7		1.95 (br s)
25′	16.2		1.19 (d, J=6.7)

hydroxyl groups were found to be located at C-4, C-13′, C-14′ and C-17′ by analyzing the ^{1}H NMR spectra in $CDCl_3$ of **2**. Thus, hydroxy protons at 5.12, 3.95, 3.78 and 2.45 ppm disappeared upon addition of D_2O, and the oxymethine signals at 4.47 (17′), 4.27 (14′), 4.11 (13′) and 3.79 (4) ppm were collapsed to yield sharp signals.

Consequently, the remaining two chlorine atoms were placed at C-11′ and C-18′ by elimination. The substitution with a chlorine atom at C-11′ as well as a methyl group at C-6′ is consistent with the bathochromic shift of 12 nm observed in the UV spectrum taken at low temperature as compared with the absorption behavior of 2,4,6,8,10-dodecapentaenoic acid[4]. The ester linkage at the C-3 position of the cyclohexane ring was determined from the chemical shift of 3-H (5.21 ppm) and the larger J_{C-H} value of C-3 (150.7 Hz)[9,10]. The stereochemical relationship of the substituents on the cyclohexane ring was determined by comparing the splitting patterns and coupling constants of 1-H, 3-H and 4-H. 1-H (dddd, J=11.6, 11.6, 3.5 and 3.5 Hz) and 4-H (ddd, J=9.4, 6.4 and 2.9 Hz) were axial protons, while 3-H (dm, J=4.3 Hz) was equatorially oriented. Therefore, the carboxy and hydroxyl groups at C-1 and C-4 are equatorially oriented and the acyloxy group at C-3 is axially positioned on the chair-formed cyclohexane ring.

Fig. 4. ^{1}H NMR spectra of ENX IIa (**1**) and its methyl ester (**2**).

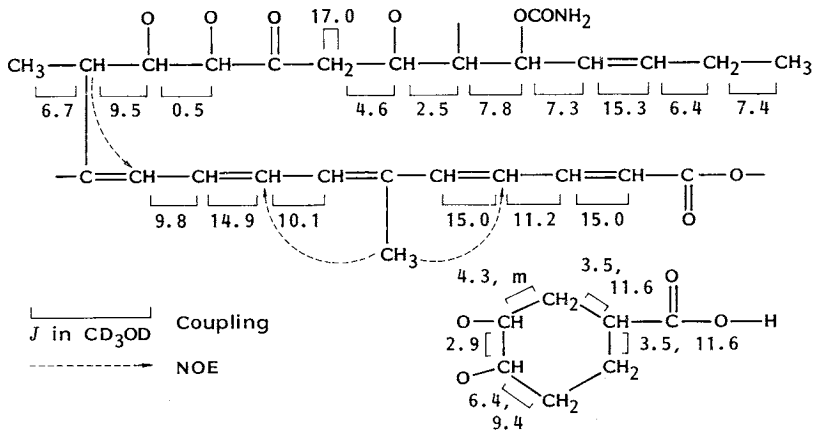
A: ENX IIa (**1**) (in CD_3OD, 500 MHz); B: ENX IIa methyl ester (**2**) (in $CDCl_3$, 400 MHz), C: expansion of olefinic region of **2** (in C_6D_6, 400 MHz).



The geometries of double bonds were determined from the ^{1}H NMR spectral analysis of **2** obtained in C_6D_6 solution. Most of olefinic proton signals were observed separately under the influence of magnetic anisotropy of the benzene ring and their splitting patterns could be easily analyzed as shown in Fig. 4. The *trans*-geometries of all olefinic units were unambiguously established by comparison of the coupling constants of the olefinic protons and the observation of NOE between 12′-H and 10′-H, 24′-H and 8′-H, and 24′-H and 4′-H.

In conclusion, the chemical structure of the novel antibiotic, enacyloxin IIa was determined to be

Fig. 5. Partial structures of ENX IIa.



(2′*E*,4′*E*,6′*E*,8′*E*,10′*Z*,20′*E*)-3-(19′-carbamoyloxy-11′,18′-dichloro-6′,12′-dimethyl-15′-oxo-13′,14′,17′-trihydroxytricosa-2′,4′,6′,8′,10′,20′-hexaenoyloxy)-4-hydroxy-1-cyclohexanecarboxylic acid (Fig. 1). Structure **1** is unique among antibiotics[11]. Free 3,4-dihydroxycyclohexanecarboxylic acid has been isolated as one of the metabolites of the genus *Lactobacillus*[12,13], but this derivative with a multi-functionalized acyl group is the first example among the known antibiotics.

As mentioned previously[1], the bacterium *Frateuria* sp. W-315 produces, together with enacyloxin IIa, several structurally related congeners depending upon the cultural conditions. Compound **1** may be produced through the quinoprotein-mediated dehydrogenative process in the culture medium. Details of biosynthetic studies on ENXs will be published later.

Experimental

General Procedure

TLC was carried out on Silica gel 60 plates (Merck) or Silica gel 60 silanised plates (Merck). UV spectra were recorded using a Jasco Uvidec-510 spectrometer. IR spectra were recorded on Jasco IRA-1 and IR-810 spectrometers. Optical rotation was measured on a Jasco digital polarimeter DIP-4. ^1H and ^{13}C NMR spectra were recorded on Jeol GSX-270, GSX-400 and GSX-500 spectrometers. Mass spectra were obtained on a Jeol DX-303HF spectrometer using *m*-nitrobenzyl alcohol as a matrix for FAB-MS.

Enacyloxin IIa (1)

Production, extraction and purification of **1** were carried out by the methods described previously[1,2]. The compound is a yellow powder. $[\alpha]_D$ +29.1° (*c* 0.653, MeOH); Rf 0.25 (Silica gel 60, $CHCl_3$-MeOH-AcOH, 65:5:1), 0.20 (silanised Silica gel 60, MeCN-AcOH-H_2O, 40:0.4:60). IR ν_{max} (KBr) cm^{-1} 3400 (st, br), 2940 (st), 1710 (st), 1620 (sh), 1575 (sh), 1455, 1405 (br), 1380, 1340, 1310, 1280, 1250 (sh), 1210, 1150, 1135, 975 (sh). UV λ_{max} (MeOH-glycerol, 1:4) nm ($E^{1\%}_{1cm}$): (at room temperature) 268 (105), 365 (910), 383 (sh, 840); (in liquid nitrogen) 338 (610), 357 (935), 375 (1,254), 396 (1,130). Other physicochemical properties are described in the text.

Enacyloxin IIa Methyl Ester (2)

A small amount of enacyloxin IIa was dissolved in MeOH and treated with distilled diazomethane solution in ether. After concentration of the solution at room temperature, the residue was purified by preparative TLC on Silica gel 60 developed with CH_2Cl_2-EtOAc (2:1) to yield a yellow oil. FAB-MS (+NaCl) *m/z* 738 $(M+Na)^+$. HRFAB-MS (+NaCl) *m/z* 738.2386 ($(M+Na)^+$, calcd for $C_{34}H_{47}O_{11}NCl_2$-

Na=738.2412). ^{1}H NMR ($CDCl_3$) δ 2.64 (1H, dddd, J=11.0, 11.0, 3.9 and 3.9 Hz, 1-H), 1.77 (1H, ddd, J=16, 15 and 3.9 Hz, 2-H), 2.23 (1H, dm, J=16 Hz, 2-H), 5.27 (1H, m, 3-H), 3.79 (1H, ddd, J=10.7, 4.0 and 3.1 Hz, 4-H), 1.71 (1H, dddd, J=16.0, 15.0, 15.0 and 4.0 Hz, 5-H), 1.87 (m, 5-H), 1.55 (dddd, J=16.0, 15.0, 15.0 and 4.2 Hz, 6-H), 2.05 (1H, m, 6-H), 5.95 (1H, d, J=15.3 Hz, 2'-H), 7.38 (1H, dd, J=15.3 and 11.2 Hz, 3'-H), 6.40 (1H, dd, J=15.0 and 11.0 Hz, 4'-H), 6.67 (1H, d, J=15.0 Hz, 5'-H), 6.35 (1H, br d, J=10.7 Hz, 7'-H), 6.65 (1H, dd, J=14.7 and 10.7 Hz, 8'-H), 6.70 (1H, dd, J=14.7 and 10.4 Hz, 9'-H), 6.38 (1H, d, J=10.4 Hz, 10'-H), 2.93 (1H, dq, J=9.4 and 7.0 Hz, 12'-H), 4.11 (1H, br d, J=9.3 Hz, 13'-H), 4.27 (1H, br s, 14'-H), 2.72 (1H, dd, J=16.5 and 4.0 Hz, 16'-H), 3.09 (1H, dd, J=16.5 and 8.2 Hz, 16'-H), 4.47 (1H, ddd, J=8.1, 4.0 and 2.0 Hz, 17'-H), 3.86 (1H, dd, J=8.7 and 2.0 Hz, 18'-H), 5.30 (1H, dd, J=8.1 and 7.9 Hz, 19'-H), 5.52 (1H, ddt, J=15.0, 7.9 and 1.5 Hz, 20'-H), 5.93 (1H, dt, J=15.2 and 6.2 Hz, 21'-H), 2.11 (2H, qdd, J=7.5, 7.8 and 1.3 Hz, 22'-H), 1.01 (3H, t, J=7.5 Hz, 23'-H), 1.93 (3H, s, 24'-H), 1.25 (3H, d, J=7.0 Hz, 25'-H). ^{13}C NMR ($CDCl_3$) δ 37.4 (C-1), 175.5 (C-1-COO-), 28.5 (C-2), 71.8 (C-3), 70.1 (C-4), 28.5 (C-5), 26.2 (C-6), 167.2 (C-1'), 120.1 (C-2'), 145.7 (C-3'), 126.6 (C-4'), 145.5 (C-5'), 136.4 (C-6'), 135.7 (C-7'), 130.0 (C-8'), 130.9 (C-9'), 127.9 (C-10'), 138.4 (C-11'), 46.6 (C-12'), 72.8 (C-13'), 77.4 (C-14'), 209.2 (C-15'), 42.6 (C-16'), 65.6 (C-17'), 66.2 (C-18'), 75.1 (C-19'), 123.8 (C-20'), 139.5 (C-21'), 25.3 (C-22'), 13.0 (C-23'), 12.7 (C-24'), 15.7 (C-25'), 156.6 (C-19'-$OCONH_2$), 51.8 (C-1-$COOCH_3$).

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