Structure and Absolute Stereochemistry of Fusaproliferin, a Toxic Metabolite from *Fusarium proliferatum*

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Fusaproliferin is a toxic sesterterpene isolated from *Fusarium proliferatum*, a widespread pathogen of cereals. Its absolute configuration has been determined by single crystal X-ray diffraction analysis. Fusaproliferin is considered to be a sesterterpene with a new ring skeleton having four C═C double bonds and four chiral atoms. The configurations of the four chiral atoms C10, C14, C15, and C19 are (*R*), (*S*), (*R*), and (*S*), respectively. In the solid state the macrolide shows a concave hydrophobic surface and hydrophilic convex face. The absolute configuration of C14 and C15 is the same as that observed for retigeranic acid, consistent with fusaproliferin being formed via a sesterterpenic-type biosynthetic pathway.

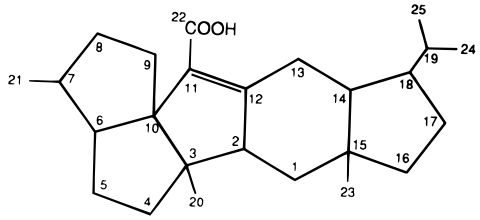
Our group has been involved in the screening of the toxic activity of fungi of the *Fusarium* species, using several bioassays. Recently we proposed, mainly using 2D-NMR methods,[1] the chemical structure and a possible stereochemistry for fusaproliferin (**1**), namely 18-[1-(acetoxymethyl)-2-methylethyl]-10,17-dihydroxy-3,7,11,15-tetramethylbicyclo[13.3.0]octadeca-*trans*,*trans*,*trans*,*cis*-2,6,11,17-tetraen-16-one.

Fusaproliferin was originally named proliferin.[1] The name was changed to fusaproliferin because the trivial name proliferin had already been used. It was isolated from a strain of *Fusarium proliferatum* (Hyphomycetes) collected from infected corn plants in northern Italy. This fungus is a member of the Liseola Woll section, which occurs worldwide as a pathogen on host plants.[2−5] *F. proliferatum* is known to produce toxins such as beauvericin, moniliformin, and fumonisins, whose toxic activities have been widely described.[6−8] Fusaproliferin is highly toxic toward *Artemia salina* L.[2] with an LD_{50} of 0.4 μg/mL, and additional cytotoxic activities have been observed.[9]

It is hypothesized that compound **1** is a sesterterpene[10] because it is a rather hydrophobic compound and apparently is derived from five isoprenic units. This hypothesis was strengthened by the observation that its carbon skeleton is similar to that of retigeranic acid (**2**).[11] The differences between the two compounds would originate from the lack of the final internal cyclization in the biosynthetic pathway of **1** leading to formation of a macrolide ring. In order to support this view, we wanted to determine the absolute stereochemistry of C14 and C15, atoms that are common to both molecules. The scaling up in production and purification procedures of **1**, herein described, allowed us to grow single crystals for a complete X-ray crystal structure analysis.

Results and Discussion

An X-ray analysis was performed to determine the absolute stereochemistry of the four chiral carbons and to compare the absolute structure of **1** with those of other biosynthetically related molecules.



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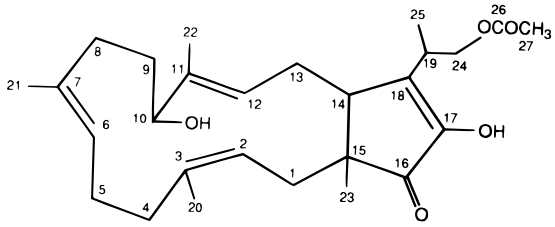

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The extraction procedure described in the Experimental section increased by 50% in weight the recovery of the toxin in the crude fraction. Moreover, the use of *n*-hexane gave a starting material enriched in the toxin, as shown by the TLC pattern. Hence, our standard isolation procedure[2] provides pure **1** in quantities suitable for crystallization.

^{1}H-NMR spectroscopy of **1** was obtained in $CDCl_3$. The ^{1}H-NMR spectrum of **1** showed some diagnostic peaks, such as the six methyl proton resonances between 0.99 and 2.02 ppm and the three olefinic protons at 5.12, 5.24, and 5.38 ppm, while the other signals were overlapped in the 1.8−2.4 ppm range. The proposed structure of **1** was determined from 2D experiments and particularly from the inv41plrnd sequence,[12] namely a 2D ^{1}H-^{13}C correlation in inverse detection that allowed elucidation of the structure of the smaller ring where only one H atom is present.

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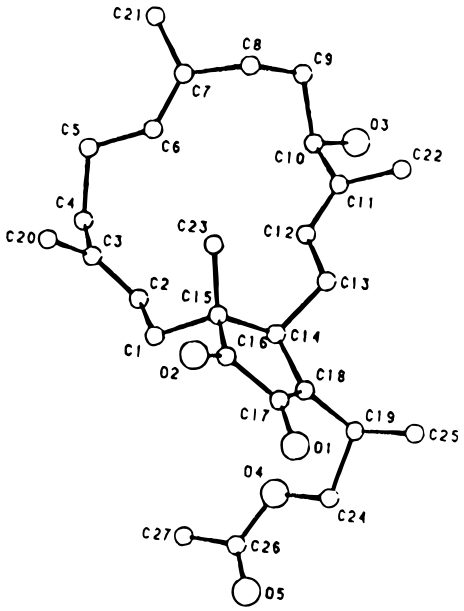

Figure 1. Molecular model of fusaproliferin as determined by X-ray diffraction analysis, together with the numbering scheme adopted for the atoms.

The quality of the X-ray diffraction data obtained, and consequently of the structural parameters, is reflected in the bond distances and bond angles, which show values in excellent agreement with similar literature data for single and double bonds involving carbon and oxygen atoms. The molecular structure of **1**, drawn using the PLUTO[13] computer program, is illustrated in Figure 1, the numbering of the atoms is indicated according to the isoprenoid biosynthetic rule.

Final atomic parameters and equivalent thermal factors for atoms of the **1** molecule together with their standard deviations are reported in Table 1. An ORTEP[14] stereo drawing of **1** is shown in Figure 2.

The absolute configurations of the four chiral carbon atoms C10, C14, C15, and C19 in the molecule were determined by the statistical test carried out on the observed structure factors of the two possible enantiomorphous solutions of the crystal structure analysis. Because the molecule has four chiral carbon atoms the crystal structure determined represents only one of the two possible enantiomorphs. For one enantiomorph the final R and R_w values were 0.045 and 0.042, respectively. The second enantiomorph (with inverted coordinates and opposite configurations of the chiral atoms) was also refined, and in this case, the final R and R_w values were 0.048 and 0.046, respectively. According to the Hamilton test,[15] we could reject, at a significance level less than 0.005, the hypothesis that the absolute configuration of the four chiral centers is that corresponding to the second enantiomorph. In the final difference Fourier synthesis for the correct enantiomorph, the maximum and minimum electron densities were 0.347 and −0.350 e/Å^3, respectively. The configurations determined for the C10, C14, C15, and C19 carbon atoms are established as *R*, *S*, *R*, and *S*, respectively. These results confirm the hypothesis proposed on the basis of molecular dynamics calculations and partially by 2D-NMR data. The configurations of the three C═C double bonds of the macrocyclic

Table 1. Final Positional and Equivalent Isotropic Thermal Parameters for Fusaproliferin (**1**) (esd's in parentheses)

position	*x*/a	*y*/b	*z*/c	*B*(eq)[a]
O1	0.6327(3)	0.4150(0)	0.4881(2)	4.67(7)
O2	0.7986(2)	0.2047(3)	0.5615(2)	4.92(7)
O3	0.6775(3)	0.3745(3)	1.2757(2)	4.94(7)
O4	0.3090(2)	0.3432(3)	0.6124(2)	5.00(7)
O5	0.1919(4)	0.3221(5)	0.4367(2)	9.07(13)
C1	0.6363(4)	0.0898(4)	0.7271(3)	4.09(9)
C2	0.6137(4)	0.0353(4)	0.8384(3)	3.77(8)
C3	0.6719(4)	−0.0662(4)	0.8894(3)	3.71(8)
C4	0.6440(4)	−0.1071(4)	1.0031(3)	4.34(9)
C5	0.7713(4)	−0.1132(4)	1.0972(3)	4.72(10)
C6	0.8369(4)	0.0117(4)	1.1162(3)	4.57(10)
C7	0.9162(4)	0.0536(4)	1.2109(3)	4.01(9)
C8	0.9669(4)	0.1840(4)	1.2155(3)	4.36(10)
C9	0.8851(4)	0.2725(4)	1.2755(3)	4.58(10)
C10	0.7481(4)	0.3059(4)	1.2037(2)	3.91(9)
C11	0.7559(3)	0.3778(4)	1.0975(2)	3.57(8)
C12	0.7025(3)	0.3329(4)	0.9962(2)	3.64(8)
C13	0.6962(4)	0.3963(4)	0.8828(2)	3.81(9)
C14	0.6290(3)	0.3173(4)	0.7810(2)	3.42(8)
C15	0.7157(3)	0.2130(4)	0.7419(2)	3.42(8)
C16	0.7314(3)	0.2545(4)	0.6230(3)	3.62(8)
C17	0.6480(3)	0.3629(4)	0.5925(2)	3.61(8)
C18	0.5865(3)	0.3945(4)	0.6759(2)	3.42(8)
C19	0.4804(4)	0.4937(4)	0.6700(3)	4.21(9)
C20	0.7702(5)	−0.1418(5)	0.8418(3)	6.01(12)
C21	0.9628(5)	−0.0223(5)	1.3157(4)	5.62(12)
C22	0.8182(5)	0.5029(5)	1.1106(3)	6.45(13)
C23	0.8569(4)	0.1927(4)	0.8142(3)	4.42(10)
C24	0.3596(4)	0.4590(5)	0.5786(3)	5.13(11)
C25	0.5318(5)	0.6208(5)	0.6437(4)	6.27(14)
C26	0.2226(4)	0.2828(5)	0.5323(3)	5.47(12)
C27	0.1723(6)	0.1688(5)	0.5769(5)	7.07(16)

[a] Anisotropic thermal factors for refined atoms are given in the form of the isotropic equivalent displacement parameter defined as: $B(\text{eq}) = 4/3[a^2B(1,1) + b^2B(2,2) + c^2B(3,3) + ab\cos(\gamma)B(1,2) + ac\cos(\beta)B(1,3) + bc\cos(\alpha)B(2,3)]$.

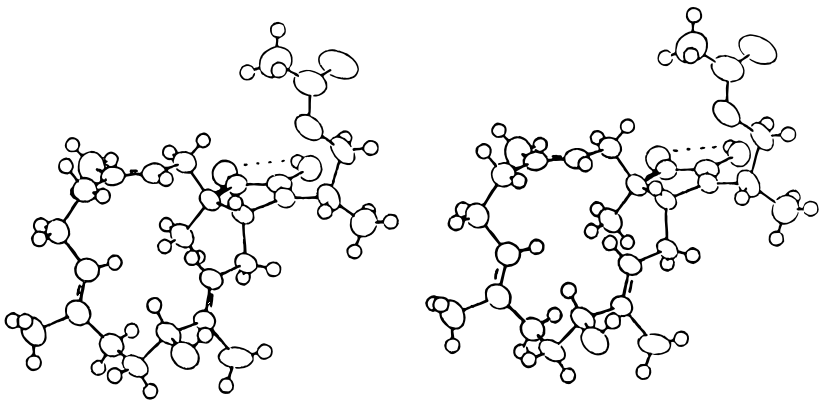

Figure 2. Stereo drawing of the fusaproliferin molecule. The larger circles refer to oxygen atoms; the smaller circles, to the hydrogen atoms. The intramolecular H-bond interaction is indicated as a dashed line.

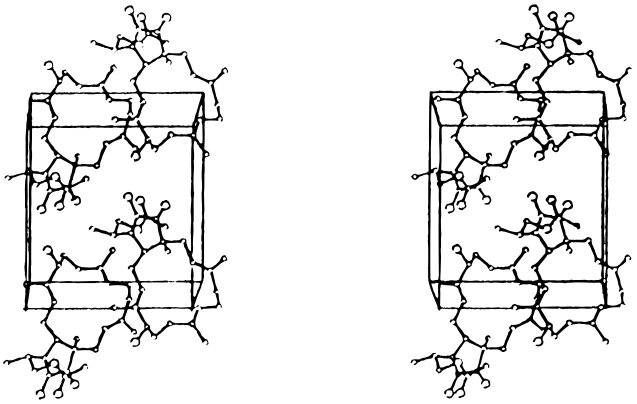

Figure 3. Stereo drawing of the mode of packing of the fusaproliferin molecules along the *c* axis.

ring, namely C2═C3, C6═C7, and C11═C12 are all *E*. This result, in contrast to that proposed in an earlier solution study[1] where a *Z* configuration for the C11═C12 double bond was claimed, confirms the molecular dynamics results[16] and is fully compatible with an isoprenoid biosynthetic pathway. The planes of two double bonds, C6═C7 and C11═C12, together with their substituents are nearly co-planar (the angle between the two average planes is 18°). Furthermore, these planes run roughly parallel to the average plane of the 15-membered macrolide.

However, both of these planes are nearly orthogonal to that involving the third double bond (C2═C3), forming with it angles of 86° and 79°. The fused five-membered cyclopentanone ring is at an angle of 54° from the average plane of the macrolide ring. The 15-membered macrolide ring is quite puckered. The four methyl substituents on C3, C7, C11, and C15 protrude from the slightly concave face of the ring system on the same side, thus forming, together with the C-H and CH_2 groups, a rather hydrophobic environment. One methyl group, C23, points directly inside the ring system, while the other three point outside the ring.

Conversely, the hydroxyl group O3-H protrudes from the convex face of the macrolide ring. The fused cyclopentanone ring is nearly equatorial with respect to the average plane of the sesterterpene ring. This side of the molecule is much more hydrophylic because of the presence of the C16═O2 carbonyl, the O1-H hydroxyl, and the O4−C26═O5 ester groups. The presence of two sides of different polarity in the molecule may explain the "detergent like" effect that **1** has on some biological membranes (unpublished results).

In the macrocyclic ring the trans (180°), gauche+ (+60°), gauche− (−60°), skew+ (+120°), and skew− (−120°) conformations occur five, one, four, three, and two times, respectively. The O1-H hydroxyl group is intramolecularly H-bonded to the carbonyl C16═O2 group (O1···O2 distance 2.88 Å), while the O3-H group is intermolecularly H-bonded to the O1 atom of a molecule translated along the *c* axis (O3···O1 distance 2.70 Å). This latter interaction stabilizes the structure in the solid state, together with dipole−dipole interactions between polar groups and van der Waals forces between hydrophobic moieties, as shown in the stereo drawing of the mode of packing illustrated in Figure 3.

Finally, X-ray-data analysis shows that the stereochemistry of the common chiral carbon atoms, namely C14 and C15, is the same in both **1** and retigeranic acid.[11,17] This molecular feature strongly suggests that: (a) the two molecules derive from the common precursor geranyl farnesol; (b) the first cyclization step could be common to both molecules; (c) the differences in the carbon skeleton (i.e., five rings for retigeranic acid and two rings for **1**) could be due to the lack in the biosynthesis of **1** of the successive cyclization steps (Figure 1); (d) the structure of both molecules is fully compatible with the biosynthetic pathway proposed by Kaneda for retigeranic acid.[11]

It is also worth noting that the absolute configuration of C19 in **1** is the same as that reported for the fungal toxin fusicoccin.[18] The chirality of this carbon atom, which is present in the identical lateral chain of both compounds, has its biosynthetic origin from an hydroxylation of one of the two methyls of an isopropyl group.

Experimental Section

Production. *F. Proliferatum* was isolated from ear-rot-infected corn in northern Italy and the strain (no. 1494) deposited at the collection of the Istituto Tossine e Micotossine da parassiti vegetali (ITEM), Bari, Italy. Fusaproliferin was obtained by inoculation of autoclaved yellow corn kernels with *F. proliferatum*.

Isolation. Purification of **1** was performed as follows: 300 g of lyophylized culture filtrate was resuspended in 1.3 L of MeOH−1% aq NaCl solution (55/45 v/v). Fusaproliferin was extracted from the aqueous residue with 3.250 L of *n*-hexane and concentrated under reduced pressure. Successive steps of the purification were carried out as previously reported.[2] The crystalline product, $[\alpha]^{25}_D$ −35° (*c*0.7, $CHCl_3$), had a T_m = 103 °C with a ΔH_m = 61.1 J/g as obtained by differential scanning calorimetry using a Perkin-Elmer DSC7 apparatus. Samples were examined under a dry N_2 flow with a temperature scanning rate of 10 K/min. The transition temperature reported was measured at the onset of DSC endotherm.

Brine Shrimp Lethality. The brine shrimp lethality assay was performed as described by Bottalico et al.[19] yielding an LD_{50} = 0.4 μg/mL.

NMR Analysis. Crystalline **1** (1 mg) was dissolved in $CDCl_3$, and proton NMR spectra were run on a Bruker AMX600 spectrometer operating at 600.13 MHz. ^{1}H-NMR ($CDCl_3$, 600.13 MHz): δ 1.70 (1H, s, J = 6.6, 13.6 Hz, H-1′), 2.38 (1H, m, J = 10.6, 13.6 Hz, H-1″), 5.24 (1H, m, H-2), 2.30 (1H, m, H-4′), 2.01 (1H, m, H-4″), 2.30 (1H, m, J = 8.8 Hz, H-5′), 2.13 (1H, m, J = 4.5 Hz, H-5″), 5.12 (1H, m, H-6), 2.11 (1H, m, H-8′), 1.78 (1H, m, H-8″), 1.77 (1H, m, J = 4 Hz, H-9′), 1.68 (1H, m, J = 10 Hz, H-9″), 4.05 (1H, dd, H-10), 5.38 (1H, m, H-12), 2.40 (1H, m, J = 3, 17, 2.5 Hz, H-13′), 1.92 (1H, m, J = 6−7, 17, 11.1 Hz, H-13″), 2.67 (1H, dd, J = 11,1 Hz, H-14), 2.78 (1H, m, H-19), 1.64 (3H, s, H-20), 1.64 (3H, s, H-21), 1.56 (3H, s, H-22), 0.99 (3H, s, H-23), 4.28 (2×1H, m, J = 7.6, 10.6 Hz, H-24′), 4.25 (1H, m, J = 6.9 Hz, H-24″), 1.31 (3H, d, J = 2 Hz, H-25), 2.02 (3H, s, H-27), 5.56 (1H, s, OH-17). ^{13}C-NMR ($CDCl_3$, 150.92 MHz,): δ 207.86 (C-16), 170.91 (C-26), 147.27 (C-17), 146.71 (C-18), 138.20 (C-3), 136.54 (C-11), 132.93 (C-7), 128.89 (C-12), 124.31 (C-6), 121.38 (C-2), 76.51 (C-10), 66.43 (C-24), 49.56 (C-14), 49.01 (C-15), 40.33 (C-4), 39.14 (C-1), 34.93 (C-8), 33.71 (C-19), 29.72 (C-9), 28.72 (C-13), 23.83 (C-5), 16.19 (C-23), 15.55 (C-20), 15.32 (C-21), 14.52 (C-25), 10.38 (C-22).

Table 2. Crystal Data for Fusaproliferin (**1**)

molec formula	$C_{27}H_{40}O_5$
formula wt, D	444.6
crystal system	monoclinic
space group	$P2_1$
Z, molecules/unit cell	2
a, Å	10.118(4)
b, Å	10.908(3)
c, Å	11.970(4)
β, deg	101.01(2)
V, Å^3	1296.9
d (calcd), g/cm^3	1.138
radiation, Å	Cu Kα ($\lambda = 1.5418$)
independent reflections	2719
reflns used $I > 3.0\sigma(I)$	2169
R	0.045
R_w	0.042
esd of obsd unit wt	0.540

X-ray Diffraction. Colorless single crystals of **1** were obtained by slow evaporation from a MeOH solution. A crystal, 0.3, 0.2, 0.3 mm in size, was used for cell determination and intensity data collection ($\theta_{max} = 70°$) on a turbo-CAD4 Enraf Nonius automated diffractometer, using graphite-monochromated, Ni filtered, Cu Kα radiation ($\lambda = 1.541\,78$ Å). Crystallographic details are reported in Table 2. A total of 2169 reflections with $I \geq 3.0\sigma(I)$ were classified as observed and used for structure determination and refinement. All intensities were corrected for Lorentz and polarization factors, but not for absorption ($\mu = 5.9$ cm^{-1} for Cu Kα radiation); also, no correction for extinction was applied.

The structure was solved by direct methods using the SIR92 package[20] and subsequent difference Fourier syntheses. Refinement of the structure was performed by a full matrix least-squares procedure minimizing the quantity $\Sigma w(F_o - F_c)^2$, with $w = 1/\sigma(F_o)^2$. At convergence H atoms were located on successive Fourier maps. All non-H atoms were refined anisotropically. Hydrogen atoms were introduced in the calculations with isotropic thermal factors equal to the Beq of the carrier atom, and their parameters were not refined. The scattering factors for all atomic species were calculated from Cromer and Waber.[21]

All calculations were performed on the VAX 3100 Digital Computer of the Biocrystallography Research Centre of the CNR at the Chemistry Department of the University of Naples "Federico II," using the SDP software package.[22]

Final atomic parameters and equivalent thermal factors for all atoms with their standard deviations are reported in Table 1.

Tables of bond lengths, bond angles, torsion angles, and hydrogen atom parameters have been deposited with the Cambridge Crystallographic Data Centre. Copies may be obtained from the Centre, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, United Kingdom.

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Supporting Information Available: Tables of final positional and equivalent isotropic thermal parameters of hydrogen atoms, bond distances and angles, and torsion angles for fusaproliferin (5 pages). Ordering information is given on any current masthead page.

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