COMMUNICATION TO THE EDITOR

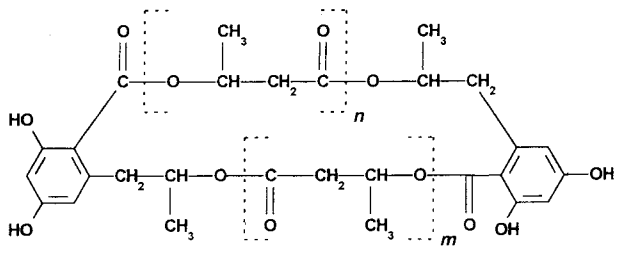
Orbuticin, a New Secondary Metabolite from *Acremonium butyri*†

Sir:

During the course of our screening for new biologically active secondary metabolites from fungi, a new cell-wall active compound named orbuticin was isolated from *Acremonium butyri* (HIL Y-87,1745). Herein, we report the fermentative production, isolation and structure elucidation of orbuticin[1].

The producing organism HIL Y-87,1745 was isolated from a soil collected from Kashet ghat, Alibagh, Maharashtra, India and was identified as *A. butyri* (van Beyma) Gams††. A few loopfuls of the sporulated culture from a slant maintained on SABOURAUD's dextrose agar were transferred to 500 ml wide mouth Erlenmeyer flasks containing 100 ml seed medium. The medium contained: soluble starch 15 g; soyabean meal 15 g; glucose 5 g; $CaCO_3$ 2 g; NaCl 5 g; yeast extract 2 g; corn steep liquor 1 g; $ZnSO_4 \cdot 7H_2O$ 0.22 mg; $CaCl_2$ 0.55 mg; $MnCl_2 \cdot 4H_2O$ 0.5 mg; $FeSO_4 \cdot 7H_2O$ 0.5 mg; $CuSO_4 \cdot 5H_2O$ 0.16 mg; $CoCl_2 \cdot 6H_2O$ 0.16 mg in 1000 ml demineralized water and the pH of the medium was adjusted to 6.5 before autoclaving for 20 minutes at 121°C. The seed culture was grown on a rotary shaker at 200 rpm for 48 hours at 26°C (±1°C). This was used as a seed culture when good growth was observed.

The production medium contained: Beef extract 3 g; tryptone 5 g; glucose 10 g; soluble starch 24 g; yeast extract 5 g; $CaCO_3$ 4 g; $ZnSO_4 \cdot 7H_2O$ 0.22 mg; $CaCl_2$ 0.55 mg; $MnCl_2 \cdot 4H_2O$ 0.5 mg; $FeSO_4 \cdot 7H_2O$ 0.5 mg; $CuSO_4 \cdot 5H_2O$ 0.16 mg; $CoCl_2 \cdot 6H_2O$ 0.16 mg in 1000 ml demineralized water and the pH of the medium was adjusted to 6.5 before autoclaving for 20 minutes at 121°C. The medium was distributed in 200 ml amounts in 1000 ml Erlenmeyer flasks and inoculated with 1% (v/v) of the seed culture. The fermentation was carried out on a rotary shaker at 200 rpm for 90 hours at 26°C (±1°C).



1 : $m = 0$; $n = 3$; **1'** : $m = 1$; $n = 2$

The production as well as the purification of orbuticin were monitored by the modified method[2] of KIRSCH and LAI using *Neurospora crassa* SGF 18 strain. Orbuticin gave turbid zone and an unidentified orange pigment at 37°C after 18~36 hours incubation of the strain when tested. Microscopic observation revealed enlarged and ballooned hyphae and sphaeroplast/protoplast like bodies in addition to some normal hyphae around the turbid zone.

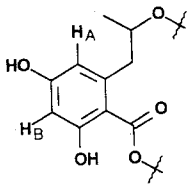
The culture broth (10 liters) was centrifuged and separated from the mycelium. The filtrate was passed through a column of Diaion HP-20 (1 liter). The column was successively washed with 10 liters each of demineralized water, 1 M aqueous NaCl solution and demineralized water. The column was then eluted with 50% acetone in water (7 liters), 75% acetone in water (4 liters) and acetone (3 liters). The active eluates containing orbuticin eluted out in 75% acetone in water and were combined, concentrated under reduced pressure and lyophilized to get crude orbuticin (200 mg). The mycelium was extracted with methanol (3 × 1 liter) and the methanol was removed under reduced pressure. The residual aqueous solution was diluted with water to 1 liter and chromatographed over a column of Diaion HP-20 (500 ml) in a manner similar to that used for the filtrate. The active eluates were combined, concentrated under reduced pressure and lyophilized to get crude orbuticin (250 mg). The combined crude orbuticin was subjected to medium pressure liquid chromatography (MPLC) on RP-18 (40~65 μ). The column was eluted with a 5% step-wise gradient of acetonitrile in 0.02 M sodium phosphate buffer (pH 7.0) at a flow rate of 25 ml/minute. The column eluates, collected in 50 ml fractions, were monitored by their activity against *N. crassa*. Orbuticin eluted out in 35% acetonitrile in sodium phosphate buffer. The active eluates were concentrated under reduced pressure and desalted by extraction with ethyl acetate (2 × 75 ml). The combined extracts were concentrated to dryness under reduced pressure to yield semi-pure orbuticin (50 mg).

Semi-pure orbuticin was finally purified by preparative HPLC on a 16 mm × (30 + 120) mm ODS-Hypersil column (10 μm) using 50% CH_3CN in 0.02 M sodium phosphate buffer, pH 7.0 at a flow rate of 2 ml/minute and detection at 220 nm. The fraction, containing orbuticin, was concentrated under reduced pressure and extracted with ethyl acetate (3 × 10 ml). The combined ethyl acetate extract was then concentrated under reduced pressure to get pure orbuticin (15 mg) as a colourless solid, MP 240°C (decomp), $[\alpha]_D$ −19.5° (*c* 0.04, MeOH). It was found to be soluble in MeOH, EtOAc and $CHCl_3$.

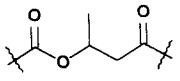
† Dedicated to Prof. E. NEGISHI of Purdue University, U.S.A. on the occassion of his 60th birthday.

†† The microorganism has been deposited as a reference with the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (DSM No. 6243)

The alkali induced UV bathochromic shift (298 nm to 302 nm) indicated phenolic OH groups. The IR frequencies at 3300 and 1720~1730 cm^{-1} revealed the presence of hydroxyls and carbonyls. The molecular formula of orbuticin was determined to be $C_{32}H_{38}O_{14}$ by HRFAB-MS [Found: 647.2332 $(M+H)^+$; Calcd. for $C_{32}H_{39}O_{14}$: 647.2335]. The ^{13}C chemical shifts along with a DEPT-135 spectrum showed the presence of $5\times CH_3$ [δ_C 19.72, 19.79, 19.98, 20.20 and 20.28], $5\times CH_2$ [δ_C 41.24, 41.64 ($\times 2$), 42.06 and 42.57], $5\times OCH$ [δ_C 69.24, 70.24, 70.33, 73.46 and 73.99], $4\times =CH$ [δ_C 102.87, 102.98, 112.87 and 113.44], $8\times =C$ [δ_C 106.70, 107.40, 143.09, 143.37, 163.58, 163.80, 165.17 and 165.65] and 5 carbonyls [δ_C 171.24, 171.38 ($\times 3$) and 171.54]. This accounted for $C_{32}H_{34}O_{10}$ to which four phenolic hydroxyl groups had to be added to make up the observed molecular formula. Orbuticin (**1**) afforded a poor quality ^1H NMR spectrum in $CDCl_3$ wherein the phenolic hydroxyls were non-observable. However, from a careful analysis of the 400 MHz ^1H NMR spectrum in CD_3OD and a phase-sensitive HH shift correlated COSY spectrum, two spin systems **2** and **3** could be extracted.



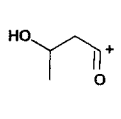
(2)



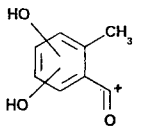
(3)

Four aromatic proton doublets at δ 6.20, 6.25 and 6.21, 6.23 each having a coupling constant of $\approx$2.5 Hz indicated two units of a modified orsellinic acid (**2**). The relatively upfield shifts of these protons excluded any isomer of **2** with $-CO_2-$ group in *ortho* orientation to these hydrogens. Additionally, the doublets at δ 6.25 and 6.23 exhibited a long-range coupling of $\approx$0.5 Hz to methylene protons having typically benzylic shifts. The ^1H NMR spectrum showed five CH multiplets appearing at δ 5.0 (2H), 5.25 (1H) and 5.5 (2H) indicating their α-acyloxy nature, five CH_2 multiplets (δ_H 3.46/2.76, 3.36/2.90, 2.84/2.68, 2.81/2.65, 2.60/2.56) and five CH_3 doublets with coupling constants of 6.5 Hz (δ_H 1.14, 1.17, 1.25, 1.40 and 1.41), thereby implying further that orbuticin had three units of **3**.

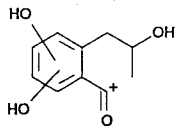
The connectivity pattern of **2** and **3** was established by the analysis of DCI-MS spectrum of orbuticin which showed diagnostic fragmentations. Base peaks at *m/z* 87, 151 and an intense peak at *m/z* 195 could be attributed to the structural units shown below.



m/z 87



m/z 151



m/z 195

Additionally, a peak of moderate intensity at *m/z* 173 could be assigned to a dimeric species $[HO-CH(CH_3)-CH_2C(O)OCH(CH_3)CH_2CO]^+$. The serial mass losses of 86 amu (probably a combined loss of two neutrals CH_3CHO and CH_2CO originating from substructure **3**) starting from two low intensity signals at *m/z* 453 and 409 could be interpreted in a manner leading to structure **1** for orbuticin. The ion spectrum could also be analyzed invoking remote-charge-site fragmentation[3] leading to an isomeric structure **1'**. Paucity of sample precluded further analysis.

Notwithstanding the structural ambiguity, orbuticin represents a hitherto unreported unusual cyclic polylactone formed by two modified orsellinic acid units and three β-hydroxybutyric acid residues. The absolute stereochemistry of the asymmetric centres was not established.

Orbuticin exhibited weak *in vivo* activity against a few phytopathogens such as *Phytophthora infestans*, *Pyricularia oryzae* and *Erysiphe graminis p.v. hordei*.

Acknowledgment

We thank Mr. M. S. PHANSALKAR and Mr. J. BHAMBANI for technical assistance. We acknowledge the support of Dr. P. K. INAMDAR for providing us some of the physico-chemical data. We are grateful to Dr. B. SACHSE, AgrEvo, Frankfurt, Germany for "*in vivo*" testing.

KIRITY ROY*
SUGATA CHATTERJEE*
S. K. DESHMUKH
E. K. S. VIJAYAKUMAR
B. N. GANGULI
H.-W. FEHLHABER†

Research Centre, Hoechst Marion Roussel Limited, Mulund (W), Mumbai - 400 080, India
†Pharma Synthese, Hoechst AG, Frankfurt M (80), Germany

(Received April 26, 1996)

References

1) KIRITY, R.; S. CHATTERJEE, B. N. GANGULI, J. BLUMBACH & H.-W. FEHLHABER: A process for the production of a new cell wall inhibitor named orbuticin from a fungal culture *Acremonium butyri* (van Beyma) Gams (Culture Number Hoechst India Limited Y-87,1745. Indian Patent No. 175429 dated January 6, 1994. European Patent Appl. No. 92121986.1 dated December 24, 1992
2) KIRSCH, D. R. & M. H. LAI: A modified screen for the detection of cell wall-acting antifungal compounds. J. Antibiotics 39: 1620~1622, 1986
3) TOMER, K. B. & M. L. GROSS: Fast atom bombardment and tandem mass spectrometry for structure determination: remote site fragmentation of steroid conjugates and bile salts. Biomed. Environ. Mass Spectrom. 15: 89~98, 1988