Biosynthesis of 1-N-methylalbonoursin by an Endophytic Streptomyces sp. Isolated from Perennial Ryegrass

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BIOSYNTHESIS OF 1-*N*-METHYLALBONOURSIN BY AN ENDOPHYTIC *STREPTOMYCES* SP. ISOLATED FROM PERENNIAL RYEGRASS

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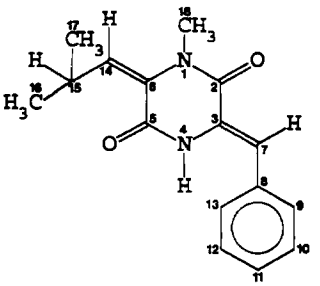
ABSTRACT.—A *Streptomyces* sp. has been isolated from perennial ryegrass seedling tissues from which it emerged in liquid culture after surface sterilization of seed. In submerged fermentation the *Streptomyces* produced 1-*N*-methylalbonoursin [**1**], a fluorescent and weakly antibiotic metabolite which was identified by ms and X-ray crystallography and further characterized by, uv, ^{1}H-, and ^{13}C-nmr spectroscopy. The biosynthesis of the diketopiperazine skeleton of compound **1** from leucine and phenylalanine was demonstrated. A close affinity of the *Streptomyces* sp. with *Streptomyces albus*, from which this metabolite was first isolated, is implied. The possibility that the *Streptomyces* sp. should be recognized as an endophyte of ryegrass is discussed.

During studies on *Acremonium* endophytes of perennial ryegrass (*Lolium perenne* L.) the standard isolation of endophytic fungi was from submerged seedlings grown from surface-sterilized seed, free from pericarp fungi. *Acremonium* endophyte in embryos characteristically emerges from the expanded plumule tissue of such seedlings. From seed of an Irish ecotype of *L. perenne*, filamentous colonies arose at discrete loci on the hypocotyl region and/or roots of seedlings. At first sight, these colonies resembled *Acremonium* both in form and slow rate of growth. Similarly, the organism slowly gave microcolonies on potato dextrose agar (PDA) used routinely for *Acremonium* and grew somewhat like these endophytes in submerged fermentation in shaken flasks. Submerged cultures produced a principal metabolite which fluoresced yellow at 350 nm. The data obtained from hrms indicated that the compound was not a known fungal metabolite. X-ray crystallography showed that this compound was 1-*N*-methylalbonoursin [**1**], fully characterized by this technique as an antibiotic metabolite of a streptomycete designated as *Streptomyces albus* (1,2). Reappraisal at the International Mycological Institute of the ryegrass isolate (IMI 351155) producing compound **1** showed that it conformed to *Streptomyces* Waksman Henrici and was similar to IMI 349029. The ready production by the *Streptomyces* sp. of an alkaloid of unusual structure for a prokaryote has facilitated study of its biosynthesis using ^{14}C-labelled putative precursors. Recognition, apparently for the first time, of an endophytic *Streptomyces* sp. from ryegrass has stimulated consideration of its biological significance.



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EXPERIMENTAL

ISOLATION OF *STREPTOMYCES* SP. FROM *LOLIUM PERENNE*.—Seeds of perennial ryegrass harvested from permanent pasture in County Kerry, Ireland in 1989 were surface-sterilized in 0.1% $HgCl_2$ for 5 min, washed in sterile distilled H_2O, and incubated on PDA plates to germinate and check for contamination. After 5 days, clean seedlings (coleoptile<1.0 cm) were transferred to tubes containing glucose (4%), peptone (1%), and yeast extract (0.5%) medium (GPYE) and examined at

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weekly intervals for the appearance of white/cream mycelium. After 2 months, mycelium was subcultured to PDA slopes or directly into submerged culture.

SUBMERGED CULTURE OF *STREPTOMYCES* SP.—Submerged cultures were inoculated with mycelium from a single seedling into 500-ml Erlenmeyer flasks containing 100 ml GPYE and incubated on a rotary shaker (200 rpm) at 23° for 1 week. This provided a finely-pelleted inoculum for experimental shaken cultures. Fermentations were monitored by sampling flasks in triplicate at intervals for up to 14 days. Samples were filtered under vacuum and the cells lyophilized.

ISOLATION OF 1-*N*-METHYLALBONOURSIN.—Lyophilized cells were extracted twice with 50 ml $CHCl_3$-MeOH (2:1). Filtrates were extracted twice with an equal volume of $CHCl_3$. Extracts were reduced to dryness in vacuo. Tlc on 0.25 mm Si gel plates, with fluorescent indicator UV_{254}, in $CHCl_3$–*n*-hexane–Me_2CO (10:10:1) revealed the principal compound (R_f 0.67) as yellow fluorescent under uv light at 350 nm. Hplc employing a normal phase Si gel column with an eluent of CH_2Cl_2-MeCN (9:1) at a flow rate of 1.5 ml/min resolved compound **1** (Rt 4.2 min), which was detected spectrophotometrically at 265 nm and also by fluorescence (excitation at 265 nm, emission at 450 nm).

CHARACTERIZATION OF 1-*N*-METHYLALBONOURSIN.—Ten flasks were harvested after 10 days, and compound **1**, isolated by preparative tlc and hplc (yield 14.2 mg), was subjected to ms, uv, and ^{1}H- and ^{13}C-nmr spectroscopy (including DEPT 135 and ^{1}H-^{13}C correlation experiments). X-ray analysis was performed on material crystallized from CH_2Cl_2 in an *n*-hexane atmosphere.

ANALYTICAL.—Compound **1** was quantified in 20-μl aliquots of fermentation extract, by hplc as above, with respect to a standard curve over the range 0.1–10 μg.

BIOSYNTHESIS.—Submerged *Streptomyces* cultures were given aliquots of [1-^{14}C]acetate (57 mCi/mmol), *RS*-[2-^{14}C]mevalonic acid (54 mCi/mmol), [U-^{14}C]glycine (56 mCi/mmol), L-[U-^{14}C]leucine (342 mCi/mmol), L-[U-^{14}C]phenylalanine (475 mCi/mmol), and [^{14}C-carboxyl]-anthranilic acid (10.5 mCi/mmol) on days 4 and 5 of the fermentations, corresponding to the early linear phase of the accumulation of compound **1**. A total of 10 μCi of putative precursor was given, except for leucine (0.4 μCi). Cultures were harvested after 10 days and the alkaloid extracts prepared as above. Chromatograms were autoradiographed for up to 1 month to reveal radiolabel. Extracts were also analyzed by hplc and eluate corresponding to the compound **1** peak collected; percentage incorporation of radiolabel was determined by scintillation counting.

RESULTS AND DISCUSSION

CHARACTERIZATION OF COMPOUND **1**.—The principal idiolyte in submerged fermentations of the *Streptomyces* sp. was shown to be compound **1** by comparison of our analyses with reported data (1,2). Found $[M]^+$ 270.1363 ($C_{16}H_{18}N_2O_2$ requires 270.1368); uv max (MeOH) 315 nm, (ε 29150); ^{1}H nmr (500 MHz, $CDCl_3$) δ 1.06 (6H, d, Me-16, -17), 3.26 (3H, s, NMe), 3.74 (1H, m, H-15), 5.60 (1H, d, H-14), 6.85 (1H, s, H-7), 7.32 (1H, t, H-11), 7.43 (2H, t, H-10, -12), 7.52 (2H, t, H-9, -13), 8.95 (1H, s, NH). The absolute configuration as 3*Z*,6*E* was confirmed by X-ray crystallography. In addition, new ^{13}C-nmr (125 MHz, Me_2CO-d_6) spectroscopic data was consistent with this structure: δ 23.3 (2×Me, C-16, -17), 27.3 (CH, C-15), 31.0 (NCH_3, C-18), 115.4 (CH, C-7), 127.3, 129.5 (2×q, C-3, C-6 but may be interchanged), 128.9 (aryl CH, C-11), 129.7 (aryl CH, C-9, -13), 129.8 (aryl CH, C-10, -12), 133.8 (CH, C-14), 134.8 (q, C-8), 158.3, 158.6 (2×C=O, C-2, C-5 but may be interchanged). The DEPT 135 experiment showed that there were no CH_2 signals in the spectrum, and unequivocal assignments were readily apparent from the ^{1}H-^{13}C correlation spectrum.

Compound **1** is unique among naturally occurring 2,5-piperazinediones in possessing the 3*Z*,6*E* configuration, in contrast to albonoursin (3) and (3*Z*,6*Z*)-dibenzylidene-2,5-piperazinedione, an analogue containing two phenylmethylene moieties (4). These metabolites, and the related compound neihumicin produced by *Micromonospora neihuensis* (5), possess the 3*Z*,6*Z* configuration and are variously antimicrobial or cytotoxic (4–6). Compound **1**, although of different configuration, is weakly active against *Staphylococcus aureus* (confirmed during the present study) and dermatophytes (2).

FERMENTATION KINETICS.—Biomass and dynamics of compound **1** in *Strepto-*

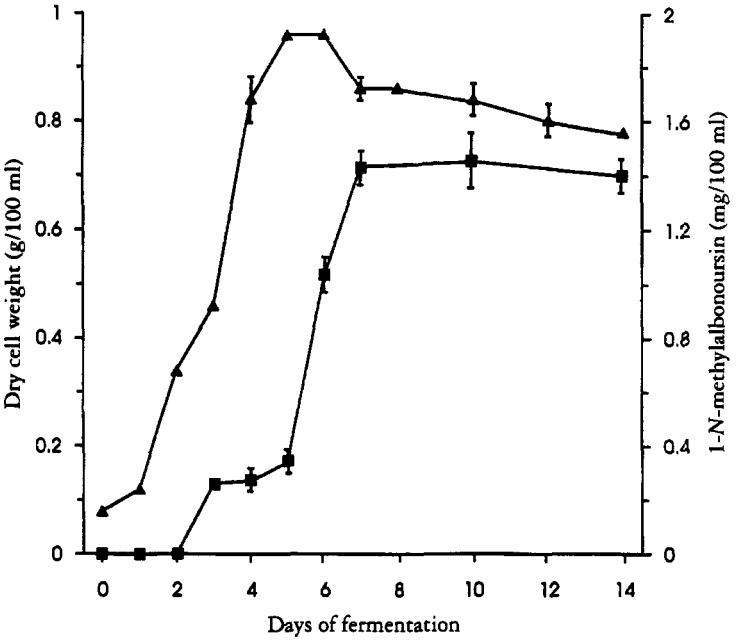

FIGURE 1. Progress of submerged fermentation of the *Streptomyces* sp. for the production of 1-*N*-methylalbonoursin [**1**]. Biomass (▲) and compound **1** (■) data are means ±SE (n=3).

myces submerged fermentation are shown in Figure 1. In this typical pattern it is evident that replicatory growth was complete before radiolabelled putative precursors were added on days 4 and 5. However, biosynthesis of compound **1** commenced during trophophase but occurred principally after growth. The maximum titer of alkaloid was sustained for at least 2 weeks. The total yield was typically 1.0–1.5 mg/100 ml, but the distribution between cells and filtrate varied widely in individual cultures (15:1 to 1:2) in otherwise apparently well-replicated flasks.

BIOSYNTHESIS.—The results of biosynthetic experiments (Table 1), augmented by autoradiography, showed compelling evidence for direct involvement of leucine and phenylalanine in forming the diketopiperazine of compound **1**, the incorporation values being orders of magnitude greater than that of another amino acid, glycine. The glycine value was attributed solely to metabolic scrambling. The case for mevalonate providing the C_5 unit (C-14–C-17) was exceptionally weak even when recalculated to account for less than half of the added ^{14}C-mevalonic acid being taken up. Anthranilic acid was also poorly taken up, but the evidence of radiolabel in compound **1** was shown to be an artifact attributed to co-chromatography of the selected hplc peak with a known radiolabelled impurity in the anthranilic acid. No radiolabelled band corresponding with compound **1** was seen on the autoradiograph. Weak labelling by acetate, most evident from the autoradiograph, was consistent with its role in leucine biosynthesis. For completeness, the assumption must be that the *N*-methyl group of compound **1** is derived from *S*-adenosylmethionine.

Autoradiographs of extracts from cultures supplied with ^{14}C-phenylalanine showed another yellow fluorescent, prominently radiolabelled metabolite at R_f 0.34. This could be the dibenzylidene analogue of albonoursin described by Khoklov *et al.* (4), compounding the simi-

TABLE 1. Incorporation of ^{14}C from Putative Biosynthetic Precursors into 1-*N*-Methylalbonoursin [**1**] in Submerged Fermentation.[a]

Experimental precursor	^{14}C in culture filtrate (%)	^{14}C incorporation into 1-*N*-methylalbonoursin (%)
[1-^{14}C]acetate	4.7	0.002
RS-[2-^{14}C]mevalonic acid	60.0	0.0004
[U-^{14}C]glycine	13.2	0.002
L-[U-^{14}C]leucine	10.0	0.53
L-[U-^{14}C]phenylalanine	9.0	0.41
[^{14}C-carboxyl]anthranilic acid	60.1	0.027

[a]Culture (100 ml) was given radiolabelled precursor on days 4 and 5, total 10.0 μCi (0.4 μCi for leucine), and harvested after 10 days.

larities between this *Streptomyces* sp. and their *Str. albus*.

Concerning mechanism, it should be noted that the hypothesis concerning intermediacy of dehydroamino acids in the biosynthesis of dialkylidene-2,5-piperazinedione microbial metabolites has been extended to compound **1** (7).

BIOLOGICAL SIGNIFICANCE.—Although close association of streptomycetes with plants seemed limited to the nitrogen-fixing *Frankia* and certain phytopathogenic types, a recent report showed that *Streptomyces* spp. may be endophytic in a wide range of plants, including grasses (8). Streptomycetes are noted for their propensity to produce antimicrobial compounds. Compound **1** possesses weak in vitro antibiotic activity, but organisms from which it has been isolated [*Str. albus* and *Streptomyces noursei* (3,4)] also produce potent antimicrobials such as nystatin and cycloheximide. However, preliminary studies on agar media have not revealed any growth promotory or antagonistic interactions between the *Streptomyces* sp. and an *Acremonium* from *L. perenne*.

The isolation of an endophytic *Streptomyces* sp. from perennial ryegrass has not been reported previously, although two fungal endophytes, *Acremonium lolii* and a *Gliocladium*-like species, are commonly recognized (9). The *Streptomyces* sp. was isolated on several separate occasions from seeds surface-sterilized by $HgCl_2$. This treatment, as expected, was lethal to the streptomycete, which therefore could not have been superficial. Endophytic hyphae have been observed in embryos of seed (M. do Valle Ribeiro, personal communication) similar to that used in the present study (about 20% infection) and in leaf sheath tissue, and noted as being even narrower than *Acremonium* and staining poorly with aniline blue. It is an open question as to whether this was an observation of streptomycete mycelia. The occurrence of *Streptomyces* spp. in perennial ryegrass may have been overlooked, as isolation media for *Acremonium* typically employ anti-prokaryote antibiotics to reduce contamination. Additionally, the shyness of the *Streptomyces* sp. in growing on agar media suggests that isolation from plant tissue on agar would be precluded. Since in the present study the *Streptomyces* sp. was isolated also from seeds stored cool or frozen, the robust association and persistence, coupled with a potential for antimicrobial biosynthesis, may be of agricultural significance.

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