Munumbicins E-4 and E-5: novel broad-spectrum antibiotics from *Streptomyces* NRRL 3052

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**Abstract**

*Streptomyces* NRRL 3052 was originally isolated as an endophyte from *Kennedia nigriscans*, snakevine, in the Northern Territory of Australia. This plant has been used for centuries by Aboriginal peoples to treat open bleeding wounds to prevent sepsis. A solvent extract of the crude fluid from cultures of this endophyte possesses wide-spectrum antibiotic activity. Some of the bioactivity is associated with the appearance of actinomycins X2, D, and Xoβ, the first two of which had been previously designated munumbicins A and B, respectively. Other novel compounds bearing wide-spectrum antibiotic activity are also produced by *Streptomyces* NRRL 3052, and these are designated munumbicins E-4 and E-5. Mass spectrometric analyses of these peptide antibiotics show that they have identical masses (1445.00) but different retention times on HPLC. Both compounds showed activity against gram-positive and gram-negative bacteria. The plant pathogenic fungus, *Pythium ultimum* is sensitive to both munumbicins at 5.0 µg mL⁻¹. The malarial parasite, *Plasmodium falciparum* has IC₅₀ values of 0.50 ± 0.08 and 0.87 ± 0.026 µg mL⁻¹ for E-4 and E-5, respectively. It appears that other bioactive compounds, related to E-4 and E-5, are also produced making it the most biologically active endophytic *Streptomyces* spp. on record.

**Introduction**

The majority of the world’s antibiotics have been obtained from soil-borne *Streptomyces* spp. (Waksman, 1967; Arai, 1976). It is now obvious that these filamentous bacteria can be found as endophytes within the living tissues of certain higher plants (Castillo et al., 2002; Kunoh, 2002). It has been suggested that this novel source of *Streptomyces* spp. may result in bioactive compounds previously not isolated from soil microbes (Strobel & Daisy, 2003; Strobel, 2003). A search for plants hosting such endophytic streptomycetes has now begun (Castillo et al., 2002; Kunoh, 2002). One of the methods used to narrow the search is to take an ethnobotanical approach, that is, to utilize the knowledge of native people who have relied on plants as medicines for centuries or even millennia. Thus, it may be possible that the bioactive compounds within the plant may not be the product of the plant, but products of the endophytes that live therein.

The longest held continuous human culture is the Aboriginal Australians. Several years ago a tribal leader, Reggie Munumbie, had guided us to the snakevine plant (*Kennedia nigriscans*), as a medicinal source that they use to treat open, bleeding wounds to prevent sepsis. From this plant was isolated *Streptomyces* NRRL 3052 (Castillo et al., 2002). This endophyte produces a series of wide-spectrum antibiotics initially termed the munumbicins after this tribal leader. Recently, at least 39 different *Streptomyces* spp. have been obtained from several snakevine plants collected in various places in the Northern Territory, Australia (Castillo et al., 2005). This discovery has demonstrated that the world’s rainforests are a novel source of endophytic streptomycetes (U. Castillo and G. Strobel, unpublished). In addition, many of the snakevine endophytic streptomycetes possess antimicrobial activity (Castillo et al., 2005). This result makes even more reasonable the prospect that the intended Aboriginal uses of this plant for the treatment of skin lesions may be the
direct result of the antibiotics present in the tissues having been produced by endophytic streptomycetes.

Originally, we proposed that the munumbicins A, B, C, and D were unique antibiotics with wide-spectrum activities (Castillo et al., 2002). Most recently we have learned that some of munumbicins are identical to some of the better-known antibiotics, the actinomycins. Now, this report describes the isolation of several novel antibiotics from Streptomyces NRRL 30562 with wide-spectrum biological activity that are termed munumbicin E-4 and E-5.

Materials and methods

Streptomyces NRRL 30562, product isolation, and chromatographic methods

This bacterium was originally obtained as an endophyte from a snakevine plant located near the Manyallaluk Aboriginal village in the Northern Territory of Australia using standard techniques (Castillo et al., 2002). The organism was grown for two weeks in a 10 L fermenter with stirring at 150 rpm, pH adjusted to 7.0 and a temperature of 27 °C. The initial steps in antibiotic isolation were as previously described (Castillo et al., 2002). The conditions for HPLC separation and product isolation employed a Microsorb 60–8, 250 × 10 mm Dynamax C-18 column (Varian Inc., Palo Alto, CA) held at 25 °C. The elution solvent was 20% tetrahydrofuran (THF)/80% water programmed for 70 min to a final concentration of 60% THF/40% water and then held isocratically for 135 min. The elution profile from the HPLC column was comparable to that previously described (Castillo et al., 2002). The last significant peak to emerge from the column was designated munumbicin A (c. 130 min) while the second to last peak to emerge was designated munumbicin B (c. 82–92 min) (Castillo et al., 2002). Two other peaks, possessing biological activity and appearing earlier in the elution sequence, before munumbicins C and D, at 55 and 60 min (munumbicins E-4 and E-5) were individually subjected to further purification on a Jupiter 4 μm 90’A column 250 × 4.60 mm (Phenomenex, Torrence, CA) held at 30 °C. Fractions were collected at 1 mL min⁻¹ beginning with 20% acetonitrile and 80% water at 0 time then programmed up to 60:40 volume in volume (v/v) up to 70 min and finishing with 100% acetonitrile at 90 min.

For thin-layer chromatographic analysis (TLC) the samples were placed on Merck TLC plates and subjected to separation in Solvent A: chloroform, acetonitrile, methanol 10:1.5:1 v/v and Solvent B: n-butanol, acetic acid, water 3:1:1 v/v.

Spectroscopic analyses

Liquid chromatography/mass spectroscopy (LC/MS) data were acquired on munumbicins E-4 and E-5 on a Bruker Esquire 3000 system (Bruker, Karlsruhe, Germany) with a flow rate of 5 μL min⁻¹ (Castillo et al., 2003). For independent confirmation, mass spectral data were also obtained on a Bruker Biflex matrix assisted laser desorption ionization (MALDI) mass spectrometer under the conditions previously described (Castillo et al., 2002). For NMR analyses, the samples of actinomycins (munumbicins A and B) were dissolved in 99.95% deuterated acetonitrile and data obtained in a Bruker 600 MHz instrument at 300 K. Samples of actinomycins (munumbicins A and B) were dissolved in both 99.95% deuterated acetonitrile and 99.95% deuterated methanol. The munumbicins E-4 and E-5 were dissolved in 99.95% deuterated methanol.

Amino acid analysis

Samples for amino acid analysis were dissolved in 50% methanol–water and subjected to hydrolysis and analysis, essentially as described (Castillo et al., 2002, 2003). Briefly, the released amino acids were derivatized with 6-aminoquinolyl-N-hydroxysuccinimyl carbamate (AQC) and their identities and amounts determined relative to standard amino acids using reverse phase HPLC on an Alliance / Millenium HPLC system (Waters, Milford, MA) equipped with an AccQ-Tag amino acid analysis column. The AccQ-Tag chemistry and subsequent chromatography were performed according to manufacturer’s instructions.

Minimum inhibitory concentrations and antimalarial assays

Microbroth dilution assays of human associated bacteria were performed as described in the NCCLS. Unless otherwise noted, the majority of the bacteria isolates tested were obtained from the ATCC. The assays were performed in sterile 96-well plates, and the total volume per well was 100 μL. The bacterial inoculum was prepared to give c. 10¹⁻¹⁰⁵ CFUs per well and the compounds were tested at concentrations from 0.0625 to 64 μg mL⁻¹ in two-fold step dilution. The actual CFUs per well was confirmed by plating onto Mueller–Hinton or blood agar. Two wells were inoculated for a given concentration. The plates were incubated for 16–20 h at 35 °C unless otherwise specified with or without 5% CO₂. The minimum inhibitory concentration (MIC) was defined as the minimum concentration of compound resulting in no visible growth of the test organism. The MICs were determined by visually observing the plates following incubation.

The antimalarial assay was performed according to established protocols and procedures (Castillo et al., 2002). Details of the use of labelled phenylalanine and the Giemsa staining procedures have been summarized in a recent publication (Castillo et al., 2002). Data are reported as IC₅₀ values.
Results and discussion

Isolation and characterization of the munumbicins A and B

Munumbicins A and B were prepared as outlined and then subjected to accurate nuclear magnetic resonance (NMR) spectroscopic analysis based on one-dimensional and two-dimensional experiments (Braun et al., 1998). In addition, these compounds were subjected to amino acid, comparative high-performance liquid chromatography (HPLC), TLC and UV analyses. The results unequivocally demonstrate that these two compounds are identical to actinomycin X₂ and actinomycin D, respectively, and are not novel antibiotics as previously implied (Castillo et al., 2002). Furthermore, using column chromatographic (silica gel) and HPLC methods, actinomycin X₀β was also isolated from the culture fluid of this endophytic streptomycete (U. Castillo et al. unpublished data). Its amino acid composition was identical to that reported (Chapman & Hall Dictionary of Natural products on CD ROM). It also appears that munumbicin C with a retention time of 77 min on the Dynamax-Microsorb HPLC column is also another known actinomycin having a mass of 1269 (Castillo et al., 2002).

These results have broad implications in tropical herbal medicine as used by the Australian Aborigines and others. Now, for the first time, it is realized that the antibiotic activities in a medicinal plant extract may arise from one or more endophytic microbes existing within the plant. Furthermore, the scope of microbial bioactivity associated with this plant is broad given that many endophytic associations occur in Kennedia nigriscans (Castillo et al., 2005). It is beginning to seem obvious why the snakevine extract is so potent as a treatment for open wounds since it is a source of so many bioactive compounds.

The novel antibiotics munumbicins E-4 and E-5

The original HPLC separation of the extract of NRRL 3052 demonstrated two peaks at 55 min (munumbicin E-4) and 60 min (munumbicin E-5), respectively (Castillo et al., 2002). The ingredients of each of these peaks were subjected to final purification methods on an HPLC system that utilized a Jupiter column as described in the Materials and methods section. Munumbicin E-4 possessed a retention time of 47.9 min and munumbicin E-5 had a retention of 46.1 min. Compounds E-4 and E-5 both have a mass of 1445.00 Da as determined by MALDI-MS and LC/MS (Table 1). The LC/MS/MS revealed that the molecules are different since E-4 went to major ions other than those of E-5 and their TLC–R_f values were different (Table 1). Bioactive compounds with identical masses, but with different chemistries are not unusual, especially among microbes that make families of similar antibiotics. Interestingly, still other biologically active compounds are present in the culture fluid of this streptomycete.

Amino acid analysis and LC–MS of the derivatized free amino acids were done to further characterize the E-4 and E-5 compounds. Chromatographic analysis revealed that both compounds produced peaks consistent with the presence of Gly, Sar, Pro, Val, and N-methyl-Val. An additional peak eluting near the position of a Tyr standard was observed. Normalization of the derived mole percent data to Val produced similar compositions (Table 1). Amino acids also were identified using LC-MS of AQC-derivatives. Masses were observed consistent with those predicted for Sar, Pro, Val, and N-methyl-Val. A Gly signal was seen clearly in the E-5 sample, but not in the E4 sample. In addition, peaks with elution times and masses similar, but not identical, to those of AQC-Tyr were observed. Summation of the numbers of moles of each amino acid per mole of antibiotic reveals 10.6 for E-4 and 9.8 for E-5. The major difference (0.5 mole) was Gly. The LC–MS data suggest that the

| Table 1. Chemical characteristics and chromatographic properties of munumbicins E-4 and E-5 |
|------------------------------|------------------------------|
| **lc/ms molecular ion**      | Munumbicin E-4               | Munumbicin E-5               |
| 1445.00                      | 1445.00                      |
| Major ions in LC/MS/MS       | 1315 > 1270.9 > 819.0 > 775.6 > 662.5 | 1431.1 > 1251.0 > 1431.1 > 923.7 > 810.6 > 530.4 |
| Amino acid composition (pmoles) | Gly, 204 [1.2]; Sar, 373 [2.2]; Pro, 415 [2.5]; Val, 169 [1]; N-methyl-Val, 535 [3.2]; X₁, 86 [0.5] | \begin{align*} 
\text{Gly, 307 [0.7]; Sar, 926 [2.1]; Pro, 1127 [2.5]; Val, 450 [1]; N-methyl-Val, 1252 [2.8]; X₁, 317 [0.7]}
\end{align*} |
| LC–MS identification of AQC-derivatives | Sar, Pro, Val, N-methyl-Val, X₁ | Sar, Pro, Val, N-methyl-Val, X₁ |
| UV absorption               | 250 nm, ε = 4.68            | 250 nm, ε = 5.96            |
|                            | 280 nm, ε = 3.70 (shoulder) | 280 nm, ε = 3.70 (shoulder) |
|                            | 360 nm, ε = 3.46            | 360 nm, ε = 4.60            |
| Color of methanolic solution | Light yellow                | Light yellow                |
| R₁ in Solvent A             | 0.07                        | 0.09                        |
| R₁ in Solvent B             | 0.65                        | 0.66                        |

LC, liquid chromatography; MS, mass spectrometry.

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amount of Gly in E-4 determined by amino acid analysis is artefactually high and therefore it is not considered as a component amino acid of either E-4 or E-5. The rationale is that Gly is ubiquitous and this type of anomalous datum is not uncommon. Therefore, it appears that both E-4 and E-5 contain a minimum of 10–11 amino acid residues. Interestingly, N-methyl valine appears as two moles per molecule in every actinomycin that has been characterized (see Chapman & Hall). There appear to be three residues in each of the munumbicins examined here. Furthermore, considering the increased mass of E-4 and E-5 to 1445, it would appear that these compounds may contain two amino acid residues more than such compounds as actinomycin D (having 10) as do many of its related antibiotics.

The chromophore of E-4 and E-5 is not the same adduct as that on the actinomycins since the color is different (light yellow vs. intense reddish orange), the UV spectrum is notably different and the single sharp intense nuclear magnetic resonance at 8.5 ppm does not occur in actinomycin D or any of its related compounds (Castillo et al., 2002) (Fig. 1, Table 1). However, both the NMR and UV data (280 absorbance shoulder peak) strongly suggest the presence of aromaticity in E-4 and E-5 (Table 1; Fig. 1). This resonance, at 8.5 ppm, is most likely associated with the protons on one or two substituted benzene rings that are associated with the chromophores of both E-4 and E-5 (Pouchert & Campbell, 1974). Furthermore, E-4 and E-5 are not soluble in acetonitrile in contrast with the actinomycins that are soluble (Table 1). Presently, the exact structures of E-4 and E-5 are not known, but there is no question of their novelty given that the Chapman & Hall database shows no compounds listed with a mass of 1445. Both E-4 and E-5 can be considered as chromophoric peptides whose structures are uniquely different from the actinomycins. In turn, although these compounds share the same mass, they are different as shown by their unique amino acid compositions, their NMR spectra (especially in the regions of 2.8–3.2 and 7.4–8.6 ppm) the MS/MS of the parent molecules, and their TLC values (Table 1).

**Comparative biological activities of munumbicins E-4 and E-5**

Munumbicins E-4 and E-5 were tested on a comparative basis with other antibiotics. These compounds were effective in the same range of biological activity against some drug resistant *Staphylococcus aureus* isolates as the well-known drug vancomycin (Table 2). Both compounds were effective against gram-negative and gram-positive bacteria in this test (see *Escherichia coli* and *Bacillus subtilis*) and they were also effective against the plant pathogenic fungus *Pythium ultimum* at a low level, while not demonstrating any activity against *Rhizoctonia solani* at the concentrations tested (Table 2). When tested against *Plasmodium falciparum*, the malarial parasite, E-5 was more effective than E-4 as measured by the LD$_{50}$s, however both E-4 and E-5 were significantly less active than the actinomycins against this parasite (Castillo et al., 2002). Also, the munumbicins E-4, E-5, along with vancomycin were not effective against a known drug resistant emerging bacterial pathogen, *Burkholderia thailandensis*. The comparative bioactivities of actinomycins D and X$_2$ (munumbicins B and A) have been previously recorded against many of these target microbes and in contrast to munumbicins E-4 and E-5 show no activity against *E. coli* (Table 2) (Castillo et al., 2002). On the other hand, they are active against *Pythium ultimum* and *Plasmodium falciparum* (Castillo et al., 2002).

**Table 2. Inhibition of various pathogenic organisms as MICs (µg mL$^{-1}$) of munumbicins E-4 and E-5 and LD$_{50}$s against the malarial parasite Plasmodium falciparum**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Munumbicin E-4</th>
<th>Munumbicin E-5</th>
<th>Vancomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Burkholderia thailandensis</em></td>
<td>192</td>
<td>256</td>
<td>&gt;128</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>&gt;16</td>
<td>16</td>
<td>128</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 29213</td>
<td>8</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> 43000 (MRSA)</td>
<td>8</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> clinical isolate # 1</td>
<td>32</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td><em>Pythium ultimum</em></td>
<td>5</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>5</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td><em>Rhizoctonia solani</em></td>
<td>&gt;80</td>
<td>&gt;80</td>
<td>ND</td>
</tr>
<tr>
<td><em>Plasmodium falciparum</em></td>
<td>2.94 ± 0.32</td>
<td>0.50 ± 0.08</td>
<td>ND</td>
</tr>
</tbody>
</table>

MIC, minimum inhibitory concentration; LD$_{50}$, lethal dose 50%; ND, not done.
Conclusions

It appears that in addition to a number of actinomycins being produced by endophytic Streptomyces sp. 3052, at least two other, previously undescribed, antibiotics are also produced, namely munumbicin E-4 and E-5. These compounds are chromophoric peptides sharing similarities to other antibiotics such as the actinomycins but are unique. Other biologically active compounds are also produced by this isolate of Streptomyces sp. 30562 (U. Castillo, unpublished), making it one of the one of the most biologically active endophytic streptomycetes ever isolated. It seems evident that this one host plant, the snakevine, with its complex of streptomycetes, alone can act as a veritable pharmacy to native peoples using it as a source plant to treat open bleeding wounds. There seems to be an unlimited number of other bioactive Streptomyces spp. associated with this interesting native plant (Castillo et al., 2005). The biological activity of the munumbicins E-4 and E-5 is quite broad and includes both gram-positive and gram-negative bacteria (Table 2). However, the antimalarial activity of munumbicins E-4 and E-5 is about two orders of magnitude above that of chloroquine (Table 2) (Castillo et al., 2002). The prospects of being able to find just one or two plants out of hundreds of various plant species in an area with antibiotic properties would be quite low if one did not have the assistance of native peoples (Castillo et al., 2002). Nevertheless, it does seem that there would be a myriad of other plants in various locations in the world that are host to bioactive endophytic streptomycetes.

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