

ANTIBIOTICS IN MICROBIAL ECOLOGY
Isolation and Structure Assignment of Several New
Antibacterial Compounds from the Insect-Symbiotic
Bacteria *Xenorhabdus* spp.

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Abstract—Nine strains of the terrestrial bacterial genus *Xenorhabdus*, all isolated as symbionts of nematodes, were examined for their abilities to produce substances with antibiotic activities when grown in pure culture. All nine produced measurable antibiotic activities against one or more of the test strains utilized. The inhibition patterns indicated that different compounds were being produced by the various bacteria. Two of the species that showed particularly strong inhibition patterns were studied in detail. The inhibitory compounds were purified and identified. Strain R produced a mixture of active substances, the major components of which were hydroxyl- and acetoxy-bearing indole derivatives, presumably produced via tryptophan. Strain Hb, on the other hand, produced only two antibiotics, 4-ethyl- and 4-isopropyl-3,5-dihydroxy-*trans*-stilbenes, which are presumed to arise via polyketide pathways.

Key Words—Bioluminescent bacteria, symbiosis, *Xenorhabdus* spp., antibacterials, 3-(2'-acetoxy-3'-keto-4'-methylpentyl)-indole, 3-(2'-hydroxy-3'-keto-4'-methylpentyl)-indole, 3-(2'-acetoxy-3'-keto-4'-methylhexyl)-indole, 3-(2'-hydroxy-3'-keto-4'-methylhexyl)-indole, 3,5-dihydroxy-4-ethyl-*trans*-stilbene, 3,5-dihydroxy-4-isopropyl-*trans*-stilbene.

INTRODUCTION

The role that inhibitory compounds play in microbial ecology is not well understood, although it is apparent that many organisms have the capacity to produce one or more chemicals with antibiotic activities. These compounds have often been found to be of great use to humans, while their purposes in the

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environment, with regard to naturally occurring inhibitions of microbes, have remained mysterious. Part of the problem in assigning roles to the production of such compounds is that the places where they might be effective are not obvious; presumably the producing organism must be in high concentration and in an environment where it is of some advantage to inhibit invading microbes.

One such environment has been described in the remarkable symbiosis-parasitism exhibited by members of the bacterial genus *Xenorhabdus* (Thomas and Poinar, 1979). These bacteria live as symbionts in the guts of parasitic nematodes. The symbionts are carried here until the nematode finds a suitable host, which may be any of a wide variety of insects, including fleas, beetles, bees, or others; in laboratory experiments caterpillars are commonly used. The nematode, after entering the animal's gut tract via the mouth, penetrates the gut wall, thus entering the hemocoel. In response to factors in the hemolymph, the nematode then releases the bacterial symbionts, which grow in the hemolymph, kill the insect, and allow the nematode to complete its life cycle in the hemolymph. Without the bacteria, the nematode can kill the insect but cannot complete its life cycle. This cycle, which has been described by several authors (Khan and Brooks, 1977; Poinar et al., 1977), is outlined in Figure 1.

One of the curious features of the cycle is that the infected insects do not putrefy, as usually occurs after infection and degradation of organic substrates by bacteria. One explanation for the lack of putrefaction is that the bacteria that are degrading the parasitized animal produce substances with antibacterial activities, and these substances keep putrefying bacteria from invading, thus preserving the environment for the life cycle of the nematode. We report here the presence of inhibitory compounds in a variety of the nematode bacterial isolates tested and the chemical characterization of the active compounds from two different isolates.

METHODS AND MATERIALS

Bacteria. Bacteria were obtained from Dr. G. Poinar (University of California, Berkeley) and Dr. Williams Brooks (North Carolina State University). The strains, designations, and sources are listed in Table 1. The bacterial strains used as indicators were obtained from our own culture collection. Bacteria from nematodes were maintained on nutrient agar, or SWCLS medium. This consisted of 25% sea water, 3 g glycerol, 5 g bactopectone, 3 g yeast extract per liter of 25% sea water. Solid medium contained 12 g of agar per liter.

Sensitivity Tests. For screening of antibiotic activities the strains were cross-streaked on agar plates; inhibition was seen at the interfaces of the

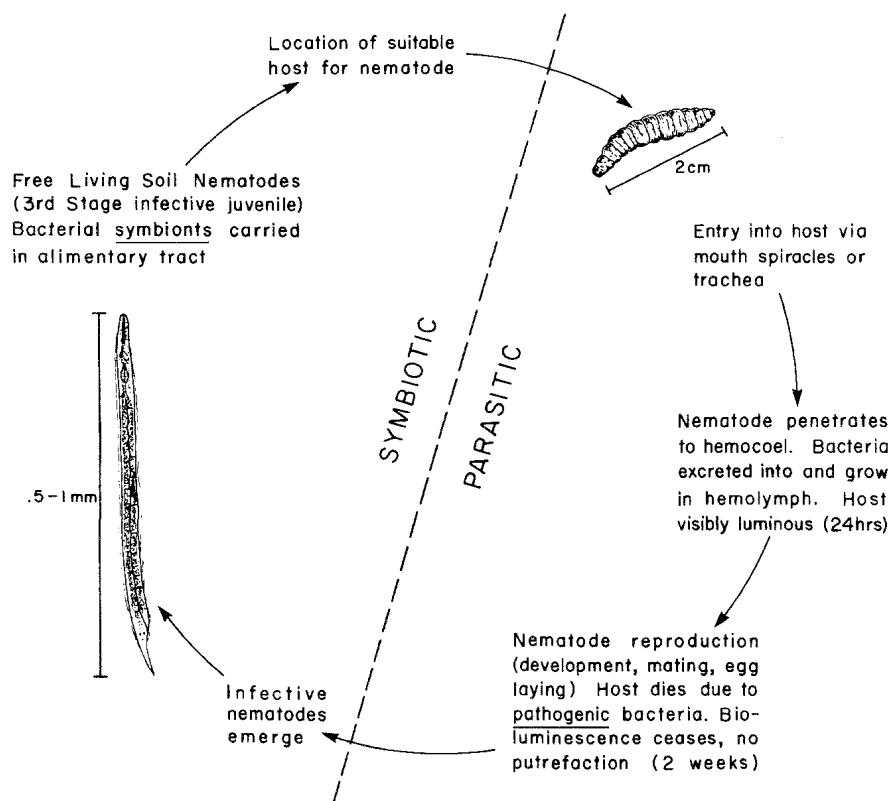


FIG. 1. Involvement of luminous bacteria in the nematode/insect life cycle.

cultures. Cells to be assayed for antibiotic production were also grown to stationary phase in liquid medium, the cells removed by centrifugation and filtration, and the growth medium tested. For these tests, a filter paper disk was first saturated with the growth medium, then placed, after it was allowed to dry in the air at room temperature, on a freshly streaked lawn of sensitive bacteria. This latter method was used for all of the purification procedures to assay the biological activity of the various fractions.

Antibiotic Extraction and Purification. Fifteen liters of cell-free media from strains R and Hb were extracted three times with 3-liter portions of ethyl acetate. For each strain the extracts were combined and reduced in vacuo to 100 ml. The extracts were then dried over anhyd. magnesium sulfate, filtered and further evaporated to yield a viscous residue. Strain R yielded 500 mg, and strain Hb 550 mg of extract. In each case the oily residue was applied to a column of silica gel (Grace grade 62, 60-200 mesh) (2.5×60 cm) packed using isooctane. Subsequent elution of the column with isooctane, methylene chloride, and ethyl acetate mixtures led to the collection of 30 fractions. For strain R antibiotic activity was detected in fractions eluted with 10% methylene chloride in isooctane and with fractions eluted with 20% methylene

TABLE 1. ANTIBACTERIAL ACTIVITIES^a OF *Xenorhabdus*^b STRAINS

Strains from nematodes ^d	Test strains ^c										
	H98	B392	PL721	B398	NZ11D	B378	B332	MJ-1	B404	B334	B333
R	+	-	-	+	+	+	-	+	+	-	-
Hb	+	-	+	+	+	-	-	+	+	-	-
NL	+	-	±	+	+	-	-	±	+	-	-
AT	+	-	+	+	+	-	-	+	+	±	-
B1	+	-	+	+	+	+	-	+	+	+	-
B2	+	-	+	+	+	+	-	+	+	-	-
A11	+	-	+	+	+	-	+	+	+	-	-
Hm	-	-	+	+	+	-	-	+	+	-	-
DN	-	-	-	+	+	-	-	-	+	+	-

^aThese activities determined by both cross-streaking and disk inhibition assays.

^bThis is a new genus of nematode symbionts (Thomas and Poinar, 1979).

^cThese are luminous bacteria from our collection. *Vibrio* (*Beneckea*) spp. (H98, B392, B378, B332, B334, B398, MJ-1; *Photobacterium* spp. NZ11D, PL721). Strains are described in Reichelt and Baumann (1973) and Hastings and Nealson (1977).

^dSome of the bacterial isolates from nematodes were kindly supplied by Dr. G. Poinar. AT, R, A11, and DN are isolates of *X. nematophilus*, with AT indicating the ATCC type species #19601. Hb and Hm are strains of *X. luminescens*, while NL is a spontaneous nonluminous isolate of Hb. B1 and B2 were supplied by Dr. William Brooks. B1 was designated as *Achromabacter nematophilus* while B2 was designated NC-19 (Khan and Brooks, 1977).

chloride in isooctane. For strain Hb antibiotic activity was detected in fractions eluted with 20% methylene chloride in isooctane.

Subsequent purification of antibiotic column chromatography fractions was accomplished by high-pressure liquid chromatography (HPLC) on a 16 mm × 50 cm preparative silica gel column. For the purification of all compounds, mixtures of ethyl acetate in isooctane gave good results.

Instrumental Analyses. Infrared spectra were recorded on a Perkin Elmer model 137 spectrophotometer. Ultraviolet spectra were recorded on a Beckman Acta M-VI spectrophotometer. Optical rotations were obtained using a 10-cm microcell on a Perkin Elmer model 141 polarimeter and low resolution mass spectra were recorded on a Hewlett-Packard 5930A instrument. High resolution mass measurements were obtained through the Department of Chemistry, University of California, Los Angeles. Nuclear magnetic resonance spectra were recorded on a Varian HR 220 spectrometer with Nicolet FT system (¹H) and on a Varian CFT-20 spectrometer (¹³C). All solvents were distilled in glass prior to use.

RESULTS

When the nematode symbionts were examined by either cross-streaking or filter paper methods, it was seen that many had the ability to produce compounds with antibiotic activity (Table 1). The various strains differed

both with respect to the number and types of sensitive strains, suggesting that there were several different compounds involved; only two of the isolates, Hb and NL, showed identical patterns of inhibition of the test strains.

Two of the more active strains, R and Hb, were chosen for further study, and the antibiotic components, excreted into the culture medium, were isolated, purified (as described above), and structurally defined.

Strain R Antibiotics. The antibiotic metabolites from strain R were found to consist of a mixture of the homologous indole derivatives I–IV (Figure 2). Each compound inhibited the test strains used and showed the following spectral features:

Compound I, 3-(2'-hydroxy-3'-keto-4'-methylpentyl)-indole, was isolated as 4% of the extract, and showed $[\alpha]_D = 65.4^\circ$ (c 1.2, CHCl_3). The mass spectral fragmentation showed $M^+ m/e = 231$ for $\text{C}_{14}\text{H}_{17}\text{NO}_3$. ^1H NMR (CDCl_3): δ 8.08, 1H, bs; 7.63, 1H, d, $J = 7$ Hz; 7.36, 1H, d, $J = 7.5$ Hz; 7.16, 2H, m; 7.10, 1H, s; 4.62, 1H, dd, $J = 7.5$ Hz; 3.33, 1H, dd, $J = 15.4$ Hz; 3.05, 1H, dd, $J = 15.7$ Hz; 2.90, 1H, m; 1.10, 3H, d, $J = 7$ Hz; 0.98, 3H, d, $J = 7$ Hz. ^{13}C NMR (benzene- d_6): 190.2, 136.1, 128.0, 121.2, 120.1, 117.7, 117.0, 109.5, 109.2, 73.4, 34.4, 28.2, 17.2, 15.4.

Compound II, 3-(2'-acetoxy-3'-keto-4'-methylpentyl)-indole, was isolated as the major antibiotic produced (20% extract) and showed $[\alpha]_D = 39.5^\circ$ (c 1.0, CHCl_3). The mass spectrum showed $M^+ m/e = 273$ which analyzed for $\text{C}_{16}\text{H}_{19}\text{NO}_3$, addition of $\text{C}_2\text{H}_2\text{O}$ to compound I. ^1H NMR (CDCl_3): δ 8.28, 1H, bs; 7.63, 1H, d, $J = 8$ Hz; 7.33, 1H, d, $J = 8$ Hz; 7.16, 2H, m; 7.0, 1H, s; 5.47, 1H, dd, $J = 7.5$ Hz; 3.23, 2H, m; 2.72, 1H, m; 2.05, 3H, s; 1.09, 3H, d, $J = 7$ Hz; 0.98, 3H, d, $J = 7$ Hz. ^{13}C NMR (acetone- d_6): 183.0(s), 170.5(s), 137.0(s), 128.0(s), 124.0(d), 122.4(d), 120.0(d), 119.3(d), 112.4(d), 110.4(s), 79.2(d), 38.0(d), 27.0(t), 21.0(q), 19.1(q), 17.8(q). UV (MeOH): λ_{max} 289, 279, 273 nm, $\epsilon = 5900, 7050, 6700$. IR (CHCl_3): 3600, 1728 cm^{-1} (broad).

Compound III, 3-(2'-hydroxy-3'-keto-4'-methylhexyl)-indole, was isolated as 3% of the organic extract and showed $[\alpha]_D = 112.5^\circ$ (c 0.8, CHCl_3). ^1H NMR (CDCl_3): δ 8.08, 1H, bs; 7.63, 1H, d, $J = 7$ Hz; 7.36, 1H, d, $J = 7.5$ Hz; 7.16, 2H, m; 7.10, 1H, s; 4.57, 1H, dd, $J = 7.5$ Hz; 3.32, 1H, dd, $J = 15.4$ Hz; 3.03, 1H, dd, $J = 15.7$ Hz; 2.76, 1H, m; 1.66, 1H, m; 1.45, 1H, m; 0.87, 3H, t, $J = 7$ Hz. ^{13}C NMR (benzene- d_6): 190.2, 136.1, 128.0, 121.0, 120.2, 117.7, 117.9, 109.5, 109.0, 73.7, 42.2, 29.0, 26.8, 15.2, 11.4.

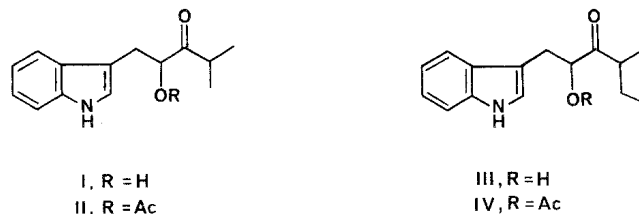


FIG. 2. Molecular structures of antibiotics isolated from *Xenorhabdus* sp. (strain R).

Compound IV, 3-(2'-acetoxy-3'-keto-4'-methylhexyl)-indole, of the extract showed $[\alpha]_D = 58.2^\circ$ (c 1.0, CHCl_3): ^1H NMR (CDCl_3): δ 8.29, 1H, bs; 7.53, 1H, d, $J = 7$ Hz; 7.32, 1H, d, $J = 8$ Hz; 7.16, 2H, m; 7.0, 1H, s; 5.46, 1H, dd, $J = 7.5, 5$ Hz; 3.24, 2H, m; 2.61, 1H, m; 2.04, 3H, s; 1.90, 1H, m; 1.70, 1H, m; 0.98, 3H, d, $J = 6$ Hz; 0.87, 3H, m. ^{13}C NMR (benzene- d_6): 192.4(s), 170.0(s), 136.5(s), 128.0(s), 123.3(d), 122.3(d), 119.7(d), 119.0(d), 111.8(d), 110.2(s), 78.0(d), 44.4(d), 26.8(t), 26.5(t), 20.3(q), 15.2(q), 11.4(q). UV (MeOH): λ_{max} 289, 280, 273 nm, $\epsilon = 6200, 6990, 6560$. IR (CHCl_3): 3600, 1725 cm^{-1} .

Strain Hb Antibiotics. The antibiotic metabolites from strain Hb were found to be the homologous *trans*-stilbene derivatives V and VI (Figure 3). The following spectral features were exhibited by these compounds:

Compound V, 3,5-dihydroxy-4-isopropyl-*trans*-stilbene, isolated as 20% of the extract, was analyzed for $\text{C}_{17}\text{H}_{18}\text{O}_2$ by high-resolution mass spectrometry (obs. $M^+m/e = 254.1288$; calc. 254.1302). ^1H NMR (CDCl_3): δ 7.46, 2H, d, $J = 7$ Hz; 7.25, 3H, m; 7.00, 1H, d, $J = 16$ Hz; 6.90, 1H, d, $J = 16$ Hz; 6.50, 2H, s; 4.90, 2H, bs (D_2O exc.); 3.45, 1H, heptet, $J = 7$ Hz; 1.30, 6H, d, $J = 7$ Hz. ^{13}C NMR (MeOH- d_4): 2×158.4 (s), 139.8(s), 137.7(s), 130.7(d), 2×130.4 (d), 2×129.1 (d), 2×128.1 (d), 122.9(s), 2×109.5 (d), 26.4(d), 2×21.8 (q). UV (MeOH) of the diacetate derivative: λ_{max} 283 nm, $\epsilon = 29,500$. IR (CHCl_3): 3700, 3500, 1620, 1590 cm^{-1} .

Compound VI, 3,5-dihydroxy-4-ethyl-*trans*-stilbene, isolated as 6% of the extract, was analyzed for $\text{C}_{16}\text{H}_{16}\text{O}_2$ by low-resolution mass spectrometry ($M^+m/e =$ obs. 240). ^1H NMR (CDCl_3): δ 7.46, 2H, d, $J = 7$ Hz; 7.25, 3H, m; 7.00, 1H, d, $J = 16$ Hz; 6.90, 1H, d, $J = 16$ Hz; 6.70, 2H, s; 4.90, 2H, bs (D_2O , exc.); 2.60, 2H, q, $J = 7$ Hz; 1.35, 3H, t, $J = 7$ Hz. UV (MeOH): λ_{max} 288 nm, $\epsilon = 16,500$. IR (CHCl_3): 3700, 3500, 1620, 1590 cm^{-1} .

Transformations of Compound VI. As part of the structure elucidation of the ethylated stilbene VI, the metabolite was acetylated ($\text{Ac}_2\text{O}/\text{py}/25^\circ$) and the bisacetate was hydrogenated ($\text{Pt}/\text{Et}_2\text{O}$). The derivatives obtained had the following spectral features:

3,5-Diacetoxy-4-ethyl-*trans*-stilbene: ^1H NMR (CDCl_3): δ 7.50, 2H, d, $J = 6$ Hz; 7.35, 3H, m; 7.15, 2H, s; 7.10, 2H, d, $J = 2$ Hz; 2.50, 2H, q, $J = 7$ Hz; 2.40, 6H, s; 1.20, 3H, t, $J = 7$ Hz.



FIG. 3. Molecular structures of antibiotics isolated from *Xenorhabdus* sp. (strain Hb).

1-Phenyl-2-(3',5'-diacetoxy-4'-ethylphenyl)ethane: MS, low resolution, $M^+ m/e = 326$ for $C_{20}H_{22}O_4$; UV (MeOH) λ_{max} 256, 220 nm; $\epsilon = 550, 4300$; [^1H]NMR ($CDCl_3$): δ 7.20, 5H, m; 6.70, 2H, s; 2.90, 4H, s; 2.40, 2H, q, $J = 7$ Hz; 2.30, 6H, s; 1.10, 3H, t, $J = 7$ Hz.

DISCUSSION

The structure elucidations of compounds I-VI followed readily from interpretation of their spectral data, including comparisons with suitable spectral standards. The indoles I-IV showed UV and NMR spectra virtually superimposable with indole itself (Scott, 1964), and the substitution pattern on indole and the composition of the side chains were easily confirmed by proton spin-decoupling NMR experiments. In each case the methylene protons at the C-1' position were related to the C-2' methine proton, and the C-4' methine proton could be related to the adjacent methyl groups (compounds I, II) or adjacent methyl and ethyl substituents (compounds III, IV). The alcohols I and III were converted to II and IV, the major antibiotics, upon treatment with acetic anhydride in pyridine. Although chiral metabolites, no attempt was made to determine the absolute stereochemistries at C-2'.

Assignment of the structures for IV and V based upon spectral analysis posed several interesting problems. The molecular formulae were readily established by mass spectrometry; however, due to the twofold symmetry of these derivatives, both the ^{13}C and ^1H NMR spectra were not initially readily interpretable. The ^{13}C spectrum of V, for example, exhibited only 12-carbon resonances for the 17-carbon atom structure, indicating the degenerate methyl groups of the isopropyl substituent, and the degenerate carbons C-2,6, C-3,5, C-2',6', and C-3',5' on the aromatic nuclei. Once the symmetry of these compounds was established, the data quickly allowed the formulation of structures V and VI. The Hb antibiotics were assigned as *trans*-stilbene derivatives based upon the 16-Hz olefin coupling constants, and alternative structures with the hydroxyl groups substituted at C-2' and C-6' were considered. While this alternative could not be excluded based upon ^1H NMR data, calculations of the predicted ^{13}C bands clearly eliminated this alternative (Wehrli and Wirthin, 1978).

As part of the confirmation of structures V and VI, the ethyl derivative VI was acetylated and the bisacetate obtained was catalytically hydrogenated. One mole of H_2 was smoothly taken up, and the product, 1-phenyl-2-(3',5'-diacetoxy-4'-ethylphenyl)-ethane, was purified by HPLC. The ^1H NMR features of this compound, and its classical benzenoid UV absorption at 256 and 220 nm (Pasto and Johnson, 1969), confirmed the structure as proposed.

The antibiotics isolated in this study from the two strains of *Xenorhabdus*

are, curiously, of quite dissimilar structure types. Compounds I-IV would appear to be degradation products of tryptophan, a nutrient in the culture medium. The stilbenes V and VI, however, are not clearly derived from simple precursors, nor are stilbenes considered common isolates from bacteria. Rather, groups of stilbenes have been isolated from terrestrial plants (Devon and Scott, 1972), and these compounds are thought to be derived via polyketide (fatty acid) biosynthesis (Geissman and Crout, 1969). The different patterns of inhibition noted in those strains not chemically investigated would suggest further variations in antibiotic composition. The pathways for biosynthesis of the antibiotics described here have not been investigated, but it should be a fairly straightforward problem to explore using mutant strains and defined media.

It has been noted that the infected insects in the life cycle shown in Figure 1 do not putrefy, and it has been speculated that the bacteria may prevent putrefaction by the production of inhibitory substances. While this has not been proven, the observation that inhibiting substances are present in all the media assayed, and the successful isolation of discrete antibiotics from the two strains studied, strongly supports these speculations. It should also be noted that both *X. luminescens* and *X. nematophilus* have been recently reported to contain defective phages that may be responsible for lysis of *Bacillus cereus* (and possibly other bacteria) (Poinar *et al.*, 1980a). Thus, several antibacterial mechanisms may be operating in this system.

The final point in question is whether any of these mechanisms operate under natural conditions. Are these compounds, or the defective phages discussed above, effective agents in preventing putrefaction in the infected insects? Perhaps this system is one of the best for the study of natural activities and uses of compounds with antibacterial activities.

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