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BIOLOGICALLY ACTIVE METABOLITES FROM *XENORHABDUS* SPP., PART 1. DITHIOLOPYRROLONE DERIVATIVES WITH ANTIBIOTIC ACTIVITY

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ABSTRACT.—Five related antibiotic compounds, named xenorhabdins, were isolated from cultures of *Xenorhabdus* spp., bacteria symbiotically associated with insect-pathogenic nematodes. Their chemical structures were elucidated by X-ray crystallography, nmr, and mass spectral analyses to be *N*-acyl derivatives of either 6-amino-4,5-dihydro-5-oxo-1,2-dithiolo [4,3-*b*] pyrrole (compounds **1–3**) or 6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo [4,3-*b*] pyrrole (compounds **4** and **5**). They are previously unreported members of the pyrrothine family of antibiotics. Antimicrobial and insectidical activities were found. These metabolites are specific to phase one *Xenorhabdus*.

Insect pathogenic, soil-dwelling nematodes of the families Heterorhabditidae and Steinernematidae are known to be symbiotically associated with bacteria of *Xenorhabdus* spp. (Enterobacteriaceae) (1–3), which are carried monoxenically within the intestine of the infective stage of the nematode (4). After invading the hemocoel of the host insect, the nematodes release a toxin (5,6) and an inhibitor of the insect immune system (7), as well as releasing their bacterial symbiont. These bacteria proliferate in the killed host and favor reproduction of the nematodes by providing nutrients and by producing antibiotics which inhibit the growth of other microbial flora in the insect cadavers (8,9).

Xenorhabdus spp. produce two phases, designated primary and secondary, when cultured in vitro. The phases can be distinguished by colony morphology, adsorption of dyes from agar media, and antibiotic production (10,11). Both phases are pathogenic to insect larvae, but phase one has superior ability to provide nutrients for the nematodes in insects and artificial media (11–13).

Xenorhabdus spp. phase one metabolites, but not phase two, exhibit a wide spectrum of antibiotic activity (9). There have been two previous reports of the isolation of *Xenorhabdus* spp. antimicrobial metabolites. These were indole and hydroxystilbene derivatives isolated from *Xenorhabdus bovienii* and *Xenorhabdus luminescens*, respectively (14,15). As part of our studies leading to the commercial development of entomogenous nematodes for use as insecticides, we investigated the antibiotic metabolites of *Xenorhabdus* spp. This paper describes the production by fermentation, isolation, characterization, and biological activity of a series of dithiolopyrrolone compounds, the xenorhabdins. Another series of compounds, the xenocoumacins, isolated from *Xenorhabdus* spp., are described in Part 2. These compounds are novel and are unrelated to the indole and hydroxystilbene derivatives previously reported.

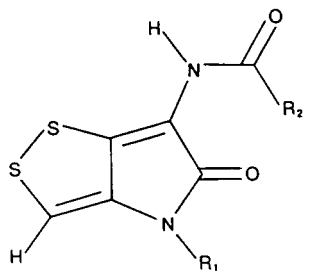
RESULTS AND DISCUSSION

Three fermentation processes were used to generate xenorhabdins from *Xenorhabdus*

spp. Initially, *X. bovienii* strain T319 (2) was cultured on a solid medium of foam rubber impregnated with homogenized chicken offal. *Xenorhabdus* spp. are grown on artificial media by this procedure during the mass production of nematodes with which they are symbiotically associated (13). The whole culture was extracted with EtOH and after lyophilization further extracted with EtOAc and Me_2CO. The crude extract was then subjected to Si gel chromatography followed by size exclusion chromatography in MeOH. Fractionation was guided by an antibacterial bioassay using *Micrococcus luteus* as the test organism. Activity was found in a yellow fraction which was further purified by reversed-phase hplc to yield three yellow compounds of similar activity, xenorhabdins 1 [**1**], 2 [**2**], and 3 [**3**].

In subsequent cultures, batch (48 h) and continuous liquid fermentation processes were used. Under continuous liquid culture of strain Q1, an undescribed *Xenorhabdus* sp. (3,10), xenorhabdin production reached a steady-state concentration of 10.8 mg/liter of **1** and 36.4 mg/liter of **2** to give a total productivity of 2.4 mg/liter per hour. Significant reversion of the culture to the non-producing secondary form was observed after 140 h, when xenorhabdin productivity had fallen to 30% of the initial steady-state level and the secondary form accounted for 31% of the viable cell population. This instability is a significant problem when culturing this organism in liquid culture.

The higher productivity and less complex media of the liquid fermentation processes enabled a simpler purification procedure to be developed. The xenorhabdins were extracted from the culture with EtOAc and the crude extract subjected to size exclusion chromatography. The xenorhabdins were then purified to homogeneity by reversed-phase hplc. Because of the low solubility of the xenorhabdins in the culture broth, both the broth and the cell pellet were extracted with EtOAc or, alternatively, the whole culture was extracted. As well as compounds **1**, **2**, and **3**, another yellow, active compound, named xenorhabdin 4 [**4**], was isolated from the culture of *Xenorhabdus* sp. strain Q1. From a batch liquid fermentation of *X. bovienii* strain Umea (10) a further compound, xenorhabdin 5 [**5**], was isolated together with **1**, **2**, and **4**.



1 R_1=H, R_2=*n*-C_5H_{11}
2 R_1=H, R_2=$(CH_2)_3CH(Me)_2$
3 R_1=H, R_2=*n*-C_7H_{15}
4 R_1=Me, R_2=*n*-C_5H_{11}
5 R_1=Me, R_2=$(CH_2)_3CH(Me)_2$
6 R_1=xanthydryl, R_2=*n*-C_5H_{11}

Xenorhabdins were produced by phase one (10) but not phase two of *Xenorhabdus* spp. This is in agreement with the results of previous studies, which found that only phase one had antibiotic activity (9). Care was exercised to ensure that all cultures were in phase one.

From an analysis of ^1H- and ^{13}C-nmr spectra, mass spectra, and X-ray crystallographic data the molecular structures of the five xenorhabdins were assigned as **1–5**, re-

spectively. They are *N*-acyl derivatives of either 6-amino-4,5-dihydro-5-oxo-1,2-dithiolo [4,3-*b*] pyrrole (compounds **1–3**) or 6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo [4,3-*b*] pyrrole (compounds **4** and **5**). The uv and ir spectra supported these assignments. The uv spectra revealed that the five xenorhabdin compounds have a common chromophore characterized by a dominant maximum at 387 nm.

The hreims of **1** shows a molecular ion at *m/z* 270 with a molecular formula of $C_{11}H_{14}N_2O_2S_2$. This was confirmed by chemical ionization studies. Loss of the elements of $C_6H_{10}O$ leads to the principal daughter ion at *m/z* 172 (composition $C_5H_4N_2OS_2$). The calculated isotopic distributions for $C_{11}H_{14}N_2O_2S_2$ and $C_5H_4N_2OS_2$ are in agreement with those found in the mass spectrum.

Xenorhabdin 2 was found to have a molecular formula of $C_{12}H_{16}N_2O_2S_2$ $[M]^+$ (*m/z* 284) and xenorhabdin 3 a molecular formula of $C_{13}H_{18}N_2O_2S_2$ $[M]^+$ (*m/z* 298) by hrms. Each has a base peak at *m/z* 172 and a fragmentation pattern similar to that of **1**. This supported the conclusion that they are members of a homologous series with a common nucleus of $C_5H_4N_2OS_2$. Additional methylenes are incorporated into the $C_6H_{10}O$ portion of **1** to give **2** and **3**. The base peak (*m/z* 172) of each mass spectrum shows only limited tendency for further fragmentation, which indicates that the ion of composition $C_5H_4N_2OS_2$ is heterocyclic.

^{1}H-nmr spectral analysis, including spin decoupling experiments, indicated the presence of a hexanamido moiety in **1**, a 5-methylhexanamido moiety in **2**, and an octanamido moiety in **3**. Together with the mass spectral data, this suggested structures containing a common heterocyclic ring to which is attached an alkyl side chain via an amide linkage. The ^{1}H-nmr spectrum also indicated one olefinic proton (δ 6.9) and two amidic protons (which are exchangeable). The ^{13}C-nmr spectrum of **1** provides confirmation of the structure of the side chain; the six carbons ranging from δ 14.0 to δ 34.7 have chemical shift values and multiplicities in accord with the proposed partial structure. The ^{13}C-nmr spectrum also confirmed the presence of eleven carbons in **1**, including two carbonyl carbons (δ 171.9 and δ 168.0). Three singlet carbons (δ 134.1, 133.6, and 115.4) and a doublet at δ 110.7 together with one of the carbonyl carbons are contained in the heterocyclic nucleus.

Xenorhabdins 4 and 5 have molecular formulae of $C_{12}H_{16}N_2O_2S_2$ $[M]^+$ (*m/z* 284) and $C_{13}H_{18}N_2O_2S_2$ $[M]^+$ (*m/z* 298), respectively, as determined by hrms. That is, they are isomers of **2** and **3**, respectively. The mass spectra show that **4** and **5** differ from the other xenorhabdins in having a base peak at *m/z* 186, suggesting an extra methyl group on the heterocyclic ring. The ^{1}H-nmr spectra of **4** and **5** are similar to those of **1** and **2**, respectively, but show loss of an amidic NH signal and an additional signal (3H, s) at δ 3.28 due to N-Me. The ^{1}H-nmr spectra suggest a hexanamido moiety is present in **4** and a 5-methylhexanamido moiety in **5**. That is, **4** and **5** have the same *N*-acyl side chains as **1** and **2**, respectively, but differ in having a methyl substituent on the amidic-N of the heterocyclic ring system.

The above spectral data were consistent with the basic molecular structure of a heterocyclic nucleus with a substituent *N*-acyl hydrocarbon side chain of 6–8 carbons. However, the data were insufficient to resolve completely the structure of the heterocyclic nuclei, $C_5H_3N_2OS_2$ for **1–3** and $C_6H_5N_2OS_2$ for **4** and **5**. To achieve this end, a single crystal X-ray study was performed on the xanthydrol derivative **6** of **1**. Hrms was used to confirm the composition of this derivative as $C_{24}H_{22}N_2O_3S_2$.

The results of the X-ray crystallographic study of **6** are shown in Figures 1 and 2 and Table 1. Assignment of the stoichiometry and molecular structure as shown takes into account the above spectral evidence, particularly in regard to the stoichiometry of the molecular "tail," where thermal motion is very high, possibly associated with minor disorder, with some consequent unreality in the associated interatomic distances and

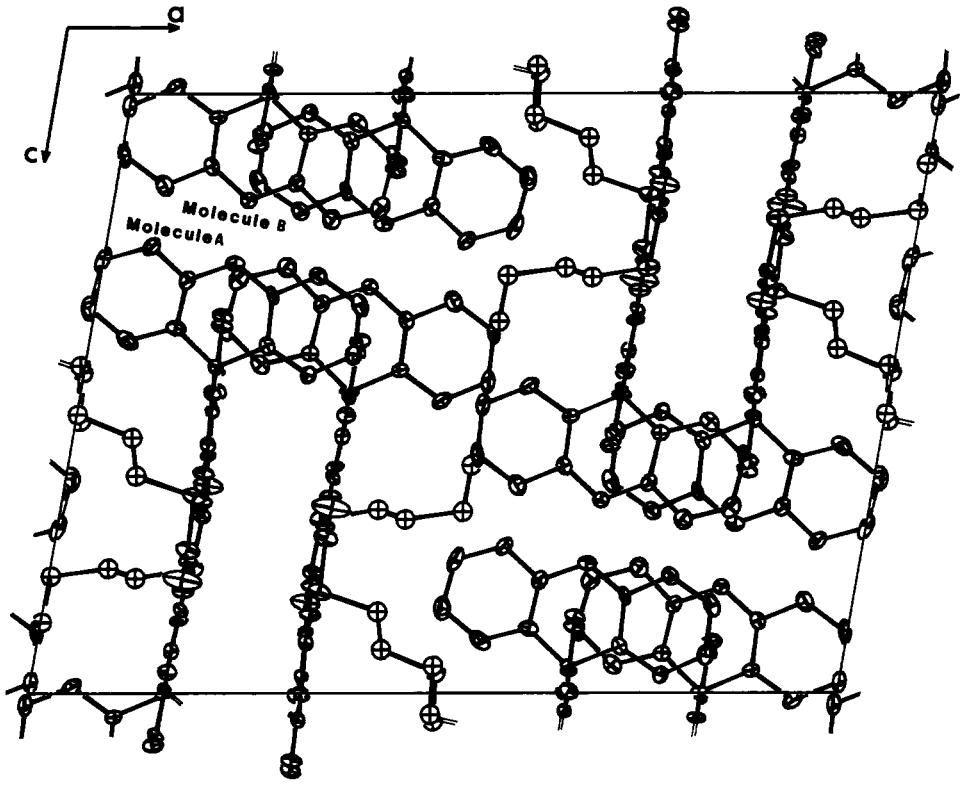


FIGURE 1. Unit cell contents down *b*; 20% thermal ellipsoids are used for the non-hydrogen atoms, excepting those in the molecular tail, where isotropic values are adopted.

angles. The asymmetric unit of the structure comprises two distinct molecules; the packing of these and associated molecular fragments contains elements of pseudo-symmetry in consequence of the disposition of the molecule into planar segments disposed parallel to the crystallographic planes. Within each molecule, the xanthydryl groups,

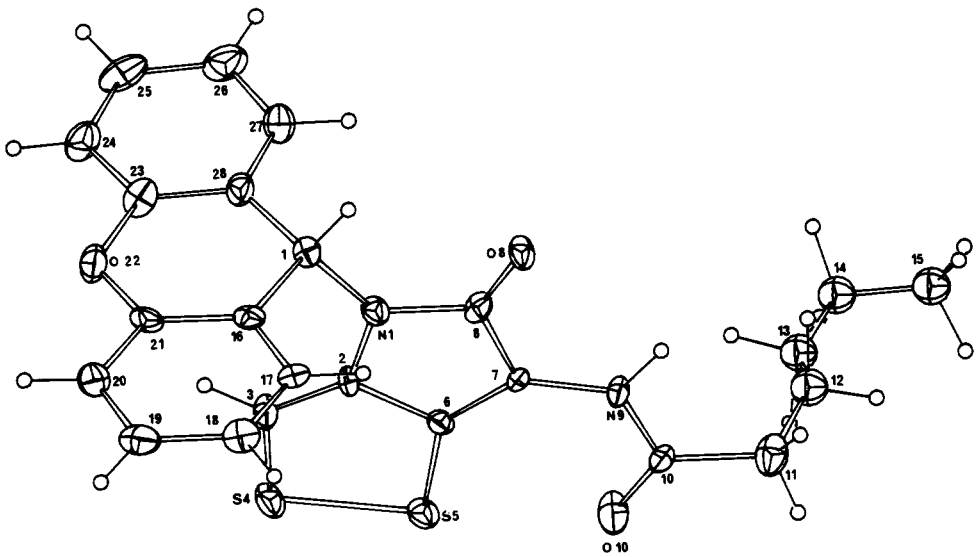


FIGURE 2. A projection of molecule A. Hydrogen atoms have an arbitrary radius of 0.1 Å.

TABLE 1. Non-Hydrogen Atom Coordinates for Compound **6**.

Atom	Molecule A			Atom	Molecule B		
	x	*y*	*z*		*x*	*y*	*z*
N-1A	0.1625(9)	0.576(1)	0.499(1)	N-1B	0.1680(8)	0.536(1)	−0.0396(9)
C-2A	0.1634(9)	0.678(2)	0.463(1)	C-2B	0.1685(9)	0.430(2)	−0.007(1)
C-3A	0.159(1)	0.711(2)	0.388(1)	C-3B	0.1672(9)	0.395(2)	0.071(1)
S-4A	0.1591(3)	0.8559(5)	0.3718(3)	S-4B	0.1657(3)	0.2536(5)	0.0862(3)
S-5A	0.1677(3)	0.8977(4)	0.4961(3)	S-5B	0.1676(3)	0.2122(4)	−0.0363(3)
C-6A	0.1672(9)	0.762(1)	0.531(1)	C-6B	0.1680(9)	0.347(1)	−0.071(1)
C-7A	0.1711(8)	0.703(2)	0.603(1)	C-7B	0.1704(9)	0.404(2)	−0.139(1)
C-8A	0.167(1)	0.586(2)	0.584(1)	C-8B	0.169(1)	0.523(2)	−0.123(1)
O-8A	0.1687(7)	0.509(1)	0.6326(8)	O-8B	0.1682(7)	0.600(1)	−0.1714(7)
N-9A	0.1734(7)	0.744(1)	0.6821(8)	N-9B	0.1698(8)	0.366(1)	−0.2195(9)
C-10A	0.178(1)	0.854(2)	0.704(1)	C-10B	0.166(1)	0.257(2)	−0.241(1)
O-10A	0.177(1)	0.926(1)	0.6538(9)	O-10B	0.166(1)	0.187(1)	−0.190(1)
C-11A	0.174(1)	0.878(2)	0.792(1)	C-11B	0.162(1)	0.229(2)	−0.331(1)
C-12A[a]	0.115(1)	0.896(2)	0.809(2)	C-12B[a]	0.086(1)	0.218(2)	−0.360(1)
C-13A[a]	0.065(2)	0.905(4)	0.788(3)	C-13B[a]	0.074(2)	0.195(3)	−0.435(2)
C-14A[a]	0.000(1)	0.941(3)	0.806(2)	C-14B[a]	−0.001(1)	0.196(2)	−0.467(2)
C-15A[a]	0.002(2)	0.953(3)	0.884(2)	C-15B[a]	−0.005(1)	0.152(2)	−0.547(1)
C-1A	0.1605(9)	0.470(2)	0.456(1)	C-1B	0.169(1)	0.644(2)	0.002(1)
C-16A	0.221(1)	0.452(1)	0.420(1)	C-16B	0.226(1)	0.653(1)	0.068(1)
C-17A	0.278(1)	0.455(2)	0.469(1)	C-17B	0.285(1)	0.654(2)	0.049(1)
C-18A	0.329(1)	0.448(2)	0.435(1)	C-18B	0.336(1)	0.661(2)	0.106(2)
C-19A	0.326(1)	0.434(2)	0.353(2)	C-19B	0.330(1)	0.662(2)	0.189(1)
C-20A	0.267(1)	0.428(2)	0.305(1)	C-20B	0.272(1)	0.666(2)	0.208(1)
C-21A	0.2154(9)	0.441(2)	0.336(1)	C-21B	0.2202(9)	0.663(2)	0.149(1)
O-22A	0.1626(7)	0.434(1)	0.2839(9)	O-22B	0.1651(7)	0.665(1)	0.1773(8)
C-23A	0.1082(9)	0.439(2)	0.312(1)	C-23B	0.114(1)	0.667(2)	0.121(1)
C-24A	0.055(1)	0.426(2)	0.254(1)	C-24B	0.060(1)	0.680(2)	0.153(1)
C-25A	−0.002(1)	0.428(2)	0.277(2)	C-25B	0.004(1)	0.680(3)	0.101(2)
C-26A	−0.008(1)	0.452(3)	0.358(2)	C-26B	−0.000(1)	0.673(3)	0.020(2)
C-27A	0.047(1)	0.465(2)	0.412(2)	C-27B	0.0516(9)	0.660(2)	−0.012(1)
C-28A	0.103(1)	0.453(2)	0.392(1)	C-28B	0.1106(9)	0.658(2)	0.038(1)

[a]Atom refined with isotropic thermal parameters.

with closely coplanar skeletons (σ, 0.04, 0.07 Å), lie normal to *b*, while the dithiol moiety, with a pair of fused rings and closely coplanar amide substituents (σ, 0.02, 0.02, Å) lies at right angles (interplanar dihedral, 89.2, 89.1°) and parallel to *bc* (Figure 1). The coplanarity of the amide group with the fused ring system appears to arise less from any conjugation in the C-7–N-9 bond between them [1.37 (1), 1.41 (1) Å], than from possible interaction between O-10 and S-5, which is shorter than might be expected for a normal non-bonding contact [2.594 (5), 2.567 (1.0) Å], and is suggestive of possible mesionic contributions to the bonding in the system. Within the fused systems the S-S bonds [2.097 (5), 2.091 (4) Å] are comparable to those observed in formally single bonded systems (e.g., S_8, ca. 2.05 Å). C-2–C-3 and C-8–O-8 are fairly well localized double bonds, C-6–C-7 possibly less so, while N-1–C-2, -8 are nearer the single bond extreme; C-1 does not deviate at a high level of significance from the fused ring plane. Nevertheless, there are clearly double bond contributions in C-3–S-4 and C-6–S-5 [1.72 (1)–1.74 (1) Å, the single bond value being ca. 1.82 Å], supportive of the mesionic hypothesis.

Based on the above spectral and X-ray studies, the complete structures were assigned. The xenorhabdins contain the 1,2-dithiolo [4,3-*b*] pyrrol-5-one or pyrrothine ring system. There are a number of compounds, all isolated from *Streptomyces* spp. and with antibiotic activity, which contain the dithiolopyrrolone ring structure, including thiolutin (16), aureothricin (16), isobutyropyrrothine (17), and holomycin (18). A less precise structural description of this ring system has been previously recorded (19).

A comparison of the mass spectra of xenorhabdins with those of thiolutin and holomycin provided further confirmation of the assigned structures. Analogous ei mass

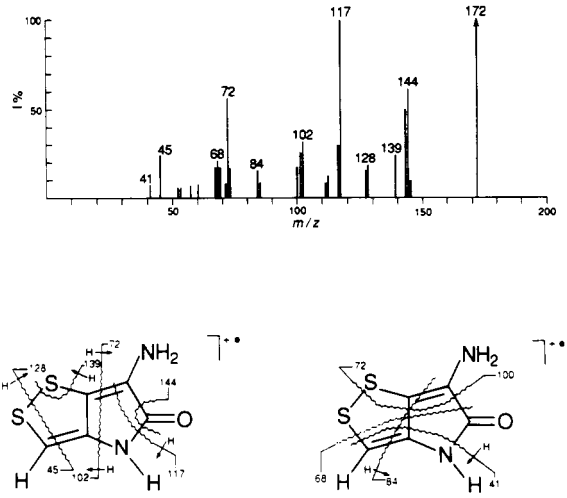


FIGURE 3. Collision-induced dissociation spectrum and inferred bond cleavages for ions of *m/z* 172.

spectra were obtained with principal fragment ions of *m/z* 172 for holomycin and *m/z* 186 for thiolutin. Moreover, diagnostic spectra were obtained by enforcing fragmentation of these ions downstream of the ion source. The resulting collision-induced dissociation spectra of the ions of *m/z* 172 in the mass spectra of **1–3** and holomycin are the same, supporting the conclusion that these compounds have a heterocyclic nucleus in common. Similarly, the collision-induced dissociation spectra for ions of of *m/z* 186 in the mass spectra of **4**, **5**, and thiolutin are the same, leading to analogous conclusions.

Collisional activation favors the occurrence of energetic bond cleavages and fast hydrogen rearrangement of the selected parent ions rather than skeletal rearrangements. Thus, collision-induced dissociation spectra are diagnostic of structure, and the spectrum for ions of *m/z* 172 is consistent with a structure derived formally from cystine (Figure 3). Likewise, the collision-induced dissociation spectrum for ions of *m/z* 186 can be rationalized in terms of the homologous structure (Figure 4).

Xenorhabdin 2 exhibits antimicrobial activity as shown in Table 2. The antimicrobial spectrum is similar to that of other dithiolopyrrolone compounds. The other xenorhabdin compounds had similar activity against *M. luteus* but were not tested against other organisms. Other pharmacological activities reported for the pyrrothines include antifungal activity, potent membrane stabilizing activity, and inhibition of platelet aggregation (20). Aureothricin has also been found to have antitrichomonal activity (21).

In our larval feeding assay against *Heliothis punctigera* larvae, xenorhabdin 2 showed insecticidal activity with 100% mortality at 150 $\mu g \cdot cm^{-2}$. The LC_{50} was 59.5 $\mu g \cdot cm^{-2}$ with the 95% confidence limits 48.2 and 73.5 $\mu g \cdot cm^{-2}$, respectively. Xenorhabdin 2 also showed a considerable effect on weight of surviving larvae. At a concentration of 37.5 $\mu g \cdot cm^{-2}$ there was only 18.8% mortality, but there was in fact a reduction in weight of survivors of 64.7% compared to controls. This effect on weight might suggest that **2** is in some way interfering with feeding by the larvae. The other xenorhabdin compounds were not tested, but thiolutin has been reported to have larvicidal activity against *Lucilia sericata* (22).

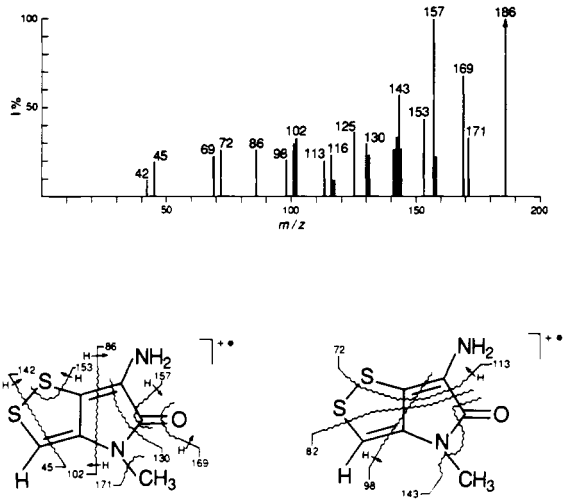


FIGURE 4. Collision-induced dissociation spectrum and inferred bond cleavages for ions of *m/z* 186.

Though there are no data on the in vivo production or efficacy of the xenorhabdins, the above biological activities suggest that these compounds may play a role in the nematode–bacteria symbiosis. Part of the function of the symbiotic bacteria is to provide antibiotics to inhibit the growth of other microorganisms, thereby preventing the putrefaction of the host insect cadaver and providing favorable conditions for the feeding and reproduction of the associated nematode (8). The significant activity against Gram-positive bacteria (as shown in Table 2) and the known antifungal activity of related compounds (23) suggest that the xenorhabdins may play a part in this antimicrobial process. Another aspect to consider is whether the xenorhabdins are involved in the mechanism of pathogenic action against the host insect through their insecticidal properties, though this activity is weak in vitro in comparison with modern insecticides such as the pyrethroids. Further study is needed to assess the significance of these compounds in the nematode–bacteria symbiosis.

TABLE 2. Antimicrobial Activity of Xenorhabdin 2 [**2**].

Organism	MIC (μg/ml)[a]
Staphylococcus aureus ATCC 6538P	20
Micrococcus luteus BTA 433	0.156
Escherichia coli K88 BTA 430	>100
Proteus mirabilis BTA 585	>100
Salmonella typhimurium BTA 438	>100
Shigella sonnei BTA 431	>100
Pseudomonas aeruginosa BTA 429	>100
Bacillus cereus BTA 432	3.13
Candida albicans ATCC 10231	>100
Streptococcus pyogenes IMR-RNSH	1.25

[a]Minimum inhibitory concentration.

The strains Pi, A24, TN6, and TP7 of *X. nematophilus* (2) have high antibiotic activity; however, hplc analysis of an organic extract (EtOAc) of the culture broth of these isolates revealed that the xenorhabdin compounds were not present. These results indicated that other, non-xenorhabdin, antibiotic compounds were being produced. Part 2 describes these compounds and their isolation.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—H_2O for culturing and fractionation was purified by a Millipore Milli-Q Water System. Si gel chromatography was performed using Merck Kieselgel 60 silica. Sephadex LH-20 was supplied by Pharmacia. Gel filtration chromatography was monitored using an LKB 2238 Uvicord SII and fractionated with an LKB 2111 Multirac. Semi-preparative hplc was performed on a Whatman Partisil-10 ODS column using a Waters hplc system incorporating a Model 6000A pump, U6K injector, and a Model 450 variable wavelength LC spectrophotometer. Analytical hplc was performed on a Brownlee RP-18 column (10 μm, 4.6 mm × 25 cm) using a system consisting of a Waters Model 6000A pump, U6K injector, and a Model 440 absorbance detector. The eluent, MeCN-H_2O (1:1), was delivered at 1.5 ml/min and monitored at 405 nm.

Liquid fermentations were carried out in E-series laboratory fermenters (B. Braun, Melsungen, FRG). Centrifugation was performed using a Beckman J2-21M centrifuge. Uv spectra were measured on a Varian DMS 90 spectrophotometer; ir spectra were measured on a Pye Unicam SP3-200 spectrophotometer. Melting points are uncorrected. Purity of the compounds was assessed by hplc and by the lack of spurious peaks in ^{1}H- and ^{13}C-nmr spectra. ^{1}H-nmr spectra of **1** and **2** were measured on a Bruker WM-400, while the spectra of **3–5** were measured on a Varian XL-200 instrument. The internal reference was Me_2CO (δ 2.04). ^{1}H-nmr assignments are from first order considerations and were confirmed by double irradiation experiments where necessary. ^{13}C-nmr spectra were measured on a Jeol FX-100 spectrometer.

Mass spectra were determined on a VG 70-70 mass spectrometer interfaced to a VG 11-250 data system. Samples were analyzed by flash volatilization from an extended gold support (24) and rapid data acquisition. This method minimized any competing thermolysis and accentuated the molecular parent ions compared with conventional probe techniques. Ei mass spectra were determined with an ionization energy of 70 eV and a trap current of 100 μA. Ci mass spectra were obtained at 50 eV ionization energy and 200 μA emission current. NH_3 at a source pressure of 60 Pa was used as a reagent gas for positive-ion ci while isobutane at a source pressure of 80 Pa was used for negative-ion ci. Source temperatures were 180–210°. High-resolution mass analyses were carried out by peak matching. Collision-induced dissociation spectra for selected parent ions fragmenting in the field-free region between the ion source and the electric sector were obtained by linked scanning of the magnetic field B and electric sector voltage E at constant B/E ratio. The estimated collision cell pressure was 0.04 Pa (helium) and was sufficient to reduce the height of the main-beam parent ion peak to 30% of that in the absence of helium.

CULTURING OF *X. NEMATOPHILUS*.—All strains of *Xenorhabdus* were kindly provided by Drs. R. Bedding and R. Akhurst and are deposited with the CSIRO Division of Entomology Culture Collection, Canberra, Australia. *Xenorhabdus* sp. strain Q1 (ATCC 39497) is an isolate from a nematode of undescribed genus (Steinernematidae) strain Q1 found in Australia. *X. bovienii* strains T319 (2) and Umea (10) are symbionts of *Steinernema bibionis* strains T319 and Umea, respectively. *X. nematophilus* strains Pi, A24, TN6, and TP7 are symbionts of *Steinernema carpocapsae* strains Pieridarum, Agriotos, and two Tasmanian isolates, respectively (2).

The initial culture of *Xenorhabdus* was on solid medium. *X. bovienii* T319 was cultured on a medium (3 kg) of 12 parts of homogenized chicken offal to 1 part of polyurethane foam (11,12). After autoclaving in an evacuated polypropylene bag, the bag was inflated with air through 0.45-μm filters and the medium inoculated. The culture was grown for 5 days at ambient temperature before harvesting.

Subsequent cultures used liquid fermentation processes. *Xenorhabdus* strains Q1, Umea, and T319 were cultured in batch fermentations. Each of the strains was grown for 48 h in 5-liter laboratory fermenters at 28°, pH 6.7, with sufficient agitation and aeration to maintain pO_2 above 50% of saturation. The following yeast extract-salts (YS) medium (3.5 liters) was used: yeast extract 5 g/liter, $(NH_4)_2SO_4$ 5 g/liter, $MgSO_4 \cdot 7H_2O$ 0.2 g/liter, KH_2PO_4 0.5 g/liter, K_2HPO_4 0.5 g/liter.

Xenorhabdus sp. Q1 was also grown using a continuous culture process. A 5-liter laboratory fermenter was equipped with an inverted weir (culture off-take) tube for a culture working volume of 2.3 liters. The temperature was maintained at 28°, the pH at 7.0, and the pO_2 at or above 40%. The medium was prepared in 18-liter batches containing: yeast extract 15 g/liter, NaCl 5 g/liter, $(NH_4)_2SO_4$ 1 g/liter, $MgSO_4 \cdot 7H_2O$ 0.5 g/liter, KH_2PO_4 1 g/liter, and K_2HPO_4 1 g/liter. After inoculation the culture was grown batchwise for 8 h; then fresh medium was pumped into the fermenter at a rate sufficient to give a dilution rate of 0.05/h. Thus, one culture volume was collected in the reservoir via the off-take tube every 20 h. The fermenter was operated continuously for 280 h.

The inocula for all cultures and strains were grown overnight in 500-ml shake flasks containing 100 ml of the YS medium at 28°, 150 rpm. A fresh culture (48–72 h old) on nutrient agar supplemented with 0.004% (w/v) triphenyltetrazolium chloride and 0.0025% (w/v) bromothymol blue (NBTA) (10) was used to inoculate these flasks. Only phase one colonies were subcultured. The fermentation culture was regularly monitored for the presence of phase two by plating-out serially diluted culture samples (in 0.85% NaCl) on NBTA plates and incubating for 48 h at 30°. The phase two colonies appear red whereas the colonies of phase one have a red core overlaid by blue.

ISOLATION OF XENORHABDINS.—The isolation was monitored by an in vitro antibacterial assay. Fractions were dissolved in DMSO and applied to a paper disc which was placed on agar previously inoculated with *M. luteus*. Zones of inhibition were measured after incubation at 37° for 20 h.

Xenorhabdins 1, 2, and 3 were initially isolated from *X. bovienii* T319 cultured on chicken offal. The culture and medium were steeped and manually mixed in EtOH (2 × 5 liters) for 10 h. The combined EtOH extracts were concentrated by evaporation in vacuo at 40° and lyophilized to yield 143 g of residue. The residue (138 g) was stirred with EtOAc (4 × 500 ml) followed by Me_2CO (2 × 500 ml). The organic extracts were decanted, filtered, dried (anhydrous Na_2SO_4), combined, and evaporated in vacuo to yield a viscous, brown oil (50 g). The oil (50 g) was dissolved in petroleum ether (40°–60°, 150 ml), Si gel (35–70 mesh, 100 ml) was added, and the mixture was evaporated. The dry, sticky residue was applied to the top of a column (900 × 50 mm) of Si gel (35–70 mesh, 1790 g) equilibrated in petroleum ether (40°–60°). The column was eluted sequentially as follows: petroleum ether (4 liters); petroleum ether-EtOAc (9:1) (2 liters), (3:1) (2 liters), (2:1) (4 liters), (1:1) (2 liters), (1:2) (4 liters), EtOAc (2 liters), MeOH (4 liters).

Antibacterial activity was found in a yellow fraction eluted in petroleum ether-EtOAc (1:2). This fraction (2.65 g) was triturated with petroleum ether (100 ml), filtered, and dried in vacuo to yield 555 mg of pale yellow solid. A solution of this pale yellow solid (80 mg) in MeOH (4 ml) was chromatographed on Sephadex LH-20 (88 × 3.0 cm) in MeOH at 1 ml/min. The eluate was monitored at 280 nm and the active fraction eluted at 433–475 ml (57.8 mg). Several batches of sample were chromatographed to afford 265 mg of yellow solid.

This fraction was further purified by semi-preparative, isocratic hplc on a reversed-phase C_{18} column (10 μm, 9.4 mm i.d. × 50 cm) using MeCN-H_2O (1:1) as the mobile phase delivered at 4 ml/min and monitored at 280 nm. Three main constituents were collected and dried to yield 3 pure, yellow, equiactive compounds: **1** (42.7 mg), **2** (8.0 mg), and **3** (4.0 mg). Retention times were 11.4, 13.3, and 17.5 min, respectively.

Xenorhabdins 1–4 were isolated from a continuous liquid fermentation of *Xenorhabdus* sp. strain Q1. The fermentation liquor (10 liters) was centrifuged (9,000 rpm, JA-10 rotor, 15 min) and the culture broth extracted with EtOAc (2 × 1.5 liters) in batches of 3 liters. The combined organic extracts were washed sequentially with saturated NaCl solution and H_2O, dried (Na_2SO_4), and evaporated in vacuo to yield 2.09 g of residue. Hplc analysis showed the extract to contain **1** (28 mg), **2** (35 mg), and **4** (38 mg). The cell pellet was slurried in H_2O (40 ml) and EtOAc (100 ml), sonicated (not disrupted) for 15 min, and centrifuged (12,000 rpm, JA-14 rotor, 15 min), and the organic phase was decanted. The aqueous phase was extracted further with EtOAc (3 × 300 ml), and the extracts were combined, dried (Na_2SO_4), and evaporated in vacuo to yield a crude extract of xenorhabdins (1.325 g). The extract contained **1** (15.9 mg), **2** (83.1 mg), **3** (3.3 mg), and **4** (27.4 mg) by hplc analysis. The crude extract (1.3 g) was chromatographed on Sephadex LH-20 (94 × 5 cm) in MeOH at a flow rate of 5 ml/min. The eluent was monitored continuously at 280 nm. Xenorhabdin 4 eluted between 920 and 1106 ml, whereas xenorhabdins 1, 2, and 3 eluted in the fraction 1106–1636 ml. The xenorhabdins were purified to homogeneity by semi-preparative, isocratic, reversed-phase hplc as described above. The retention time of **4** was 14.6 min.

Xenorhabdin 5 was isolated from a batch liquid fermentation of *X. bovienii* strain Umea together with **1**, **2**, and **4**. The isolation procedure employed was similar to the above procedure for *Xenorhabdus* sp. strain Q1. The hplc retention time of **5** was 16.4 min.

Xenorhabdin 1 [**1**].—Mp 192–193°; uv λ max (MeOH) 387 nm, 300, 242 (s); ir (Nujol) 3250 (br), 1670, 1640, 1600, 1555 cm^{-1}; ^{13}C nmr (Me_2SO-d_6) 171.9 (s), 168.0 (s), 134.1 (s), 133.6 (s), 115.4 (s), 110.7 (d), 34.7 (t), 30.9 (t), 24.9 (t), 21.9 (t), 14.0 (q); ^1H nmr [$(CD_3)_2CO$] 9.64 (1H, s br, CO-NH), 8.64 (1H, s br, CO-NH), 6.93 (1H, s, H-3), 2.45 (2H, t, CO-CH_2, J = 7.2 Hz), 1.64 (2H, m, CH), 1.32 (4H, m, CH_2-CH_2), 0.88 (3H, t, Me); eims *m/z* (%) 272 (2), 271 (3), $[M]^+$ 270 (20), (calcd for $C_{11}H_{14}N_2O_2S_2$, 270.0498, found 270.0496), 174 (10), 173 (10), 172 (100) (calcd for $C_5H_4N_2OS_2$, 171.9766, found 171.9759), 171 (4), 144 (2), 143 (2), 117 (2), 99 (2), 72 (2), 71 (5), 55 (3), 45 (4), 43 (24). Analytical hplc Rt 4.6 min.

Xenorhabdin 1 xanthydrol derivative [**6**].—A solution of 730 μg of **1** in HOAc (50 μl) was added to a solution of xanthydrol (9-hydroxyxanthene) (3.3 mg) in HOAc (50 μl). Crystals formed overnight at room temperature. The crystals were washed (2 × 50 μl) with cold HOAc and recrystallized from a mixture of MeOH (1 ml), HOAc (50 μl), and MeCN (50 μl) at 85° to give the xanthydrol derivative

6 of xenorhabdin 1 which was used without further purification: eims *m/z* (%) $[M]^+$ 450 (3), (calcd for $C_{24}H_{22}N_2O_3S_2$, 450.1072, found 450.1094), 182 (22) 181 (100), 172 (23), 152 (9), 86 (6), 60 (8), 45 (9), 44 (6), 43 (11), 41 (7), 40 (28).

Xenorhabdin 2 [**2**].—Mp 210–213°; uv λ max (MeOH) 387 nm, 300, 244 (s); ^{1}H nmr [$(CD_3)_2CO$] 9.66 (1H, s br, CO-NH), 8.66 (1H, s br, CO-NH), 6.94 (1H, s, H-3), 2.45 (2H, t, CO-CH_2, J = 7.4 Hz), 1.67 (2H, m, CH_2), 1.58 (1H, m, CH), 1.25 (2H, q, CH_2), 0.88 (6H, d, Me, J = 6.5 Hz); eims *m/z* (%) 286 (2), 285 (3), $[M]^+$ 284 (20) (calcd for $C_{12}H_{16}N_2O_2S_2$, 284.0653, found 284.0659), 174 (10), 173 (13), 172 (100) (calcd for $C_5H_4N_2OS_2$, 171.9766, found 171.9771), 171 (3), 143 (2), 95 (4), 69 (4), 55 (3), 45 (3), 43 (18), 41 (9). Analytical hplc Rt 6.0 min.

Xenorhabdin 3 [**3**].—Mp 360°; uv λ max (MeOH) 387 nm, 300, 244 (s); ^{1}H nmr [$(CD_3)_2CO$] 9.7 (1H, s br, CO-NH), 8.7 (1H, s br, CO-NH), 6.96 (1H, s, H-3), 2.44 (2H, t, CO-CH_2, J = 7.4 Hz), 1.64 (2H, m, CH_2), 1.3 [8H, m, $(-CH_2-)_4$], 0.86 (3H, t, Me); eims *m/z* (%) 300 (2), 299 (3), $[M]^+$ 298 (19) (calcd for $C_{13}H_{18}N_2O_2S_2$, 298.0810, found 298.0815), 174 (10), 173 (10), 172 (100) (calcd for $C_5H_4N_2OS_2$, 171.9766, found 171.9769), 57 (12), 55 (4), 45 (2), 43 (9), 41 (8). Analytical hplc Rt 9.6 min.

Xenorhabdin 4 [**4**].—Mp 165°; uv λ max (MeOH) 390 nm, 312, 245 (s), ir (KBr) 3260, 3050, 2935, 1680, 1650, 1610, 1540, 1440, 1240, 830 cm^{-1}; ^{1}H nmr [$(CD_3)_2CO$] 8.85 (1H, s br, CO-NH), 7.08 (1H, s, H-3), 3.28 (3H, s, N-Me), 2.43 (2H, t, CO-CH_2, J = 7 Hz), 1.64 (2H, m, CH_2), 1.30 (4H, m, CH_2-CH_2), 0.86 (3H, t, Me); eims *m/z* (%) 286 (3), 285 (4), $[M]^+$ 284 (25) (calcd for $C_{12}H_{16}N_2O_2S_2$, 284.0653, found 284.0654), 188 (10), 187 (11), 186 (100), 185 (5), 157 (2), 86 (3), 71 (3), 55 (3), 45 (2), 43 (17), 42 (7), 41 (6). Analytical hplc Rt 6.6 min.

Xenorhabdin 5 [**5**].—Uv λ max (MeOH) 388 nm, 308, 246 (s); ^{1}H nmr [$(CD_3)_2CO$] 8.90 (1H, s br, CO-NH), 7.10 (1H, s, H-3), 3.29 (3H, s, N-Me), 2.42 (2H, t, CO-CH_2, J = 7 Hz), 1.62 (3H, m, CH_2, CH) 1.25 (2H, m, CH_2), 0.87 (6H, d, Me, J = 6.5 Hz); eims *m/z* (%) 300 (2), 299 (4), $[M]^+$ 298 (20) (calcd for $C_{13}H_{18}N_2O_2S_2$, 298.0810, found 298.0807), 188 (10), 187 (13), 186 (100), 95 (3), 86 (3), 69 (4), 55 (5), 45 (3), 43 (19), 42 (7), 41 (9). Analytical hplc Rt 9.0 min.

X-RAY CRYSTALLOGRAPHIC STUDY OF **6** .—*Crystal data.*—$C_{24}H_{22}N_2O_3S_2$, M = 450.6, monoclinic, space group $P2_1/n$ (C_{2h}, No. 14), a = 22.074 (9), b = 11.99 (1), c = 16.59 (1) Å, β = 100.04 (5)°, U = 4323(6) Å^3, D_c (Z = 8) = 1.38 g·cm^{-3}. F(000) = 1888, monochromatic Mo Kα radiation, λ = 0.7106$_9$ Å, μ = 2.7 cm^{-1}. T = 295 K.

Structure determination.—After several recrystallizations, we obtained a specimen which yielded data of minimal quality to afford a structure solution; 7865 independent reflections were measured within the limit 2θ max = 50° using a Syntex $P2_1$ four-circle diffractometer in conventional 2θ/θ scan mode. Of these, 1790 with $I > 3\sigma(I)$ were considered observed and used in the 9 × 9 block diagonal least squares refinement after solution of the structure by direct methods. Also included in the refinement were 1332 reflections with $I_{calcd} > 3\sigma(I)$. No absorption correction was applied. Anisotropic thermal parameters were refined for the non-hydrogen atoms, excepting those associated with C-11–C-15 in each molecule, where thermal motion (or disorder ?) was such as to permit meaningful isotropic refinement only; these atoms were assigned as carbon on the basis of chemical information, but irregular geometry throughout the string shows that the model adopted is not entirely satisfactory in this respect. Hydrogen atoms (x, y, z, U_{iso}) were included at estimated values. At convergence, residuals were (R,R′) 0.068, 0.066, reflection weights being $(\sigma^2(F_o) + 0.0005(F_o)^2)^{-1}$. Neutral atom complex scattering factors were used (25). Computation used the X-RAY 76 program system (26) implemented by S.R. Hall on a Perkin-Elmer 3240 computer. Non-hydrogen atom labeling within each molecule is shown in Figure 2.[1]

ANTIMICROBIAL ASSAY.—The minimum inhibitory concentration (MIC) of **2** was determined using a broth dilution method against 10 organisms (Table 3). The compound was dissolved in DMSO and diluted to a starting concentration of 2.5%. Inocula were prepared from fresh Tryptone Soya (TS) agar plates and grown in TS broth at 37° until the absorbance (525 nm) was 0.5 units, then diluted 10^{-4} for the test. The assay was performed in multi-dishes (24-well) (Linbro) with a medium of TS broth (Oxoid) and incubated at 37° for 18 h. Inhibitory activity was determined by inspection of turbidity and verified by inoculation onto TS agar. The MIC was defined as the last dilution at which 20 or fewer colonies were produced on the plate.

[1]Atomic coordinates for this structure have been deposited with the Cambridge Crystallographic Data Centre and can be obtained on request from Dr. Olga Kennard, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK.

LARVAL FEEDING ASSAY.—Tissue culture plates (24-well) (Flow Laboratories) were filled with molten artificial diet (27) to the depth of 1 cm and allowed to cool. Xenorhabdin 2, dissolved in DMSO at the desired concentration, was applied (30 μl) to the surface of the diet and spread with a clean glass rod according to the method of Teakle *et al.* (28). Control plates were treated with DMSO alone. Once the surface of the diet was dry, a single first instar larvae of the Australian native budworm *Heliothis punctigera* Wallengren was added to each well (16 wells per dose) using a fine camel hair brush, and the well was closed with a polypropylene ball. The plates were then incubated for 7 days at 28°, after which they were examined individually for mortality and surviving larvae were weighed. The LC_{50} was determined using a probit analysis program.

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