Biologically Active Metabolites from Xenorhabdus Spp., Part 2. Benzopyran-1-one Derivatives with Gastroprotective Activity

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BIOLOGICALLY ACTIVE METABOLITES FROM *XENORHABDUS* SPP., PART 2. BENZOPYRAN-1-ONE DERIVATIVES WITH GASTROPROTECTIVE ACTIVITY

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ABSTRACT.—Two novel antibiotic compounds, named xenocoumacins 1 [**2**] and 2 [**3**], with potent antiulcer activity were isolated from cultures of *Xenorhabdus* spp. Both compounds exhibit antibacterial activity and potent antiulcer activity against stress-induced ulcers when dosed orally. In addition, **2** has antifungal activity. Their chemical structures were determined by extensive ^{1}H-nmr, ^{13}C-nmr, and mass spectral studies to be 3,4-dihydro-8-hydroxy-1*H*-2-benzopyran-1-one derivatives.

The bacteria of the genus *Xenorhabdus* (Enterobacteriaceae) are known to exhibit antibiotic activity (1) and to be symbiotically associated with the insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae (2,3). After invading the hemocoel of the host insect, the nematodes release a toxin (4,5) and an inhibitor of the insect immune system (6), as well as releasing their bacterial symbiont. These actions either cause pathogenicity directly or predispose the insect to the development of septicemia. The bacteria proliferate in the insect host and enhance nematode reproduction by providing nutrients and by producing antibiotics which inhibit the growth of other microbial flora in the insect cadavers (1,7).

We have investigated the antibiotic metabolites of *Xenorhabdus* spp. as part of our studies leading to the commercial development of insect pathogenic nematodes for use as bioinsecticides. In Part 1, we reported the isolation, structures, and biological activity of a series of five dithiolopyrrolone derivatives, xenorhabdins, which were isolated from *Xenorhabdus bovienii* and an undescribed *Xenorhabdus* sp. There have been two previous reports on the isolation of antimicrobial metabolites from *Xenorhabdus* spp. (8,9). These were indole and hydroxystilbene derivatives isolated from *X. bovienii* and *Xenorhabdus luminescens*, respectively.

During the isolation of the xenorhabdins it was observed that there was antibiotic activity associated with an H_2O-soluble fraction. The fraction differed from the xenorhabdins in solubility and lacked the distinctive yellow color and the characteristic uv-visible absorbance spectrum of the xenorhabdins. The presence of other antibiotic compounds had been suspected because there were a number of strains of *Xenorhabdus* spp. whose culture broths had high antibiotic activity although no xenorhabdins were evident.

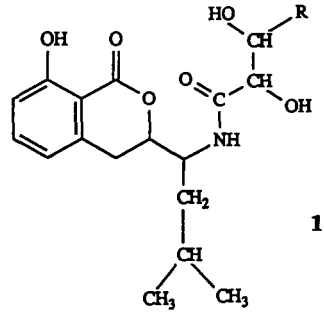
In this paper we report the isolation, structural determination, and pharmacological activity of two benzopyran-1-one (isocoumarin) derivatives, named xenocoumacins, from the culture broth of *Xenorhabdus* spp. These compounds have novel structures and display antibacterial, antifungal, and interestingly, potent antiulcer activity. They belong to the same class of compounds with regard to structure and pharmacological ac-

tivity as the amicoumacins (10–12) and AI-77 series of compounds (13–15), although they have been isolated from a different source.

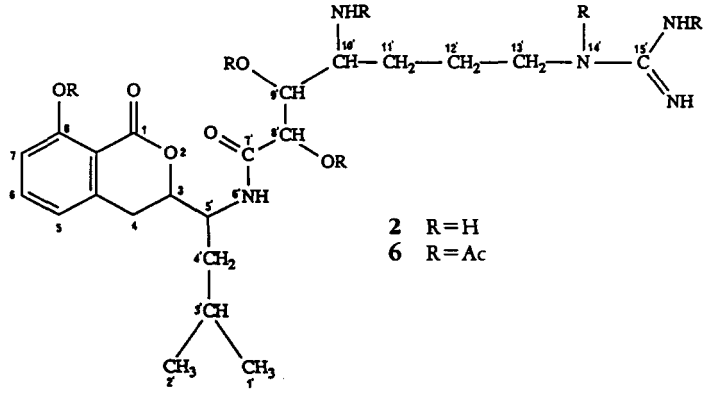
RESULTS AND DISCUSSION

The initial isolation of an H_2O-soluble, non-xenorhabdin, antibiotic compound was achieved from a culture of *Xenorhabdus* sp. strain Q1 (16). This strain is also a producer of xenorhabdins. The culture broth was subjected to adsorption cc (Amberlite XAD-2 resin) and the crude active component eluted in an aqueous MeOH fraction. After EtOAc extraction, the fraction was further purified by size exclusion chromatography. The active component failed to elute from the column in H_2O but was subsequently eluted with aqueous HOAc (10%). This fraction was purified to homogeneity by isocratic reversed-phase hplc to give a single active compound named xenocoumacin 1 [**2**]. The above isolation procedure was guided by an antibacterial bioassay using *Micrococcus luteus* as the test organism.

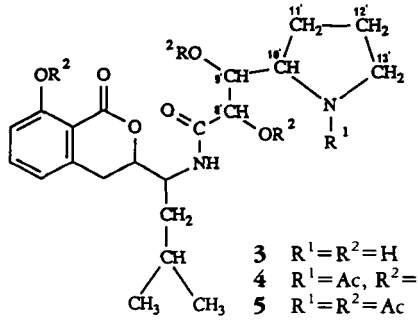
Another active compound, named xenocoumacin 2 [**3**], was isolated from a fermentation of *Xenorhabdus nematophilus* strain All (2). This strain was subsequently used for the production of material for pharmacological studies. Yields of up to 300 mg/liter of



1



2 R=H
6 R=Ac



3 $R^1=R^2=H$
4 $R^1=Ac$, $R^2=H$
5 $R^1=R^2=Ac$

xencoumacin 1 and 100 mg/liter of xenocoumacin 2 were obtained from cultures of the All strain in TSB medium.

The capacity and efficiency of the isolation procedure described above were improved, and gram quantities of both xenocoumacins were produced. Flash chromatography with octadecyl silica instead of XAD resin cc was employed as the initial purification step. This was followed by size exclusion chromatography with aqueous HOAc (0.5%) as the eluent and a final purification using reversed-phase hplc.

Xenocoumacins were produced only by phase one (16,17) of *Xenorhabdus* sp. strain Q1 and *X. nematophilus* strain All and not phase two. This is in agreement with the results of Akhurst (1) who showed that only phase one had antibiotic activity.

Xenocoumacins 1 and 2 exhibited ir and uv spectra (in neutral and alkaline solutions) characteristic of 3,4-dihydro-8-hydroxyisocoumarin, suggesting a relationship with the amicoumacins and AI-77 series of compounds which have the general structure 1, with an amino acid derived fragment attached at C-9′. Interpretation of the high field ^{1}H- and ^{13}C-nmr spectra and mass spectra defined the structures of xenocoumacin 1 as **2** and xenocoumacin 2 as **3**.

Xenocoumacin 2, $C_{21}H_{30}N_2O_6$, formed a neutral, *N*-acetyl derivative **4** and a tetraacetyl derivative **5** in which three hydroxyl groups were also acetylated. The *N*-acetyl derivative **4** showed in its ^{1}H-nmr spectrum the presence of a strongly H-bonded hydroxyl proton (at C-8) (δ 10.8) and an amidic NH (δ 7.9) coupled to H-5′. The ^{1}H-nmr spectrum (Table 1) of xenocoumacin 2 was totally resolved except for coincidental overlap of signals from H-8′/H-5′ (δ 4.17) and H-11′a/H-12′a (δ 1.92). Decoupling experiments established the connectivities H-5 to H-7 and H-4, -3, -5′, -4′, -3′, and -1′, -2′. An nOe enhancement of the signal from H-5 (δ 6.78) on irradiation of H_2-4 completed the connectivity chain. Likewise, the connectivity chain H-8′ to H_2-13′ was completely established by decoupling experiments. The ^{13}C-nmr spectra provided confirmation of the structure **3**; the chemical shift values, multiplicities (determined by DEPT methods), and coupling constants were all in accord with the environments postulated in **3**. A ^{1}H-^{13}C correlation spectrum allowed the specific ^{13}C assignments shown in Table 2. In particular a CH (δ 62.5) and a CH_2 (δ 46.6), preferentially deshielded, are consistent with the presence of the pyrrolidine ring. The ^{13}C assignments are essentially in agreement with the corresponding values reported for the amicoumacins (12) and AI-77 compounds (14), but our correlation spectrum indicated a reversal of assignment for C-4 and C-4′ from the amicoumacins and a reversal of assignment for C-3′ and C-4′ from the AI-77 compounds.

The ^{1}H-nmr spectrum of the *N*-acetyl derivative **4** was similar to that of **3** except that the signals from H-10′ and H_2-13′ had shifted downfield significantly, in keeping with the introduced amide character. In the spectrum of the peracetate **5** the signals from H-8′ and H-9′ were also shifted downfield; in this case a conformational change has also occurred, resulting in a small value for $J_{8',9'}$.

The connectivities in the spectra of **4** and **5** were confirmed completely by decoupling experiments and by a 2D COSY spectrum of **5**. The ei and positive and negative ion ci mass spectra of **5** support the structures for xenocoumacin 2.

The structure **2** for xenocoumacin 1 was determined by analysis of the ^{1}H- and ^{13}C-nmr spectra of the compound and of the hexaacetyl derivative **6**. The ms data supported these postulations. It was found that the highly basic guanidino group in xenocoumacin 1 [**2**] led to retention of HOAc used in the isolation procedure. Because of this there were spurious signals in initially obtained spectra which made interpretation of the spectra difficult.

The ^{1}H-nmr spectra obtained initially in D_2O and DMSO-d_6 solvents contained broad peaks, presumably because of association effects, and were not suitable for analy-

TABLE 1. ^{1}H-nmr Data for Xenocoumacins and Derivatives.

Proton	Compound									
	2[a]		6[b]		3[c]		4[d]		5[d]	
	δ	Multiplicity, J (Hz)	δ	Multiplicity, J (Hz)	δ	Multiplicity, J (Hz)	δ	Multiplicity, J (Hz)	δ	Multiplicity, J (Hz)
H-3	4.59	ddd, 8.2,4.5,4.0	4.09	ddd, 12.6,3.5,2.8	4.76	td, 7,4.8	4.60	dt, 12.7,2.4,2.0	4.50	ddd, 12.5,2.5,1.5
H-4A	2.94	dd, 16.8,8.2	2.53	dd, 16.8,2.8	2.96	d, 7	2.83	dd, 16.8,2.4	3.30	dd, 16.8,13
H-4B	2.98	dd, 16.8,4.5	2.97	dd, 16.8,12.6	2.96	d, 7	3.11	dd, 16.8,12.7	2.89	dd, 16.8,2.5
H-5	6.80	d, 8.0	6.84	d, 8.0	6.78	d, 8	6.71	d, 8	7.03	d, 8
H-6	7.45	t, 8.0	7.20	t, 8.0	7.44	t, 8	7.92	t, 8	7.52	t, 8
H-7	6.82	d, 8.0	6.86	d, 8.0	6.81	d, 8	6.71	d, 8	7.12	d, 8
H-1′	0.82	d, 6.6	0.89	d, 6.6	0.82	d, 6.5	0.93	d, 6.6	0.93	d, 6.6
H-2′	0.90	d, 6.6	0.91	d, 6.6	0.89	d, 6.6	0.96	d, 6.6	0.97	d, 6.6
H-3′	1.58	m[e]	1.66	m[e]	1.56	m	1.68	m	1.7	m
H-4′A	1.41	dd, 13.2,7.2,4	1.20	ddd, 13.5,9.1,4.2	1.39	ddd, 14,9.5,3.6	1.50	ddd, 13.5,8.6,5	1.48	ddd, 13.5,8.5,5.5
H-4′B	1.64	m[e]	1.81	ddd, 13.5,9.1,4.2	1.69	ddd, 14,10.5,4.5	1.78	m	1.88	ddd, 13.5,10,5.5
H-5′	4.20	dt, 9.8,4,4	4.24	tdd, 9.1,9.1,3.5,4.2,1.4	4.17	ddd, 10.5,4.8,3	4.35	tdd, 10,10,5,2	4.34	m, 8.5,8.5,5,1.5
H-6′	—	—	9.00	d, 9.1	—	—	7.9	d, 10	8.69	d, 8.5
H-8′	4.27	d, 6.1	5.32	d, 1.7	4.16	d, 6.6	3.90	d, 7.8	5.21	d, 1.5
H-9′	4.11	dd, 6.1,4.0	5.09	dd, 7.2,1.7	4.10	dd, 6.64	3.70	dd, 7.8,4.4	5.14	dd, 9.9,1.5
H-10′	3.47	ddd, 8.4,4.0,3.5	4.43	qd, 7.4,7.4,7.4,2	3.70	ddd, 9,7,4	4.21	ddd, 9,7,4.4	4.56	t(br), 7.5,2
H-11′A	1.70	m[e]	1.37	m[e]	1.92	m	1.91	m	1.63	m
H-11′B	1.83	m[e]	1.50	m[e]	2.10	m	2.27	m	1.7	m
H-12′A	1.7	m[e]	1.50	m[e]	1.92	m	1.88	m	2.01	m
H-12′B	1.7	m[e]	1.50	m[e]	2.06	m	2.04	m	1.7	m
H-13′A	3.19	t, 6.4	3.39	q, 6	3.27	dd, 8,5	3.53	ddd	3.45	q(b) 10
H-13′B	3.19		3.39		3.27		3.63	ddd	3.54	td, 10,10,3
			[f]				[g]		[h]	

[a]B.Cl in D_2O; dioxan δ 3.70.
[b]In $CDCl_3$-C_6D_6 (3:2).
[c]In D_2O; dioxan δ 3.70.
[d]In $CDCl_3$.
[e]Overlapping signals.
[f]Other signlas: H-14′ (δ 9.00, t, 6), NH-10′ (δ 6.59, d, 9.8), Ac (δ 1.81, 1.86, 1.89, 1.94, 2.13, 2.23).
[g]Other signals: Ac (δ 2.12), 8′-OH, 9′-OH (broad envelope, δ 3.6), 8-OH (δ 10.8).
[h]Other signals: Ac (δ 1.95, 2.10, 2.11, 2.40).

sis. Spectra taken in $CDCl_3$ containing several drops of DMSO-d_6 gave satisfactory spectra except that the region δ 2.9–3.9 was largely obscured by an envelope from OH/NH. Nevertheless, the spectrum was useful for assigning the protons of the isocoumarin portion and H-1′ to H-6′; these data parallel those found for xenocoumacin 2. In particular only one amidic proton (H-6′, δ 7.53, coupled with H-5′) was indicated. Addition of MeOD caused removal of the latter signal and narrowing of the OH/NH envelope through exchange; the previous assignments could then be confirmed by decoupling experiments.

It was found that a sample of xenocoumacin 1 could be obtained free of HOAc by evaporation of an aqueous solution containing HCl. This sample (HCl salt) gave, unlike previous samples, a well-resolved ^{1}H-nmr spectrum in D_2O solution. The spectrum could be completely assigned by means of decoupling experiments, as shown in Table 1. Noteworthy in the spectrum were the signals from the deshielded (protonated N effect) H-10′ and H_2-13′; H-10′ showed coupling to three protons (H-9′ and H_2-11′), and H_2-13′ gave a triplet, from H_2-12′. The signals from H_2-11′ and H_2-12′ partly overlapped with those of H_2-4′ and H-3′ in the δ 1.5–1.9 region, but their presence was proved by decoupling. The presence of H_2-12′ was indicated, since the protons vicinally coupled to H-10′ were not coupled to H_2-13′.

The ^{1}H-nmr spectrum [$CDCl_3$-C_6D_6 (3:2) for best dispersion] of the peracetate **6** showed three (exchangeable) signals from amide NH. In particular H_2-13′ now gave rise to a quartet (δ 3.39) because of an additional vicinal coupling, with H-14′ (triplet, δ 9.0). The spectrum was fully assigned by decoupling experiments and by a ^{1}H 2D COSY correlation spectrum. The ^{13}C-nmr spectrum of xenocoumacin 1 paralleled that

TABLE 2. ^{13}C-nmr Data for Xenocoumacins 1 [**2**] and 2 [**3**].[a]

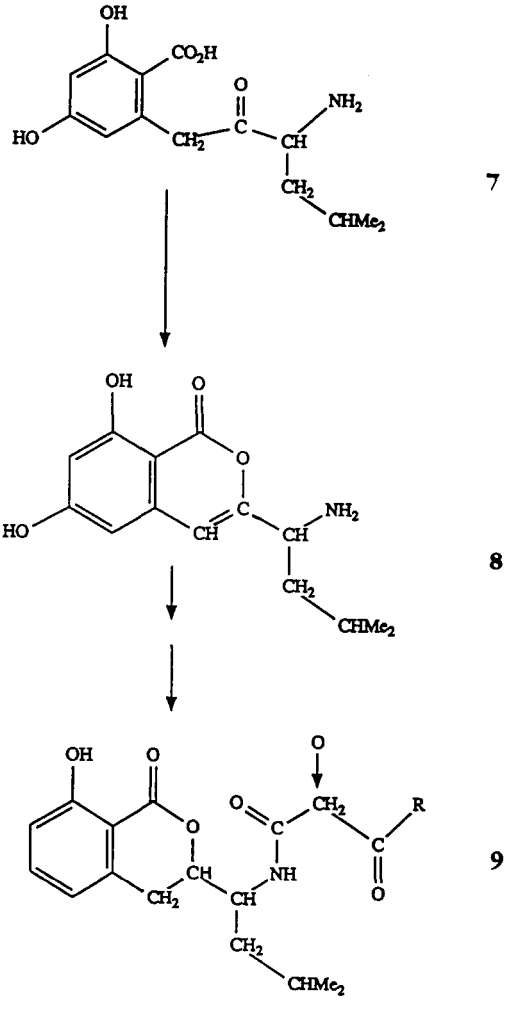
Carbon	Compound			
	2		**3**	
	δ	$J_{C\text{-}H}$ (Hz)	δ	$J_{C\text{-}H}$ (Hz)
C-1	170.9	s	171.1	s
C-3	82.5	d 151	82.6	d 152
C-4	30.4	t 132	30.4	t 130
C-4a	141.2	s	141.2	s
C-5	120.5	dd 162, 6	120.4	dd 157, 4
C-6	138.3	d 161	138.3	d 161
C-7	116.8	dd 162, 7	116.9	dd 164, 7
C-8	161.4	d 9.6	161.6	d 9.6
C-8a	109.1	s	109.3	s
C-1′	22.2	q 126	22.2	q 125
C-2′	23.6	q 126	23.8	q 125
C-3′	25.5	d 126	25.6	d 126
C-4′	40.0	t 124	40.0	t 126
C-5′	50.9	d 141	50.8	d 140
C-7′	174.5	s(br)	174.4	s(br)
C-8′	73.7	d 144	74.4	d 147
C-9′	72.1	d 144	70.7	d 145
C-10′	55.1	d 141	62.8	d 145
C-11′	25.7	t 126	25.8	t 130
C-12′	26.0	t 126	24.6	t 130
C-13′	42.0	t 138	46.9	t 148
C-15′	158.0	t (br)3		

[a]100.62 MHz, D_2O solution; dioxan δ 67.8.

of xenocoumacin 2 (see Table 2). The additional feature in the spectrum of **2** was the singlet at δ 158 due to the guanidine carbon. The assignments were made on the basis of multiplicities from DEPT experiments, ^{1}H-coupled spectra, and a ^{1}H-^{13}C correlation spectrum.

A high resolution mass measurement of the $[M-H]^-$ ion obtained in a fab mass spectrum of xenocoumacin 1 indicated its molecular formula is $C_{22}H_{35}N_5O_6$. Ei and positive ion and negative ion ci spectra of the hexaacetyl derivative **6** supported this formula. A principal fragment ion of *m/z* 184 in the ei and pci mass spectra of **6** has the composition $C_8H_{14}N_3O_2$ and contains the guanidino group. Ions of *m/z* 163, 206, and 249 arise from the isocoumarin moiety of xenocoumacin 1. The presence of the guanidine residue in xenocoumacin 1 was confirmed by a positive reaction to the Sakaguchi test.

The novel isocoumarin system present in the xenocoumacins and the amicoumacins/AI-77s can be considered to arise from leucine and four acetate units to form a polyketide chain which is cyclized to give an acylated orsellinic acid system **7** and thence the isocoumarin **8**; at some point deoxygenation occurs at C-6, and reduction at C-3, C-4, to produce the final dihydroisocoumarin system **9** (Scheme 1). The amino



SCHEME 1

group of **8** is acylated by a unit derived from HOAc and an amino acid, arginine in the case of xenocoumacin 1 and proline in the case of xenocoumacin 2. In contrast the amicoumacins/AI-77s presumably involve aspartic acid. In all cases oxidation occurs at C-8′ and reduction at C-9′ to produce the diol system.

Pharmacological studies on the xenocoumacins have shown that both compounds have antibacterial activity and potent antiulcer activity. Xenocoumacin 1 also has antifungal activity. The xenocoumacins were tested for antibacterial activity against a wide range of bacterial organisms. A summary of the results can be found in Table 3. There was high activity, especially by xenocoumacin 1, against most Gram-positive bacteria including *Staphylococcus* and *Streptococcus* species and also against some strains of *Escherichia coli*. However, most enterobacteria and *Pseudomonas aeruginosa* were resistant, as was the multi-resistant V573 strain of *Staphylococcus aureus*.

TABLE 3. Summary of Antibacterial Activity of Xenocoumacins 1 [**2**] and 2 [**3**].

Organism	MIC (μg/ml)[a]	
	2	**3**
Escherichia coli ESS	0.5	2.5
Escherichia coli 10418	2.5	50
Escherichia coli DCO	10	>100
Other enterobacteria[b]	>100	>100
Pseudomonas aeruginosa 1771P	>100	>100
Acinetobacter lwoffii BRL 2400	2.5	5
Bacillus megaterium NCIB 9376	2.5	5
Corynebacterium xerosis NCTC 9755	1	5
Sarcina lutea ATCC 8740	1	5
Staphylococcus aureus Oxford NCTC 6571	10	5
Staphylococcus aureus V573	25	>100
Streptococcus pyogenes CN10	1	5
Streptococcus sanguis ATCC 10556	10	10

[a]Minimum inhibitory concentration.

[b]*Enterobacter cloacae* N1, *Klebsiella aerogenes* A, *Citrobacter freundii* ES, *Salmonella typhimurium* CT10, and *Proteus mirabilis* C977.

High antifungal activity was seen for xenocoumacin 1 against *Cryptococcus neoformans* where MIC (minimum inhibitory concentration) values were 0.125 μg/ml against the light inoculum and 0.5 μg/ml for the heavy inoculum using a complex medium (the values were four-fold higher with the defined medium). Moderate to weak activity was seen against *Aspergillus niger* (10 μg/ml), *Aspergillus fumigatus* (100 μg/ml), *Trichophyton mentagrophytes*, and *Trichophyton rubrum* (2–8 μg/ml, defined medium, 32 μg/ml, complex medium with the heavy inoculum), but **1** was inactive (at 128 μg/ml) against *Candida albicans* and *Candida parapsilosis*. Xenocoumacin 2 was inactive (at 128 μg/ml) against the above fungi.

Both xenocoumacins 1 and 2 display potent antiulcerogenicity against experimental, stress-induced gastric ulcers in rats. As shown in Table 4, both compounds are effective when administered orally, with xenocoumacin 2 having superior activity and significantly inhibiting ulcers at 10 mg/kg (po).

The range of pharmacological activities demonstrated by the xenocoumacins is similar to that of the AI-77 (13,15,18) and amicoumacin (10,11) compounds. These compounds, as shown above, are structurally related to the xenocoumacins. They have a similar spectrum of antibacterial activity, and potent gastro-protective activity has

TABLE 4. Antiulcer Activity of Xenocoumacins 1 [2] and 2 [3] Against Stress-Induced Gastric Ulcers in Rats.

Compound	Dose mg/kg, po	Protective Value (%)
Xenocoumacin 1	25	74[b]
	10	8 NS
Xenocoumacin 2	25	70[a]
	10	61[a]
	5	26 NS

[a]$P<0.05$.
[b]$P<0.01$.

been reported for amicoumacin A, AI-77-A, AI-77-B, and AI-77-C2. It has been shown that the antiulcer activity of AI-77-B is not associated with central suppressive, anticholinergic, and antihistaminergic effects (19). Like the AI-77s toxicity may be a problem with the use of the xenocoumacins as a therapeutic agent.

Though the xenocoumacins belong to the same class of compounds with regard to structure and pharmacological activity as the AI-77/amicoumacin compounds, the latter were isolated from a taxonomically distant organism, *Bacillus pumilus*. This phenomenon was also seen with the xenorhabdin antibiotics in Part 1 of this study, where these dithiolopyrrolone compounds isolated from *Xenorhabdus* spp. had previously been isolated only from *Streptomyces* spp.

In general, the diversity of the bioactive metabolites produced by *Xenorhabdus* spp. is remarkable. Two diverse classes of metabolites were isolated, the xenorhabdins and xenocoumacins, with antimicrobial activity as well as other pharmacological properties. Other antibiotic compounds have previously been isolated: indole derivatives from *X. bovienii* strain R, and stilbene derivatives from *X. luminescens* strains Hb and HK (8,9).

The presence of many different antibiotic classes and their variation in occurrence within strains of *Xenorhabdus* spp. helps to explain the strain-to-strain variation in antibiotic spectrum observed by Akhurst (1). It also explains how one strain can inhibit another. It is unclear, however, whether the production of xenorhabdins, xenocoumacins, and the other antibiotic compounds isolated from *Xenorhabdus* spp. is sufficient to explain all the variation. It is possible that there are other antibiotics being produced, and further work is needed in this area. Additional studies are also needed to survey the relative abundance of the xenorhabdins and xenocoumacins within *Xenorhabdus* spp.

EXPERIMENTAL

GENERAL PROCEDURE.—Flash chromatography was performed using Whatman octadecyl silica (50–70 μm). The gel filtration media, Sephadex G-10 and G-25, were supplied by Pharmacia and the Amberlite XAD-2 resin by Rohm and Haas. The gel filtration chromatography was monitored at 254 nm using an LKB 2238 Uvicord SII, and fractions were collected in an LKB 2211 Superrac. Hplc was performed using Whatman Partisil 10 ODS columns for reversed-phase chromatography and a Whatman Partisil 10 Silica column for normal phase. A Waters hplc system was employed incorporating a Model 590 pump, U6K injector, and a Model 480 variable wavelength LC spectrophotometer. Tlc was carried out on Merck precoated Kieselgel F254 plates and visualized under uv light (254 nm).

Fermentations were carried out in E-series laboratory fermenters (B. Braun, Melsungen, FRG). Centrifugation of the fermentation culture was performed using a Beckman J2-21M centrifuge equipped with a JA-10 rotor. Uv spectra were obtained on a Varian DMS 90 spectrophotometer, and ir spectra were obtained on a Pye Unicam SP3-200 spectrophotometer.

^{1}H-nmr spectra were measured at 400 MHz for $CDCl_3$ solutions (unless otherwise stated) on a Bruker WM-400 instrument. The reference was TMS unless otherwise stated. NOe spectra were obtained in the difference mode. Chemical shifts and coupling constants were derived from first-order considerations and

were confirmed by double and/or triple resonance experiments wherever possible. 2D shift-correlated (COSY) spectra were obtained by the general method of Bax and Freeman (20) with a 90°-t_1-45° pulse sequence using appropriate phase cycling to ensure quadrature detection in the F_1 dimension. Either 32 or 48 transients (preceded by two dummy scans) were collected after delays of 1 sec; 2×2^{10} data points were collected in the F_2 dimension over 2500 Hz and 512 increments collected in the F_1 dimension (zero filled to 1×2^{10}), giving a $2^{10} \times 2^{10}$ matrix for absolute value calculation. A shifted sine-bell window was usually applied in both dimensions.

^{13}C-nmr spectra were measured in the noise-decoupled mode with a Bruker WM-400 (100.62 MHz) instrument. Coupled spectra with gated decoupling for nOe and with selective low-power ^{1}H decoupling were obtained by procedures previously described. Attached proton spectra were determined at 100.62 MHz with the DEPT pulse sequence. ^{1}H-^{13}C heteronuclear correlation spectra were obtained by the method of Rutar (21) with refocusing pulses to remove non-geminal ^{1}H-^{1}H couplings.

Mass spectra were determined on a VG 70-70 mass spectrometer interfaced to a VG 11-250 data system. Compounds were analyzed by flash volatilization from an extended gold support (22) with rapid data acquisition. Ei mass spectra were determined with an ionization energy of 70 eV and 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 chemical ionizaton (pci), while isobutane at a source pressure of 80 Pa was used for negative-ion chemical ionization (nci). Source temperatures were 180–210°. High-resolution mass analyses were obtained by peak matching.

Purity of the compounds was assessed by hplc or tlc and by the lack of spurious peaks in ^{1}H- and ^{13}C-nmr spectra.

CULTURING OF *X. NEMATOPHILUS*.—All strains of *Xenorhabdus* were kindly provided by Drs. R. Bedding and R. Akhurst, CSIRO Division of Entomology, Canberra, Australia.

X. nematophilus strain All (ATCC 53200), symbiont of *Steinernema feltiae* All, was cultured for 48 h in 10-liter batch fermentations at 28°, pH 7.0, with sufficient agitation and aeration to maintain pO_2 above 40% of saturation. The following medium was used: glycerol 5 g/liter, yeast extract 15 g/liter, $MgSO_4 \cdot 7H_2O$ 1.25 g/liter, $(NH_4)_2SO_4$ 2 g/liter, KH_2PO_4 0.7 g/liter, K_2HPO_4 0.9 g/liter, Na_2SO_4 1.3 g/liter. An alternative medium was Trptone Soya Broth (TSB, Oxoid).

Xenorhabdus sp. strain Q1 (ATCC 39497) is an isolate from a nematode of undescribed genus (Steinernematidae) strain Q1 found in Australia. The following medium was found to be suitable for the culture of *Xenorhabdus* sp. Q1: glycerol 20 g/liter, yeast extract 10 g/liter, $MgSO_4 \cdot 7H_2O$ 2.5 g/liter, $(NH_4)_2SO_4$ 2 g/liter, KH_2PO_4 10 g/liter, $CaCl_2 \cdot 2H_2O$ 0.29 g/liter, $FeSO_4 \cdot 7H_2O$ 27.8 mg/liter, $MnSO_4 \cdot H_2O$ 8.45 mg/liter, $ZnSO_4 \cdot 7H_2O$ 14.4 mg/liter, $CoCl_2 \cdot 6H_2O$ 0.10 mg/liter and $CuSO_4 \cdot 5H_2O$ 0.19 mg/liter. The culture was grown for 6 days before harvesting.

Cultures were grown overnight in 500-ml shake flasks containing 100 ml of the above 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) (16) 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 the phase one have a red core overlaid by blue.

ISOLATION OF XENOCOUMACINS.—The isolation was monitored by an in vitro antibacterial bioassay. 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.

Xenocoumacin 1 [**2**] was initially isolated from the culture broth of *Xenorhabdus* sp. Q1. The cells were separated from the culture broth by centrifugation (9000 rpm, 15 min). The culture broth (136 liters) was applied to a column of Amberlite XAD-2 resin (150 mm × 1 m, 17.1 liters) at 500 ml/min. After washing the column with 20 liters of H_2O, MeOH was pumped onto the column at 500 ml/min and the eluate collected in 20-liter aliquots. From the first aqueous MeOH fraction, 500 ml was lyophilized, extracted with EtOAc (3 × 200 ml), and chromatographed on Sephadex G-25 (2.5 cm × 76 cm) in H_2O. After thorough washing with H_2O, the active component was eluted with aqueous HOAc (10% v/v) and lyophilized. This fraction was further purified by semi-preparative, isocratic hplc on a reversed-phase C_{18} column (10 mm i.d. × 50 cm). The mobile phase of MeCN-NH_4OAc (30:70) (0.2 M, pH 4.5) was delivered at 4 ml/min and monitored at 254 nm. The retention time of **2** was 19 min.

From a culture of *X. nematophilus* All **2** and **3** were isolated. The cells from the fermentation culture were separated from the broth by centrifugation (9000 rpm, 15 min). The culture broth was then subjected to flash chromatography. A sintered glass funnel was uniformly packed with dry octadecyl silica (50–70 μm, 6 cm × 25 cm diameter) and covered with filter paper. Solvent flow was induced by a vacuum (10 kPa) and the eluate collected in a filter flask. The silica was washed with MeOH (1 liter) and H_2O (4 liters), and the culture broth (5 liters) was applied followed by H_2O (2 liters) and MeCN-NH_4OAc (1:1) (0.2 M, pH

4.5) (2 liters). This latter fraction was evaporated in vacuo to yield a crude mixture of xenocoumacins (3%, 21 g).

A solution of this mixture (5 g) in H_2O (20 ml) was chromatographed on Sephadex G-10 (84 × 5 cm, 1650 ml) in aqueous HOAc (0.5%) at a flow rate of 3.2 ml/min. The eluate was monitored continuously at 254 nm, and absorbances corresponding to **2** and **3** occurred at 1150–1400 ml and 1300–1600 ml, respectively. A total 3.7 g (20%) of xenocoumacins was recovered as a brown solid.

The raw xenocoumacins were subjected to preparative, isocratic hplc on a reversed-phase C_{18} column (22 mm i.d. × 25 cm) using MeCN-NH_4OAc (40:60) (0.2 M, pH 4.5) as the mobile phase delivered at 12 ml/min and monitored at 254 nm. Compounds **2** and **3** eluted at 26–30 min and 32–36 min, respectively.

Xenocoumacin 1 [**2**].—$[\alpha]^{23}D$ −57° (c = 0.7, H_2O); uv λ max (H_2O) 314 nm (ε 3300), 249 (5460); uv λ max (0.1 N NaOH) 347 nm, 248 (sh); ir (KBr) 3500–3000 (br), 2960, 1660, 1550, 1465, 1410, 1230, 1165, 1110, 1045, 805 cm^{-1}; negative ion fabms 464.2574 (calcd for $C_{22}H_{34}N_5O_6$ $[M-H]^-$ 464.2509); eims no $[M]^+$, major peaks at *m/z* 249, 206, 163, 135, 86, 60, 43, 32.

Xenocoumacin 1 hexaacetate [**6**].—Compound **2** (9.3 mg) was dissolved in Ac_2O (200 μl) and pyridine (200 μl) and left stand 16 h at room temperature. The reaction mixture was evaporated to dryness to give the crude product. Purification by isocratic reversed-phase hplc eluted with MeCN-H_2O (1:1) gave the hexaacetylated xenocoumacin [**6**]: negative ion cims *m/z* (%) $[M]^-$ 717 (25), $[M-CH_2CO]^-$ 675 (52), 657 (42), 615 (52), 403 (5), 361 (20), 254 (10), 163 (38), 150 (100); eims *m/z* (%) $[M]^+$ 717 (2), 702 (4), 675 (15), 658 (10), 632 (10), 616 (10), 512 (20), 470 (15), 341 (5), 267 (28), 184 (90) (calcd for $C_8H_{14}N_3O_2$, 184.1086, found 184.1076), 170 (28), 112 (30), 86 (50), 70 (100); positive ion cims *m/z* (%) $[M+H]^+$ 718 (100), $[MH-CH_2CO]^+$ 676 (98), 658 (8), 634 (10), 616 (8), 592 (14), 512 (10), 470 (8), 267 (14), 184 (25), 70 (14), 60 (46).

Xenocoumacin 2 [**3**].—$[\alpha]^{23}D$ −75° (c = 0.7, H_2O); uv λ max (H_2O) 314 nm (ε 3280), 249 (5530); uv λ max (0.1 N NaOH) 347 nm, 248 (sh); ir (KBr) 3500–3000 (br), 2950, 1525, 1460, 1400, 1230, 1165, 1110, 1045, 805 cm^{-1}.

Xenocoumacin 2 monoacetate [**4**].—Compound **3** (3.5 mg) was dissolved in MeOH (200 μl) and Ac_2O (200 μl) and left to stand 16 h at room temperature. The reaction mixture was evaporated to dryness and gave a single component (no starting material) on tlc [$CHCl_3$-MeOH (9:1)], R_f 0.8. The *N*-acetyl derivative **4** of xenocoumacin 2 was used without further purification.

Xenocoumacin 2 tetraacetate [**5**].—Compound **3** (18.1 mg) was dissolved in Ac_2O (1.0 ml) and pyridine (1.0 ml) and left to stand 3 days at room temperature. The reaction mixture was evaporated to dryness to give the crude product. Purification by preparative, normal phase hplc, 1% MeOH in $CHCl_3$ mobile phase, gave **5**, the tetraacetylated xenocoumacin 2: negative ion cims *m/z* (%) $[M-H]^-$ 573 (45), $[M-CH_2CO]^-$ 531 (100), 514 (8), 454 (18), 412 (12), 394 (12), 265 (10), 222 (10), 163 (8), 150 (10); eims *m/z* (%) $[M]^+$ 574 (10), 369 (10), 242 (11), 196 (10), 154 (12), 135 (5), 112 (35), 70 (100); positive ion cims *m/z* (%) $[M+H]^+$ 575 (100), $[MH-CH_2CO]^+$ 533 (12), 369 (10), 242 (8), 196 (12), 154 (8), 112 (15), 70 (17).

ANTIBACTERIAL ASSAY.—MIC versus each test organism was determined using a blood agar screen with a medium containing DST agar and 10% defibrinated horse blood. Inocula were undiluted overnight broth cultures, and the plates were incubated aerobically at 37° overnight.

ANTIFUNGAL ASSAY.—MIC versus each test organism was determined using a defined medium (Yeast Nitrogen Base broth + 1% glucose) and a complex medium (Sabouraud's glucose broth). The test compounds were dissolved in DMSO and diluted into broth to give a final DMSO concentration of <1%. Activity against *Candida* spp. and *Cr. neoformans* was determined using a microtiter method (twofold dilutions) with inocula of 10^3 and 10^5 cells/ml. Incubation was for 2 days at 37°. For *Aspergillus* spp. a tube dilution method (tenfold dilutions, i.e. 100 to 0.1 μg/ml) was used with an inoculum of 10^4 spores/ml. Incubation was for 2 days at 37°. For *Trichophyton* spp. a microtiter method (twofold dilution) was used with inocula of spore/hyphal homogenate in PBS diluted until approximately equivalent (by eye) to the turbidity of 0.5 mg/ml $BaSO_4$. This suspension was diluted to give final inocula of 10^{-2} and 10^{-4}. Incubation was for 11 days at 26°.

GASTROPROTECTIVE ASSAY.—Male wistar rats, 160–275 g body weight, were deprived of food for 18 h and then weighed, dosed orally, and placed in Bollman restraining cages. The test compounds were suspended in 1% methyl cellulose. Animals were allocated to groups using a Latin square system and a minimum group size of 7. Thirty min after dosing, the restraining cages were stood on end in open cages and placed in a cold room at 4° for 2.5 h. At the end of the stress period the animals were sacrificed and the stomach of each was removed, distended with 10 ml H_2O, incised along the greater curvature and examined under a binocular microscope. The damage was assessed according to a subjective scoring system

based on the severity of ulceration. Stomachs without ulceration were rated 0, a rating of 9 was defined as having deep damage in most parts of the glandular stomach, often in streaks; others were rated 1 to 8 depending on severity. The test scores were compared to controls using the Wilcoxon rank sum test.

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