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NEW CYANOCYCLINES FROM A CYANIDE-TREATED BROTH OF *STREPTOMYCES LUSITANUS*[1]

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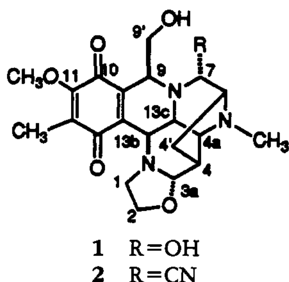
ABSTRACT.—Organic extracts of *Streptomyces lusitanus*, the producer of the anticancer antibiotic naphthyridinomycin [**1**], were found to contain two additional compounds active in an antibiotic screen. As with **1**, these reacted with NaCN added at the end of the fermentation. One of the addition products has been named cyanocycline B [**3**] and is derived from *N*-desmethylnaphthyridinomycin [**4**], while the other has been named cyanocycline C [**5**], and is derived fromthe hydroquinone **6** of **1**. Cyanocycline C is unstable and was characterized after conversion to the dimethyl derivative with CH_2N_2 in the presence of TFA. The implications of these metabolites for the biosynthesis of **1** are discussed. A third new antibiotic, cyanocycline D [**8**] was isolated from the cyanide-treated broth and proved to be an artifact in which the oxazolidine ring had been opened by cyanide. The potential relevance of the formation of **8** to the reaction of **1** with DNA is also discussed.

Naphthyridinomycin [**1**] is a broad spectrum antibiotic and potent antitumor agent produced by *Streptomyces lusitanus* AY B-1026 (1). Its structure was established by X-ray crystallography (2). Naphthyridinomycin is labile in solution and when stored as a solid but can be stabilized by conversion to the nitrile **2** with addition of NaCN to the fermentation broth during workup (3). Compound **2** has also been isolated as a natural product from cultures of *Streptomyces flavogriseus*, in which case it was named cyanocycline A (4), and its structure was confirmed by X-ray crystallography (5). A variety of other structurally related microbial metabolites have since been isolated from various organisms, including the saframycins as represented by saframycin A (6), quinocarcin (DC-52) (7), and quinocarcinol (DC-52d) (8). The biosynthesis of the novel ring system of naphthyridinomycin has been studied in considerable detail (9–11), although the origin of the C-9/C-9′ unit still remains obscure. In our continuing studies with *S. lusitanus*, we have observed by bioautography of thin layer chromatograms the presence of additional antibiotics, the R_f's of which increased, after treatment with cyanide, similar to that observed in the conversion of **1** to **2**. We now report the structures of two new naturally occurring naphthyridinomycins as well as a cyanocycline that is produced as an artifact of the workup with cyanide.

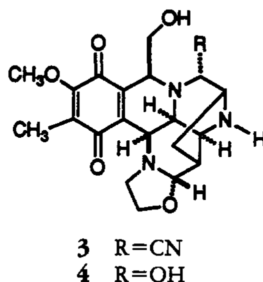
RESULTS AND DISCUSSION

S. lusitanus was grown in liquid culture as previously described (1), and tlc (10% MeOH/$CHCl_3$) examination of EtOAc or CH_2Cl_2 extracts of broths, followed by bioautography on sterile agar overlaid with a suspension of *Bacillus subtilis* ATCC 6633 spores, revealed three bioactive metabolites, one of which was **1**. A new growth medium was developed that produced significantly higher titers of the two new metabolites; however, blackstrap molasses was an essential ingredient in both media. With extracts of either broth, the least polar spot on tlc was due to **1** (R_f 0.64), and the two unknowns had R_f's of 0.41 and 0.16. In experiments where the broth was treated with cyanide before extraction, the three zones of inhibition were at R_f 0.72, 0.46, and 0.21. The increased R_f's suggested that the two new metabolites were related to **1**, and their derivatives were named cyanocycline B and cyanocycline C, respectively.

[1] This manuscript is dedicated to the memory of Professor Edward Leete.



1 R=OH
2 R=CN



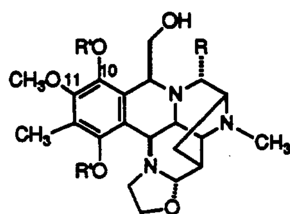
3 R=CN
4 R=OH

CYANOCYCLINE B.—Cyanocycline B was purified with a slight modification of the protocol previously used for isolation of **2**. The final step in that protocol, preparative layer chromatography (plc) on reversed-phase C_{18} plates, led to degradation, and this was replaced with plc on normal phase Si gel plates. The new cyanocyclines are minor metabolites, and only 8 mg of cyanocycline B could routinely be obtained from 10 liters of fermentation broth.

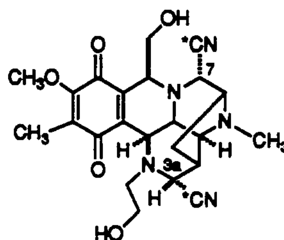
The uv spectrum of cyanocycline B showed maxima at 270 and 370 nm, indicating the presence of a benzoquinone moiety (e.g., **2**). The ^{1}H-nmr spectra of the two compounds were remarkably similar, with the key difference being lack of the *N*-Me resonance at δ 2.4. The ^{13}C-nmr spectra also were nearly identical, but now the *N*-Me carbon resonance at δ 41 was missing. Thus, cyanocycline B appeared to be *N*-desmethylcyanocycline A [**3**] and would be derived from *N*-desmethyl-naphthyridinomycin [**4**]. Hrfabms confirmed the molecular formula $C_{21}H_{24}N_4O_5$, and analysis of a COSY spectrum confirmed all the expected proton-proton couplings. Three separate spin systems could be identified: H-3a–H-4–H-4″–H-4′–H-6–H-7, H-1–H-1–H-2–H-2, and H-13a–H-13b–H-13a. A long-range coupling between H-3a and H-13b was also detected. This had been observed in the COSY spectrum of **2** (S.J. Gould and V.A. Palaniswamy, unpublished results).

CYANOCYCLINE C.—Purification of cyanocycline C proved to be much more difficult. It was extremely unstable and readily decomposed to a greenish-brown mixture that included **2**, even at −20°. Chromatography using Bio-Beads SM-7, followed by hplc on a reversed-phase cyano column, appeared to yield a homogeneous compound. However, after lyophilization both ^{1}H-nmr and hplc analysis revealed the sample had decomposed. Centrifugal countercurrent chromatography (ccc) proved to be a milder method, and a relatively pure sample could be obtained using iPrOH-MeOH-$CHCl_3$-H_2O (10:10:3:10). Immediate tlc and bioautography revealed only one spot that was bioactive. However, attempts to crystallize this material were unsuccessful, and re-examination of the sample by tlc and bioautography revealed that a significant amount of **2** was now present. It was now possible to observe that solutions of cyanocyclinc C did not have the distinctive color of the benzoquinones (**2** is orange-red and **4** is orange) but did have the typical cyanocycline yellow color on tlc. A hydroquinone was suspected to be present, and a new sample was prepared by ccc and the ^{1}H-nmr spectrum immediately recorded. The spectrum contained three methyl singlets, but the resonance of the ring methyl was now shifted downfield from δ 1.94 to 2.20 and the methoxy methyl resonance was shifted upfield from δ 4.02 to 3.80, consistent with a hydroquinone structure. Reduction of cyanocycline A with either sodium dithionite or catalytic hydrogenation gave **5** with the same spectrum. Hydroquinone **5** would be derived from dihydronaphthyridinomycin A [**6**].

Derivatization of **5** with Ac_2O in pyridine or with NaOAc gave only partially acetylated material at room temperature, and at elevated temperature decomposition



5 R=CN, R′=H
6 R=OH, R′=H
7 R=CN, R′=Me



8 *=12
9 *=13

was observed. Treatment of **5** with CH_2N_2 in Et_2O or in Et_2O/EtOH gave no reaction unless a trace of TFA was added. This was apparently necessary to break the strong hydrogen bonding between the oxygen at C-11 and the phenol at C-10. Under these conditions, methylation yielded **7**. A low yield of the same product could be obtained by methylating partially purified naturally occurring cyanocycline C obtained from chromatography on Bio-Beads SM-7. The ^{13}C-nmr spectrum of **7** lacked resonances for quinone carbons, but six aromatic resonances between δ 123 and 154, as well as the two additional methyl resonances at ca. δ 45, were observed. Analysis of a COSY spectrum confirmed the proton assignments. The long-range H-3a–H-13b coupling was again observed.

CYANOCYCLINE D.—A third minor component in the original cyanide-treated CH_2Cl_2 extract was initially overlooked because its R_f was nearly identical to that of **4**. During workup of a large-scale fermentation, a considerable quantity of this new compound could be discerned. While **4** and this new compound, cyanocycline D, could be separated from other metabolites with a Chromatotron®, further separation of these two was very inefficient by either normal or reversed-phase Si gel chromatography. However, once again careful tlc and bioautography experiments revealed that cyanocycline D could also be converted to cyanocycline A.

The ^{1}H-nmr spectrum of cyanocycline D showed all the resonances similar to those of **2**. The major differences were the chemical shifts assigned to the protons at C-1 and C-2, which indicated a change in the oxazolidine ring. Hrfabms gave an $[M+1]^+$ ion with the molecular formula of $C_{22}H_{24}N_5O_6$ at *m/z* 236, equivalent to the addition of HCN to **2**. Apparently, the oxazolidine ring had been opened. The ^{1}H-nmr spectrum now contained two exchangeable resonances at δ 2.5 and 4.5. A COSY spectrum showed that the δ 4.5 resonance was coupled to one of the protons at C-9′, and was assigned to 9′-OH, while the δ 2.5 resonance was coupled to the C-2 protons. Consistent with cleavage of the O–C-3a bond, the δ 93 resonance of the oxazolidine of cyanocyclines A–C was missing and new resonances were present at δ 55 and 118.

While cyanocycline D appeared to be structure **8**, the chemical shift for H-3a at δ 4.67 seemed unusually deshielded and was similar to that of H-3a in **2**. In contrast, the H-7 resonance always lies between δ 3.6 and 3.9 in the cyanocyclines. It had, in the meantime, been established that **2** could be converted to **8** by treatment with excess cyanide, and **9** was now prepared using sodium [^{13}C]cyanide. The ^{1}H-nmr spectrum of the labeled sample showed a ^{13}C-3a–H-3a coupling with $^2J_{CH}$=7.8 Hz. A long-range HETCOSY (12) spectrum of this sample showed not only this coupling, but also a weaker signal for ^{13}C-7–H-7 coupling due to partial exchange of the original cyano group of **2** in the conversion to **9**. These experiments provided confirmation of the structure of cyanocycline D. The H-3a–H-13b long-range coupling was observed in the COSY spectrum of **8**, revealing that the stereochemistry at H-3a was again *R*.

BIOLOGICAL ACTIVITIES.—The two new metabolites, **4** and **6**, and the three new cyanocyclines, **3**, **5**, and **8**, are active against a variety of bacteria, while dimethylcyanocycline C [**7**] is not. The hydroquinone could not be tested quantitatively due to its instability. Bioautography of tlc plates containing **7** did show activity, but this may have been due to prior oxidation to **2** on the Si gel. The data are given in Table 1.

TABLE 1. Minimum Inhibitory Concentrations (μg/ml) of Cyanocyclines A, B, and D Against Selected Microorganisms.

Test Organism	Compound		
	2	**3**	**8**
Escherichia coli ATCC 10536	0.125	0.250	0.500
Proteus vulgaris[a]	0.250	1.000	0.500
Klebsiella pneumoniae A-"AD"[b]	0.125	0.031	0.125
Serratia marcescens ATCC 13880	0.500	0.500	0.250
Bacillus subtilis ATCC 6633	0.031	0.016	0.061
Micrococcus luteus ATCC 4698	0.031	0.063	0.125
Streptococcus faecalis ATCC 29212	0.250	0.250	0.250

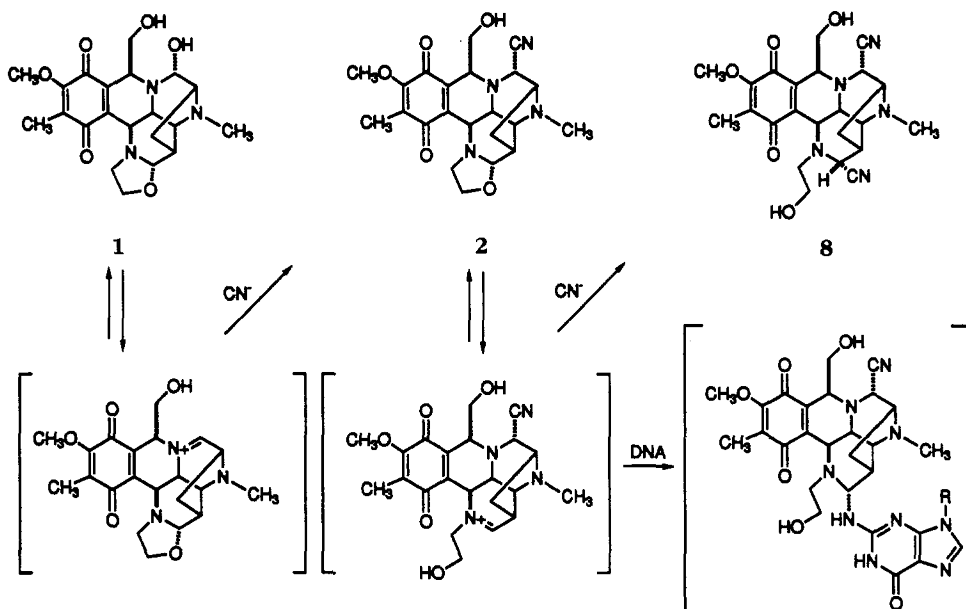
[a]Obtained from Oregon State University Department of Microbiology.
[b]Obtained from Lederle Laboratories.

The oxazolidine ring of cyanocycline has been shown to open reversibly upon treatment with dilute HCl or HBr (5). Thus, it is clear that **8** was produced from **2** during the workup with NaCN. The carbinolamines **4** and **6**, from which compounds **3** and **5** were derived, however, are new metabolites of *S. lusitanus* and shed light on the last steps in the biosynthesis of naphthyridinomycin. The presence of the hydroquinone indicates that oxidation to the quinone may be the last step in the pathway. Air oxidation of **6** yields numerous products besides **2**, and the relative simplicity of fermentation extracts suggests that in vivo oxidation of **6** is enzymatic. *N*-Methylation yielding **6** is apparently the penultimate step, and it would seem only coincidental that the norcyanocycline hydroquinone has not yet been isolated from cyanide-treated broths. Additional, quite minor bioactive spots have been occasionally observed on tlc plates.

The lack of biological activity for dimethylcyanocycline C supports the view that the benzoquinone moiety of naphthyridinomycin antibiotics plays an important role in the mechanism of action of DNA binding (13). Computer modeling studies have indicated that the hydroquinone or quinone is involved in hydrogen bonding inside the minor groove of DNA. The loss of binding to DNA by the dimethyl derivative of the hydroquinone moiety is consistent with the tight fit. Cyanocycline D, derived from cyanocycline A by cyanide treatment during the isolation process, represents a new semisynthetic antibiotic in the naphthyridinomycin family. Bioactivity of this compound supports an alternative mechanism of action involving the oxazolidine ring for this type of antibiotic, which has been proposed by Remers' group from other modeling studies (14). Thus, the mechanism of drug and DNA binding at C-3a can be written as shown in Scheme 1. In this mechanism, when a nucleophilic reagent such as cyanide is available in the medium, cyanocycline D or an analogue could form. Due to the specific configuration of the iminium ion intermediate, only an approach from the α face (the convex side) is possible, which results in an *R* configuration at C-3a.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Ir spectra were recorded on a Nicolet 5DXB Ft-ir spectrometer. Uv spectra were obtained on an IBM-9420 Uv-Vis spectrometer or with a Waters 990+photodiode



SCHEME 1

array hplc detector (see below for hplc conditions). ^{1}H-nmr spectra were determined on either a Bruker AM 400 spectrometer or a Bruker AC 300 spectrometer and referenced with TMS at δ 0.00 unless indicated otherwise. ^{13}C-nmr were determined on either a Bruker AM-400 at 100.61 MHz or on a Bruker AC-300 at 75.47 MHz with TMS (δ 0.00 ppm) or $CDCl_3$ (δ 77.0 ppm) as an internal reference. High resolution fab mass spectra were obtained on Kratos MS 50 TC spectrometer with 3-nitrobenzyl alcohol or glycerol as a matrix. Melting points were determined on a Buchi Melting Point apparatus and are uncorrected. Hplc was performed on either an IBM LC/9533 or on a Waters 600E or Waters 6000A system with a 4.6×250 mm cyano column, eluting with MeCN-MeOH-H_2O (0.5% TFA) (70:10:20) (retention times: **5** 15 min, **2** 17 min, **3** 25 min). Tlc was done on EM-Reagents Si gel aluminium-backed plates or glass-backed plates. Reversed-phase tlc was done on Whatman C-18 tlc plates (1 mm or 0.5 mm). Centrifugal tlc was done on a Chromatotron® with a 1 mm or 2 mm Si gel plate. Centrifugal countercurrent chromatography was done on an Ito Model 1 Multilayer Coil Separator-Extractor. Bio-Beads SM-7 were obtained from BioRad Laboratories.

BIOASSAY AND BIOAUTOGRAPHY.—*Bioassay*.—Bioassays were done by the agar diffusion method (15). Petri dishes (150 mm) were prepared with a 25 ml base layer of trypticase peptone (5.0 g) and Bacto agar (15.0 g) in distilled H_2O (1000 ml) without pH adjustment. A 15 ml seed layer was overlaid on the base layer. To prepare the seed layer, an aliquot (ca. 0.45 ml) of a 1:50 dilution in saline of *B. subtilis* ATCC 6633 spore suspension (Baltimore Biological Laboratories, BBL) was mixed with 15 ml of agar at 50°. These plates could be kept at 4° for up to 1 week. Up to eight stainless steel cylinders (5 mm i.d., 7 mm tall) were arranged on the agar plate. To test the antibiotic activity of a fermentation broth, a sample (1 ml) was centrifuged for 4 min (Beckman microfuge), and an aliquot (150 μl) was transferred into each cylinder. The assay plates were incubated at 37 ° for 12 h, and the quantity of the antibiotic produced correlated to the size of the inhibition zone.

Bioautography.—All bioautography experiments were done on Merck Si gel plates with an aluminum backing. The test sample was applied to a tlc plate, 1×10 cm or 2×10 cm, and developed using an appropriate system. After development, the organic solvents were removed completely by drying the tlc plate first in air and then under vacuum. The colored or uv sensitive compounds on the tlc plate were marked with pencil and the tlc plate was then placed upside down for 4 min on a seeded agar plate. During that time, the tlc origins, solvent front, and the positions of colored or uv sensitive spots were marked on the back of the agar plate. The tlc plate was then removed and discarded, and the agar plate was incubated at 37° for 12 h. The inhibition zone and pattern of the bioautography results could be recorded by photocopying the back of the agar plate. If higher sensitivity was required, the developed tlc plates were taped face up in the bottom of a Petri dish, and the base layer and seed layer then were overlaid successively.

MINIMUM INHIBITORY CONCENTRATIONS.—Culture tubes (20 ml) containing brain heart infusion

media (BHI, Difco, 5 ml) were each inoculated with a loopful of bacterial suspension. After incubation at 37° for 24 h, each was transferred to a second aliquot of BHI broth (5 ml), and incubated for an additional 8 h. These cultures were diluted with sterile 0.9% NaCl to match the Macfarland 0.5 turbidity standard (ca. 10^8 cfu/ml), and then further diluted 20-fold with BHI medium. A series of antibiotic dilutions [$\log_2$ (two-fold)] was prepared in small vials, and 50 μl of each antibiotic solution and 50 μl of the BHI culture transferred into a well of a microtiter plate. The plates were incubated at 37° for 16–20 h, and the minimum inhibitory concentration calculated (16).

CYANOCYCLINE B [**4**].—A culture of *S. lusitanus* was grown in a seed medium consisting of yeast extract/malt extract broth (1 l) for 48 h at 25° and 250 rpm. The resulting seed culture was used to inoculate (2% v/v) 200 ml production media composed of 0.3% trypticase soy (BBL), 0.3% yeast extract (Difco), and 2% blackstrap molasses in 1-liter Erlenmeyer flasks incubated at 29° and 250 rpm for 36 h. Five liters of fermentation broth was filtered through cheesecloth under vacuum. The mycelial cake was washed with distilled H_2O several times and discarded. The combined filtrates were adjusted to pH 8.0 with 2% KOH, and NaCN (250 mg) was then added. The mixture was stirred at room temperature for 2 to 4 h and extracted with CH_2Cl_2 (3×1.5 liters), and the extracts were concentrated in vacuo to give a dark brown residue (250–350 mg). This residue, dissolved in the minimal volume of hexane/EtOAc (1:1), was loaded onto Bio-Beads SM-7 (10×150 mm) equilibrated with the same solvent. Fractions (5 ml) were collected while eluting with the same solvent. Bright yellow fractions were analyzed by tlc (Si gel, 10% MeOH/$CHCl_3$) and those containing cyanocycline B (R_f 0.46) were pooled. Concentration gave a mixture (35–50 mg) that was separated by centrifugal tlc (1 mm plate) eluting with CH_2Cl_2. After elution of cyanocycline A, the solvent was changed to 5% MeOH/$CHCl_3$, and fractions containing cyanocycline B were combined. Preparative tlc (10% MeOH/$CHCl_3$) then yielded 5 mg of pure cyanocycline B: λ max (85% MeCN/H_2O) 210, 266, 375 nm; ir (KBr) 3303, 2944, 2360, 2247, 1654, 1450, 1310, 912, 732 cm^{-1}; ^1H nmr ($CDCl_3$, 400 MHz) δ 4.66 (br s, 1H), 4.17 (br s, 1H), 4.03 (s, 3H), 4.03 (m, 1H), 3.91 (m, 1H), 3.88 (dd, J=2.6, 11.4 Hz, 1H), 3.81 (br d, J=2.9 Hz, 1H), 3.80 (br s, 1H), 3.73 (m, 1H), 3.63 (m, 1H), 3.48 (dd, J=3.1, 6.0 Hz, 1H), 3.03 (m, 1H), 3.01 (m, 1H), 2.79 (br d, J=3.0 Hz, 1H), 2.66 (ddd, J=6.0, 6.9, 12.9 Hz, 1H), 2.26 (ddd, J=6.9, 12.9, 12.9, 1H), 1.98 (s, 1H), 1.72 (dd, J=6.9, 12.9 Hz, 1H); ^{13}C nmr ($CDCl_3$, 100.6 MHz) δ 186.39, 181.16, 156.69, 142.94, 140.71, 128.29, 117.13, 93.12, 61.57, 61.15, 57.27, 56.40, 55.65, 53.28, 52.28, 52.80, 50.06, 47.55, 38.89, 31.61, 8.86; hrfabms calcd for $C_{21}H_{24}N_4O_5$ 412.17467, found 412.17519.

DIMETHYLCYANOCYCLINE C [**5**].—*From cyanocycline A.*—Cyanocycline A (25 mg) was treated with sodium dithionite (50 mg) in MeOH (5 ml) under an N_2 atmosphere. Distilled H_2O (100 μl) was added via syringe, and after ca. 5 min the yellow color had completely disappeared. The reaction flask was cooled to 0–5°, and CH_2N_2 (30 ml, prepared from Diazald), was added followed by addition of TFA (25 μl). The mixture was stirred at 0° for 1 h and at room temperature overnight. The mixture was concentrated in vacuo to a small volume, diluted with CH_2Cl_2 (50 ml), washed with 5% $NaHCO_3$, H_2O, and saturated NaCl successively, and then dried over anhydrous Na_2SO_4. Pure dimethylcyanocycline C (8 mg) was obtained by plc on Si gel (10% MeOH/$CHCl_3$): mp 98–100°; λ max (85% MeCN/H_2O) 216, 278 nm; ir (KBr) 3200, 2941, 2885, 1466, 1348, 1074, 730, 642 cm^{-1}; ^1H nmr ($CDCl_3$, 300 MHz) δ 4.80 (br s, 1H), 4.31 (s, 1H), 3.95 (d, J=3.1 Hz, 1H), 3.91 (m, 1H), 3.90 (dd, J=2.5, 10.3 Hz, 1H), 3.86 (s, 3H), 3.84 (s, 3H), 3.74 (m, 1H), 3.73 (d, J=7.4, 1H), 3.71 (s, 1H), 3.64 (s, 3H), 3.43 (m, 1H), 3.40 (m, 1H), 3.22 (m, 1H), 3.00 (br d, J=3.0 Hz, 1H), 2.93 (m, 1H), 2.91 (m, 1H), 2.43 (s, 3H), 2.37 (dd, J=6.9, 12.5 Hz, 1H), 2.20 (s, 3H), 1.79 (dd, J=6.8, 13.1 Hz, 1H); ^{13}C nmr ($CDCl_3$, 75.5 MHz) δ 151.74, 151.14, 146.54, 127.19, 125.59, 123.92, 117.60, 93.01, 62.47, 61.65, 61.57, 61.49, 61.03, 59.98, 59.84, 57.61, 54.78, 54.01, 49.63, 49.48, 41.25, 35.23, 28.94, 9.64; hrfabms calcd for $C_{24}H_{33}N_4O_5$ $[M+1]^+$ 457.24510, found 457.24510.

From fermentation broth.—Broth from an *S. lusitanus* fermentation (5 liters) was worked up as described for **4**. After eluting the Bio-Beads SM-7 column with 50% EtOAc/hexane, the solvent was changed to 70% EtOAc/hexane, and additional fractions (5 ml) were collected. Fractions containing cyanocycline C were pooled, concentrated to a small volume, and treated with ethanolic CH_2N_2 (30 ml) under an inert atmosphere. TFA (50 μl) was added, and the mixture was stirred overnight. Workup as described above yielded **6** (4 mg), identical with authentic material as indicated by hplc and ^1H-nmr spectroscopic analysis.

CYANOCYCLINE D [**8**].—Following the procedure for isolation of cyanocycline B, 10 liters of fermentation broth was processed through centrifugal tlc to yield a mixture of **4** and **8**. Repetition of this last step afforded 15 mg of enriched material, and pure cyanocycline D was obtained by recrystallization from EtOAc/hexane: mp 112–114°; λ max (85% MeCN/H_2O) 204, 268, 370 nm; ir (KBr) 3356, 2944, 2864, 2306, 2227, 1656, 1450, 1238, 1151, 905, 739 cm^{-1}; ^1H nmr ($CDCl_3$, 300 MHz) δ 4.67 (br s, 1H), 4.56 (br d, J=9.1 Hz, 1H, exchangeable with D_2O), 4.21 (d, J=2.6 Hz, 1H), 4.05 (s, 3H), 3.96 (d, J=3.4 Hz), 3.88 (dd, J=2.6, 11.4 Hz, 1H), 3.87 (s, 3H), 3.72 (m, 1H), 3.66 (m, 1H), 3.62 (br d, J=11.4 Hz, 1H), 3.45 (m, 1H), 3.27 (dd, J=4.4, 4.5 Hz, 1H), 2.91 (dd, J=5.6, 11.0 Hz, 1H), 2.90 (s, 1H), 2.69 (m, 1H),

2.60 (m, 1H), 2.46 (s, 3H), 2.38 (dd, J=7.1, 13.3 Hz, 1H), 2.27 (m, 1H, exchangeable with D_2O), 1.99 (s, 3H), 1.79 (dd, J=6.1, 13.0 Hz, 1H); ^{13}C nmr ($CDCl_3$, 75.5 MHz) δ 184.90, 180.80, 155.70, 140.70, 128.60, 116.70, 116.20, 62.80, 61.10, 60.90, 59.11, 59.09, 56.55, 53.63, 53.41, 53.02, 51.79, 51.73, 41.15, 36.81, 28.50, 8.87; hrfabms calcd for $C_{23}H_{28}N_5O_5$ $[M+1]^+$ 412.17467, found 412.17519.

[^{13}CN]CYANOCYCLINE D [**9**].—Cyanocycline A was treated with sodium [^{13}CN]cyanide (40 mg, Cambridge Isotope Laboratories) in MeOH (2 ml) and distilled H_2O (10 ml) at room temperature. After 30 min, the reaction was diluted with CH_2Cl_2 (50 ml), and the organic layer was washed successively with H_2O and saturated NaCl, dried over anhydrous Na_2SO_4, and concentrated in vacuo. Centrifugal tlc gave 8 mg of pure **9**: ^1H nmr ($CDCl_3$, 300 MHz) identical to **8** except for δ 4.67 ($^2J_{CH}$=7.8 Hz); ^{13}C nmr ($CDCl_3$, 75.5 MHz) intense signal at δ 116.2 (C-3a) and strong signal at δ 116.7 (C-7).

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