Structures of the Bioxalomycins and Their Relationship to Naphthyridinomycin

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Summary: Bioxalomycins α1, α2, β1, and β2 are novel compounds with potent antimicrobial and antitumor activity isolated from fermentation broths of *Streptomyces viridostaticus*. These structures are closely related to that of naphthyridinomycin and call into question the published structure of the naturally occurring form of that antitumor antibiotic.

As part of a screening program to identify new antimicrobial agents with potential utility to treat organisms resistant to clinically useful antibiotics, fermentations of *Streptomyces viridostaticus* ssp. "litoralis"[1] were found to produce four novel antibiotics called bioxalomycins α1 (**1**), α2 (**2**) ,β1 (**3**), and β2 (**4**).These compounds displayed potent antimicrobial activity against a broad spectrum of Gram-negative and -positive organisms including clinically important strains of *Staphylococcus aureus* and *Enterococcus faecium*. Subsequent biological evaluation showed that these antibiotics also displayed activity against a panel of tumor cell lines.[2] This paper describes the isolation and structural characterization of the bioxalomycins and discusses their relationship to the previously reported antitumor antibiotic, naphthyridinomycin (**5**). In addition, we propose that the structure of the naturally occurring form of naphthyridinomicin (**5**)[3-5] is identical to bioxalomycin β2 (**4**).

Broth from a 600-L fermentation was filtered through Celite and the cake washed with water. The combined cake washes along with the aqueous filtrate were chromatographed over a column of HP20 and the active principles eluted with 90% MeOH/water. Next, active freeze-dried fractions were chromatographed by HPLC using a reversed-phase column equilibrated with 0.1% trifluoroacetic acid in water. The active principles were eluted with an acetonitrile gradient to 10%. Fractions containing the α component were pooled and freeze-dried whereas fractions containing the β component were adjusted to pH 8 with solid ammonium bicarbonate and extracted with methylene chloride. Bioxalomycin β was stored in methylene chloride as drying led to rapid decomposition. Finally, crude bioxalomycin α in 0.01 M HCl was resolved into two components, α1 and α2, by chromatography over a PRP-1 reversed-phase column by elution with an acetonitrile gradient to 10%. These fractions were freeze-dried to yield off-white powders. From 600 L of fermentation broth, 350 mg of bioxalomycin α2, the predominant species accounting for 80–95% of total α, was obtained.

High-resolution FABMS of the α2 component provided a protonated molecular weight of 402.2034 corresponding to $C_{21}H_{28}N_3O_5$ (calcd 402.2029). Identical molecular weights were derived from EI, TS, ES, and CIMS. The UV spectrum of bioxalomycin α2 is characteristic of a benzhydroquinone with a maximum at 294 nm in water and 290 nm in methanol (ϵ 2729; $[\alpha]^{25}_D = +31°$ MeOH). The compound is soluble in MeOH, H_2O, and DMSO, but insoluble in methylene chloride, chloroform, hexane, and acetonitrile. Bioxalomycin α2 is soluble but unstable in acetone. The ^1H and ^{13}C NMR spectra of weakly acidic, neutral, or basic solutions exhibited a series of minor signals which were shown to be directly associated with **1** and **2** rather than with impurities. They gave negative NOESY peaks which correlated with major peaks of **1** and **2**. This would be indicative of a molecule in equilibrium between two distinct forms. The chemical shift of carbon 3a of the minor components was shifted from 92.8 ppm in the major component to 177.5 ppm while its ^1H-signal shifted from 5.4 to 8.9 ppm. These chemical shift changes are consistent with opening of the oxazolidine ring at 3a and the formation of an imine between carbon 3a and the adjoining nitrogen but not an aldehyde.[6] The same 8.9 ppm ^1H-signal showed HMBC peaks associated with minor signals in the ^{13}C-spectrum whose chemical shifts are similar to those of the corresponding carbons in the parent compound (i.e., carbons 13b, 4, and 4a), all of which are in the vicinity of the oxazolidine ring. HETCOR experiments also revealed the connectivities of the minor peaks in the ^1H-spectra and associated peaks in the ^{13}C spectra. Finally, several of these minor proton peaks displayed their own set of COSY correlations which reflected their assigned positions in the molecule. Peak intensities suggested that in any one molecule the ring was open about 15% of the time.

The molecular weight of α1 is 387 as determined by FABMS and its UV spectra and solubility properties are similar if not identical to α2. Examination of the ^1H NMR of this material revealed the absence of the peak assigned to the *N*-methyl group (C5a) in α2. All other peaks are identical to those observed in the α2 spectra.

The β2 form (**4**; MW 399; FABMS) has UV maxima at 264 and 370 nm in acidic aqueous solution and maxima at 270 and 370 nm in methanol characteristic

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(1) "Litoralis" is the latin term for shoreline signifying the origin of this organism from a Key West shoreline.

(2) The biological activity of the bioxalomycins will be the subject of a separate publication.

(3) Kluepfel, D.; Baker, H. A.; Piattoni, G.; Sehgal, S. N.; Sidorowicz, A.; Singh, K.; Vezina, C. *J. Antibiot.* **1975**, *28*, 497.

(4) (a) Sygusch, J.; Brisse, F.; Hanessian, S.; Kluepfel, D. *Tetrahedron Lett.* **1974**, 4021; errata: *Tetrahedron Lett.* **1975**, 170. (b) Sygusch, J.; Brisse, F.; Hanessian, S. *Acta Crystallogr.* **1976**, *B32*, 1139.

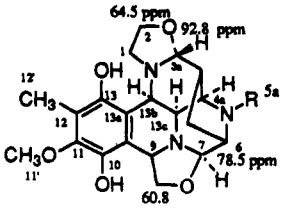
(5) For a review of naphthyridinomycin and related natural products, see: Remers, W. A. *The Chemistry of Antitumor Antibiotics*; Wiley: New York, 1988; Vol. 2, Chapter 4.

(6) A reviewer has suggested that the aldehyde instead of the iminium form might be considered for the minor component in bioxalomycin α2. However, the ^{13}C-NMR chemical shift of 177.5 ppm for carbon 3a of the α2 minor component is in excellent agreement with that of an imine carbon. The chemical shift for an aliphatic aldehyde carbonyl carbon would be expected to be around 200 ppm, and therefore, the presence of an aldehyde seems unlikely. A strong precedent for the iminium assignment comes from the following work: Hayashi, T.; Nawata, Y. *J Chem. Soc. Perkin Trans* 2 **1983**, 335. These authors prepared the identical iminium form from cyanocycline A as the bis(hydrogen chloride and bromide) salts and characterized them by X-ray crystal analysis.

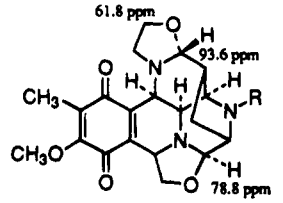
0022-3263/94/1959-4045$04.50/0

Chart 1

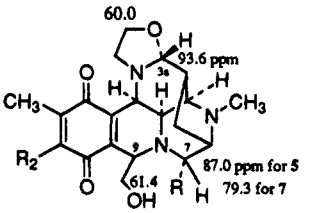
1 Bioxalomycin α1 R=H
2 α2 R=CH_3

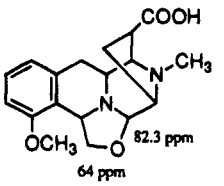


3 Bioxalomycin β1 R=H
4 β2 R=CH_3

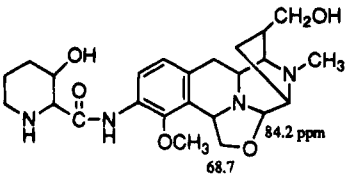


5 Naphthyridinomycin R=OH R_2=OCH_3
6 Cyanocycline R=CN R_2=OCH_3
7 SF-1739 HP R=OH R_2=OH

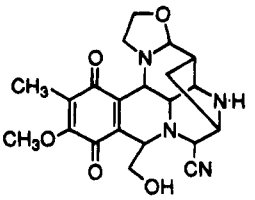




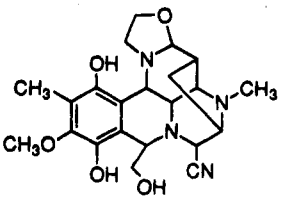
8 Quinocarcin



9 Tetrazomine



10



11

of a benzoquinone. It is soluble in MeOH, H_2O, chloroform, methylene chloride, ether, and hexane. This compound can be converted by reduction with $NaBH_4$ in low yield to a species having the identical spectral and chromatographic properties of α2. Analysis of the spectral data and molecular weight indicates that the β component is the quinone form of α. Long-range COSY correlations (measured in $CDCl_3$) were assigned to hydrogens at C7 and C9′ (4.4 ppm and 3.9 ppm) flanking the intervening oxygen atom which indicates the existence of a closed oxazolidine ring in β2. A similar correlation is seen between hydrogens on C2 and C3a in the second oxazolidine ring.

Obviously, the α and β components are closely related to the antibiotic naphthyridinomycin (**5**)[3-5] but differ by the oxazolidine ring involving carbons 7 and 9′. The position of this ring is similar to that found in the antibiotics quinocarcin (**8**)[7] and tetrazomine (**9**).[8] The solubility of the bioxalomycin β component in hexane differs most strikingly from that of naphthyridinomycin in that it lacks the propensity to crystallize readily from this and other solvents used to obtain crystals of naphthyridinomycin. The carbon at C7 resonates at 78.8 ppm compared to the published value for naphthyridinomycin of 87.0 ppm.[9] Chemical shifts for the analogous carbons in quinocarcin and tetrazomine are 82.3 and 84.2 ppm, respectively (recorded in D_2O). It is noteworthy that C7 in antibiotic SF-1739HP (**7**)[10] is assigned to a ^{13}C resonance of 79.3 ppm in D_2O. SF-1739HP is proposed to have a structure similar to naphthyridinomycin except that the methoxy group on the quinone ring is replaced by a hydroxyl group. However, the structure of this compound was only surmised from analysis of the cyano derivative.

The β component (**4**) reacted with KCN at pH 8.0 to produce a material with the same molecular weight and identical chromatographic and spectral properties as cyanocycline (**6**), the cyano adduct of naphthyridinomycin.[9,11] Cyanide added exclusively to C7 as evidenced by appearance of the cyano carbon signal at 117.0 ppm and

(7) Tomita, F.; Takahashi, K.; Shimizu, K. *J. Antibiot.* **1983**, *36*, 463. Takahashi, K.; Tomita, F. *J. Antibiot.* **1983**, *36*, 468. Hirayama, M.; Shirahata, K. *J. Chem. Soc., Perkin Trans. 2* **1983**, 1705.
(8) Suzuki, K.; Sato, A.; Morioka, M.; Nagai, K.; Abe, K.; Yamaguchi, H.; Saito, T.; Ohmi, Y.; Susaki, K. *J. Antibiot.* **1991**, *44*, 479.
(9) Zmijewski, M. Jr.; Goebel, M. *J. Antibiot.* **1982**, *35*, 524.
(10) Itoh, J.; Omoto, S.; Inouye, S.; Kodama, Y.; Hisamatsu, T.; Niida, T.; Ogawa, Y. *J. Antibiot.* **1982**, *35*, 642.
(11) Hayashi, T. Noto, T.; Nawata, Y.; Okazaki, H.; Sawada, M.; Ando, K. *J. Antibiot.* **1982**, *35*, 771.

the disappearance of the C7 signal at 78.8 ppm signal. When this material (**6**) was treated with $AgNO_3$, a compound was recovered with chromatographic and spectral properties, and molecular weight identical to those of $\beta 2$, the natural product isolated from *S. viridostaticus*. This indicates that the oxazolidine ring has been restored in a manner analogous to the final synthetic step in route to quinocarcin and was not converted to the aminal-containing structure reported for naphthyridinomycin.[12]

In order to resolve further the difference between the bioxalomycins and the published structure of naphthyridinomycin, an attempt was made to isolate naphthyridinomycin from broths of the strain originally reported to produce it, *S. lusitanus* (NRRL8034). Our isolation and purification was accomplished in a manner similar to that of the bioxalomycins. However, the only compound isolated under these mildly acidic conditions exhibited the physical and spectral characteristics of $\beta 2$. The hydroquinone form of $\beta 2$ was present in very minor amounts. Further attempts to produce naphthyridinomycin from Lederle fermentations of *S. lusitanus* by reproducing the purification conditions originally reported by Kluepfel et al. (0.1 N HCl elution of an Amberlite IRC-50 ion exchange resin)[3] were unsuccessful.

Our inability to isolate naphthyridinomycin suggests that it may be a transitory artifact of the fermentation. However, the fact that only $\beta 2$ was obtained following the previously cited Ayerst isolation procedure leaves the origin of the X-rayed compound in some doubt. On the assumption that it is a transitory artifact of the original fermentation conditions, we can only speculate whether the closed ring structure precedes or follows the biosynthesis of naphthyridinomycin. Since all subsequent literature referring to *S. lusitanus* involves the addition of cyanide to the fermentation and since we have demonstrated that the β compound produces the same cyano derivative as naphthyridinomycin when exposed to cyanide, this question is not easily answered.[13] The existence of quinocarcin and tetrazomine, however, provides a precedent for the structures of the ring-closed bioxalomycins. We believe that the isolation, structure and chemistry of the bioxalomycins strongly suggest that the true naturally occurring form of naphthyridinomycin is bioxalomycin $\beta 2$ (**4**) instead of **5** and provides a logical explanation for the difficulty in obtaining **5** by total synthesis.[12,14] A comparison of ^1H and ^{13}C NMR spectra of synthetic naphthyridinomycin (Professor Tohru Fukuyama of Rice University)[15] with those of bioxalomycin $\beta 2$ showed that the synthetic material is indeed identical to bioxalomycin $\beta 2$, (**4**).

Recently, Gould et al. reported the isolation of two new cyanocyclines, **10** and **11** from fermentation broths of *Streptomyces lusitanus* by the addition of NaCN at the end of fermentations.[16] Cyanocycline B (**10**) is obviously derived from *N*-desmethylnaphthyridinomycin, and **11** appears to be derived from the hydroquinone form of naphthyridinomycin. In view of the above results we believe that the true structures of these new naturally occurring metabolites are derived from the bioxalomycins rather than naphthyridinomycin, **5**.

Supplementary Material Available: ^1H-NMR (300 MHz), ^{13}C-NMR for the bioxalomycins, a table presenting the comparison of NMR data of the bioxalomycins with naphthyridinomycin and a, COSY and HETCOR for bioxalomycin $\beta 2$ as well as a summary of the 2D NMR experiments (COSY, NOESY, and HMBC) for $\alpha 2$ (9 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see, current masthead page for ordering information.

(12) For a review of the effort by several groups to synthesize the accepted structure of naphthyridinomycin (**5**), as well as cyanocycline A and quinocarcin see: Fukuyama, T. In *Advances in Heterocyclic Natural Product Synthesis*; Pearson, W., Ed.; JAI Press Inc.: Greenwich, CT, 1992; Vol. 2, pp 189–249. The absolute configuration of naphthyridinomycin and hence the bioxalomycins is that given in this review and is based on Fukuyama's synthetic studies as opposed to the original X-ray analysis.

(13) Zmijewski, M., Jr.; Mikolajczak, M.; Viswanatha, V.; Hruby, V. *J. Am. Chem. Soc.* **1982**, *104*, 4969.

(14) One of the reviewers has asked if our studies provide any insight whether the quinone forms or the phenolic forms of bioxalomycin might be artifacts. Given that in fermentations of *Streptomyces viridostaticus* the hydroquinones are the predominant metabolites and that they are easily converted to the quinones by air oxidation, we speculate that in this instance the quinone forms are artifacts. From *Streptomyces lusitanus*, however, only the quinone form, $\beta 2$, was obtained using the identical isolation procedure as above suggesting that in this organism the quinone is a true metabolite and not an artifact of the isolation procedure.

(15) We wish to thank Professor Fukyama of Rice University for making available copies of ^1H and ^{13}C spectra of his synthetic naphthyridinomycin to permit this comparison.

(16) Gould, S, J.; He, W.; Cone, M. C. *J. Nat. Prod.* **1993**, *56*, 1239.