Mode of Antibiotic Action of 4-Hydroxy-3-Nitrosobenzaldehyde from *Streptomyces viridans*

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The free ligand, deferroviridomycin A, and its iron(II) complex, viridomycin A, were detected in culture supernatant fluids of *Streptomyces viridans* 1671 and were structurally characterized as 4-hydroxy-3-nitrosobenzaldehyde and tris(4-hydroxy-3-nitrosobenzaldehydato- N^3,O^4)ferrate(II), respectively. We investigated the antibiotic activity of the above compounds and of the chemically synthesized bis copper(II), tris cobalt(III), and tris nickel(II) complexes against *Escherichia coli* NIHJ, *Salmonella typhimurium* LT-2Z, *Staphylococcus aureus* 209P, *Streptococcus faecium* 10541, and *Bacillus cereus* T. The free ligand and its kinetically labile copper(II) and nickel(II) complexes displayed activity against all of the above organisms, whereas the kinetically inert iron(II) and cobalt(III) complexes displayed activity only against *S. aureus* and *B. cereus*. The antibiotic activity of the substitutionally labile metal complexes was attributed to dissociation of the free ligand. The mode of antibiotic action of the free ligand against *E. coli* appears to be interference with the structural and functional integrity of the cell membrane.

Several *Actinomyces* (*Streptomyces*) species produce extracellular iron-containing green pigments which display antibiotic activity (3, 13, 16). For example, in iron-containing culture media *Actinomyces viridaris* 1876 produces viridomycin A, which inhibits the growth of gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*, but has no effect on gram-negative bacteria and fungi (3). The green product chemically prepared from 4-hydroxy-3-nitrosobenzaldehyde and iron(II) was shown by Khokhlov and Blinova to have chemical and physical properties identical to those of viridomycin A (13). Viridomycin A was structurally formulated as bis(4-hydroxy-3-nitrosobenzaldehydato- N^3,O^4)-iron(II), $\text{Fe}(\text{C}_7\text{H}_4\text{NO}_3)_2$ and was shown to be substitutionally inert (13). The free ligand, 4-hydroxy-3-nitrosobenzaldehyde (deferroviridomycin A), was isolated from the supernatant fluids of iron-limiting cultures of *A. viridaris* 1876 (3).

The structural formulation of viridomycin A seemed odd to us since the chemical structure of ferroverdin (1), the intracellular green pigment of *Streptomyces* Wak. no. A-305, had previously been confirmed to be tris(*p*-vinylphenyl-4-hydroxy-3-nitrosobenzoato- N^3,O^4)ferrate(II) (4), i.e., an octahedral iron(II) complex with three identical *o*-nitrosophenol ligands. We report here that viridomycin A should be formulated as tris(4-hydroxy-3-nitrosobenzaldehy-

dato- N^3,O^4)ferrate(II), that the nickel(II) and cobalt(III) complexes are likewise octahedral tris complexes, and that the copper(II) complex is a square-planar bis complex. The antibiotic activity of these metal complexes and the free ligand against the bacterial test strains—gram-positive *S. aureus* 209P, *Streptococcus faecium* 10541, and *Bacillus cereus* T and gram-negative *Escherichia coli* NIHJ and *Salmonella typhimurium* LT-2Z—is described. In particular, the mode of antibiotic action of the free ligand against *E. coli* NIHJ was determined.

The structural composition and substitutional lability of viridomycin A were first ascertained. Viridomycin A was isolated from the culture supernatant fluids of *Streptomyces viridans* 1671 (ATCC 19849) grown at room temperature in synthetic SR-I medium (16), which was supplemented with 0.5 g of ferric citrate and 20 g of glycerol per liter of tap water instead of glucose. The green culture supernatant fluids were saturated with ammonium sulfate, and the resulting suspension was extracted with benzyl alcohol and further treated by a previously described procedure (20). The crude material was chromatographed on kieselgel D-O silica gel (Camag Inc., New Berlin, Wis.) with 25% $\text{CH}_3\text{OH}-\text{CHCl}_3$ as the eluant according to an earlier procedure (14). Viridomycin A was converted into the sodium salt by ion-exchange chromatography on AG50W-X2 (200 to 400 mesh, sodium form)

(Bio-Rad Laboratories, Richmond, Calif.) with water as the eluant and was recrystallized from acetone-hexane at room temperature.

Alternatively, viridomycin A could be chemically prepared. A solution of 4-hydroxy-3-nitrosobenzaldehyde (see below) in ethyl acetate was washed with aqueous ferrous ammonium sulfate solution and with saturated sodium chloride solution and dried over MgSO_4 . Viridomycin A was recrystallized as described above.

Chemically prepared viridomycin A had identical elemental analyses (Table 1), R_f 's on thin-layer chromatography, migrations toward the anode on paper electrophoresis, ultraviolet-visible spectra, chemical inertness toward ethylenediaminetetraacetic acid, solubilities in organic solvents, and proton nuclear magnetic resonance spectra in CD_3COCD_3 (data not shown) as natural viridomycin A. The last four properties were indistinguishable from those reported earlier for viridomycin A (13). However, our data on the elemental analysis (N:Fe:Na of 3.0:0.9:0.8) and the paper electrophoretic behavior of viridomycin A require that it be formulated as the monoanion, tris(4-hydroxy-3-nitrosobenzaldehyde-

to- N^1,O^4)ferrate(II), $[\text{Fe}(\text{C}_7\text{H}_4\text{NO}_3)_3]^-$. Viridomycin A appeared to be substitutionally inert within 16 h as determined from a chemical exchange experiment consisting of $^{55}\text{FeSO}_4$ and viridomycin A (data not shown).

When *S. viridans* was cultured in our modified SR-I medium without iron(III), the free ligand was detected in culture supernatant fluids by the addition of iron(II) or cobalt(II), followed by isolation and characterization of viridomycin A or the cobalt(III) analog (see below), respectively (data not shown). Therefore, the free ligand was a natural product under transition metal ion-limiting conditions. The free ligand was most conveniently obtained by treatment of its copper(II) complex (see below) with aqueous acid, followed by extraction of the product with ethyl acetate, as described earlier (5). The free ligand was stable in the dry state at 4°C for 1 week, whereas an ethyl acetate solution was stable for up to 1 month at 4°C. In contrast to a previous report of the instability of the free ligand in neutral solution (3), the free ligand was ascertained to be stable at neutral pH for up to 16 h at 37°C (data not shown).

TABLE 1. Physical characteristics and MICs of various compounds^a

Compound	Elemental analysis ^b		Absorption maximum nm (ϵ), solvent	MIC (μM) against:				
	Calculated	Experimental		<i>S. aureus</i> 209P	<i>B. cereus</i> T	<i>S. faecium</i> 10541	<i>E. coli</i> NIHJ	<i>S. typhimurium</i> LT-2Z
$\text{Fe}(\text{L})_3^-$	3.0	3.2	675 (1.05×10^4), CH_3COCH_3	27	15	NE ^d at 250	NE at 250	NE at 250
$\text{Co}(\text{L})_3$	3.0	3.0	350 (2.9×10^4), CHCl_3	128	47	NE at 1,300	NE at 5,760	NE at 2,360
$\text{Cu}(\text{L})_2$	2.0	2.1	518 (8.1×10^3), CH_3CN	32	15	50	23	8
$\text{Ni}(\text{L})_3^-$	3.0	2.5	485 (1.2×10^4), CH_3CN	24	11	50	15	11
HL ^e			445 (3.65×10^3), CH_3OH	60	25	102	60	19

^a Minimal medium A (15, 23) was used for *E. coli* NIHJ (ATCC 26) and *S. typhimurium* LT-2Z (M. Luckey); medium SAM (2) was used for *S. aureus* 209P (ATCC 6938P); and modified G medium (11), which was supplemented with 1 g of Casamino Acids (Difco certified) per liter instead of yeast extract and which omitted the $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, was employed for *B. cereus* T (W. Brown). The *B. cereus* medium, which was modified to contain 1 g of peptone (Difco certified) per liter instead of Casamino Acids, was used for *S. faecium* ATCC 10541. Stock solutions of compounds were prepared immediately before testing by an earlier procedure (2), and their concentrations were determined spectrophotometrically. The MIC of each compound against the five test species was determined by a previously described method (2). The MIC was chosen to be that concentration of antibiotic at which there was no growth overnight. The temperature of incubation for all species was 37°C, except for *B. cereus*, which was incubated at 30°C. Since the cobalt(III) complex was not soluble in water, its antibiotic effects were assessed by the filter paper disk method, as previously described (12). Paper disks containing 20 μl of chloroform solutions of the cobalt(III) complex were applied to agar plates, previously seeded with the test species. Nutrient agar plates were used for *S. aureus*; Trypticase soy agar (BBL Microbiology Systems) plates were used for *B. cereus* and *S. faecium*; and medium A plates were used for *E. coli* and *S. typhimurium*. All MICs reported are for aerobically growing cells.

^b Nitrogen-to-metal ratio in the metal complex.

^c Data refer to the visible region only. Units are liters mol⁻¹ cm⁻¹.

^d NE, No effect.

^e HL, Deferroviridomycin A (4-hydroxy-3-nitrosobenzaldehyde).

Various metal complexes of 4-hydroxy-3-nitrosobenzaldehyde were prepared to determine the effect of their substitutional lability on antibiotic action. Bis(4-hydroxy-3-nitrosobenzaldehydato- N^3,O^1)copper(II) and sodium tris(4-hydroxy-3-nitrosobenzaldehydato- N^3,O^1)nickelate(II) were prepared from Baudisch reactions (5) of 4-hydroxybenzaldehyde (Aldrich Chemical Co., Milwaukee, Wis.) and were recrystallized from $CH_3OH-CHCl_3$ at room temperature. Tris(4-hydroxy-3-nitrosobenzaldehydato- N^3,O^1)cobalt(III) was prepared from $CoCl_2 \cdot 6H_2O$ and the free ligand by an earlier procedure (14). Although satisfactory elemental analyses of the nickel(II) complex (N:Ni of 2.5:1) were not obtained, this complex is formulated as an octahedral tris complex since its R_f value on thin-layer chromatography was virtually identical to that of viridomycin A. We confirmed a previous communication of the substitutional lability of the copper(II) and nickel(II) complexes and the substitutional inertness of the cobalt(III) complex (3).

The requirement of labilization of the ligand in metal complexes of 4-hydroxy-3-nitrosobenzaldehyde for antibiotic action was ascertained by testing the free ligand and substitutionally labile and inert metal complexes. Kinetically inert viridomycin A was inhibitory against gram-positive *S. aureus* 209P and *B. cereus* T, but not against gram-positive *S. faecium* 10541 or gram-negative *E. coli* NIHJ or *S. typhimurium* LT-2Z (Table 1). Similar results were reported by Blinova et al. (3). The kinetically inert cobalt(III) complex displayed a similar antibiotic spectrum. The free ligand was inhibitory against all species tested; the kinetically labile copper(II) and nickel(II) complexes were inhibitory at even lower concentrations. The effective ligand concentrations of the copper(II) and nickel(II) complexes required for growth inhibition were approximately the same as the minimal inhibitory concentration (MIC) of the free ligand for each test species. Hence the free ligand was probably the actual antibiotic agent in view of the extreme lability of the nickel(II) and copper(II) complexes. The MIC of the free ligand against anaerobically growing *E. coli* in medium A was the same as that against aerobically growing cells.

Hence, two classes of compounds were found to exhibit antibiotic activity. The first class, which consists of 4-hydroxy-3-nitrosobenzaldehyde and its substitutionally labile metal complexes, inhibited the growth of all species tested. Labile metal complexes appear to exert their antibiotic activity by rapidly releasing the free ligand, which is the actual antibiotic agent. The second class is comprised of substitutionally inert metal complexes such as viridomycin A and

its cobalt(III) analog, which were only active against certain gram-positive bacteria such as *S. aureus* and *B. cereus*. These metal complexes do not require labilization of the ligand as a prerequisite for antibiotic activity.

Since the free ligand displayed the broadest antibiotic spectrum, we focused our attention on its mode of action. The free ligand was found to be bactericidal against growing *E. coli* NIHJ in medium A at its MIC (60 μM) by an earlier procedure (22). The bactericidal concentration is defined as that concentration of antibiotic which caused a two orders of magnitude decrease in the number of viable cells within one doubling time (10). The free ligand also decreased cell viability of resting *E. coli* in medium A without glucose and ammonium sulfate.

The effect of the free ligand on protein, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) syntheses in *E. coli* was examined by measurement of the incorporation of [^{14}C]leucine, [^{14}C]uracil, and [^3H]thymidine, respectively, into trichloroacetic acid-insoluble material. The free ligand at its MIC (60 μM) completely inhibited all macromolecular synthesis (Fig. 1). RNA and DNA syntheses were inhibited by 50 and 40%, respectively, at 12 μM free ligand, whereas protein synthesis was only slightly retarded. The transport of the above radioactive precursors in the presence of the free ligand into *E. coli* was measured in parallel to the incorporation experiments. The inhibition of uptake of [^{14}C]uracil by various concentrations of the free ligand was identical to that of incorporation of [^{14}C]uracil into RNA (Fig. 1). Similar results were obtained with [^{14}C]leucine and [^3H]thymidine (data not shown). Since the rate of incorporation of [^{14}C]uracil into RNA in the presence of the free ligand could be limited by its rate of uptake, cellular RNA levels were measured directly as a function of time with the orcinol reagent (8). The inhibition of RNA synthesis by the free ligand as determined by the above procedure was identical to that determined by the incorporation of [^{14}C]uracil (data not shown). Our measured rates of protein and DNA syntheses (Fig. 1) could only be higher if the uptake of their respective precursors was rate limiting.

The effect of the free ligand on cellular adenosine triphosphate (ATP) levels in *E. coli* was determined by the luciferase firefly method (6). Cellular ATP levels remained constant at the MIC. The ATP levels declined only at free ligand concentrations greater than the MIC. The observed increase in ATP levels of control cells as a function of time was attributed to an increase in cell density.

The free ligand at 60 μM completely inhibited

FIG. 1. Effects of the free ligand on (A) incorporation of L-[U- ^{14}C]leucine ($460\ \mu\text{M}$, $3.2\ \text{mCi/mmol}$) into protein, (B) incorporation of [2- ^{14}C]uracil ($450\ \mu\text{M}$, $3.2\ \text{mCi/mmol}$) into RNA, and (C) incorporation of [methyl- ^3H]thymidine ($17\ \mu\text{M}$, $95\ \text{Ci/mmol}$) into DNA by *E. coli* (10^8 cells per ml) at 37°C in the presence of $0.1\ \text{mM}$ 2-deoxyadenosine to inhibit enzymatic conversion of thymidine into thymine (10). The rates of synthesis of all macromolecules were determined simultaneously by an earlier procedure (17). In (B) the effect of the free ligand on uptake of [2- ^{14}C]uracil ($450\ \mu\text{M}$, $3.2\ \text{mCi/mmol}$) by *E. coli* (10^8 cells per ml) at 37°C was measured in parallel to the incorporation experiment, following an established procedure (2). The amount of labeled precursor incorporated or taken up per $100\ \mu\text{l}$ of cell suspension was calculated by comparison with a control containing the precursor but no cells. The free ligand (arrow) was added at the following concentrations: $0\ \mu\text{M}$ ($\circ$), $12\ \mu\text{M}$ ($\square$), $30\ \mu\text{M}$ ($\triangle$), $60\ \mu\text{M}$ ($\bullet$). Incorporation (—), uptake (-----).

oxygen consumption by *E. coli* (Fig. 2). A similar result was obtained with $2\ \text{mM}$ KCN.

The effect of the free ligand on glucose transport in *E. coli* at room temperature was studied with the non-metabolizable analog [α -methyl glucose- ^{14}C]methyl α -D-glucopyranoside (α -methyl glucoside) ($2\ \mu\text{M}$, $300\ \text{mCi/mmol}$), as described earlier (25). Freshly grown *E. coli* in medium A, which was supplemented with $5\ \text{g}$ of glycerol per liter instead of glucose, was washed and suspended to 3×10^8 cells per ml in the same medium without $(\text{NH}_4)_2\text{SO}_4$. The free ligand at its MIC completely abolished uptake. We confirmed that $2\ \text{mM}$ cyanide stimulated α -methyl glucoside transport when compared with control cells (25). Another energy inhibitor, 2,4-dinitrophenol, was also shown to stimulate transport in the same study (25).

In summary, the free ligand at its MIC completely inhibited protein, DNA, and RNA syntheses, α -methyl glucoside transport, and oxygen consumption in *E. coli*. However, at $12\ \mu\text{M}$ free ligand RNA synthesis was preferentially inhibited when compared with DNA and protein syntheses. Stable RNA must constitute part of the total RNA whose synthesis was inhibited. The preferential inhibition of stable RNA synthesis, known as shift-down effect, is suggestive of the disruption of the cell's energy metabolism (18). The well-known energy inhibitors, cyanide and 2,4-dinitrophenol, cause shift-down (18, 19). Although the free ligand inhibited O_2 consumption in *E. coli*, it did not stimulate α -methyl

glucoside transport like cyanide and 2,4-dinitrophenol. Hence, if the free ligand were an energy inhibitor, its mode of action would appear to be different from those above. Although the MICs

FIG. 2. Oxygen consumption in *E. coli* (10^8 cells per ml) in medium A at 37°C was determined with an oxygen electrode. The free ligand (arrow) was added at the following concentrations: $0\ \mu\text{M}$ (A), $30\ \mu\text{M}$ (B), $60\ \mu\text{M}$ (C); cyanide was added at $2\ \text{mM}$ (D).

of the free ligand against aerobically and anaerobically growing *E. coli* were identical, the electron transport chain cannot be excluded as the target. The electron transport chain in *E. coli* is not well understood; parts of it appear to be present even under anaerobic conditions. However, these components appear to be different from those of aerobically grown *E. coli* (24).

The bactericidal effect of the free ligand at its MIC against growing *E. coli* suggests that the mode of action could be the inhibition of DNA or cell wall syntheses or membrane disruption (10). Moreover, the bactericidal action against resting cells indicates membrane disruption. The antibacterial circular oligopeptides of the tyrocidin and polymyxin families, known to cause interference with the structural and functional integrity of membranes (10), are bactericidal against both growing and resting cells (7, 9, 21). Hence, membrane disruption by the free ligand would affect all transport processes and the electron transport chain and would explain the inhibition of oxygen consumption and macromolecular synthesis at its MIC and the shift-down effect.

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LITERATURE CITED

1. Ballio, A., H. Bertholdt, A. Carilli, E. B. Chain, V. Di Vittorio, A. Tonolo, and L. Vero-Barcellona. 1963. Studies on feroverdin, a green iron-containing pigment produced by a *Streptomyces* Wak. species. Proc. R. Soc. London Ser. B **158**:43-70.
2. Bell, S. J., S. A. Friedman, and J. Leong. 1979. Antibiotic action of N-methylthioformohydroxamate metal complexes. Antimicrob. Agents Chemother. **15**:384-391.
3. Blinova, I. N., S. A. Egorova, I. V. Marchenko, L. I. Saulina, N. O. Blinov, and A. S. Khokhlov. 1976. New iron-containing antibiotics— isolation and properties of viridomycins A, B, and C. Chem. Nat. Compd. (USSR) **11**:506-512.
4. Candeloro, S., D. Grdenic, N. Taylor, B. Thompson, M. Viswamitra, and D. C. Hodgkin. 1969. Structure of feroverdin. Nature (London) **224**:589-591.
5. Cronheim, G. 1947. *o*-Nitrosophenols. II. New substituted *o*-nitrosophenols and characteristic properties of their inner complex metal salts. J. Org. Chem. **12**:7-19.
6. Feingold, D. S. 1970. The mechanism of colicin E1 action. J. Membr. Biol. **3**:372-386.
7. Few, A. V., and J. H. Schulman. 1953. The absorption of polymyxin E by bacteria and bacterial cell walls and its bactericidal action. J. Gen. Microbiol. **9**:454-466.
8. Fiil, N. P., and K. von Meyenburg. 1972. Accumulation and turnover of guanosine tetraphosphate in *Escherichia coli*. J. Mol. Biol. **71**:769-783.
9. Gale, E. F., and E. S. Taylor. 1947. The assimilation of amino-acids by bacteria. 2. The action of tyrocidin and some detergent substances in releasing amino-acids from the internal environment of *Streptococcus faecalis*. J. Gen. Microbiol. **1**:77-84.
10. Hahn, F. E. 1977. Modes of action of antimicrobial agents, p. 1-19. In M. J. S. Dewar (ed.), Topics in current chemistry. Springer-Verlag, Berlin.
11. Hashimoto, T., S. H. Black, and P. Gerhardt. 1960. Development of fine structure, thermostability and picolinate during sporogenesis in a *Bacillus*. Can. J. Microbiol. **6**:203-212.
12. Hassan, H. M., and I. Fridovich. 1980. Mechanism of the antibiotic action of pyocyanine. J. Bacteriol. **141**:156-163.
13. Khokhlov, A. S., and I. N. Blinova. 1974. Structure of viridomycin A—a new iron-containing antibiotic. Dokl. Biochem. **214**:186-188.
14. Leong, J., and K. N. Raymond. 1974. Coordination isomers of biological iron transport compounds. I. Models for the siderochromes. The geometrical and optical isomers of tris(N-methyl-1-menthoxyacetyldroxamato)chromium(III). J. Am. Chem. Soc. **96**:1757-1762.
15. Luckey, M., J. R. Pollack, R. Wayne, B. N. Ames, and J. B. Neilands. 1972. Iron uptake in *Salmonella typhimurium*: utilization of exogenous siderochromes as iron carriers. J. Bacteriol. **111**:731-738.
16. Marchenko, I. V., S. A. Egorova, N. O. Blinov, and N. A. Krassilnikov. 1968. The role of iron in the formation of green pigments by actinomycetes. Z. Allg. Mikrobiol. **8**:437-439.
17. Molin, S., K. von Meyenburg, O. Maaloe, M. T. Hansen, and M. L. Pato. 1977. Control of ribosome synthesis in *Escherichia coli*: analysis of an energy source shift-down. J. Bacteriol. **131**:7-17.
18. Nazar, R. N., and J. T.-F. Wong. 1969. Inhibitor-induced shift-downs in *Escherichia coli*. J. Bacteriol. **100**:956-961.
19. Nazar, R. N., and J. T.-F. Wong. 1972. Nucleotide changes and the regulation of ribonucleic acid accumulation during growth rate shifts in *Escherichia coli*. J. Biol. Chem. **247**:790-797.
20. Neilands, J. B. 1952. A crystalline organo-iron pigment from a rust fungus (*Ustilago sphaerogena*). J. Am. Chem. Soc. **74**:4846-4847.
21. Newton, B. A. 1953. The release of soluble constituents from washed cells of *Pseudomonas aeruginosa* by the action of polymyxin. J. Gen. Microbiol. **9**:54-64.
22. Olenick, J. G., T. M. Cook, and F. E. Hahn. 1971. Bactericidal action of 2-hydroxy-3-(cyclohexylpropyl)-1,4-naphthoquinone on *Bacillus megaterium*. J. Bacteriol. **107**:528-534.
23. Pollack, J. R., B. N. Ames, and J. B. Neilands. 1970. Iron transport in *Salmonella typhimurium*: mutants blocked in the biosynthesis of enterobactin. J. Bacteriol. **104**:635-639.
24. Reid, G. A., and W. J. Ingledew. 1979. Characterization and phenotypic control of the cytochrome content of *Escherichia coli*. Biochem. J. **182**:465-472.
25. Reider, E., E. F. Wagner, and M. Schweiger. 1979. Control of phosphoenolpyruvate-dependent phosphotransferase-mediated sugar transport in *Escherichia coli* by energization of the cell membrane. Proc. Natl. Acad. Sci. U.S.A. **76**:5529-5533.