PROTORUBRADIRIN, AN ANTIBIOTIC CONTAINING A *C*-NITROSO-SUGAR FRAGMENT, IS THE TRUE SECONDARY METABOLITE PRODUCED BY *Streptomyces achromogenes* var. *rubradiris*.

RUBRADIRIN, DESCRIBED EARLIER, IS ITS PHOTO-OXIDATION PRODUCT

BRIAN BANNISTER and BARBARA A. ZAPOTOCKY†

Medicinal Chemistry Research, The Upjohn Company,
Kalamazoo, MI 49001, U.S.A.

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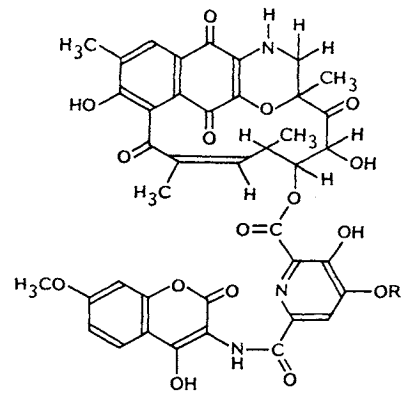
In an attempt to improve the isolation of the antibiotic rubradirin from fermentations of *Streptomyces achromogenes* var. *rubradiris*, the use of preparative reversed-phase chromatography was investigated. The product isolated was a mixture of rubradirin and a new antibiotic named protorubradirin, of extremely similar structure, which is converted into rubradirin on exposure to light and air. Methanolysis of protorubradirin in the dark yields an anomeric mixture of methyl glycosides of a *C*-nitroso-sugar, converted photo-oxidatively into the methyl rubranitrosides derived from rubradirin. Thus, protorubradirin is the *C*-nitroso-analogue of rubradirin. It is suggested that the same relationship between protorubradirin and rubradirin may apply to the anthracycline antibiotics viriplanin A and viriplanin D.

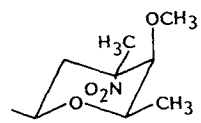
The conditions of fermentation of *Streptomyces achromogenes* var. *rubradiris*[1] which led to the production of a new antibiotic activity, and the isolation[2] of rubradirin, were reported in 1964. Rubradirin is highly active against a variety of Gram-positive bacteria both *in vitro* and *in vivo*, and its activity has been shown to be the consequence of its impairment of ribosomal functions by the selective inhibition of protein synthesis.[3]

The structure of the sugar fragment of the antibiotic, rubranitrose, was described as that of 2,3,6-trideoxy-3-*C*-methyl-3-nitro-L-xylo-hexopyranose.[4] Although the relative stereochemistry was established by X-ray crystal-structure determination, circular dichroism comparisons were used to assign the absolute stereochemistry, the correctness of which was challenged.[5~7] The syntheses of L-rubranitrose[8] and of D-rubranitrose[9] confirmed that the sugar indeed belongs to the D-series. The structure of rubradirin itself has been published,[10] although this structure includes the incorrect assignment of the sugar to the L-series. The structure

Fig. 1. Structures of rubradirin (**1**) and rubradirin aglycon (**2**).



1 Rubradirin, R=



2 Rubradirin aglycon, R=H

† Present company and address: The Bristol-Myers Squibb Company, 2400 West Lloyd Expressway, Evansville, IN 47712, U.S.A.

1 shows the correct stereochemistry of the sugar fragment.

Interest in rubradirin was rekindled by two discoveries: it was found to be highly active against the increasingly important multiply-antibiotic resistant strains of *Staphylococcus aureus*,[11] and it was found that, although rubradirin itself is moderately active *in vitro* in the inhibition of the human immunodeficiency virus (HIV) reverse transcriptase, rubradirin aglycon (**2**), while antibacterially inactive, is extremely active against this enzyme.[12]

Further investigation of these interesting biological activities imposed two requirements—that the isolation procedure be improved, and that the production of antibiotic by the fermentation be increased.

The original method[2] of isolation of the antibiotic involved extraction of the acidified beer, and countercurrent distribution of the crude material, a procedure of limited utility because of low solubility in appropriate solvents. Although extensive decomposition of the antibiotic occurred on attempted chromatography on silica, chromatography in chloroform on silica which had been treated with aqueous potassium dihydrogen phosphate, and re-activated by heating, permitted the isolation of essentially pure rubradirin.[10] However, the quantity which could be processed in this manner remained small.

Since reversed-phase columns were not commercially available at the time of previous investigations, such chromatography was now examined. Using a sample of a crude extract from an old fermentation, it was found that gradient elution on a C-18 analytical column with a mobile phase consisting of tetrahydrofuran and aqueous ammonium acetate at pH 4.0 gave a major peak not distinguished from that of a sample of rubradirin which had been isolated by "buffered silica" chromatography. Preparative reversed-phase chromatography of the crude extract using a 1:1 mixture of these solvents isocratically yielded a major fraction showing the same symmetrical peak on examination on the analytical column as that given by pure rubradirin. Isolation of material gave an amorphous red powder, the ^{13}C NMR spectrum of which was identical to that recorded,[10] thereby establishing that rubradirin could be isolated readily by preparative reversed-phase chromatography from a crude extract of the antibiotic.

It had been found in the original isolation[2] of the antibiotic that the activity is present in the filtered beer and is not retained in the mycelium. The antibiotic is produced in the fermentation only in low titer, and it was considered that this might be the consequence of a feed-back mechanism which shut off its production when a certain extra-cellular concentration of the antibiotic had been attained. Adsorption of the antibiotic on a non-ionic resin, added to the fermentation medium, therefore, might prevent this concentration from being reached, and thereby permit the greater production of the antibiotic. That this method indeed gives increased yields of rubradirin has been published.[13,14]

Preparative reversed-phase chromatography of the crude antibiotic, obtained by the elution of the separated resin, gave a major fraction which on the analytical column in the gradient system was not distinguished from the pure rubradirin isolated earlier. The ^{13}C NMR spectrum of the material isolated from this fraction showed all of the signals of rubradirin, but there were three additional signals which seemed to pair those assigned to the carbon atoms at the 1-, 3- and 5-positions of the sugar fragment in rubradirin. This implied that the material isolated from the resin contained both rubradirin and a new product of similar structure, in which the aglycon fragment was the same, but which differed in the sugar component in such a way that only the signals

Table 1. Chemical shifts in ppm, $(CD_3)_2CO$.

Sugar position assignment	Rubradirin	Second component
1-	96.16	95.64
3-	91.17	104.79
5-	70.97	72.47

of the 1-, 3-, and 5-positions were affected. That the resin played no direct role in the generation of the new component was shown by the recovery of rubradirin, of unchanged ^{13}C NMR spectrum, after adsorption onto, and elution from, the resin.

Since the previous method of isolation of rubradirin had involved chromatography on phosphate-modified silica using chloroform as the eluting solvent, it was plausible that adsorption on the somewhat acidic silica, or the presence of chloroform which might be acidic, could be responsible for the difference between the products isolated by the two methods. Chromatography over treated silica of the mixture of rubradirin and the new component obtained by reversed-phase chromatography gave material which now gave a ^{13}C NMR spectrum devoid of the three new signals, and indistinguishable from that of pure rubradirin. The chromatography was conducted on a small scale, and no material balance was examined, leaving as possibilities the conversion of the new component into rubradirin, or its loss by retention on the treated silica. In the expectation that this isolation of rubradirin only from the mixture was the result of a conversion, and that it was a consequence of solvent-pH effects, the ^{13}C NMR spectrum of this mixture, dissolved in deuteriochloroform which had been percolated through a column of treated silica, was examined automatically at intervals throughout a weekend: no change in spectrum was observed.

It was then found that the mixture of rubradirin and the new component, not resolved on the C-18 analytical column in the tetrahydrofuran - aqueous ammonium acetate-pH 4.0 system, was resolved, though poorly, in a gradient acetonitrile - aqueous ammonium acetate-pH 4.0 system. Under these conditions, rubradirin had an Rt value of 10.79 minutes and the new component 11.20 minutes. Chromatography over treated silica was repeated, and individual fractions were examined analytically in the new mobile phase. Again, no change was observed in any of the fractions, all being mixtures. On the following day, material from these fractions was isolated; examination by both ^{13}C NMR spectroscopy and reversed-phase chromatography now demonstrated this to be pure rubradirin. It became apparent, therefore, that the new component must be photosensitive, and that it is converted in the presence of light into rubradirin.

Preparative reversed-phase chromatography in an isocratic acetonitrile - aqueous ammonium acetate-pH 4.0 system allowed the isolation of the pure new component, which gave a ^{13}C NMR spectrum showing now only the new singlet signals for the 1-, 3-, 5-sugar carbon atoms. Its UV spectrum was indistinguishable from that of rubradirin.

Exposure of a solution of this pure component in tetrahydrofuran solution to daylight resulted in the generation of rubradirin; after 1.5 hours, 41% of rubradirin had been produced, after 4.5 hours, only a shoulder of the new component remained and, after 6 hours, only a symmetrical peak of rubradirin could be seen. No change in composition of the pure new component in tetrahydrofuran solution could be found after it had been kept in the dark for 24 hours. The same conversion of the new component into rubradirin on exposure to light occurred in acetonitrile solution and, more slowly, in the absence of solvent.

With the realization that this second component was converted into rubradirin on exposure to light, the isolation procedure was conducted in the dark as far as possible—though some exposure was unavoidable—and the product isolated was found to consist of 8% of rubradirin and 92% of the new component, which was named protorubradirin. The conversion of protorubradirin into rubradirin in the presence of light was accompanied by the formation of only a total of 7% of three minor, more polar, unidentified, by-products.

All of the above data indicate that the difference between rubradirin and protorubradirin lies in the sugar fragment, the aglycon fragment being identical in the two compounds. The field-desorption mass

spectrum of rubradirin had established its molecular formula as $C_{48}H_{46}N_4O_{20}$ (by means of a fleeting molecular ion of *m/z* 998), and its aglycon gave a molecular ion of *m/z* 811 (an ion also seen in the spectrum of rubradirin from the loss of the sugar fragment) corresponding to $C_{40}H_{33}N_3O_{16}$ as its molecular formula, and thus conferring a molecular formula of $C_8H_{15}NO_5$ onto the sugar fragment.[10] Neither field desorption nor FAB-MS revealed a molecular ion for protorubradirin, the ion of highest mass, *m/z* 811, corresponding to that of the aglycon, thus giving no evidence of the nature of the sugar fragment of protorubradirin.

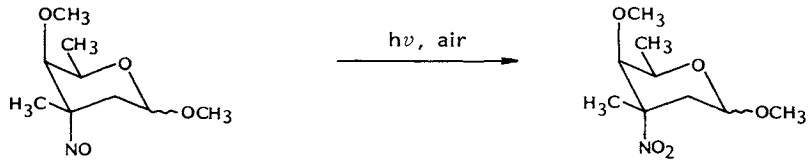
Cleavage of rubradirin in methanolic hydrogen chloride gave rubradirin aglycon and a mixture of methyl α- and β-rubranitrosides; the crystalline α-anomer corresponded in all physical properties to those of the synthetic methyl α-D-rubranitroside.[9] Methanolysis of protorubradirin was conducted similarly, but in the dark, and occurred much more rapidly than with rubradirin: its aglycon was identical to rubradirin aglycon by TLC, reversed-phase chromatography, and ^{13}C NMR spectroscopy. In an attempt to isolate the sugar fragment of protorubradirin from the filtrate of its aglycon, a trace of only methyl α- and β-rubranitrosides could be isolated.

The low solubility of protorubradirin (or rubradirin) in methanol resulted in the cleavages' being conducted at high dilutions, at which the sugar fragment could not be detected by TLC. The methanolysis of protorubradirin was therefore investigated in the dark in chloroform solution containing a small amount of methanol and acetyl chloride, giving a twenty-fold higher concentration. TLC of the reaction mixture showed two zones, on charring with sulfuric acid, which were distinguished from the anomeric methyl rubranitrosides, and which therefore corresponded to the sugar fragment of protorubradirin. Dilution of the reaction mixture with cyclohexane permitted the separation of the aglycon; removal of the solvent *in vacuo* from the filtrate gave a red syrup.

In the lack of knowledge of the difference between the sugar fragments of rubradirin and protorubradirin, it had been assumed that the photo-conversion of protorubradirin into rubradirin had been the result of the absorption of energy at some wavelength(s) corresponding to the λ_{max} value(s) of the chromophore(s), with the transmission of this energy to the sugar fragment—that is, that the equivalent of an intramolecular photosensitization was involved. As a consequence, the methanolysis of protorubradirin having been conducted in the dark, there seemed to be no need to exclude light in the isolation of the anomeric methyl glycosides. Accordingly, the crude red syrup, dissolved in ethyl acetate-*n*-hexane, was applied to the top of a column of silica equilibrated in the same mobile phase. In the process of transferring this "solution" to the column, a flocculent red precipitate formed, revealing a bright blue supernatant solution. A blue band travelled down the column, and blue fractions were collected. None of the fractions was pure; TLC showed that all now gave four zones on charring with sulfuric acid, the two lower zones not being distinguished from methyl α- and β-rubranitrosides.

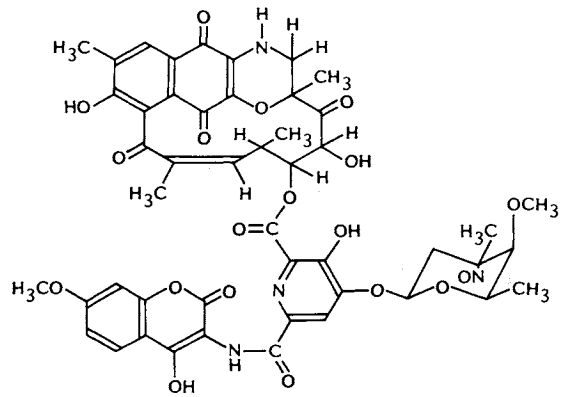
This deep blue color of a small molecule, expected to be colorless, suggested that the sugar fragment of protorubradirin was a nitroso-sugar. Repetition of the TLC, followed by spraying with periodate-permanganate, immediately showed the presence of the upper two zones, the lower zones being seen only on charring with sulfuric acid. As the fractions stood, they became noticeably lighter in color; correspondingly, as the color lightened, TLC showed that the lower pair of zones increased in intensity at the expense of the upper pair. GC-MS of a blue fraction gave two peaks, the one of shorter retention time showing ions of *m/z* 203, 172, and 141, corresponding to M^+, $M^+ - HNO$, and $M^+ - HNO - OCH_3$ for the nitroso-analogue of methyl rubranitroside. The second peak showed no molecular ion, but ions

Fig. 2. Structures of the anomeric methyl rubranitrososides (**3**) and methyl rubranitrosides (**4**).



3 4

Fig. 3. Structure of protorubradirin (**5**).



of m/z 188 ($M^+ - OCH_3$) and 141 ($188 - HNO_2$), not distinguished from the GC-MS results under the same conditions obtained with methyl α-rubranitroside.

Deliberate irradiation of a blue fraction by placing it close to a lighted incandescent bulb caused the rapid fading of color. On continued exposure to light, the blue color disappeared completely; only the lower pair of zones could be seen by TLC, and only the peak of longer retention time could be seen by GC-MS. Removal of the solvent from the originally blue fractions and chromatography on silica gave products not distinguished from authentic methyl α- and β-rubranitrosides. Thus, the loss of color is caused by the oxidation of a *C*-nitroso-group to a *C*-nitro-group; there was no indication of dimerization of the *C*-nitroso-compound to a colorless azodioxy-compound, and this is in accordance with the ready dimerization only of primary and secondary nitroso-alkanes, the tertiary analogues tending to remain monomeric.[15,16]

Hence, the sugar fragment obtained on methanolysis of protorubradirin is a mixture of methyl rubranitrososides (methyl α,β-2,3,6-trideoxy-3-*C*-methyl-3-nitroso-D-xylo-hexopyranosides (**3**)), which undergoes oxidation in the presence of light and air to the methyl rubranitrosides (**4**). Hence, protorubradirin has the structure **5**.

The literature[15~17] indicates that *C*-nitroso-compounds are photo-labile, resulting from the excitation of the weak absorption band at 680 nm of the nitroso-group, and this is presumably the wavelength responsible for the excitation permitting the oxidative conversion of the methyl rubranitrososides into the methyl rubranitrosides. Whether this is true, too, of the wavelength involved in the conversion of protorubradirin into rubradirin, or whether intramolecular photosensitization involving the chromophore(s) of the aglycon fragment is involved, has not been investigated.

That the production of protorubradirin in the fermentation was not a consequence of the presence of the non-ionic resin was shown by the demonstration of the production of protorubradirin in a fermentation conducted in the absence of the resin. Thus the true secondary metabolite[18] of the fermentation of *Streptomyces achromogenes* var. *rubradiris* is not rubradirin but protorubradirin, and it is interesting to speculate on the role of protorubradirin *vs.* rubradirin in the evolution of its producing microorganism.[19,20]

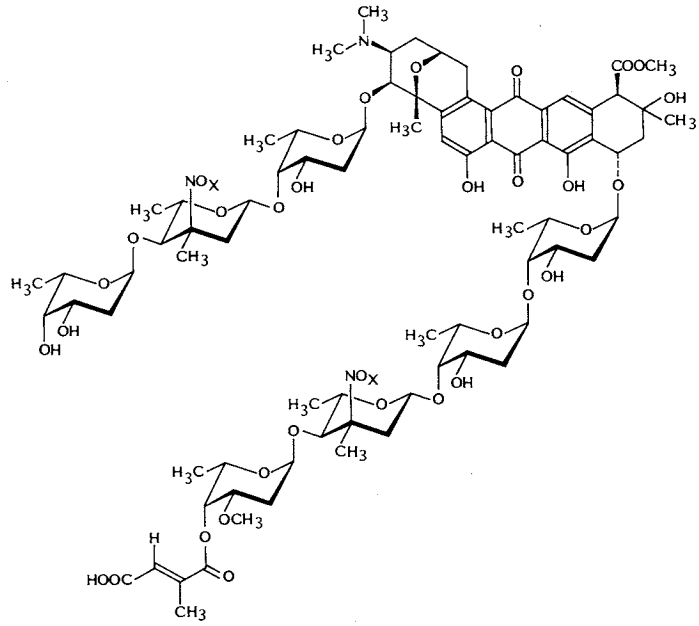
It is reported[21] that in fermentation, *Micromonospora saitamica* sp. nov. Routien produces rubradirin, together with three other antibiotics of unknown structure. Since *Streptomyces achromogenes* var. *rubradiris* lacks an enzyme capable of oxidizing a tertiary *C*-nitroso-group to a *C*-nitro-group, a process which is accomplished, non-microbially, only on exposure to light and air, it was of interest to see whether the *Micromonospora* spp. similarly lacked such an enzyme. The rubradirin had been isolated following the same conditions of extraction and chromatography on "buffered" silica gel as had been used by HOEKSEMA *et al.*,[10] and thus had involved exposure to both light and air. Addition of non-ionic resin to the *Micromonospora* spp. fermentation increased the production of antibacterial activity over that obtained under the conditions quoted in the patent. Elution of antibiotics from this resin, with as little exposure to light as possible, gave a crude complex which showed a symmetrical peak by analytical reversed-phase chromatography in the aqueous pH 4.0 ammonium acetate - tetrahydrofuran system described earlier, not distinguished from rubradirin, but resolved in the aqueous buffer - acetonitrile system into a minor component corresponding to rubradirin and a major component not distinguished from protorubradirin. Exposure of this crude extract in tetrahydrofuran solution to light and air resulted in the increase in the area of the peak ascribed to rubradirin and the disappearance of that ascribed to protorubradirin. Thus the *Micromonospora spp.*, too, produces protorubradirin as the secondary metabolite, and lacks the enzymatic ability to oxidize it to rubradirin.

In addition to rubradirin, several other antibiotics contain methyl-branched nitro-sugars, and result from fermentations involving microorganisms of different genera of the order Actinomycetales. Others are the everninomicins B, C, and D (from *Micromonospora carbonacea*),[22] kijanimicin (from *Actinomadura kijaniata*),[23] tetrocarcins A and B (from *Micromonospora chalcea*),[24] arugomycin (from *Streptomyces violochromogenes*),[25] and decilorubicin (from *Streptomyces virginiae*).[26] Not unexpectedly, none of these antibiotics had been isolated under conditions which excluded light and air, and the intriguing possibility exists, therefore, that the *C*-nitroso- and not the *C*-nitro-compound is produced as the actual secondary metabolite by these microorganisms also.

A distinct possibility along these lines is presented by the antibiotic viriplanin A (from *Ampullariella regularis*)[27] which was stated to have been isolated satisfactorily only with the exclusion of light. Methanolysis of viriplanin A gave an anomeric mixture of three identified methyl glycosides, one of which was that of decilonitrose. However, although the absorption band in the IR spectrum at 1540 cm^{-1} of the *C*-nitro-group allowed the presence of this sugar fragment to be detected in the intact decilorubicin and arugomycin, this absorption was not detectable in the spectrum of viriplanin A, and the authors stated that the methyl decilonitroside must have been an artifact of methanolysis. In addition, they reported that viriplanin A in solution was unstable to light, being converted into a single, more polar, product, viriplanin D, *via* two intermediates, viriplanins B and C, of polarities intermediate between those of A and D. The structure of the aglycon was determined, but no structure for viriplanin A was proposed.[27]

In a later publication by this group,[28] the conversion of viriplanin A into viriplanin D by irradiation

Fig. 4. Suggested structures of viriplanins A and D.



6 Viriplanin: A, X=1
D, X=2

in methanol, followed by methanolysis and the isolation of methyl decilonitroside (incorrectly[29] considered to be of the D-series) was reported. On the basis of unquoted data, the structure of viriplanin D (incorporating the incorrect absolute stereochemistry of decilonitrose) was given, and it was stated that viriplanin D is a photo-oxidation product of viriplanin A. The structure of viriplanin A has not been reported: on the basis of our investigation into the relationship between protorubradirin and rubradirin, we propose that viriplanin A has the analogous *C*-nitroso-structure. On the assumption that the disclosed structure of viriplanin D is correct, except for the absolute stereochemistry of the decilonitrose fragment, this gives viriplanins A and D the structures (**6**): Since there are two decilonitrose fragments in viriplanin D, it is reasonable to suggest that the photo-labile intermediates, the viriplanins B and C, represent the two possible mono-*C*-nitroso-mono-*C*-nitro-compounds.

In the examples in the literature[15~17] concerning the photo-lability of *C*-nitroso-compounds, the homolysis of the C–N bond to give alkyl and nitric oxide radicals dominates overwhelmingly, in the primary photochemical act, with ensuing reactions being mainly additions of the radicals to nitroso-alkanes, followed by ionic or radical reactions of the adducts. Oxidations have been observed, but these are usually to nitrate esters; the formation of *C*-nitro-compounds as major products of the photo-oxidation of *C*-nitroso-compounds has not been reported. In the formation of nitrate esters from *C*-nitroso-compounds, stereochemical integrity is lost, but it is maintained in the generation of nitro-compounds, so that C–N bond scission does not occur in this oxidation.

ABRAMOVITCH *et al.*[30] reported that the tertiary *C*-nitroso-compounds 2-nitroso-2-phenylpropane and 2-(2-biphenylyl)-2-nitrosopropane are "extremely susceptible to atmospheric oxidation to give mostly the nitro-compound," but they make no reference to whether or not this oxidation is photo-induced.

Fig. 5. Tertiary *C*-nitroso-sugars subject to spontaneous oxidation.

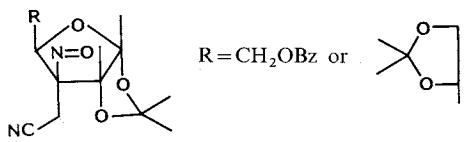


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Table 2. Antibacterial activity of protorubradirin and rubradirin.

Organism	Culture No.	Minimum inhibitory concentration (μg/ml)	
		Proto-rubradirin	Rubradirin
Staphylococcus aureus	76	0.125	0.125
S. aureus	6675	0.5	0.5
S. aureus	6685	0.5	0.25
Streptococcus pneumoniae	41	0.06	0.25
Streptococcus pyogenes	152	0.06	0.25
Streptococcus viridans	30641	0.125	1.0

Similarly, TRONCHET *et al.*[31] observed that *C*-nitroso-compounds of the type 7 obtained by the periodate oxidation of the corresponding tertiary *N*-hydroxyamino-compounds, "could not be isolated in a pure form as they spontaneously oxidized to the corresponding nitro-compounds" but again, no reference is made to any involvement of light. To make this question of the stability of tertiary *C*-nitroso-compounds to air ± light further uncertain is the report by GANGULY *et al.*[32] that the tertiary *C*-hydroxyamino-compound hydroxyaminoeverninomicin D "undergoes aerial oxidation very easily to the corresponding nitroso-compound." However, neither experimental data nor evidence for the structure of the oxidation product is given.

Biological Activities of Protorubradirin

Protorubradirin shows the same moderate inhibition of the activity of the HIV reverse transcriptase enzyme as is shown by rubradirin.

In vitro, protorubradirin possesses activity against strains of *Staphylococcus aureus* comparable to that of rubradirin, and is somewhat more active than rubradirin against species of *Streptococcus*. *In vivo* in the infected mouse, following subcutaneous administration, protorubradirin was exceptionally active against three strains of *Staphylococcus aureus*, one of which is multiply-antibiotic resistant and one methicillin resistant; its ED_{50} values are not significantly different from those of rubradirin. In contrast, following oral administration, protorubradirin was significantly less active than rubradirin. It was noted earlier that the methanolytic cleavage of the *C*-nitroso-sugar from protorubradirin occurs more readily than that of the *C*-nitro-sugar from rubradirin. The aglycon produced, the same in each case, is antibacterially inactive. It is possible that the greatly reduced activity of protorubradirin administered orally is a reflection of the more extensive cleavage of the antibiotic to aglycon in the acidic environment of the stomach than is the case with rubradirin.

Experimental

General

Optical rotations were measured with a Perkin-Elmer 241 polarimeter. ^{13}C NMR spectra were recorded with a Varian XL-300 spectrometer operating at 75 MHz using TMS as a reference. TLC was conducted on Analtech silica gel GF plates. Flash chromatography utilized EM silica G 60 (230 ~ 400 mesh). Analytical reversed-phase chromatography employed a Hewlett-Packard 1084A liquid chromatograph with a Brownlee Spheri-10 RP-18 cartridge, dimensions 4.6 × 100 mm. Preparative reversed-phase chromatography employed a Waters Prep LC/System 500A chromatograph with either a single Waters PrepPak 500-C-18 column, or two such columns in series. Gas chromatography-mass spectrometry studies utilized a Hewlett-Packard 5990A GC/MS instrument. Diaion HP-21 resin is a product of Mitsubishi Chemicals, Ltd.

Isolation of Rubradirin from an Old Crude Extract

A solution of the crude extract (1.0 g) in tetrahydrofuran (10 ml) was injected onto the PrepPak column which had been equilibrated with a 1 : 1 mixture of 0.05 M aqueous ammonium acetate, adjusted to pH 4.0 by the addition of glacial acetic acid, and tetrahydrofuran. Elution with the same solvent at a flow rate of 150 ml/minute, monitoring at 365 nm, gave early fractions consisting of unknown materials and then rubradirins B and C,[10] followed by a major fraction of Rt *ca.* 1 hour (2.5 liters). Fractions were analyzed using the Brownlee C-18 column in a gradient system of aqueous 0.05 M ammonium acetate at pH 4.0 and tetrahydrofuran of 20% to tetrahydrofuran up to 50% at 10 minutes and held there. The major fraction gave a single symmetrical peak of Rt 12.12 minutes, not distinguished from that of a sample of pure rubradirin isolated earlier from "buffered silica" chromatography in chloroform. Volatile solvent was removed from this fraction on a rotary evaporator at 35°/house vacuum to give a red aqueous suspension which was extracted with chloroform. Removal of the solvent *in vacuo* gave an amorphous solid which smelled slightly of acetic acid; this was removed by dissolving the solid in acetonitrile and again taking to dryness. A solution of this residue in chloroform was diluted with *n*-hexane to give a deep red powder, which was removed by filtration and allowed to dry in the air, giving 400 mg of rubradirin, $[\alpha]_D^{25}$ +777° (*c* 0.028, $(CH_3)_2CO$) (literature +920° (*c* 0.25, $(CH_3)_2CO$),[2] +571° (*c* 0.0205, $(CH_3)_2CO$)[10]). The ^{13}C NMR spectrum was in agreement with that in the literature.[10]

Isolation of Mixed Rubradirin-protorubradirin from a Fermentation Conducted in the Presence of Diaion HP-21 Resin

The dark brown resin (*ca.* 1.5 liters), which had been screened away from both beer and mycelia, was placed in a sintered glass funnel (4-liter), and excess water was removed under suction. Fatty material was eluted by washing the resin with cyclohexane - methylene chloride (4 : 1 v/v, 3 × 2 liters), initially under gravity and finally under suction. These washes were discarded. Similar elution of the resin with ethyl acetate (10 liters), removal of the solvent *in vacuo*, re-dissolution of the residue in ethyl acetate and dilution with *n*-hexane (4 volumes) gave a red powder (7.3 g after drying *in vacuo*), showing a major peak analytically of Rt 12.09 minutes.

This solid, dissolved in tetrahydrofuran (20 ml), was transferred *via* the pumps to two Waters PrepPak columns in series, pre-equilibrated in the 1 : 1 mixture used above, and then was eluted at 150 ml/minute. Monitoring was conducted at 365 nm, and fractions were collected accordingly. Fractions were submitted to analytical reversed-phase chromatography, as before. Fraction No. 4 assayed 92% of "rubradirin" with two minor components of slightly shorter Rt values. Fractions Nos. 5, 6, and 7 gave single symmetrical peaks of Rt's 12.03, 12.04, and 12.02 minutes, respectively; removal of volatile material *in vacuo* gave an aqueous suspension of a red solid which was collected on a sintered glass funnel under suction and washed with water. The solid was dissolved in chloroform, dried (Na_2SO_4), and diluted four-fold with *n*-hexane, precipitating a red solid which was removed by filtration, and dried. This material (1.29 g) was not distinguished from the sample of pure rubradirin above by HPLC in the gradient system of ammonium acetate - tetrahydrofuran analytically, but it was revealed to be a mixture by ^{13}C NMR as indicated in the discussion section.

Separation of Protorubradirin from Rubradirin

The above material was shown on the analytical column in a gradient system of 40% acetonitrile and 60% aqueous 0.05 M ammonium acetate at pH 4.0 to 60% acetonitrile at 10 minutes and held there, to be a mixture of two poorly separated components of Rt values 10.79 and 11.20 minutes. Pure rubradirin gave a 10.79 minutes peak; the ratio in the mixture of rubradirin to protorubradirin was 34 : 66. This solid (4.25 g) was dissolved in tetrahydrofuran (20 ml), diluted with acetonitrile (20 ml) and drawn *via* the pumps onto two PrepPak columns in series, pre-equilibrated in 65% acetonitrile - 35% aqueous ammonium acetate, pH 4.0, and eluted isocratically with this same solvent. Again, fractions were examined on the analytical column using the above gradient system. Fraction No. 5 contained 64% rubradirin : 36% protorubradirin; fraction No. 8, 64% : 36%; fraction No. 10, 49% : 51%; fraction No. 12, 43% : 57%; fraction No. 14, almost pure protorubradirin; fractions Nos. 15 and 16, pure protorubradirin.

Fractions Nos. 15 and 16 were combined, volatile solvent was removed *in vacuo*, and the suspended solid isolated as before to give protorubradirin (717 mg) as an amorphous red solid, giving a ^{13}C NMR

spectrum in $(CD_3)_2CO$ differing from that of rubradirin as indicated in the discussion section, $[\alpha]_D^{25}$ +921° (*c* 0.0273, $(CH_3)_2CO$).

Anal Calcd for $C_{48}H_{46}N_4O_{19}$: C 58.64, H 4.73, N 5.70.
Found: C 58.79, H 4.73, N 5.61.

Methanolysis of Rubradirin

Rubradirin (1.0 g) was added to methanol (2 liters) which had been stirred with acetyl chloride (14 ml), and stirring was continued till all of the solid had dissolved. After 60 minutes, no rubradirin remained by HPLC (20% to 50% tetrahydrofuran - 0.05 M ammonium acetate, pH 4.0, in 10 minutes and held there), and a single symmetrical peak, Rt 9.84 minutes, had formed. Removal of the solvent *in vacuo* gave a purple-black residue which was dissolved in tetrahydrofuran and diluted with cyclohexane to give the aglycon as an amorphous solid (750 mg); its ^{13}C NMR spectrum in DMF-d_7 corresponded with the data in the literature.[10]

Removal of solvent *in vacuo* from the above mother-liquors gave a red syrup showing two zones by TLC (ethyl acetate - *n*-hexane, 1 : 3) of Rf 0.42 (minor) and 0.35 (major). Flash chromatography in the same mobile phase gave the minor component as a colorless oil (60 mg)—which could not be induced to crystallize (methyl β-rubranitroside, $[\alpha]_D^{25}$ +21° (*c* 0.761, $CHCl_3$)). The major component (120 mg) crystallized from *n*-hexane to give colorless needles of methyl α-rubranitroside, mp 91~92°, $[\alpha]_D^{25}$ +172° (*c* 0.426, $CHCl_3$) (literature[9] mp 92~93°, $[\alpha]_D^{25}$ +172° (*c* 0.8, $CHCl_3$)), and giving a ^1H NMR spectrum ($CDCl_3$) in agreement with the literature.[9]

Methanolysis of Protorubradirin

a. In Methanol

Methanolysis was carried out exactly as for rubradirin, except that it was conducted in the dark. HPLC analysis after 10 minutes showed the cleavage to be complete (a methanolysis with rubradirin run in parallel showed 60% of the rubradirin still to be present at this time). Isolation as before gave an aglycon not distinguished from rubradirin aglycon by either HPLC or ^{13}C NMR in DMF-d_7. TLC (ethyl acetate - *n*-hexane, 1 : 3) of the concentrated filtrate showed two zones, not distinguished from those of the anomeric methyl rubranitrosides. Column chromatography in the same system gave the major, crystalline, α-anomer in low yield.

b. In Chloroform Containing Methanol and Acetyl Chloride

To chloroform (90 ml), stirred magnetically, was added methanol (9 ml), followed by acetyl chloride (1.0 ml). After 10 minutes, this solution was placed in a dark room, and protorubradirin (1.0 g) was added, with continued stirring. After 45 minutes, TLC (ethyl acetate - *n*-hexane, 1 : 4) showed two zones on charring with sulfuric acid, of Rf 0.44 and 0.41: the anomeric methyl rubranitrosides have Rf values in this system of 0.30 and 0.20. Dilution with cyclohexane (300 ml) gave a red precipitate which was removed by filtration, washed with cyclohexane, and dried, giving the aglycon (775 mg), identical with that from the reaction in methanol.

Concentration of the filtrate and washings *in vacuo* gave a red syrup (190 mg) which was dissolved in the 1 : 3 solvent system and applied to the top of a silica column equilibrated in the same solvent. During this transference, a red flocculent precipitate separated and revealed a bright blue solution. On elution, a bright blue band passed down the column, and blue fractions were collected. TLC of typical fractions showed that the two new anomers had not been resolved, but they were now accompanied by two zones of lower Rf, not distinguished from the anomeric methyl rubranitrosides. As the fractions stood, the depth of color diminished, and the ratio of the two lower zones increased as the upper zones decreased. In the course of time, these fractions all became colorless, and showed only the lower pair of zones by TLC. Combination of the originally blue fractions, evaporation to dryness, and chromatography as before resulted in the isolation of the two materials, identified as the methyl α- and β-rubranitrosides by TLC and ^1H NMR spectra.

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