(19) T. C. Bruice and F. H. Marquardt, *J. Am. Chem. Soc.*, **84**, 365 (1962).
(20) G. O. Dudek and G. P. Volpp, *J. Org. Chem.*, **30**, 50 (1965).
(21) H. Muxfeldt, G. Grethe, and W. Rogalski, *J. Org. Chem.*, **31**, 2429 (1966).
(22) The NMR spectra of **40** under a variety of conditions are reproduced in the microfilm edition.
(23) H. Muxfeldt, G. Buhr, and R. Bangert, *Angew. Chem.*, **74**, 213 (1962).
(24) H. Meerwein, G. Hinz, P. Hofmann, E. Kroning, and E. Pfeil, *J. Prakt. Chem.*, **147**, 257 (1937).
(25) R. F. Borch and H. D. Durst, *J. Am. Chem. Soc.*, **91**, 3996 (1969). A more complete discussion is to be found in the Ph.D. Thesis of R. Stojda, Cornell University, 1971.
(26) We thank Mr. Roland Plude of Chas. Pfizer Medical Research Laboratories for performing the bioassay on synthetic *dl*-terramycin.
(27) L. F. Fieser, *J. Am. Chem. Soc.*, **59**, 1018 (1937).
(28) Satisfactory analytical data were obtained.
(29) H. H. Inhoffen, H. Muxfeldt, H. Schaefer, and H. Kramer, *Croat. Chem. Acta*, **29**, 329 (1957).
(30) E. Vedejs, Ph.D. Thesis, University of Wisconsin, 1966.
(31) The best procedure for preparation of pure 2-phenylthiazolinone is the PBr_3 method.[13] The alternative method using dicyclohexylcarbodiimide for dehydration of thiohippuric acid gives product which is contaminated with dicyclohexylurea.
(32) We are grateful to Dr. I. A. Solomons and Dr. L. H. Conover of Chas. Pfizer Medical Research Laboratories for supplying us with authentic terramycin.

Biosynthesis of the Antibiotic Granaticin

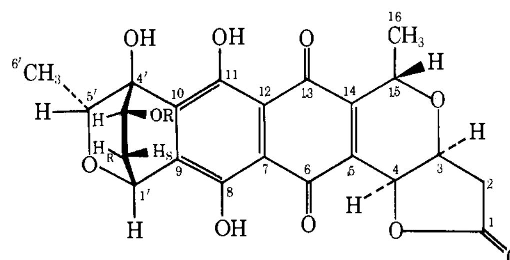
Carl E. Snipes, Ching-jer Chang, and Heinz G. Floss*

Contribution from the Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907. *Received June 12, 1978*

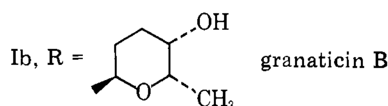
Abstract: The antibiotic granaticin (Ia) is synthesized by *Streptomyces violaceoruber* from eight acetate units, which are assembled into a benzoisochromane quinone moiety, and a molecule of glucose, which is converted into a 2,6-dideoxyhexose and attached to the aromatic moiety by carbon–carbon linkages at C-1 and C-4. Conversion of glucose into the 2,6-dideoxyhexose moiety proceeds with retention of H-1, H-2, H-4, and the hydrogens at C-6 and loss of H-3 and H-5. The hydroxyl group at C-6 of glucose is replaced with inversion of configuration by a hydrogen which is transferred intramolecularly from C-4, indicating operation of the dTDP-glucose oxidoreductase reaction as the first pathway-specific step. The hydroxyl group at C-2 of the hexose is replaced by hydrogen with retention of configuration. The last step in the biosynthesis of granaticin seems to be formation of the five-membered lactone ring; a cell-free extract of *S. violaceoruber* was shown to catalyze formation of granaticin from dihydrogranaticin (IIa) without incorporation of ^{18}O from $^{18}O_2$.

Introduction

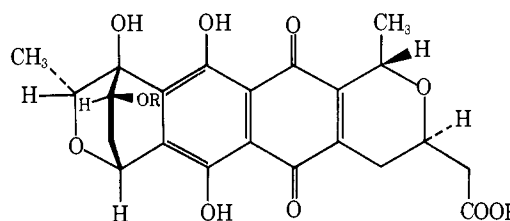
The antibiotic granaticin (Ia)[1] was first isolated from *Streptomyces olivaceus*,[2] but was also detected in a number of other actinomycetes, e.g., in *S. violaceoruber*, in which it co-occurs with the glycoside granaticin B (Ib),[3] and in *S. litmogenes*.[4] The structures of Ia and Ib were established by a combination of chemical degradations[5] and an X-ray analysis.[6] Granaticin B is the α-L-rhodinoside of granaticin. Granaticin belongs to a class of microbial metabolites which is characterized by the presence of a benzoisochromane quinone system and which also includes kalafungin,[7] frenolicin,[8] actinorhodin,[9] the nanaomycins,[10,11] the naphthocyclinones,[12,13] and the griseusins.[14] Recently, three cometabolites of granaticin were isolated and their structures were assigned as dihydrogranaticin (IIa) and the anthraquinones IIIa and IIIb.[15]



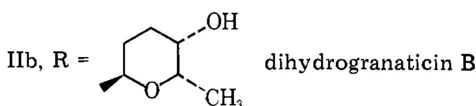
Ia, R = H, granaticin



Ib, R = granaticin B



IIa, R = H, dihydrogranaticin



IIb, R = dihydrogranaticin B

Granaticin is active against Gram-positive bacteria and protozoa, but has little or no activity against Gram-negative bacteria, mycobacteria, fungi, or yeasts.[16,17] It has some activity against P-388 leukemia[4] and granaticin B inhibits various transplanted tumors in rodents (cf. footnote 1 in ref 18). Granaticin inhibits RNA synthesis and to a lesser extent DNA and protein synthesis.[19] The antibiotic blocks the charging of Leu-tRNA by inhibiting leucyl-tRNA synthetase;[19] it also inhibits RNA-dependent DNA synthesis, but by an interaction with the template rather than with the enzyme, reverse transcriptase.[18]

In the present communication we report results which establish the overall biosynthetic origin of granaticin and which provide detailed information on certain aspects of its mode of formation.

Results

The biosynthesis of granaticin was studied in shake cultures of *S. violaceoruber* strain Tü 22,[3] which were grown in complex media based on peanut meal/glucose (acetate feeding experiments) or malt extract/yeast extract/glucose (cell-free experiments) or in a synthetic medium containing mannose

0002-7863/79/1501-0701$01.00/0 © 1979 American Chemical Society

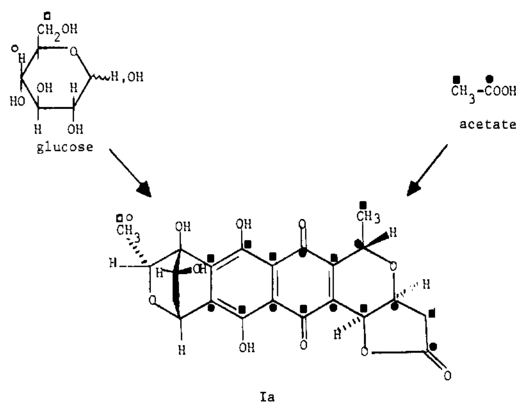
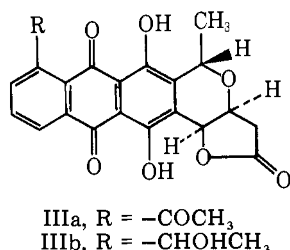


Figure 1. Biosynthetic origin of granaticin.



and ammonium sulfate as carbon and nitrogen sources (glucose feeding experiments). Based on time-course studies, addition of labeled precursors 24 h after inoculation and harvest of the cultures 24 h later were chosen as standard conditions for feeding experiments. Granaticin was isolated from the acidified culture filtrate in yields of 100–150 mg/L by extraction with chloroform and was purified by column or preparative layer chromatography on oxalic acid treated silica gel.[12]

Based on biosynthetic theory and on precedent in the biosynthesis of the nanaomycins[10] and α-naphthocyclinone[20] it was expected that the benzoisochromane quinone moiety comprising carbon atoms 1–16 arises from eight acetate units by way of the polyketide pathway. To test this notion, sodium [1-^{13}C]- and [2-^{13}C]acetate were each fed to eight 100-mL shake cultures of *S. violaceoruber* at a concentration of 300 mg/L. Optimization experiments had indicated that at this concentration a total ^{13}C enrichment in granaticin of about 24–40% or 3–5% per (presumably) labeled carbon could be expected. The ^{13}C distribution in the two labeled samples was then analyzed by ^{13}C NMR spectroscopy. Since granaticin and its tetraacetate gave relatively poor ^{13}C NMR spectra, the samples were converted to dihydrogranaticin methyl ester by hydrogenolysis over Adams catalyst and methylation with methanolic HCl.[15] The complete assignment of the ^{13}C NMR spectrum of dihydrogranaticin methyl ester (Table I) rests on chemical shift and multiplicity analysis, chemical shift comparison with several derivatives and analogues, single-frequency proton decoupling experiments, and NOE measurements. The critical distinction between the pairs of related carbon atoms in the naphthazarin moiety (C-5/C-14, C-6/C-13, C-7/C-12, C-8/C-11, and C-9/C-10) was made primarily on the basis of a spectral comparison of dihydrogranaticin methyl ester with its 3′-keto and 11-*O*-methyl derivatives.[21] Analysis of the two ^{13}C-labeled samples of dihydrogranaticin methyl ester indicated the presence of eight enriched carbons in each specimen. The data in Table I show that carbon atoms 1–16 are labeled by C-1 and C-2 of acetate in the alternating pattern predicted by the polyketide pathway (Figure 1). No significant ^{13}C enrichment is seen in carbon atoms 1′–6′.

Since the six-carbon bridged cyclic moiety is not labeled by acetate, a hexose derived from glucose seemed a likely alter-

Table I. ^{13}C NMR Analysis of Dihydrogranaticin Methyl Ester Biosynthesized from [^{13}C]Acetate

carbon atoms	chemical shift, δ ppm	atom % ^{13}C present in dihydrogranaticin methyl ester biosynthesized from: sodium [1-^{13}C]acetate	sodium [2-^{13}C]acetate
6	174.9	1.1[a]	2.4
13	174.8	2.2[a]	1.1
1	170.8	2.8	1.0
11	168.5	2.7	1.0
8	162.5	0.8	4.3
14	144.7	0.9	4.2
9	142.0	2.9	1.0
5	140.3	3.2	1.0
10	136.1	1.1	3.5
12	110.3	1.1[a]	2.2
7	110.1	2.0[a]	1.1
4′	80.4	1.1	1.0
5′	72.6	1.2	1.2
3′	70.7	1.3	1.0
15	67.4	3.5	1.1
3	63.1	3.5	1.1
1′	61.8	1.3	1.1
O-Me	51.8	1.1[b]	1.1[b]
2	40.3	1.1	4.6
2′	35.7	1.1	1.1
4	27.6	1.1	4.8
16	19.1	1.1	4.8
6′	16.4	1.2	1.1

[a] The signals for C-6 + C-13 and C-7 + C-12 were poorly resolved in this spectrum; C-13 and C-7 clearly were enriched. The ^{13}C abundance in C-13 and C-7 is calculated assuming that C-6 and C-12 are unlabeled. [b] The *O*-methyl carbon was chosen as reference signal; all other signal intensities are given as % abundance relative to OCH_3 = 1.10% natural abundance. Signal intensities were determined by integration of the area under each peak using the step height of the integration curves recorded on scale-expanded spectra.

native precursor. To test this possibility, the incorporation of glucose labeled with ^{14}C and tritium in various positions was examined. To increase the efficiency of glucose incorporation these experiments were carried out using a synthetic culture medium not containing glucose as a carbon source and the labeled precursors were fed in tracer quantities (less than 0.1 mmol/L) to 25-mL shake cultures of *S. violaceoruber*. The purified granaticin was subjected to a Kuhn–Roth oxidation to give acetic acid from C-6′ plus C-5′ and C-16 plus C-15. The tritium content of this acetic acid reflects the amount of tritium present in the two *C*-methyl groups, whereas its ^{14}C contents represents the ^{14}C present in all four carbon atoms. The results of the experiments are summarized in Table II. [6-^{14}C,6-^{3}H]Glucose is incorporated into granaticin with a substantial decrease in the $^{3}H/^{14}C$ ratio. However, the high recovery of tritium in the acetate from the Kuhn–Roth oxidation—essentially quantitative when corrected for the yield of acetate—strongly suggests that the tritium from C-6 of glucose is incorporated specifically into C-6′ of granaticin. The low $^{3}H/^{14}C$ ratio of granaticin and the relatively low recovery of ^{14}C, compared to ^{3}H, in the acetic acid from the Kuhn–Roth oxidation indicates very substantial metabolism of glucose to acetate, with at least partial loss of tritium from C-6, which is then incorporated into the polyketide portion of granaticin. The same is evident in experiment 2 with [6-^{14}C,4-^{3}H]glucose. To circumvent this problem, the subsequent experiments (3–7, Table II) were carried out with [3,4-^{14}C]glucose as the ^{14}C reference label, since C-3 and C-4 of glucose are not as readily converted into acetate as the remaining four carbons and should therefore label the hexose moiety more specifically. The results of experiments 4–7 clearly indicate that glucose is incorporated into granaticin with retention of the hydrogens at

Table II. Incorporation of ^{14}C- and Tritium-Labeled Glucoses into Granaticin

expt no.	positions of label in glucose fed	$^3H/^{14}C$ of precursor	incorporation rate into granaticin, % ^3H	^{14}C	isotope ratio of granaticin $^3H/^{14}C$	acetate from Kuhn–Roth oxidation of granaticin yield, %	$^3H/^{14}C$	% of radioactivity of granaticin isolated as acetate ^3H	^{14}C
1	[6-^{14}C,6-^3H]	3.43	0.15	0.76	0.62	70	2.57	82	24
2	[6-^{14}C,4-^3H]	5.96	0.16	0.76	1.19	96	3.29	90	33
3	[3,4-^{14}C,4-^3H]	7.3	0.14	0.33	3.09	86		89	4.6
4	[3,4-^{14}C,1-^3H]	2.33	0.46	0.45	2.39	88		7.7	2.7
5	[3,4-^{14}C,2-^3H]	12.6	4.06	0.73	15.8	90		3.8	3.2
6	[3,4-^{14}C,3-^3H]	7.81	0.01	0.12	0.76	66		2.6	3.0
7	[3,4-^{14}C,5-^3H]	3.52	0.01	0.14	0.29				

C-1 and C-2, but without the hydrogens at C-3 and C-5. The hydrogen at C-4 of glucose is incorporated into granaticin and appears at C-6′ (and/or C-16) of the antibiotic. The results leave little doubt that the intact glucose molecule, with the exception of H-3 and H-5, gives rise to the six-carbon moiety of granaticin which is not derived from acetate (Figure 1). This conversion must involve removal of the oxygen functions from C-6 and C-2 of glucose.

Some of the data presented in Table II—retention of H-6, loss of H-5, transfer of H-4 to carbon 6 of the hexose moiety—are indicative of the involvement of a hexose nucleotide oxidoreductase reaction, e.g., the dTDP-glucose oxidoreductase reaction, in the conversion of glucose to the hexose moiety of granaticin, probably as the first pathway-specific step. Extensive studies on dTDP-glucose oxidoreductase from *E. coli* and CDP-glucose oxidoreductase from *Salmonella typhimurium* have shown (cf. ref 22) that the reaction with both enzymes involves loss of the hydrogen from C-5 of glucose and an intramolecular transfer of the hydrogen from C-4 of the substrate to C-6 of the product. In a recent study using the *E. coli* enzyme we confirmed the intramolecularity of this 4 → 6 hydrogen shift and established the steric course of the replacement of the OH group at C-6 of the hexose by the hydrogen from C-4.[23] To gain further evidence for or against the involvement of a nucleotide-glucose oxidoreductase reaction in granaticin biosynthesis and to establish the steric course of this reaction in a streptomycete, we fed samples of (6*R*)- and (6*S*)-D-[4-^2H,6-^3H,6-^{14}C]glucose to cultures of *S. violaceoruber*. These compounds were labeled in such a way that every tritiated molecule also contained deuterium; they were obtained by alkaline phosphatase cleavage of the corresponding glucose 6-phosphates which were prepared as described earlier.[23] The granaticin samples from the experiments were degraded by Kuhn–Roth oxidation to give acetate which was analyzed for the chirality of the methyl group by the method of Cornforth et al.[24] and Arigoni and co-workers.[25] In this analysis procedure acetate, as acetyl-coenzyme A, is condensed with glyoxylate in the presence of malate synthetase to give malate, which is then incubated with fumarase. Loss of more than 50% of the tritium (80% for chirally pure acetate) in the fumarase reaction indicates *S* configuration of the acetate; retention of more than 50% of the tritium indicates *R* configuration.

The results of this experiment are summarized in Table III. The data clearly show that a chiral methyl group has been formed from C-6 of glucose. The glucose sample of 6*R* configuration has produced an *S* methyl group and that of 6*S* configuration an *R* methyl group, indicating that the hydroxyl group at C-6 of the glucose has been replaced by H-4 with inversion of configuration at C-6. The low apparent chiral purity of at least one of the acetate samples is not surprising since some incorporation of ^{14}C and tritium from C-6 of glucose into C-16 of granaticin can be expected; however, the breakdown of glucose into acetate followed by utilization via the polyketide pathway would not lead to incorporation of deuterium from C-4 of glucose into C-16 of granaticin. Thus any tritiated acetate from C-16 plus C-15 would not be chiral; it would, however, lower the apparent chiral purity of the acetate from C-6′ plus C-5′. The results also indicate that the 4 → 6 hydrogen shift in the transformation of glucose into the 2,6-dideoxyhexose moiety of granaticin is intramolecular. This follows from the fact that the labeled glucose fed to the cultures was diluted many times (over 100-fold) by endogenously synthesized nonlabeled glucose. Thus any intermolecular crossover in the transfer of H-4 would have resulted in transfer of ^2H to nontritiated molecules and almost exclusive incorporation of ^1H into the tritiated molecules. Since a methyl group is only chiral if ^1H, ^2H, and ^3H are present in the same molecule, the fact that a chiral methyl group is formed proves that the hydrogen migration must be predominantly or exclusively intramolecular.[26]

Table III. Biosynthesis of Granaticin from (6*R*)- and (6*S*)-[4-^2H,6-^3H,6-^{14}C]Glucose, and Chirality Analysis of the Methyl Group at C-6′

	configuration at C-6 of glucose 6*R*	6*S*
dpm ^3H in glucose	1.40×10^7	1.19×10^7
dpm ^{14}C in glucose	1.21×10^6	1.53×10^6
$^3H/^{14}C$ of glucose	11.63	7.79
dpm ^3H in granaticin	9.12×10^4	1.38×10^4
dpm ^{14}C in granaticin	3.24×10^4	7.36×10^3
$^3H/^{14}C$ of granaticin	2.82	1.87
dpm ^3H in acetate	2.92×10^4	7.45×10^3
dpm ^{14}C in acetate	9.86×10^3	1.71×10^3
$^3H/^{14}C$ of acetate	6.01	4.36
$^3H/^{14}C$ of malate	5.72,[a] 5.42[a]	3.05
$^3H/^{14}C$ of fumarate	2.14,[a] 1.98[a]	2.37
tritium retention in fumarase reaction	37.4%,[a] 36.5%[a]	77.7%
configuration at C-6′ of granaticin	*S*	*R*

[a] Results of two independent analyses.

We next determined the overall stereochemical course of the replacement of the hydroxyl group at C-2 of glucose by a hydrogen to generate the 2-deoxy function. As shown in Table II, H-2 of glucose is retained in the process. We therefore prepared [2-^2H]glucose by equilibration of fructose 6-phosphate with D_2O in the presence of glucose 6-phosphate isomerase, followed by cleavage with alkaline phosphatase and chromatographic separation of the resulting glucose and fructose. The [2-^2H]glucose (50 mg, 68% D_1) was fed to a 25-mL culture of *S. violaceoruber* and the resulting granaticin (~75% D_1 by CI mass spectrometry) was analyzed by ^1H NMR spectroscopy. The signals for the H_R and H_S at C-2′ are not well separated from other signals in the spectrum and are therefore not easily analyzed. However, the location of deuterium at C-2′ can be readily deduced from the coupling pattern of H-3′ ($CDCl_3$, δ 4.06 ppm, dd, $J_{3',2'S} = 1.9$, $J_{3',2'R} = 8.5$ Hz). As shown in Figure 2, the spectral pattern observed for

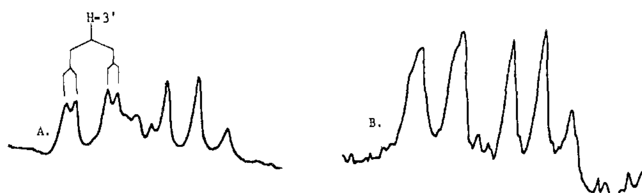


Figure 2. ^{1}H NMR spectral pattern for H-3′ in granaticin: (A) unlabeled sample; (B) granaticin biosynthesized from [2-^{2}H]glucose.

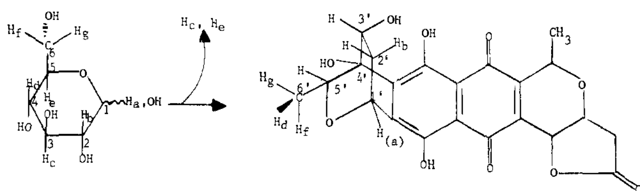


Figure 3. Overall stereochemistry and fate of hydrogens in the conversion of glucose into the 2,6-dideoxyhexose moiety of granaticin.

the signal in deuterated granaticin closely matches that expected for partial replacement of H-2′*S* by deuterium, i.e., the small coupling is reduced whereas the large coupling is retained, and is quite different from that expected for partial replacement of H-2′*R* by deuterium. Thus, the deuterium incorporated from C-2 of glucose occupies the pro-2′*S* position in granaticin and the replacement of the hydroxyl group at C-2 of glucose by ^{1}H must proceed with retention of configuration. The overall stereochemical relationships in the conversion of glucose into the 2,6-dideoxyhexose moiety of granaticin are summarized in Figure 3.

Some information was obtained on the terminal step in the biosynthetic sequence. Pyrek et al.[15] noted that the formation of granaticin was preceded by the appearance of dihydrogranaticin (IIa) in the cultures. In addition we found that one of the minor constituents in the culture extracts was identical with dihydrogranaticin B (IIb) prepared by platinum-catalyzed hydrogenolysis of granaticin B (Ib). This suggests that oxidative formation of the five-membered lactone ring may be the last step in the reaction sequence. Conversion of IIa to Ia occurs nonenzymatically in air[15] and similar stereospecific oxidative cyclizations have been reported in the nanaomycin[11] and griseusin[14] series. A likely mechanism for this conversion[14] is shown in Figure 4. To test whether the organism nevertheless contains an enzyme to carry out this conversion more efficiently we incubated shake cultures of *S. violaceoruber* in the synthetic medium and parallel flasks containing uninoculated medium with dihydrogranaticin prepared from Ia.[15] Analysis of the pigment mixture 12 h later clearly indicated much higher conversion of IIa into Ia in the cultures than in the controls. Cell-free extracts of the mycelium of *S. violaceoruber* converted IIa into Ia about five to ten times more rapidly than boiled controls. Thus there is little doubt that this conversion takes place by an enzymatic process. The enzyme may operate by a similar direct cyclization mechanism as postulated for the nonenzymatic process or, alternatively, it may catalyze a benzylic hydroxylation followed by lactone formation with loss of water. In the direct cyclization oxygen would only be required for the reoxidation of the hydroquinone, not for the enzymatic cyclization itself, and no ^{18}O from $^{18}O_2$ should be incorporated during the reaction. The hydroxylation/lactonization mechanism would require oxygen during the enzymatic reaction itself and one atom of ^{18}O from $^{18}O_2$ should be incorporated into the lactone oxygen. In an attempt to distinguish between these possibilities, we compared the conversion IIa → Ia with cell-free extract and boiled controls under aerobic and anaerobic conditions. The workup in all cases was carried out in air. Both boiled controls showed about the same small

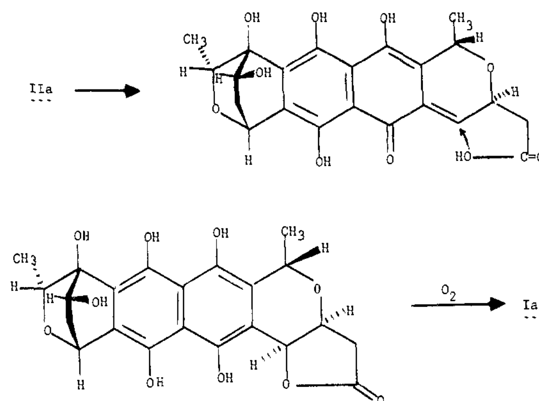


Figure 4. Presumed mechanism for the chemical and enzymatic conversion of dihydrogranaticin into granaticin.

amount of conversion. The cell-free extract under argon showed almost no conversion whereas the cell-free extract in air showed the usual five- to tenfold higher conversion than the boiled control. While on the face of it this result would indicate a requirement for oxygen during the enzymatic reaction itself, any mechanistic conclusions are negated by the additional observation that the cell-free extract under argon showed very pronounced bleaching. Presumably, therefore, IIa is enzymatically reduced to the hydroquinone under anaerobic conditions and is therefore not available as substrate for the cyclization. In a second experiment the conversion of IIa into Ia was carried out in an atmosphere of 90% enriched $^{18}O_2$. Mass spectral analysis of Ia from the incubations with cell-free extract and boiled enzyme showed no incorporation of ^{18}O in either case. While these results are certainly not yet conclusive and further evidence is required to unequivocally establish the mechanism of the enzymatic conversion of IIa into Ia, they do favor the direct cyclization rather than the hydroxylation/lactonization mechanism.

Discussion

The results presented here clearly show that granaticin is biosynthesized from eight acetate units and from a six-carbon sugar directly derived from glucose (Figure 1). The formation of the noncarbohydrate portion of granaticin by the polyketide pathway closely parallels that of nanaomycin[10] and of the monomeric precursor units of α-naphthocyclinone;[20] in analogy with the other benzoisochromane quinones it seems likely that the aromatic precursor of granaticin lacks an oxygen function at C-9. The observation of an enzymatic activity catalyzing the conversion of IIa into Ia suggests, but does not prove, that formation of the five-membered lactone ring is the last step in the biosynthetic sequence and that modification of the original polyketide and attachment of the sugar moiety occur at the stage of the open-chain compounds.

It seems likely that the carbohydrate moiety is attached in the form of a nucleotide derivative of 2,6-dideoxy-4-keto-D-glucose. The mode of elaboration of this sugar nucleotide from glucose has been traced in some detail and the results are compatible with and support the following sequence. The first pathway-specific step is a glucose nucleotide oxidoreductase reaction; based on precedent in other streptomycetes[27] the enzyme most likely is dTDP-glucose oxidoreductase. The evidence presented here shows that the 4 → 6 hydride shift encountered in this reaction proceeds intramolecularly and with inversion of configuration at C-6, in complete analogy to the results obtained with the *E. coli* enzyme.[23] It is known that in all the oxidoreductases studied this hydride shift is mediated by a tightly enzyme-bound pyridine nucleotide, in the reduced form of which the hydride occupies the pro-*S* position (cf. ref 22). As we have pointed out elsewhere,[23] intramolecularity of

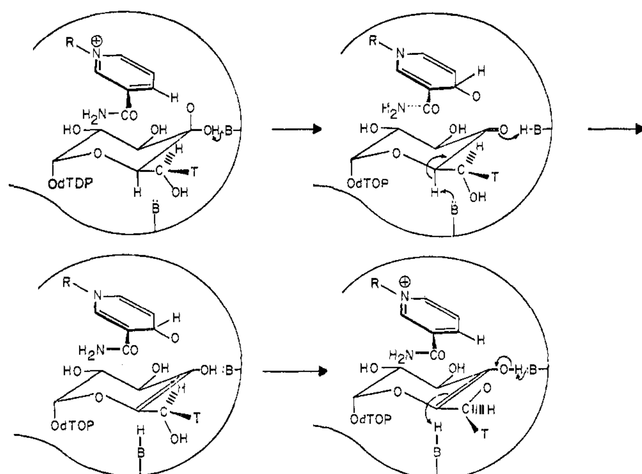


Figure 5. Stereochemical mechanism of the dTDP-glucose oxidoreductase reaction.

the reaction implies that the hydride transfer is suprafacial and suggests a conformation around the C-5/C-6 bond in which H-5 and the OH group at C-6 are syn oriented (Figure 5). This in turn renders a concerted elimination of water from the initial 4-keto intermediate unlikely, since such a process would presumably require an anti conformation. The most plausible alternative as outlined in Figure 5 is removal of H-5 by enolization followed by loss of OH^- and addition of H^- at C-6, either as a stepwise or a concerted process. Final tautomerization would then give the product dTDP-4-keto-6-deoxyglucose. Such a mechanism and the stereochemistry deduced for the reaction can be accommodated by a very plausible model for the active site of the enzyme (Figure 5).

The further modification of the product from this reaction to remove the OH group at C-2 could in principle occur by a similar sequence of steps, i.e., removal of H-3 by enolization, loss of OH^- from C-2 prior to or concerted with introduction of H^-, in this case from an external hydride source, and tautomerization to give dTDP-4-keto-2,6-dideoxyglucose (cf. ref 28 and 29). However, in light of Strominger's findings on the formation of CDP-4-keto-3,6-dideoxyglucose[30] another mechanism, outlined in Figure 6, is more likely. Transposition of the keto group from C-4 to C-3 would be followed by Schiff's base formation with pyridoxamine phosphate. The resulting ketimine would undergo 1,4-elimination of water to give a product formally resembling the α-aminoacrylate Schiff's bases which are intermediates in pyridoxal phosphate catalyzed α,β-elimination and β-replacement reactions.[31] Reduction of this intermediate and hydrolysis of the Schiff's base followed by transposition of the keto group back into the 4 position would complete the sequence. Obviously, more experiments, probably at the enzymatic level, will be necessary to prove or disprove such a mechanism, but at least the data available, e.g., the loss of the hydrogen from C-3, are compatible with it. In particular the finding that the OH group at C-2 is replaced by a hydrogen with retention of configuration supports this mechanism, because all pyridoxal phosphate catalyzed β-replacement and α,β-elimination reactions studied so far proceed with retention of configuration at the β-carbon atom.[32-34]

The attachment of the carbohydrate moiety to the aromatic system can be considered to involve *C*-glycoside formation with a hydroquinone–quinone system followed by an aldol-type condensation between C-10 and the C-4′ keto group in the boat form of the sugar (Figure 7). This requires that the enzyme recognizes the substitution pattern at the other end of the aromatic system in order to achieve regiospecific annelation of the sugar. As an alternative, the regiospecific attachment of

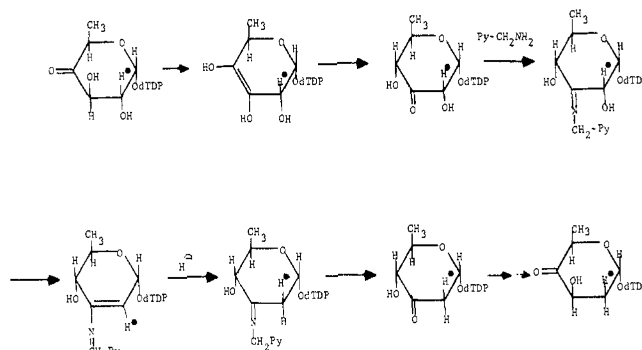


Figure 6. Proposed mechanism for the conversion of dTDP-4-keto-6-deoxyglucose into dTDP-4-keto-2,6-dideoxyglucose.

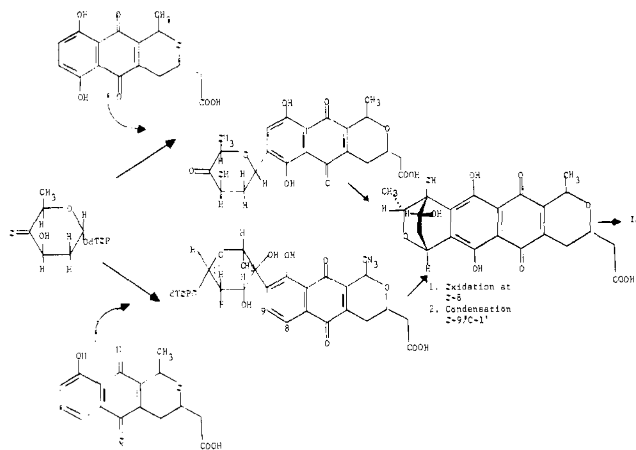


Figure 7. Two possible modes of attachment of the dideoxyhexose moiety to the aromatic system in the biosynthesis of granaticin.

the carbohydrate moiety may be the result of bond formation between C-10 and C-4′ at the stage of an earlier aromatic precursor not yet oxidized at C-8 as outlined in the alternative sequence shown in Figure 7. Experiments in progress are designed to answer this question.

Experimental Section

General Methods and Materials. NMR spectra were measured on a JEOL PFT-100 system interfaced to an EC-100 Fourier transform computer with 20K memory. ^{13}C NMR spectra were generally recorded at a pulse width of 20 μs and a repetition time of 5 s using $CDCl_3$ as solvent. IR spectra were obtained on a Beckman IR 4230 spectrometer and UV spectra on a Perkin-Elmer 124 or Cary 17 instrument. Mass spectra were measured on a Du Pont 21-492 BR mass spectrometer using either EI or chemical ionization. Deuterium enrichments in glucose and granaticin were calculated from the intensities of the molecular ion and its isotope satellites by the method of Campbell.[35] $^{18}O_2$ gas (90% enrichment) was obtained from Mound Laboratories, Monsanto Chemical Co., and ^{13}C- and ^{2}H-labeled compounds from Merck Sharp and Dohme. (6*R*)- and (6*S*)-[4-^{2}H,6-^{3}H]glucose was obtained by alkaline phosphatase cleavage of the corresponding glucose 6-phosphates available from earlier work.[23] [3,4-^{14}C]Glucose was purchased from New England Nuclear; all other radioactively labeled compounds were obtained from Amersham-Searle. Enzymes and biochemicals were obtained from Sigma and materials for culture media from Difco Laboratories. *S. violaceoruber* Tü 22 was kindly provided by Professor H. Zähner, University of Tübingen. Analytical TLC was carried out on 0.25-mm silica gel plates, 5 × 20 cm (Merck, precoated), which were sprayed before use with 0.5 N oxalic acid in 50% aqueous methanol, allowed to air dry, and activated for 1 h at 110 °C. Preparative TLC was done on 20 × 20 cm plates coated with silica gel P/UV 254 + 366 (Macherey-Nagel), prepared as directed by the manufacturer except that 0.5 N oxalic acid was used in place of water to prepare the slurry. The solvent in both cases was chloroform–ethyl acetate (60:40). Column

chromatography was carried out with oxalic acid treated silica gel as the sorbent and a step gradient of chloroform-ethyl acetate as solvent. The sample was applied as a chloroform solution, and the concentration of ethyl acetate was increased from 0 to 30% in 5% increments as required to elute a particular band from the column. Oxalic acid treated silica gel was prepared as described by Zeeck and Mardin.[12]

Culture Conditions. *S. violaceoruber* Tü 22 was maintained on agar slants composed of 1.0% malt extract, 0.4% yeast extract, 0.4% glucose, and 1.5% agar. Seed cultures were prepared by inoculating 100-mL medium, containing 1.0% peanut meal, 1% glucose, 0.5% sodium chloride, and 0.35% calcium carbonate, with spores from an agar slant. The cultures, contained in 500-mL Erlenmeyer flasks carrying three indentions, were incubated at 27-28 °C on a New Brunswick rotary shaker at 300 rpm for 24 h. Production medium was inoculated 5% v/v with vegetative inoculum from seed cultures. The production medium for large-scale production of granaticin and for sodium acetate feeding experiments was the same as that used to prepare inoculum. The production medium for glucose feeding experiments contained 0.1% ammonium sulfate, 0.5% mannose, 0.01% $FeSO_4 \cdot 7H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.2% KH_2PO_4, 0.5% $CaCO_3$, and 0.2% v/v trace element solution. Each 125-mL Erlenmeyer flask carrying three indentions contained 25 mL of this medium. The trace element solution consisted of 15 mg of $AlK(SO_4)_2$, 3.0 mg of KI, 3.0 mg of KBr, 40.0 mg of $MnCl_2 \cdot 4H_2O$, 6.0 mg of $ZnSO_4$, 6.0 mg of $CaSO_4$, 7.0 mg of $CoCl_2$, 3.0 mg of $(NH_4)_6Mo_7O_{24}$, 1.0 mg of $K_2Cr_2O_7$, and 3.0 mg of $CuSO_4 \cdot 5H_2O$ in 100 mL of distilled water. Cells used for the preparation of cell-free extracts were grown for 36 h on a medium consisting of 1% malt extract, 0.4% yeast extract, and 0.4% glucose in distilled water.

Isolation of Granaticin. The cultures were filtered and the culture filtrate was acidified to pH 3 with 1 M HCl and extracted three or four times with 0.3 volumes of chloroform. The combined chloroform extracts were dried over sodium sulfate, filtered, and evaporated to dryness in a rotary evaporator. TLC at this point indicated the presence of granaticin (R_f 0.26) and the following additional components: R_f 0.22 (dihydrogranaticin, IIa), R_f 0.17 (granaticin B, Ib), R_f 0.14 (dihydrogranaticin B, IIb, identical with material obtained by hydrogenolysis of Ib under the conditions described by Pyrek et al.[15] for the conversion of Ia to IIa), R_f 0.08 (unknown). The presence of several additional trace components is evident.

The residue was dissolved in a minimal volume of ethanol and then diluted with an equal volume of 2 N sulfuric acid. This solution was allowed to stand at room temperature for 2 h to hydrolyze granaticin B to granaticin. The solution was added to five volumes of distilled water and extracted three times with 0.3 volumes of chloroform. The combined chloroform extracts were dried over sodium sulfate, filtered, and evaporated to dryness in a rotary evaporator. Granaticin and dihydrogranaticin were purified from the residue by column chromatography on oxalic acid treated silica gel. Granaticin was hydrogenolyzed to dihydrogranaticin, combined with IIa isolated from the same experiment, and converted to the methyl ester, all as described by Pyrek et al.,[15] which was dissolved in $CDCl_3$ for ^{13}C NMR spectroscopy. Preparative TLC on oxalic acid silica gel in chloroform-ethyl acetate (60:40) was used to purify granaticin from glucose feeding experiments. The granaticin isolated was quantitated by its absorbance at 532 nm (ϵ 9670) and an aliquot was taken for radioactivity analysis.

Enzymatic Conversion of IIa to Ia. The cells from four 100-mL cultures of *S. violaceoruber* in malt extract/yeast extract medium were collected by filtration, resuspended in 80 mL of 0.05 M Tris buffer, pH 7.5, and broken open by passage through a French pressure cell at 10 000 psi. The extract was centrifuged for 30 min at 20 000$\cdot g$ to remove cell debris. One milligram of dihydrogranaticin dissolved in water was incubated for 5 h with 10 mL of cell-free extract. The incubations were carried out in sealed tubes either under air, $^{18}O_2$ gas, or argon. In each case a control incubation with boiled cell-free extract was carried out under identical conditions. Following the incubation the solution was acidified with 3 drops of 1 N HCl and extracted with three 5-mL portions of chloroform. The combined chloroform extracts were dried over sodium sulfate, filtered, and evaporated to dryness in a rotary evaporator.

The amount of conversion to granaticin was estimated by visual comparison of the spots for granaticin, R_f 0.26, and dihydrogranaticin, R_f 0.22, on analytical TLC plates. The quantities of granaticin and dihydrogranaticin were determined by their absorbance at 532 nm after purification by preparative TLC.

[2-^{2}H]Glucose. Fructose 6-phosphate (500 mg) was incubated with 50 units of phosphoglucose isomerase in 20.0 mL of deuterated triethanolamine buffer, pD 7.5, for 12 h. After the solution was boiled for 5 min in a water bath, the D_2O was removed by lyophilization and replaced with water. The pH was adjusted with 1.0 N NaOH to 10.0 and 50 units of alkaline phosphatase was added. The pH was monitored with a pH meter and adjusted with 1.0 N NaOH as required. After 12 h the solution was passed through a 1 × 15 cm column of Dowex 1-X8 (OH^- form). The eluate was evaporated to dryness in a rotary evaporator, and the glucose was separated from fructose by preparative TLC on 20 × 20 cm MN 300-50 cellulose plates (Macherey-Nagel) developed twice in methyl ethyl ketone-*tert*-butyl alcohol-formic acid-water (8:6:3:3).

Acknowledgments. We thank Professor H. Zähner, Tübingen, for the culture of *S. violaceoruber* Tü22 and for reference samples of granaticin and granaticin B and Mr. John Kozlowski, Purdue, for recording NMR spectra and for carrying out spectral simulations. Support of this work by the U.S. Public Health Service through NIH Research Grant AI 11728 is gratefully acknowledged.

References and Notes

(1) As a matter of convenience we are using a numbering system for granaticin which is based on the biosynthetic origin of this class of compounds, rather than the systematic numbering.
(2) R. Carbaz, L. Ettlinger, E. Gäumann, J. Kalvoda, W. Keller-Schierlein, F. Kradolfer, B. K. Maunkian, L. Neipp, V. Prelog, P. Reusser, and H. Zähner, *Helv. Chim. Acta*, **40**, 1262 (1957).
(3) S. Barcza, M. Brufani, W. Keller-Schierlein, and H. Zähner, *Helv. Chim. Acta*, **49**, 1736 (1966).
(4) C.-j. Chang, H. G. Floss, P. Soong, and C.-t. Chang, *J. Antibiot.*, **28**, 156 (1975).
(5) W. Keller-Schierlein, M. Brufani, and S. Barcza, *Helv. Chim. Acta*, **51**, 1257 (1968).
(6) M. Brufani and M. Dobler, *Helv. Chim. Acta*, **51**, 1269 (1968).
(7) H. Hoeksema and W. C. Krueger, *J. Antibiot.*, **29**, 704 (1976), and references cited therein.
(8) G. A. Ellestad, H. A. Whaley, and E. L. Patterson, *J. Am. Chem. Soc.*, **88**, 4109 (1966).
(9) H. Brockmann, A. Zeeck, K. van der Merwe, and W. Müller, *Justus Liebigs Ann. Chem.*, **698**, 209 (1966).
(10) H. Tanaka, Y. Koyama, T. Nagai, H. Marumo, and S. Omura, *J. Antibiot.*, **28**, 868 (1975).
(11) S. Omura, H. Tanaka, Y. Okada, and H. Marumo, *J. Chem. Soc., Chem. Commun.*, 320 (1976).
(12) A. Zeeck and M. Mardin, *Justus Liebigs Ann. Chem.*, 1063 (1974).
(13) A. Zeeck, H. Zähner, and M. Mardin, *Justus Liebigs Ann. Chem.*, 1100 (1974).
(14) N. Tsuji, M. Kobayashi, Y. Terui, and K. Tori, *Tetrahedron*, **32**, 2207 (1976).
(15) J. St. Pyrek, O. Achmatowicz, Jr., and A. Zamojski, *Tetrahedron*, **33**, 673 (1977).
(16) P. Soong, Y. Y. Jen, Y. S. Hsu and A. A. Au, *Rep. Taiwan Sugar Exp. Stn.*, **34**, 105 (1964).
(17) T. Krzywy, M. Mordarski, B. Orlowska, and A. Tkaczowa, *Arch. Immunol. Ther. Exp.*, **17**, 54, 63 (1969).
(18) M. L. Sethi, *J. Pharm. Sci.*, **66**, 130 (1977).
(19) W. Kersten and A. Ogilvie, *Z. Klin. Chem. Klin. Biochem.*, **13**, 371 (1975), and references cited therein.
(20) K. Schröder and H. G. Floss, *J. Org. Chem.*, **43**, 1438 (1978).
(21) A detailed discussion of the ^{13}C NMR analysis of this group of compounds will be published elsewhere.
(22) O. Gabriel, "Carbohydrates in Solution", R. Gould, Ed., *Adv. Chem. Ser.*, **No. 117**, 387-410 (1973).
(23) C. E. Snipes, G.-U. Brillinger, L. Sellers, L. Mascaro, and H. G. Floss, *J. Biol. Chem.*, **252**, 8113 (1977).
(24) J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckel, and C. Gutschow, *Eur. J. Biochem.*, **14**, 1 (1970).
(25) J. Lüthy, J. Rétey, and D. Arigoni, *Nature (London)*, **221**, 1213 (1969).
(26) This conclusion would only be invalid if one were to make the extremely unlikely assumptions that all the added labeled glucose is converted into granaticin at one time without mixing with endogenous pools and that all the nonlabeled material is synthesized at a different time.
(27) H. Matern, G.-U. Brillinger, and H. Pape, *Arch. Mikrobiol.*, **88**, 37 (1973).
(28) Z. Vanek and J. Majer in "Antibiotics", Vol. II, D. Gottlieb and P. D. Shaw, Ed., Springer-Verlag, West Berlin, 1967, pp 154-188.
(29) N. L. Blumson and J. Baddiley, *Biochem. J.*, **81**, 114 (1961).
(30) P. A. Rubenstein and J. L. Strominger, *J. Biol. Chem.*, **249**, 3776, 3782 (1974).
(31) L. Davis and D. E. Metzler in "The Enzymes", Vol. 7, 3rd ed., P. D. Boyer, Ed., Academic Press, New York, N.Y., 1972, pp 33-74.
(32) M. D. Tsai, J. Weaver, H. G. Floss, E. E. Conn, R. K. Creveling, and M. Mazelis, *Arch. Biochem. Biophys.*, **190**, 553 (1978).
(33) M. D. Tsai, E. Schleicher, R. Potts, G. E. Skye, and H. G. Floss, *J. Biol. Chem.*, **253**, 5344 (1978).
(34) J. C. Vederas, E. Schleicher, M. D. Tsai, and H. G. Floss, *J. Biol. Chem.*, **253**, 5340 (1978).
(35) I. Campbell, *Bioorg. Chem.*, **3**, 386 (1974).