BIOSYNTHESIS OF THE STREPTOVARICINS: 3-AMINO-5-HYDROXYBENZOIC ACID AS A PRECURSOR TO THE *META*-C_7N UNIT†

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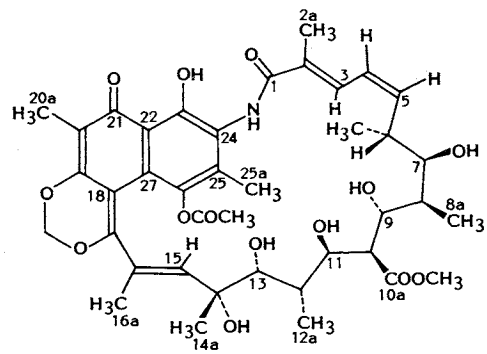
[*Carboxy*-^{14}C]-3-amino-5-hydroxybenzoic acid (AHBA) has been shown to be incorporated by *Streptomyces spectabilis* to the extent of greater than 0.1% (35 : 1 dilution) in the ansamycin antibiotic streptovaricin C, the major component of the streptovaricin complex. When [*carboxy*-^{13}C]AHBA was similarly administered, C-21 (quinone methide carbonyl at 188.3 ppm) of streptovaricin C was specifically labeled (at twenty one times natural abundance). In preparation for the ^{13}C incorporation study the ^{13}C NMR spectrum of streptovaricin C was investigated, making extensive use of short- and long-range HETCOR. These assignments revise some of those proposed earlier for streptovaricin C.

The *meta*-C_7N units in the aromatic chromophores of several ansamycin antibiotics have been shown to be biosynthetically derived from 3-amino-5-hydroxybenzoic acid (AHBA) as a discrete biological intermediate. Rifamycin,[1] actamycin,[2] geldanamycin,[3] the ansamitocins,[4] and the mitomycins[5] have all been shown to incorporate AHBA as a precursor. Although the biosynthesis of AHBA from primary metabolic precursors has not yet been completely determined, there is evidence that the compound comes from a branch of the shikimic acid pathway.[6] We report here the biosynthetic incorporation of ^{13}C- and ^{14}C-labeled AHBA into the naphthoquinoid chromophore of streptovaricin C (**1**), thus providing additional evidence that AHBA is a common progenitor of the ansamycin antibiotics. In addition, we have revised some of the previously reported ^{13}C NMR assignments of **1** based on new 2D heteronuclear correlation experiments.[7] Our continued interest in the biosynthesis of the streptovaricins is based in part on reports of their derivatives' antiviral activities,[8] most recently against the HIV virus.[9] Streptovaricin analogues with modified biological activities could conceivably be produced through mutasynthetic modifications based on a knowledge of the biosynthetic origin of these compounds.[10]



Streptovaricin C (**1**)

Results and Discussion

Incorporation Studies

[*Carboxy*-^{14}C]AHBA (0.49 μCi, 26.9 μCi/

† Dedicated to the memory of the late ROGER ADAMS on the occasion of the hundredth anniversary of his birth January 1989.

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mmol) was synthesized by the method of HERLT *et al.*[11] and was fed to *Streptomyces spectabilis* at 84 hours after inoculation. At 7 days the antibiotic was harvested and the major component was isolated and purified by reversed-phase medium-pressure liquid chromatography (MPLC), followed by normal phase gradient elution HPLC. A total of 0.48 mg of radiochromatographically pure material was thus obtained. One-half of this sample (0.24 mg) was found by liquid scintillation counting to have an activity of 0.24 nCi (530 dpm after background subtraction, 0.05% incorporation, 0.77 μCi/mmol). An incorporation of at least 0.1% (*i.e.*, 0.05% for one-half of the isolated material, plus more unisolated material) and a dilution factor of 35 (ratio of specific activities of the precursor to product, *i.e.*, 26.9/0.77) was thus established in this one component, which was found to be streptovaricin C by FAB-MS (*m/z* 770, M+H) and TLC analysis. Although the total radioactivity of the product is low, the incorporation indicated that [*carboxy*-^{14}C]AHBA is a direct, specific precursor for the streptovaricins and suggested that the administration of ^{13}C-labeled AHBA was feasible.

[*Carboxy*-^{13}C]AHBA (106 mg, 688 μmol of AHBA, 93% ^{13}C, 640 μmol of ^{13}C label) was synthesized by the same route as [*carboxy*-^{14}C]AHBA and was added to 1 liter of culture broth (ten flasks of 100 ml of each). The cultures were incubated and extracted as usual, providing 2.1 mg of [^{13}C]streptovaricin C after purification by HPLC (silica gel). A ^{13}C NMR spectrum of this material showed only one strong signal (at 188.3 ppm), consistent with C-21 (quinone carbonyl) of streptovaricin C. Very weak signals for the other resonances of streptovaricin C could be discerned after accumulation of 84,000 transients. A 1.2-mg sample of this labeled material was diluted with 9.3 mg of unlabeled streptovaricin C (10.5 mg total, 11.4% labeled material, 9-fold dilution), and a ^{13}C NMR spectrum of this material was acquired. The spectrum showed an enrichment factor of 2.3 (the ratio of the absolute intensity of the 188.3-ppm signal in the labeled sample relative to a natural abundance sample, normalized to the *O*-methyl ^{13}C signal at 52.0 ppm). Extrapolating to the sample of undiluted [^{13}C]streptovaricin C, this 2.3-fold signal enrichment for the C-21 carbonyl resonance translates to an overall enrichment factor of 21 (*i.e.*, $9 \times 2.3 = 21$).

An estimate of the incorporation rate was made as follows. Because ^{13}C occurs naturally at a rate of 1.1%, a 21-fold enrichment factor in the ^{13}C NMR spectrum corresponds to (approximately) 20% of the molecules in the sample bearing a label derived from the added precursor. Thus, of the 3.1 μmol of streptovaricin C isolated (2.4 mg, MW 769), 0.62 μmol (3.1×0.20) of the compound was labeled by [*carboxy*-^{13}C]AHBA. Since 640 μmol of ^{13}C label was added, the approximate incorporation rate would be 0.62/640, or 0.097%. This result is consistent with the feeding experiments involving [*carboxy*-^{14}C]-AHBA, which was incorporated at a rate of 0.1%. These ^{13}C data again indicate that AHBA is a definite, specific precursor for the *meta*-C_7N unit of the streptovaricin chromophore.

The poor incorporation rate of AHBA into the streptovaricins is somewhat surprising in view of the exceptional incorporations of the same precursor into geldanamycin (57%)[3] and rifamycin S (10%).[12] This result is in large measure due to the poor yield of purified streptovaricin from the culture broths (approximately 1% that of geldanamycin on a molar basis). However, the low dilution factor of 35 for [*carboxy*-^{14}C]AHBA incorporation and the strong enrichment factor of 21 for streptovaricin C from incorporation of [*carboxy*-^{13}C]AHBA indicate a direct metabolic role for AHBA in the biosynthesis of streptovaricins.

The biosynthetic origins of the *ansa* chain and chromophore of the streptovaricins, derived from acetate, propionate, methionine, and AHBA precursors, are indicated in Scheme 1.[13,14]

Scheme 1.

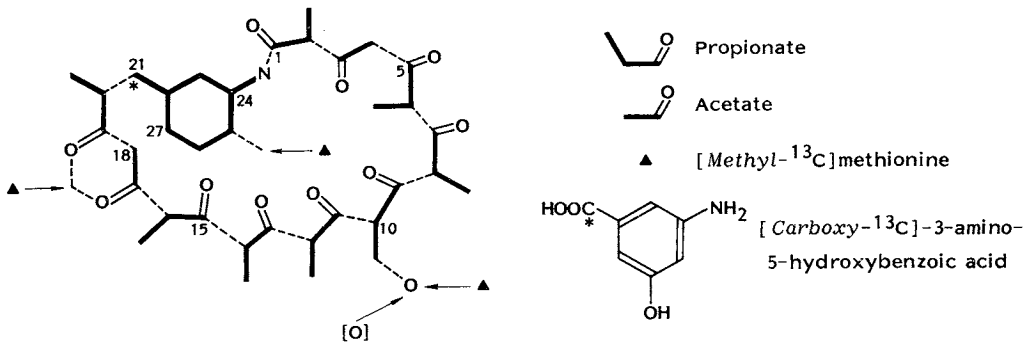


Table 1. ^{1}H NMR spectral data (500 MHz) for streptovaricin C.[a]

Proton	δ (ppm, vs. TMS)	Multiplicity	J, Hz	Proton	δ (ppm vs. TMS)	Multiplicity	J, Hz
3-H	7.74	d	12.1	10-H	2.86	br s	
4-H	6.48	t	11.5	16-CH_3	2.265	s	
OCH_2O	5.82	d	4.5	CH_3COO	2.260	s	
5-H	5.72	t	9.9	2-CH_3	2.20	s	
15-H	5.65	s		8-H	2.18	m[b]	
OCH_2O	5.15	d	4.5	25-CH_3	1.99	s	
9-H	4.11	br dd	8.0, 6.2	12-H	1.98	m[c]	
11-H	4.04	dd	7.5, 6.3	20-CH_3	1.93	s	
OCH_3	3.66	s		6-CH_3	1.18	s	
13-H	3.52	d	4.6	14-CH_3	1.09	s	
7-H	3.39	br dd	6.6, 1.8	8-CH_3	0.93	d	6.1
6-H	3.05	br m		12-CH_3	0.92	d	6.1

[a] 0.26 M solution in CD_2Cl_2, after exchange with D_2O.
[b] Signal is a broad multiplet on the upfield shoulder of the methyl singlet at 2.20 ppm.
[c] Signal is buried between the methyl singlets at 1.99 and 1.93 ppm.

^{1}H and ^{13}C Assignments of Streptovaricin C

The previously reported spectral assignments for the streptovaricins[15,16] were based on a comparison of the spectra of the various streptovaricin components and their degradation products and involved a number of ambiguous assignments. We have re-examined the ^{1}H and ^{13}C NMR spectra of streptovaricin C using 2D high-field NMR experiments and can now provide unambiguous assignments for all of the proton signals and for 36 of the 40 carbon signals.[7]

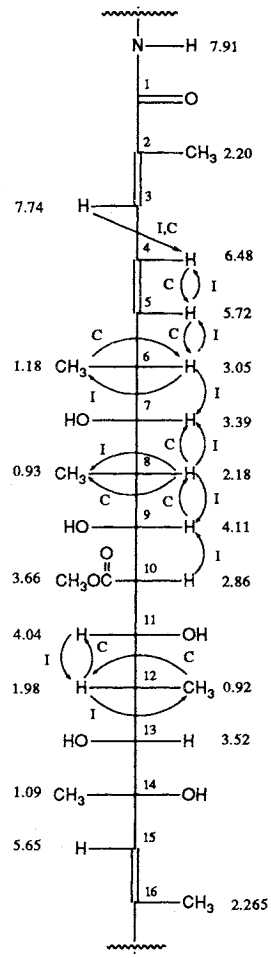
Based on the known structure of streptovaricin C,[17,18] our presently reported spectral assignments were made from chemical shift and coupling arguments and are summarized in Tables 1 and 2. The ^{1}H NMR spectrum was assigned after analysis of extensive homonuclear decouplings and COSY, and the observed coupling connectivities are summarized in Fig. 1. Starting with the easily identified olefinic protons for 3-H, 4-H, and 5-H, a complete connectivity for the *ansa* chain, including appended methyl groups, could be established from C-3 to C-10. A smaller fragment involving either C-11/C-12 or C-12/C-13 was also noted, although a firm assignment could not be made at this stage for these resonances. Ambiguities at this point existed among the methyl protons (all singlets) at 1.93, 1.99, 2.20, and 2.26 ppm, and between the methine protons at 3.52 and 4.04 ppm (*i.e.*, 11-H and 13-H). All of these ambiguities were ultimately

Table 2. ^{13}C NMR spectral data (125 MHz) for streptovaricin C.[a]

Carbon	δ^{b}	Carbon	δ^{b}
C-21	188.7	OCH_2O	89.8
10-COOMe	173.6	C-7	82.7
C-1†	169.3	C-9	77.5
C-17†	169.2	C-14	77.4
OCOMe	169.1	C-13	74.2
C-19	159.7	C-11	70.7
C-15	154.0	OCH_3	52.0
C-23	153.7	C-10	47.8
C-5	143.9	C-8	41.5
C-26†	136.7	C-6	38.8
C-25†	135.2	C-12	38.6
C-3	135.0	14-CH_3	22.3
C-16	130.5	6-CH_3	21.8
C-2	127.0	$OCOCH_3$	21.3
C-27	125.6	8-CH_3	16.0
C-4	124.4	25-CH_3	13.9
C-24	121.8	16-CH_3	13.2
C-22	113.4	2-CH_3	12.7
C-20	107.3	12-CH_3	10.4
C-18	102.1	20-CH_3	7.4

[a] 0.26 M solution in CD_2Cl_2.
[b] ppm downfield of TMS.
† Resonances may be interchanged.

Fig. 1. Proton couplings in the *ansa* chain observed by single-frequency irradiations (I) or homonuclear COSY (C).

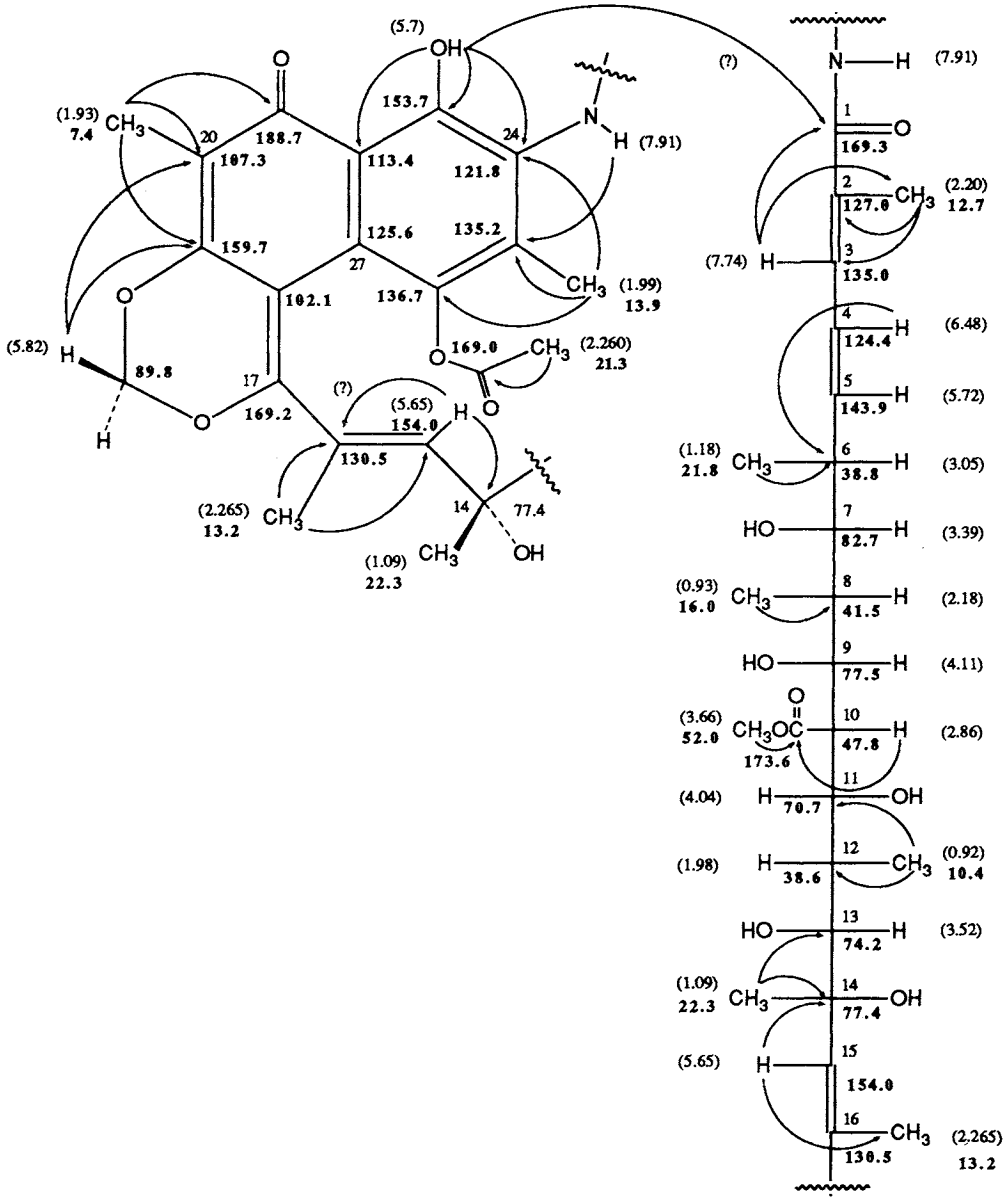


resolved by ^{1}H-^{13}C correlations. The ^{1}H NMR assignments as shown in Table 1 are in complete agreement with those previously reported and are, therefore, an independent corroboration of those earlier assignments.[16]

The ^{13}C NMR assignments for many of the *ansa* chain carbons (*e.g.*, C-3 to C-10 and the attached methyl groups) were made directly by analysis of the short-range heteronuclear correlation spectra in combination with the ^{1}H NMR assignments discussed above. The remaining resonances were assigned by chemical shift arguments (as, for example, in the case of the methylenedioxy and quinone carbonyl carbons or C-18 vs. C-27) and from analysis of the long-range heteronuclear correlations. The long-range correlations resolved the discrepancy in the 11-H/13-H assignment noted above: a strong correlation was observed between the C-14 methyl protons (1.09 ppm) and the C-13 carbon (74.2 ppm), which in turn showed short-range correlation with the 13-H proton resonance (3.52 ppm). By default, therefore, the 11-H resonance must be at 4.04 ppm, and C-11 at 70.7 ppm. Hence, a complete assignment for the remaining part of the *ansa* chain (from C-11 to C-16, including the appended methyl groups) can be made. Similarly, a combination of long-range correlations permitted the unambiguous assignment of the majority of the resonances associated with the aromatic chromophore.

All of the relevant short- and long-range correlations discussed above are summarized in Fig. 2. In the final analysis, a number of minor differences from the previously reported carbon assignments were found, but these involved simple reversals of six pairs of resonances (C-2a and C-16a; C-6a and C-14a;

Fig. 2. Long-range ^{1}H-^{13}C heteronuclear correlations for streptovaricin C.



Proton shifts are shown as (δ ppm), carbon shifts as δ ppm, and weak correlations as (?). The C-1 and C-17 assignments may be reversed. The C-23 phenolic hydroxyl resonance was observed as a "fold-over peak" at 5.7 ppm (actual chemical shift ~11.5 ppm).

C-6 and C-8; C-11 and C-13; C-20 and C-22; and C-24 and C-25). Several of these reversed assignments had indeed been noted to be interchangeable at the time of the original assignments.[15] Thus, these present results are an extension of, as well as an independent corroboration of, those earlier results.

Experimental

General Methods

^{1}H and ^{13}C NMR spectra were obtained on a Nicolet NT-360 or a General Electric GN-500

spectrometer, and chemical shifts are reported in ppm downfield of TMS as an internal standard. Heteronuclear correlation spectra were obtained on the GN-500 with the software supplied by the manufacturer (CSCM and CSCMLR), with selection for a long-range coupling constant of 10 Hz ($^{2-3}J_{H-C}=10$ Hz). FAB mass spectra were obtained on a VG ZAB-SE spectrometer in dithiothreitol-dithioerythritol matrix ("magic bullet"),[19] operating in the positive-ion mode with an Ion Tech fast atom gun and xenon atoms (8 keV). TLC analyses were performed on 0.25-mm silica plates with a UV indicator (Kieselgel 60 F-254, Merck, Darmstadt). All reagents were purchased from Aldrich, and all solvents were distilled before use. HPLC grade methanol, ethyl acetate, and acetonitrile were purchased from Burdick and Jackson. Potassium [^{14}C]cyanide was purchased from Amersham, and sodium [^{13}C]cyanide was provided by the Los Alamos Stable Isotopes Resource. HPLC of [^{14}C]AHBA was performed on an IBM ternary liquid gradient HPLC system and an Alltech Econosphere C_{18} analytical column. HPLC of streptovaricins was done on an Alltech Econosphere silica column, with a Beckman 421A solvent programmer and a Beckman 165 multiple wavelength detector set at 440 nm. Radioactivity was determined on a Tracor Betatrac 3000 liquid scintillation counter in 15 ml of AQUASOL, with ESR and SCR modes engaged. Radiochromatograms were acquired on a Radiomatic TLC Scanner Model RS with 1,024 data points.

Synthesis of Precursors

[*Carboxy*-^{13}C]AHBA and [*carboxy*-^{14}C]AHBA were synthesized by the method of HERLT *et al.*[11] from sodium [^{13}C]cyanide (93% ^{13}C) and potassium [^{14}C]cyanide (56.1 mCi/mmol), respectively. [*Carboxy*-^{13}C]AHBA was purified by recrystallization from 6 N hydrochloric acid, and [*carboxy*-^{14}C]AHBA (0.49 μCi, 26.9 μCi/mmol) was purified by reversed-phase (C_{18}) HPLC, addition of cold carrier, and reversed-phase (C_8) MPLC.[7]

Culture Conditions

All media were sterilized by autoclaving at 120°C for 20 minutes. Precursors were administered in either water or 70% ethanol-water and sterilized by filtration through 0.2-μm filters (Difco Biologicals). *S. spectabilis* (UC-2294) was provided by Miss ALMA DIETZ, The Upjohn Company, Kalamazoo, Michigan. The organism was maintained on soil stocks and on MT Medium agar slants. Incubations were conducted in 500-ml wide-mouthed Erlenmeyer flasks containing 100 ml of culture media in a New Brunswick rotary incubator at 250 rpm. Seed cultures were inoculated from the agar slants by transferring a loopful of cells to 100 ml of medium A and incubating at 32°C for 48 hours. Production cultures were prepared in medium B by inoculating with 5% of seed culture and incubating at 30°C for 5~7 days.

MT Medium: Maltose (1.0%), tryptone (Difco, 0.5%), K_2HPO_4 (0.1%), NaCl (0.1%), a trace of $FeSO_4 \cdot 7H_2O$, and agar (1.5%) in distilled water.

Medium A: NZ-Amine B (2.0%), glucose (1.0%), K_2HPO_4 (0.25%), KH_2PO_4 (0.15%), and soy sauce (1.0%) in tap water.

Medium B: Kay Soy (Archer Daniels Midland, 2.0%), corn dextrin (Pfanstiehl, 4.0%), brewers yeast (0.25%), KCl (0.3%), and $CaCO_3$ (1.2%) in tap water.

Isolation of Streptovaricins

The mature production culture was filtered through Celite, and the filter cake was washed with two 0.2-volume of water. The filtrate was acidified with 2 N sulfuric acid to pH 4.6 and extracted with ethyl acetate (three 0.5-volume). Emulsions were broken by the addition of ethanol or brine, or by refiltration through Celite. The combined organic layers were washed with water and brine, then dried (Na_2SO_4). Evaporation of the solvent provided typically 200~300 mg of crude extract per liter of culture broth. No streptovaricin could be found in an ethyl acetate extract of the filter cake. The crude product was reprecipitated from ethyl acetate-hexane and then dichloromethane-hexane to provide about 100 mg of crude streptovaricin complex per liter of culture broth.

Labeled streptovaricins from the [^{13}C]AHBA and [^{14}C]AHBA experiments were separated as follows. Passage of the extract through a silica Sep-Pak (Waters) with 95:5 chloroform-methanol removed very polar, brown materials. The fractions were assayed by TLC, and those fractions containing streptovaricins were combined, evaporated, redissolved in methanol-water (80:20, buffered at pH 4.5 with 0.01%

triethylamine-acetic acid), and passed through a C_{18} Sep-Pak (Waters). The fractions were assayed by TLC (silica, 95:5, chloroform-methanol), and those fractions containing the major streptovaricin components were combined, evaporated, and redissolved in chloroform. Silica gel HPLC (Alltech Associates Econosphere 5 μm column, 4 mm × 250 mm) using a gradient of 0 to 10% methanol in chloroform during 20 minutes at a flow rate of 1.0 ml/minute separated two main components (retention volumes of 9.0~10.8 ml and 10.8~11.8 ml), which were streptovaricins D and C, respectively. Two runs of the fractions through HPLC were sufficient to obtain 0.48 mg of [^{14}C]streptovaricin C and 2.4 mg [^{13}C]streptovaricin C.

References

1) GHISALBA, O. & J. NÜESCH: A genetic approach to the biosynthesis of the rifamycin-chromophore in *Nocardia mediterranei*. IV. Identification of 3-amino-5-hydroxybenzoic acid as a direct precursor of the seven-carbon amino starter-unit. J. Antibiotics 34: 64~71, 1981
2) KIBBY, J. J.; I. A. MCDONALD & R. W. RICKARDS: 3-Amino-5-hydroxybenzoic acid as a key intermediate in ansamycin and maytansinoid biosynthesis. J. Chem. Soc. Chem. Commun. 1980: 768~769, 1980
3) POTGIETER, M.: Biosynthetic studies on geldanamycin and pactamycin. Ph. D. Thesis, Univ. Illinois, Urbana, Illinois, 1983 [CA 101: 224902n, 1984; Diss. Abstr. Int. B 45: 195, 1984]
4) HATANO, K.; S. AKIYAMA, M. ASAI & R. W. RICKARDS: Biosynthetic origin of aminobenzenoid nucleus (C_7N-unit) of ansamitocin, a group of novel maytansinoid antibiotics. J. Antibiotics 35: 1415~1417, 1982
5) ANDERSON, M. G.; J. J. KIBBY, R. W. RICKARDS & J. M. ROTHSCHILD: Biosynthesis of the mitomycin antibiotics from 3-amino-5-hydroxybenzoic acid. J. Chem. Soc. Chem. Commun. 1980: 1277~1278, 1980
6) GHISALBA, O.: Biosynthesis of rifamycins (ansamycins) and microbial production of shikimate pathway precursors, intermediates, and metabolites. Chimia 39: 79~88, 1985
7) STALEY, A. L.: Isolation, structure, synthesis and biosynthesis of selected bioactive microbial metabolites. Ph. D. Thesis, Univ. Illinois, Urbana, Illinois, 1990
8) BYRD, D. M. & W. A. CARTER: Ansamycins: (B) Streptovaricins. *In* Selective Inhibitors of Viral Functions. *Ed.*, W. A. CARTER, pp. 329~347, CRC, 1973
9) ITO, S. & K. ONODERA (Shin-Etsu Chem.): Streptovaricin C derivatives as anti-AIDS virus agents. Eur. Pat. Appl. 273 732, July 6, 1988 [CA 110: 884f, 1988]
10) RINEHART, K. L., Jr.: Mutasynthesis of new antibiotics. Pure Appl. Chem. 49: 1361~1384, 1977
11) HERLT, A. J.; J. J. KIBBY & R. W. RICKARDS: Synthesis of unlabelled and carboxyl-labelled 3-amino-5-hydroxybenzoic acid. Aust. J. Chem. 34: 1319~1324, 1981
12) ANDERSON, M. G.; D. MONYPENNY, R. W. RICKARDS & J. M. ROTHSCHILD: Biosynthetic origins of the oxygen atoms in the ansamycin antibiotics rifamycin B, O, and S. J. Chem. Soc. Chem. Commun. 1989: 311~313, 1989
13) MILAVETZ, B.; K. KAKINUMA, K. L. RINEHART, Jr., J. P. ROLLS & W. J. HAAK: Carbon-13 magnetic resonance spectroscopy and the biosynthesis of streptovaricin. J. Am. Chem. Soc. 95: 5793~5795, 1973
14) MILAVETZ, B. I.: Biosynthesis of the streptovaricins. Ph. D. Dissertation, Univ. Illinois, Urbana, Illinois, 1975 [CA 83: 175251y, 1975]
15) KAKINUMA, K.; B. I. MILAVETZ & K. L. RINEHART, Jr.: Carbon-13 nuclear magnetic resonance spectra of the streptovaricins and related compounds. J. Org. Chem. 41: 1358~1364, 1976
16) RINEHART, Jr., K. L.; M. L. MAHESHWARI, F. J. ANTOSZ, H. H. MATHUR, K. SASAKI & R. J. SCHACHT: Chemistry of the streptovaricins. VIII. Structures of streptovaricins A, B, D, E, F, and G. J. Am. Chem. Soc. 93: 6273~6274, 1971
17) WANG, A. H.-J.; I. C. PAUL, K. L. RINEHART, Jr. & F. J. ANTOSZ: Chemistry of the streptovaricins. IX. X-ray crystallographic structure of a streptovaricin C derivative. J. Am. Chem. Soc. 93: 6275~6276, 1971
18) WANG, A. H.-J. & I. C. PAUL: Structure of a derivative of streptovaricin C triacetate. Crystal and molecular structure of the atropisomer of the cyclic *p*-bromobenzeneboronate ester of streptovaricin C triacetate: methylene dichloride 1:1 solvate. J. Am. Chem. Soc. 98: 4612~4619, 1976
19) WITTEN, J. L.; M. H. SCHAFFER, M. O'SHEA, J. C. COOK, M. E. HEMLING & K. L. RINEHART, Jr.: Structures of two cockroach neuropeptides assigned by fast atom bombardment mass spectrometry. Biochem. Biophys. Res. Commun. 124: 350~358, 1984