BIOSYNTHESIS OF CHRYSOMYCINS A AND B

ORIGIN OF THE CHROMOPHORE

GUY T. CARTER*, AMEDEO A. FANTINI, JOHN C. JAMES, DONALD B. BORDERS and RICHARD J. WHITE

Medical Research Division, American Cyanamid Company, Lederle Laboratories, Pearl River, New York 10965, USA

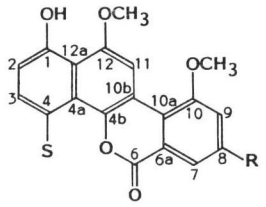
(Received for publication October 3, 1984)

The biosynthetic origin of the carbon atoms in the chromophores of chrysomycins A and B was investigated in feeding experiments using ^{13}C labeled acetates and propionate. A biosynthetic scheme is proposed involving the condensation and rearrangement of a decaketide intermediate which contains either propionate (chrysomycin A) or acetate (chrysomycin B) as the chain initiator.

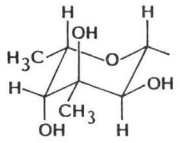
The isolation of chrysomycin was first reported in 1955 by STRELITZ and coworkers[1] who were screening for antibiotics with activity against bacteriophages. Although the physico-chemical properties of this antibiotic, including a highly characteristic absorption spectrum, were published, no structure was established. Subsequently, several related antibiotics containing very similar, if not identical, chromophores were described: toromycin[2], virenomycin[3], gilvocarcin[4] and ravidomycin[5]. The first member of this group of related antibiotics to have its structure determined was toromycin[6], which was shown to consist of a substituted naphthalene chromophore with a sugar moiety attached by a carbon-carbon glycosyl bond. Structures for other members of this group quickly followed[7~9], and all contained the same tetracyclic chromophore. Gilvocarcin V proved to be identical to toromycin as also did other antibiotics[10~12]. Although a detailed comparison has not been possible, it seems likely that virenomycin has the same structure as chrysomycin. Both chrysomycin and gilvocarcin occur in microbial fermentations as a mixture of two analogues that differ only in having a vinyl (chrysomycin A and gilvocarcin V) or methyl (chrysomycin B and gilvocarcin M) substituent on the chromophore. In the case of ravidomycin only the vinyl analogue has been described. The structures of chrysomycin, gilvocarcin and ravidomycin are shown in Fig. 1. Although toromycin is the preferred name for **3**, the only biosynthetic studies reported for this compound were done using *Streptomyces gilvotanareus*[13], hence in this paper we refer to this antibiotic as gilvocarcin V.

Recently we reported on the biosynthesis of ravidomycin[14] and proposed a scheme in which a single decaketide chain initiated by propionate gave rise to the chromophore *via* a tetracyclic intermediate from which two carbons are lost. TAKAHASHI and TOMITA[13] have published results on the biosynthesis of gilvocarcins V and M, and proposed a scheme that differs significantly from that for ravidomycin. The key difference between the two biosynthetic schemes concerns the C-8 vinyl substituent. In the case of ravidomycin, it derives from propionate, which is suggested to be a polyketide chain initiator and retains its carboxyl as part of the chromophore. Although the gilvocarcin scheme also proposes a propionate origin, it is a secondary substituent added to a pre-existing ring system with loss of its associated carboxyl group. According to this latter scheme gilvocarcin M was synthesized by substituting the chromophore with acetate rather than propionate, again with concomitant loss of the carboxyl group.

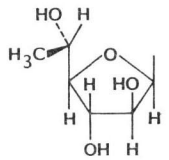
Fig. 1. Structures of the chrysomycins, gilvocarcins and ravidomycin.



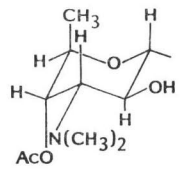
Chrysomycin A (**1**) R = $CH=CH_2$
Chrysomycin B (**2**) R = CH_3
S =



Gilvocarcin V (**3**) R = $CH=CH_2$
(Toromycin)
Gilvocarcin M (**4**) R = CH_3
S =



Ravidomycin (**5**) R = $CH=CH_2$ S =



In addition to the proposal of TAKAHASHI and TOMITA[13], there are two reasonable biochemical routes to explain the origin of the methyl side chain. Since each group of antibiotics in this family (*i.e.* chrysomycin, gilvocarcin and ravidomycin) contain a vinyl component, the methyl analog could arise through degradation of the initially formed two-carbon side chain. Alternatively, an acetate-initiated decaketide chain homologous with that proposed for ravidomycin[14], would, after condensation and rearrangement, produce the methyl substituent directly. These alternatives were explored in a series of experiments in which labeled acetate or propionate was fed to shake flask cultures of the producing organism.

Results and Discussion

Early fermentations for production of chrysomycin were done utilizing two strains of *Streptomyces flaveolus*, NRRL B1687 and NRRL B574. Detection of activity was by a prophage induction assay (BIA)[11]. Fermentation of these cultures showed minimal activity, and difficulty was encountered in repeating any of the positive results. Actually, within a few weeks no BIA activity whatsoever was detectable under any of the several fermentation conditions investigated. *S. flaveolus* (NRRL B574) was then plated, and an isolate (NS135) was found which again elicited slight activity in the BIA assay. Further fermentation studies with this isolate, however, failed to produce improved yields, and quantitation *via* HPLC indicated yields of <1 μg/ml.

Fortunately, at about this time it was discovered that the Lederle Culture Collection was in possession of a lyophile of the original *Streptomyces* species reported by STRELITZ *et al.*[1] as the chrysomycin

Table 1. ^{13}C NMR data for the aglycone portions of labeled chrysomycins A and B.

Carbon	Chemical shift[a] (δ)		Enrichment factor[b]						Coupling constant[c] (Hz)		Acetate unit
			[1-^{13}C]Acetate		[2-^{13}C]Acetate		[1-^{13}C]Propionate		[1,2-$^{13}C_2$]Acetate		CH_3CO_2H
	A	B	A	B	A	B	A	B	A	B	
1	153.8	154.3	3.59	2.87	1.07	0.617	1.09	0.561	64.2	63.1	12a-1
2	112.6	112.7	0.973	1.07	3.12	2.22	1.30	1.23	56.5	56.5	2-3
3	129.2	129.1	5.12	6.24	0.982	1.24	1.30	0.974	56.7	56.6	
4	127.0	126.6	0.760	1.38	2.53	2.67	0.978	1.16	61.4	61.4	4-4a
4a	125.1	125.0	2.89	5.16	0.689	1.17	0.939	2.13	61.1	60.9	
4b	142.5	142.0	0.586	1.07	2.86	2.06	0.882	0.795	68.2	69.7	4b-10b
6	161.1	161.0	0.707	1.25	2.98	3.33	0.780	1.14	nc[e]	nc	
6a	122.2	121.7 (121.5)[d]	3.14	(5.23)	0.701	(1.00)	1.13	[f]	62.6	(61.1)	7-6a
7	119.8	122.0 (121.1)	0.615	1.20	2.57	2.72	1.00	1.70	62.0	(61.9)	
8	138.9	140.3	2.22	6.34	1.55	1.26	24.1	1.69	nc	45.2	
9	114.2	118.4	0.753	0.993	2.58	2.21	1.05	1.59	nc	nc	
10	157.3	156.9 (156.8)	4.19	3.67	0.739	0.885	1.17	1.40	70.0	(69.7)	10a-10
10a	123.6	121.7 (120.8)	0.464	(1.00)	2.14	(2.75)	0.734	[f]	69.6	(68.9)	
10b	113.8	114.0	2.50	7.30	0.726	1.44	1.04	1.51	[g]	69.4	
11	102.0	102.1	0.817	1.20	3.13	2.54	1.34	1.28	74.6	75.0	11-12
12	152.1	152.1	3.54	4.42	0.807	0.766	1.13	1.56	74.5	75.2	
12a	115.6	115.4	0.894	0.785	2.93	1.81	1.26	1.13	64.2	63.3	
CH=	135.0	—	0.659	—	2.11	—	0.923	—	nc	—	
$=CH_2$	116.5	—	0.891	—	2.46	—	1.50	—	nc	—	
8-CH_3	—	21.6	—	1.49	—	3.21	—	1.50	—	45.0	8CH_3-8

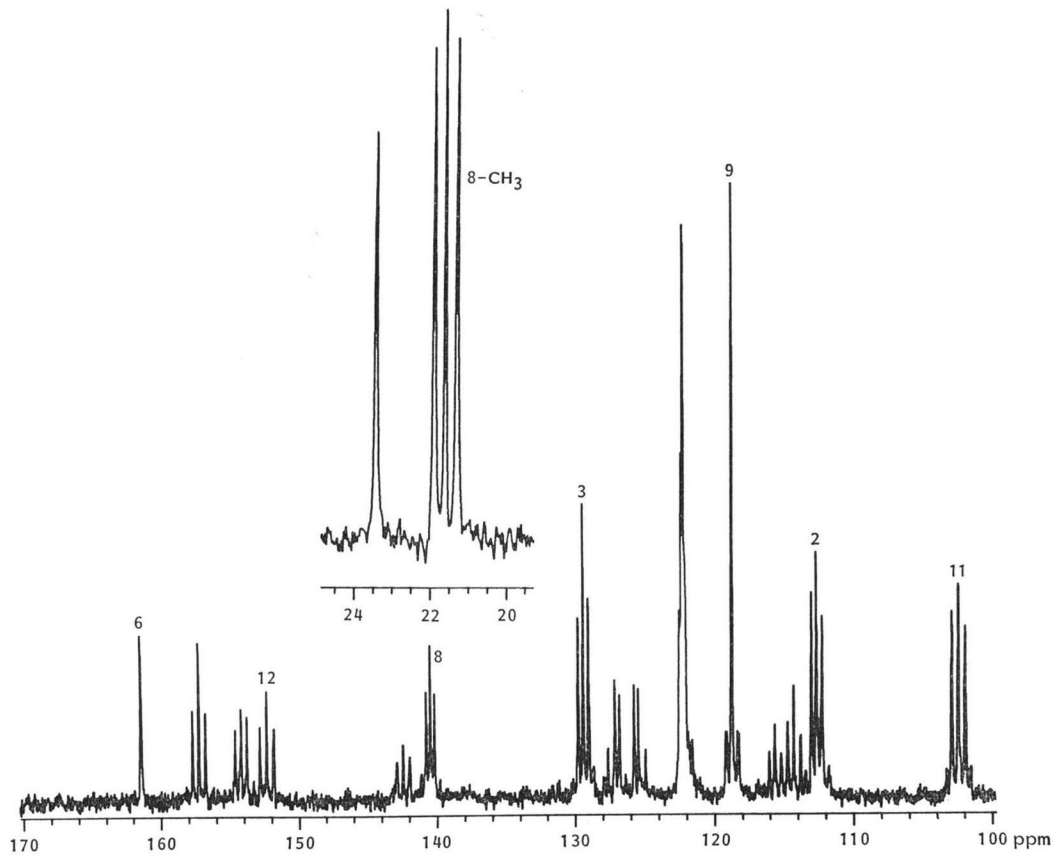
[a] In $CDCl_3/CD_3OD$, ppm downfield from TMS, ± 0.1 ppm.
[b] Ratio of normalized signals between labeled and natural abundance spectra.
[c] Values are ± 0.5 Hz.
[d] Values in parentheses were obtained in DMSO-d_6.
[e] nc denotes a signal without significant coupling.
[f] Signals were unresolved in $CDCl_3$, however, no incorporation was observed.
[g] Half of the doublet is obscured by the resonance at 114.2.

producer. This culture, Lederle Number LL-AD0819, was used in all the studies here reported. The first fermentation with culture LL-AD0819 produced readily detectable activity (BIA), and a subsequent run gave a yield of 3 μg/ml as determined by HPLC. In an effort to improve yields to a workable level for isolation and NMR analysis, culture LL-AD0819 was plated and a number of single colonies were isolated. An isolate designated NS44 was eventually selected as suitable for the biosynthetic experiments. This isolate, when grown in fermentation medium F3, produced approximately equal titers of chrysomycins A and B, permitting direct comparison of incorporation data for these compounds in the same fermentation.

The results of the feeding experiments are presented in Table 1. With the exception of C-8, the labeling pattern of the ring carbons by 1 and 2 ^{13}C-enriched acetates was identical for chrysomycins A and B. Thus carbons 1, 3, 4a, 6a, 10, 10b and 12 showed enhanced ^{13}C NMR signals when the antibiotics were produced with [1-^{13}C]acetate fortified medium, while carbons 2, 4, 4b, 6, 7, 9, 10a, 11 and 12a were enriched by [2-^{13}C]acetate. These results are consistent with the earlier reports on gilvocarcin[13] and ravidomycin[14].

For ring carbon 8 the incorporation data demonstrate different patterns of enrichment for chrysomycins A and B. In the case of chrysomycin A, a low level of enrichment by [1-^{13}C]acetate was observed. This result is over-shadowed by the 24-fold signal enhancement obtained in the [1-^{13}C]propionate

Fig. 2. A portion of the ^{13}C NMR of **2** labeled with [1,2-$^{13}C_2$]acetate (inset shows coupling observed for 8-CH_3 signal).

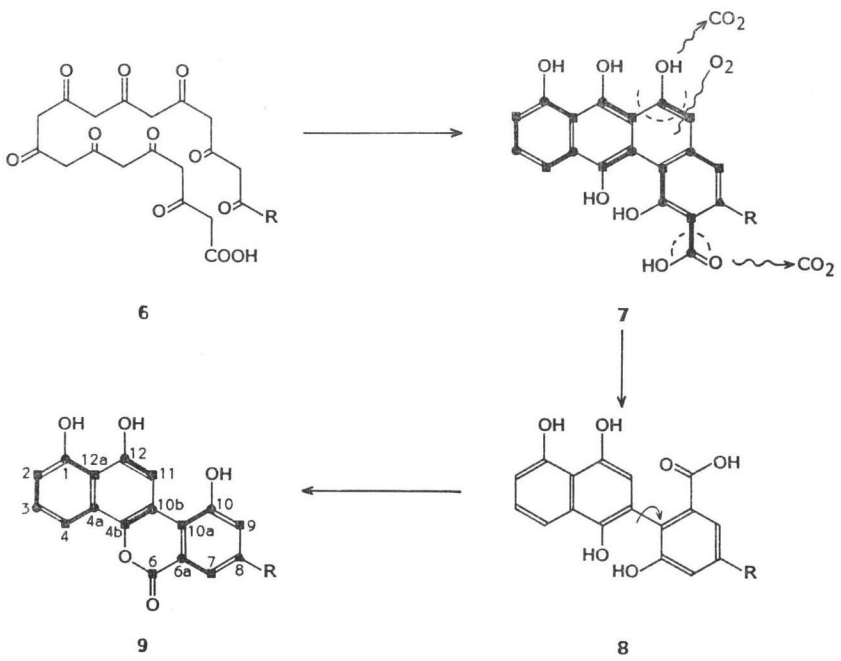


labeling experiment. Chrysomycin B showed a very high degree of enrichment at C-8 by [1-^{13}C]acetate and the C-8 methyl group is clearly labeled by [2-^{13}C]acetate; C-8 shows essentially zero signal enchancement by [1-^{13}C]propionate. The lack of incorporation of propionate into chrysomycin B clearly refutes the degradative hypothesis for its formation. The acetate incorporation observed at C-8 for chrysomycin A no doubt results from the TCA cycle mediated conversion of acetate to propionate. This metabolic argument can also be applied to explain the enrichments observed for the vinyl carbons of chrysomycin A by [2-^{13}C]acetate.

Individual "acetate units" were established in an incorporation experiment using [1,2-$^{13}C_2$]acetate. The coupled pairs indicated in Table 1 define intact acetates incorporated into the antibiotics. As shown in Fig. 2, the 8-CH_3 group of chrysomycin B produced in this experiment gives rise to a signal at δ 21.6 which clearly shows coupling. This coupling between C-8 and the C-8 methyl group precludes the secondary addition mechanism postulated by TAKAHASHI and TOMITA[13] for gilvocarcin. The uncoupled signals either represent fragmented acetate units (C-6 and C-9) or carbons for which acetate is not a direct precursor. The biosynthetic scheme depicted in Fig. 3 is fully consistent with these results. As shown, the substituents at the 8 position are introduced by the chain starter unit. Although the real sequence of events is not known, the further processing of the decaketide chain **6** could follow the proposed scheme for ravidomycin[14]. Thus, **6** is cyclized, deoxygenated at C-3 and oxidized at C-4b to produce the tetracyclic intermediate **7**. Oxidative ring cleavage and decarboxylation affords intermediate **8**, which upon lactonization gives the intact chromophore **9**. This precursor would then be further processed by methylation and glycosylation (and dehydrogenation for chrysomycin A) to yield the antibiotics.

It seems unlikely that the chromophores of ravidomycin and chrysomycin are produced by a dif-

Fig. 3. Proposed biosynthesis of the chromophore of chrysomycins A and B.



R = CH_3 or CH_2CH_3, ▲ = C-1 of chain starter, ● = C-1 acetate, ■ = C-2 acetate, ●—■ = Acetate unit

ferent biosynthetic route from gilvocarcin. We suggest that the key result reported for gilvocarcin on the incorporation of [1-^{13}C]acetate into C-8 of the vinyl form, is due to a non-specific labeling arising from the metabolic conversion of acetate to propionate, and not from the direct incorporation of an acetate unit; our results reveal a similar non-specific incorporation of [1-^{13}C]acetate at C-8 of chrysomycin A. The critical experiment for gilvocarcin using double labeled acetate as a precursor, that could have unambiguously demonstrated to which acetate unit C-8 belonged, was apparently not carried out. On this basis we propose a common biosynthetic pathway for the chromophore of these three antibiotics.

Experimental

Fermentation

Conducted as follows: culture LL-AD0819-NS44 grown on a slant of medium ST (2% Gerber baby oatmeal, 2% glycerol, 2% agar, pH 6.8 prior to sterilization) was inoculated into medium S (3% glucose, 0.5% yeast extract, 0.5% N-Z Amine A, 0.1% $CaCO_3$, pH 6.8 prior to sterilization), 100 ml in a 500-ml Erlenmeyer flask and incubated on a rotary shaker (200 rpm) at 28°C for 72 hours. The vegetative growth obtained was then used to inoculate a second flask of the same medium and was incubated for 48 hours (two-stage seed). The latter was then used as inoculum for the fermentation media. The composition of the fermentation media proved to be an important factor in determining the ratio of chrysomycins A and B produced (Table 2).

Medium F3 was selected for this biosynthetic study as it provided essentially equal amounts of chrysomycins A and B. The incorporation of labeled precursors was done by employing 1 liter volumes of medium F3 (10 flasks, 100 ml/flask), inoculated as previously described. After incubation for 48 hours, 50 mg of the labeled precursor was added to each flask; after an additional 24-hour incubation, flasks were supplemented with an additional 50 mg of their respective labeled precursors, and the fermentation was continued for a total period of 6 days.

Labeled Precursors

The acetates and propionate used in these experiments were 99% ^{13}C enriched at the positions indicated and were obtained from Cambridge Isotope Labs.

HPLC Assay

The titers of chrysomycins A and B were determined using reverse phase HPLC with detection by ultraviolet absorbance at 254 nm. A 5 μC_{18} column (Altex) was used with a mobile phase consisting of acetonitrile - 0.1 M ammonium acetate buffer (pH 5.0), (35: 65) at 2.0 ml/minute. Titers were estimated by comparison of peak heights with standards. The retention volume for the B component is 22 ml; 24 ml for the A component. Samples were prepared by sonicating a mixture of 5 ml whole broth with 1 ml Me_2CO for 5 minutes, followed by extraction into EtOAc (4 ml). This extract was used for the HPLC analysis.

Table 2. Effect of medium on chrysomycin A/B ratio.

Medium[a]	Titer (μg/ml)	
	A	B
F1 (2% molasses, 1% glucose, 1% N-Z Amine A)	16	16
F2 (2% Gerber baby oatmeal, 2% glycerol)	82	34
F3 (6% glucose, 1% Proflo, 1% soy flour, 1% $CaCO_3$, 0.1% $(NH_4)H_2PO_4$, 0.05% $FeSO_4 \cdot 7H_2O$)	43	43

[a] pHs of all media adjusted to 6.8 prior to sterilization.

Isolation and Separation of Chrysomycins A and B

One liter of fermentation mash was mixed with Celite 545 (50 g) and filtered. The cake was extracted with Me_2CO (2×500 ml) in a Waring blender, and the combined extracts were concentrated *in vacuo* at 35°C to remove the bulk of the Me_2CO. The resulting aq concentrate was extracted (2× 250 ml) with EtOAc. The extract was evaporated *in vacuo* at 35°C to a residue, redissolved in 2 ml of THF - MeOH (1: 1) and filtered through a 0.45 μm filter prior to chromatography.

The chromatographic separation was accomplished using a C_8 reverse phase column (2.2×50 cm, Whatman) developed with acetonitrile - 0.1 M ammonium acetate buffer (pH 4.8), (37.5: 62.5) at 9.9 ml/minute. Detection was by absorbance at 405 nm. Chrysomycin B eluted between 57~70 minutes, chrysomycin A between 77~90 minutes. The antibiotics were extracted from the mobile phase with CH_2Cl_2.

^{13}C NMR

Spectra were recorded on a Nicolet NP-300 WB at 75.5 MHz. Intensities were normalized to that of the signal at 72 ppm which is assigned to C-5′. Assignments were adapted from ref 9.

Acknowledgments

The authors express their appreciation to Ms. PEGGY LEIGHTON for her skillful assistance in the precursor incorporation studies.

References

1) STRELITZ, F.; H. FLON & I. N. ASHESHOV: Chrysomycin: A new antibiotic substance for bacterial viruses. J. Bacteriol. 69: 280~283, 1955
2) HATANO, K.; E. HIGASHIDE, M. SHIBATA, Y. KAMEDA, S. HORII & K. MIZUNO: Toromycin, a new antibiotic produced by *Streptomyces collinus* subsp. *albescens* subsp. nov. Agric. Biol. Chem. 44: 1157~1163, 1980
3) BRAZHNIKOVA, M. G.; M. K. KUDINOVA, V. V. KULYAEVA, N. P. POTAPOVA & V. I. PONOMARENKO: Physicochemical characteristics of virenomycin, a new antitumorigenic antibiotic. Antibiotiki 22: 967~970, 1977
4) NAKANO, H.; Y. MATSUDA, K. ITO, S. OHKUBO, M. MORIMOTO & F. TOMITA: Gilvocarcins, new antitumor antibiotics. 1. Taxonomy, fermentation, isolation and biological activities. J. Antibiotics 34: 266~270, 1981
5) SEHGAL, S. N.; H. CZERKAWSKI, A. KUDELSKI, K. PANDEV, R. SAUCIER & C. VEZINA: Ravidomycin (AY-25,545), a new antitumor antibiotic. J. Antibiotics 36: 355~361, 1983
6) HORII, S.; H. FUKASE, E. MIZUTA, K. HATANO & K. MIZUNO: Chemistry of toromycin. Chem. Pharm. Bull. 28: 3601~3611, 1980
7) TAKAHASHI, K.; M. YOSHIDA, F. TOMITA & K. SHIRAHATA: Gilvocarcins, new antitumor antibiotics. 2. Structural elucidation. J. Antibiotics 34: 271~275, 1981
8) FINDLAY, J. A.; J. LIU, L. RADICS & S. RAKHIT: The structure of ravidomycin. Can. J. Chem. 59: 3018~3020, 1981
9) WEISS, U.; K. YOSHIHIRA, R. J. HIGHET, R. J. WHITE & T. T. WEI: The chemistry of the antibiotics chrysomycin A and B. Antitumor activity of chrysomycin A. J. Antibiotics 35: 1194~1201, 1982
10) BALITZ, D. M.; F. O. O'HERRON, J. BUSH, D. M. VYAS, D. E. NETTLETON, R. E. GRULICH, W. T. BRADNER & T. W. DOYLE: Antitumor agents from *Streptomyces anandii*: Gilvocarcins V, M and E. J. Antibiotics 34: 1544~1555, 1981
11) WEI, T. T.; J. A. CHAN, P. P. ROLLER, U. WEISS, R. M. STROSHANE, R. J. WHITE & K. M. BYRNE: Detection of gilvocarcin antitumor complex by a biochemical induction assay (BIA). J. Antibiotics 35: 529~532, 1982
12) JAIN, T. C.; G. C. SIMOLIKE & L. M. JACKMAN: Structure and stereochemistry of toromycin; studies of its acid-catalyzed rearrangement. Tetrahedron 39: 599~605, 1983
13) TAKAHASHI, K. & F. TOMITA: Gilvocarcins, new antitumor antibiotics. 5. Biosynthesis of gilvocarcins: Incorporation of ^{13}C-labeled compounds into gilvocarcin aglycones. J. Antibiotics 36: 1531~1535, 1983
14) CARTER, G. T.; A. A. FANTINI, J. C. JAMES, D. B. BORDERS & R. J. WHITE: Biosynthesis of ravidomycin. Use of ^{13}C-^{13}C double quantum NMR to follow precursor incorporation. Tetrahedron Lett. 25: 255~258, 1984