MICROMONOSPORA-PRODUCED SISOMICIN COMPONENTS

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A sisomicin fermentation carried out in the presence of (methyl-^{14}C)-L-methionine resulted in a crude mixture, composed of methyl-^{14}C-labeled sisomicin as a major component; and two 4″-C-desmethylsisomicin (66-40B and 66-40D) isomer-like components, an unidentified component and a gentamicin A-like antibiotic as minor components. When (methyl-^{14}C)-L-methionine was added in an early stage of the fermentation (24 hours), incorporation of methyl-^{14}C-label into polar components (*e.g.*, gentamicin A-like antibiotic) preceded that into sisomicin. Chromatographic evidence for the bioconversion of (methyl-^{14}C)-gentamicin A to a radioactive sisomicin-like product (possibly (3″-N-methyl-^{14}C)-sisomicin) was seen, when a *Micromonospora* blocked mutant was incubated in the presence of the former antibiotic.

Sisomicin, an unsaturated aminoglycoside, is produced by *Micromonospora inyoensis*, NRRL 3292[1], and its structure was reported[2]. Procedures for fermentative production, isolation and purification of the antibiotic were described[3], and its biological activity determined[4]. Minor components (two isomers of 4″-C-desmethylsisomicin) were isolated from a sisomicin fermentation and their structures characterized[5].

Like the gentamicins, sisomicin is a methylated aminoglycoside antibiotic. L-Methionine was shown to be an excellent methyl donor for the gentamicins[6,7]. Incorporation of label from (methyl-^{14}C)-L-methionine into sisomicin was found to be similarly high, and the radioisotope was therefore chosen to prepare labeled sisomicin. The areas examined in the current investigation were the composition of a methyl-^{14}C-labeled crude mixture of sisomicin and minor components, the mode of incorporation of methyl-^{14}C-label into different sisomicin fermentation components during the fermentation process, and the bioconversion of (methyl-^{14}C)-gentamicin A to a radioactive sisomicin-like product.

Materials and Methods

Organisms and cultivation

Micromonospora inyoensis (NRRL 3292), and an *M. inyoensis* mutant strain (1550F)[8] were used. The strain (1550F) produces sisomicin only in the presence of added 2-deoxystreptamine. Identical procedures[3] were used for cultivation of the organisms.

Chemicals

(Methyl-^{14}C)-L-methionine, purchased from New England Nuclear (Boston, Mass.), had a specific radioactivity of 40～55 mCi/m mole. (Methyl-^{14}C)-gentamicin A base, isolated from a radioactive gentamicin fermentation broth, and (methyl-^{14}C)-sisomicin base, isolated from a radioactive sisomicin fermentation broth, had a specific radioactivity of 0.28 and 1.57 μCi/mg

respectively.

Analytical methods

Identical procedures[6,7] were used for paper chromatography, bioassay, scan and measurement of radioactivity.

Fermentation for the preparation of (methyl-^{14}C)-sisomicin components

Five ml of inoculum (NRRL 3292) from the germination stage[3] was transferred to each of thirty 30-ml Erlenmeyer flasks containing 50 ml of the following medium: dextrin 50 g; dextrose, 5 g; soybean meal, 35 g; corn steep liquor, 5 g; $CaCO_3$, 7 g; $CoCl_2$, 2 mg; tap water, 1,000 ml. Following shaking at 32°C at 350 rpm for 96 hours, 100 μCi quantities of (methyl-^{14}C)-L-methionine were added into each of the 30 flasks. An additional 96 hours were allowed for continued shaking of the flasks under the same conditions.

Isolation of mixture of sisomicin components

A fifteen g portion of oxalic acid was added to the combined fermentation broth (1,800 ml including washings of the flasks), pH of the fermentation broth adjusted to 2.0 with conc. H_2SO_4, and the acidified broth stirred at room temperature for 1 hour and filtered. The filtrate (1,700 ml including washings) was neutralized with conc. NH_4OH, 45 g (wet weight) of IRC-50 resin (NH_4^+ form) added to the neutralized filtrate and the mixture (resin plus filtrate) was stirred at room temperature for 1 hour. Liquid was decanted, the resin washed twice with distilled water, and the crude solution eluted from the resin by treating it three times with 300-ml portions of 2 N NH_4OH. The combined eluates were concentrated on a flash evaporator, and the residue was redissolved in a small quantity of distilled water and lyophilized.

Column chromatographic separation of (methyl-^{14}C)-sisomicin components

Silica gel (300 g) was packed in a column (2.9 cm×116 cm) by slurrying, the above mentioned lyophilized preparation (weight, 375.5 mg; radioactivity, 351 μCi) charged onto the column, and the products eluted, with the lower phase of chloroform - methanol - conc.ammonia (1:1:1), at a flow rate of 1 ml/10 minutes. Fractions were collected every 10 minutes, and cuts based on bioautographic detection of zones (against *Staphylococcus aureus* ATCC 6538P) after paper and thin-layer chromatograpies.

Examination of radio-label in the mixture of sisomicin components

Twenty μCi of (methyl-^{14}C)-L-methionine was added to each flask, containing 50 ml medium[3], at the onset of the antibiotic biosynthesis (24 hours) and during the period of active antibiotic synthesis (96 hours), respectively. Flasks were harvested at various time-intervals, crude products were isolated[3], and the distribution of the radio-label in the mixture of sisomicin com-

Table 1. Bioactivity and radioactivity balance following 8-day fermentation of *M. inyoensis* NRRL 3292 in the presence of L-methionine (methyl-^{14}C).

Fraction	Volume or weight	Bioactivity		Radioactivity	
		mg	% Recovery[1]	μCi	% Recovery[2]
Whole broth	1,800 ml	189.9	100.0	2,879	96.0
Neutralized filtrate	1,700 ml	179.4	94.5	1,813	60.4
IRC-50 spent	1,700 ml	—	—	1,247	41.6
IRC-50 eluate	375.5 mg	153.6	80.9	351	11.7
Sisomicin	166.9 mg	144.7	76.2	262	8.7
$^{14}CO_2$[3]	—	—	—	0	0

[1] % Recovery of bioactivity is based on mg bioactivity of whole broth (189.9 mg).

[2] % Recovery of radioactivity is based on 3,000 μCi L-methionine (methyl-^{14}C) added into fermentation broths.

[3] Release of $^{14}CO_2$ was collected in three consecutive traps, each containing 1,300 ml 2 N NaOH solution, and radioactivity in the NaOH solution was measured.

ponents was examined[9].

Bioconversion of (methyl-^{14}C)-gentamicin A

(Methyl-^{14}C)-gentamicin A base (weight, 21 mg; radioactivity, 5.95 μCi) was added to a 72-hour-grown culture broth of *M. inyoensis* 1550F (a 2-deoxystreptamine-requiring mutant), and the flask (50 ml medium in a 300-ml Erlenmeyer) was shaken at 300 rpm at 28°C for an additional 96 hours. Crude products were isolated and examined[9].

Results

Radioactivity and Bioactivity-balance following a Preparative Radioactive Fermentation

Following harvest of the 30 fermentation flasks, to which a total radioactivity of 3,000 μCi was added, a series of steps including filtration, isolation of crude, and chromatographic

Table 2. Preparative chromatographic separation of sisomicin (methyl-^{14}C) fermentation products.

Fraction	Tube numbers	Component(s)	Weight (mg)	Bioactivity (mg)	Radioactivity (μCi)	Specific radioactivity (μCi/mg)
1	123~138	Sisomicin	166.9	144.7	261.80	1.57
2	149~165	P+U+B+D+S[1]	21.82	—	6.35	—
3	166~180	P+U+B[2]	21.42	—	8.08	—
4	181~205	P+U[3]	28.83	—	19.72	—
5	Methanol wash[4]	Gentamicin A	15.85	—	5.33	0.34

[1] A mixture of P (polar components): (U (unknown component)+B (66-40B)): D (66-40D): S (sisomicin)=27 : 46 : 18 : 10, based on radioactivity distribution.

[2] A mixture of P : (U+B)=47: 53.

[3] A mixture of P : U=20 : 80.

[4] The fraction was obtained by stripping the column following separation of fraction 4, washing the remaining silica gel with 500 ml MeOH, concentrating the methanol-wash to dryness, and desalting the residue (576.73 mg) by adsorption to and ammonium hydroxide-elution from 6 g (wet) IRC-50 resin.

Fig. 1.

(a) and (b) Bioautograms of authentic sisomicin and fraction (1).

(c) Radioactivity scan of fraction (1), showing a major and a minor peak representing sisomicin (S) and a trace amount of the polar components (P) respectively.

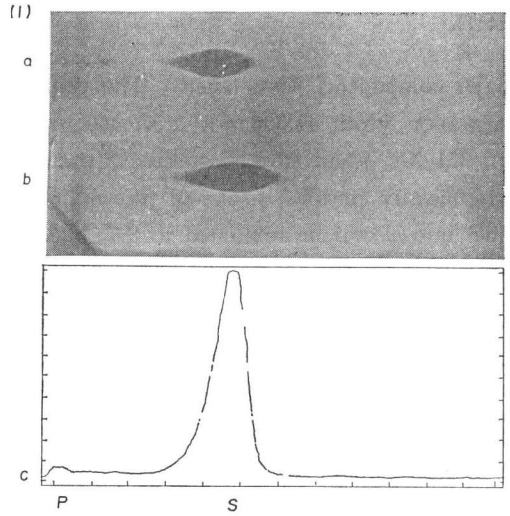
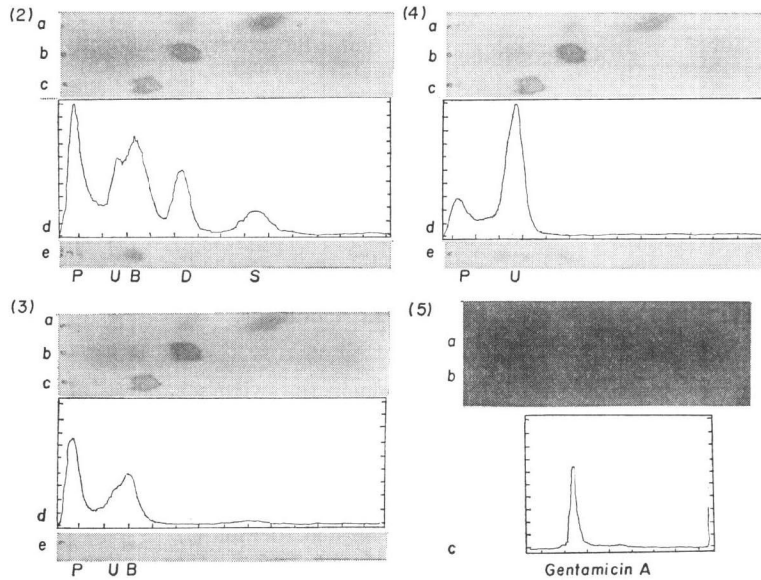


Fig. 2.* Fraction (2)
(a), (b) and (c) Ninhydrin-treated sisomicin-, 66-40D- and 66-40B-standards.
(d) Radioactivity scan of fraction (2), showing peaks representing P (polar components), U (unidentified component), B (66-40B), D (66-40D) and S (sisomicin).
(e) Ninhydrin-treated fraction (2), showing the presence of P, U, B and D predominantly.
Fraction (3)
(a), (b) and (c) Ninhydrin-treated sisomicin, 66-40D- and 66-40B-standards.
(d) Radioactivity scan of fraction (3), showing the presence of P, U and 66-40B.
(e) Ninhydrin-treated fraction (3), showing the presence of P, U and B predominantly.
Fraction (4)
(a), (b) and (c) Ninhydrin-treated sisomicin-, 66-40D- and 66-40B-standards.
(d) Radioactivity scan of fraction (4), showing the presence of P and U.
(e) Ninhydrin-treated fraction (4), showing U predominantly.
Fraction (5)
(a) and (b) Bioautograms of gentamicin A-standard and fraction (5).
(c) Radioactivity scan of fraction (5), showing a sharp peak representing gentamicin A.



* All chromatograms were developed on Whatman No. 1 paper, in the lower phase of chloroform-methanol-17% ammonia (2:1:1).

separation of sisomicin (major component) were taken. The radioactivity measured for each step was: combined fermentation broth (1,700 ml), 2,879 μCi; neutralized filtrate, 1,813 μCi; crude (IRC-50 resin eluate), 351 μCi; spent (the neutralized filtrate after removal of the crude), 1,247 μCi; and chromatographicially purified sisomicin (the major component), 262 μCi. Thus the incorporation of the label into sisomicin amounted to 8.7% (262 μCi from 3,000 μCi added). Bioactivity assayed against an authentic sisomicin standard was: neutralized filtrate, 179 mg; crude, 154 mg; and sisomicin, 145 mg (Table 1).

Chromatographic Separation of (Methyl-^{14}C)-sisomicin Components

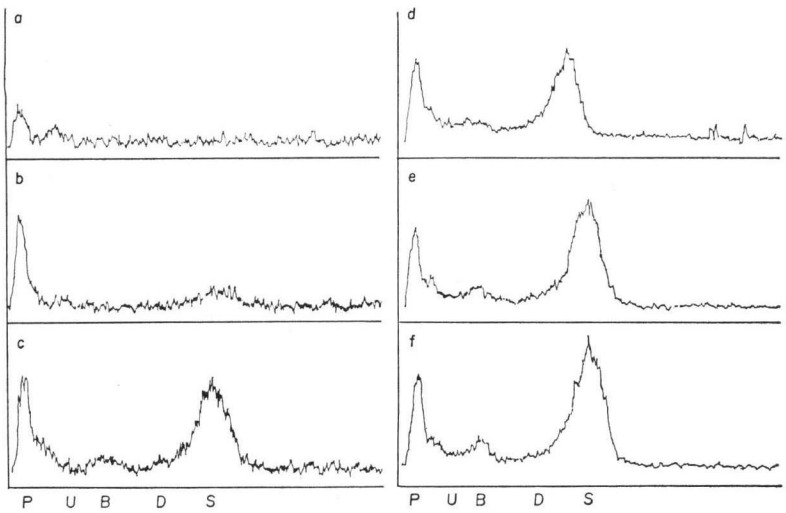
Chromatography of the above-mentioned crude yielded: fraction 1 (sisomicin), fraction 2 (a mixture of polar components, the unidentified component (U), two 4''-C-desmethylsisomicin isomer-like components and sisomicin), fraction 3 (polar components, the unidentified component (U) and a 66-40B-like component), fraction 4 (polar components and the unidentified component

Table 3. Incorporation of radioactivity from L-methionine (methyl-^{14}C), added at 24 hours, into sisomicin fermentation products.

Exposure to isotope (hours)	Radioactivity (μCi)*					Total bioactivity (mg)*
	P[1]	U[2]	B[3]	D[4]	S[5]	
0.25	0.020	0.008	0.000	0.000	0.000	0.00
6	0.090	0.011	0.000	0.000	0.000	0.00
24	0.088	0.007	0.017	0.010	0.168	0.25
48	0.380	0.080	0.128	0.100	0.806	3.24
72	0.467	0.081	0.155	0.097	1.014	3.77
96	0.414	0.080	0.151	0.109	1.264	5.51
144	0.528	0.101	0.214	0.142	1.450	6.66

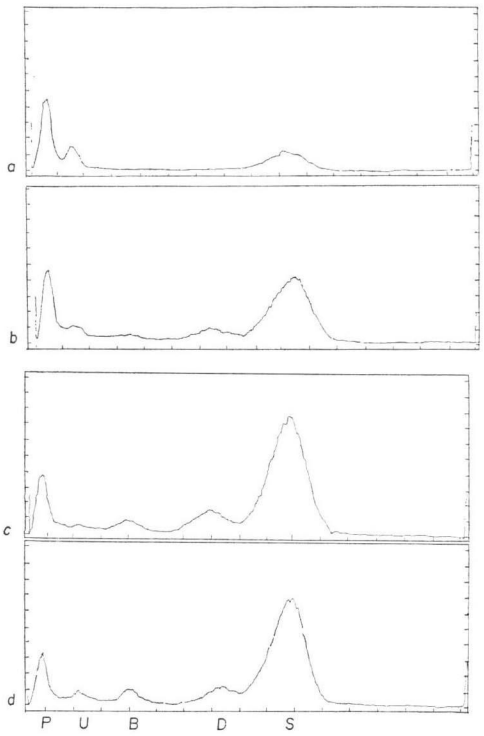
* Radioactivity and bioactivity from 50 ml fermentation broth
1) P: Polar components
2) U: Unknown component which migrates between polar components and 66-40B
3) B: 66-40B
4) D: 66-40D
5) S: Sisomicin

Fig. 3. Radioactivity scans of samples, taken after various lengths of time following addition of L-methionine (methyl-^{14}C) at 24 hours.
(a) and (b) 1/4~ and 6~ hour samples, showing exclusive incorporation of radioactivity into P.
(c), (d), (e) and (f) 24~, 48~, 96~ and 144~ hour samples, showing incorporation of radioactivity in a decreasing order of S>P>B>D>U.



(U)), and fraction 5 (a gentamicin A-like component). No attempts were made for an additional chromatographic separation and isolation of the unidentified component, and the two 4''-C-desmethylsisomicin isomer-like components from fractions 2, 3 and 4 due to limited availability of material. The two chromatographically separated sisomicin and gentamicin A-like component (1 and 5) possessed respectively, 167 mg weight, 145 mg bioactivity, 262 μCi radioactivity, 1.57 μCi/mg specific radioactivity; and 15.9 mg weight, 5.2 μCi radioactivity, 0.34 μCi/mg specific radioactivity (Table 2, Figs. 1 and 2).

Fig. 4. Radioactivity scans of samples, taken after various lengths of time following addition of L-methionine (methyl-^{14}C) at 96 hours.
(a) 1/4~ hours sample, showing radioactivity distribution in a decreasing order of P>S>U >D>B.
(b) 1~ hour sample, showing radioactivity distribution in a decreasing order of S>P>D >U>B.
(c) and (d) 6~ and 72~ hour samples, showing radioactivity distribution in a decreasing order of S>D>P>B>U.



Mode of Label Incorporation from (Methyl-^{14}C)-L-methionine into Sisomicin Components

When (methyl-^{14}C)-L-methionine was added in an early stage of the antibiotic biosynthesis (24 hours), exclusive incorporation of label into the polar components was seen up to 6-hour exposure of the organism to the radioisotope. As time for the exposure progressed to 24 hours and longer, incorporation of radioactivity into other components (the two 4''-C-desmethylsisomicin isomer-like components, sisomicin and the unidentified component (U)) was evidenced. Among these components, the incorporation of the label into sisomicin was greatest during the longer exposures. Radioactivity incorporated into all of the components increased with the progression of time (Table 3, Fig. 3).

When the isotope was added in an active biosynthetic stage (96 hours), the pattern of radioactivity incorporation was quite different. Not only did extremely rapid incorporation of label into all of the components occur, but the rate of incorporation was much greater (approximately 3 to 4-fold). The incorporation of the label into the polar components was greatest immediately after the addition of the isotope at 96 hours. As the cells were

Table 4. Incorporation of radioactivity from L-methionine (methyl-^{14}C), added at 96 hours, into sisomicin fermentation products.

Exposure to isotope (hours)	Radioactivity (μCi)*						Bioactivity (mg)
	P[1]	U[2]	B[3]	D[4]	S[5]	Total	
0.25	1.021	0.390	0.069	0.098	0.811	2.389	4.54
1	0.956	0.332	0.246	0.610	2.955	5.099	4.44
6	0.812	0.295	0.441	0.969	4.627	7.144	5.27
72	0.737	0.312	0.492	0.883	4.695	7.119	6.67

* Radioactivity and bioactivity from 50 ml fermentation broth
1) P: Polar components
2) U: Unknown component which migrates between polar components and 66-40B
3) B: 66-40B
4) D: 66-40D
5) S: Sisomicin

exposed to the isotope for 1 hour or longer, the incorporation of the label into sisomicin was found to be greatest. Interestingly, radioactivity incorporated into the polar components and the unidentified component (U) decreased with the progression of time. On the other hand, the respective radioactivity incorporated into the other components (the two 4″-C-desmethylsisomicin isomer-like components and sisomicin) increased (Table 4, Fig. 4).

Bioconversion of (Methyl-^{14}C)-Gentamicin A

Following a subsequent 96-hour incubation of the deoxystreptamine-negative *M. inyoensis* mutant (1550F) in the presence of (methyl-^{14}C)-gentamicin A, added at 72 hours, 64.2% of the added radioactivity was recovered in the isolated fermention crude. The distribution of the recovered radioactivity was 1.3% in the sisomicin-like product and 98.7% in the residual gentamicin A (Fig. 5). The small percentage of the incorporation of the label into the product may be attributable to a cell permeability barrier to the exogenously added (methyl-^{14}C)-gentamicin A.

Fig. 5. (A) Radioactivity scan of gentamicin A (methyl-^{14}C).
(B) Radioactivity scan of bioconversion sample resulted from gentamicin A (methyl-^{14}C), showing the presence of U and S besides the residual gentamicin A.
(C) Ninhydrin-treated sisomicin.
(S) Sisomicin.

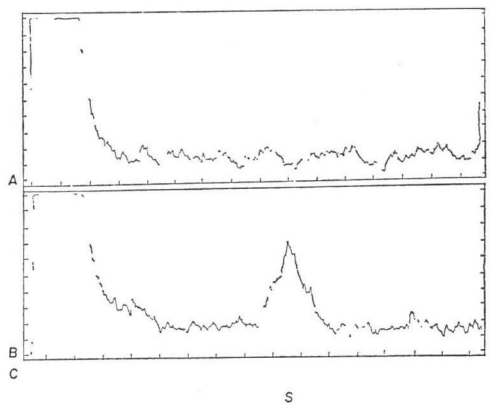
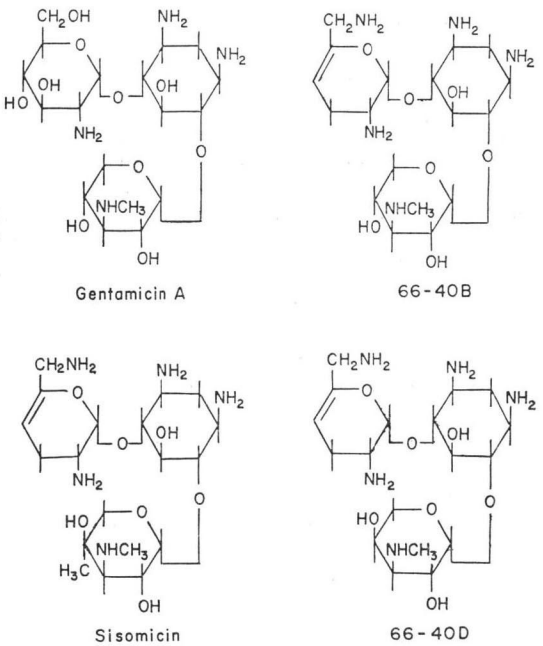


Fig. 6. Structures of sisomicin fermentation components.



Discussion

Davies *et al.*[5] isolated and characterized the two isomers of 4″-C-desmethylsisomicin from a cold sisomicin fermentation. From a (methyl-^{14}C)-sisomicin fermentation crude, we have isolated (methyl-^{14}C)-sisomicin (the first chromatographically separated fraction) and a (methyl-^{14}C)-gentamicin A-like component (the last fraction). We also evidenced the presence of the radioactive unidentified component and the two 4″-C- desmethylsisomicin isomer-like components in the middle fractions of the radioactive fermentation crude.

When (methyl-^{14}C)-L-methionine was added in an early stage of the antibiotic biosynthesis (24 hours), exclusive incorporation of the methyl-^{14}C-label into the polar components was evidenced following up to 6-hour exposure of the producing organism to the radioisotope. When the isotope was added in an active biosynthetic stage (96 hours), on the other hand, rapid incorporation of the methyl-^{14}C-label into all of the sisomicin fermentation components was observed. Following exposure of the organism

to the isotope, added at 96 hours, for 6 hours or longer, incorporation of the label into sisomicin and one of the desmethylsisomicin isomer-like components (66-40D-like) was found to be greater than that into other components. With the progression of time, the label incorporated respectively into the polar components and the unidentified component (U) decreased. On the contrary, the radioactivity which was incorporated respectively into the two desmethylsisomicin isomer-like components and sisomicin increased. Thus the data indicate that the polar components and the unidentified component (U) are produced prior to other components.

From the incubation of the deoxystreptamine-negative *M. inyoensis* mutant (1550F) in the presence of (methyl-^{14}C)-gentamicin A, the synthesis of a radioactive sisomicin-like product was evidenced. Radioactivity incorporation into the sisomicin-like product amounted to 1.3%.

As an alternative, degradation products of the added (methyl-^{14}C)-gentamicin A may have been reutilized for the synthesis of the radioactive sisomicin-like product (possibly (3''-N-methyl-^{14}C)-sisomicin).

Based on these pieces of information, it is impossible yet to determine how the above mentioned polar components (*e.g.*, the gentamicin A-like component), the unidentified component (U), and the two 4''-C-desmethylsisomicin isomer-like components are involved in the biosynthesis of sisomicin (Fig. 6).

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