# NEW MITOMYCIN ANALOGS PRODUCED
# BY DIRECTED BIOSYNTHESIS

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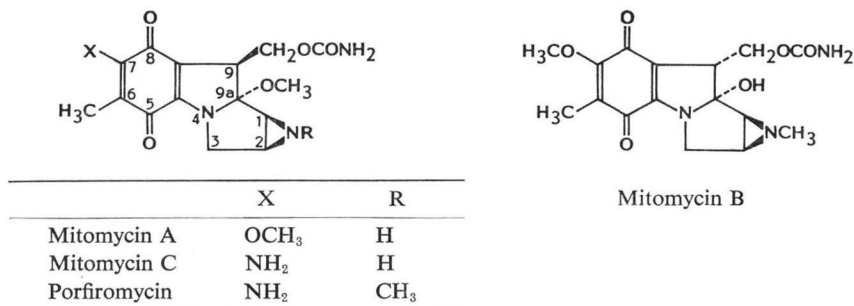D. Kimball, M. F. Kammer and J. Veitch

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When the normal fermentation medium for the production of mitomycin C with *Streptomyces caespitosus* is supplemented with a number of primary amines, two new types of mitomycin analogs described as Type I and Type II are produced. Type I analogs are related to mitomycin C with the amine substitution at position C7 on the mitosane ring. Type II analogs also contain the same substitutions at C7 but the conformation of the mitosane ring is related to mitomycin B having an OH at positions C9a and a methyl substituted aziridine. The products obtained from the supplementation of the medium with methylamine, ethylamine, propylamine, propargylamine and 2-methylallylamine were isolated and characterized. In all cases the Type I analogs are more active in a prophage induction test and against L1210 lymphatic leukemia in mice. A number of other amines have been tested and shown to yield new products that have not yet been isolated. No secondary amines are incorporated.

Mitomycin C is an antitumor antibiotic that has been widely used in the treatment of various neoplastic diseases. It was first obtained in 1956 by Hata et al.[1] from the fermentation of *Streptomyces caespitosus*, along with the closely related analogs, mitomycins A and B and porfiromycin (Fig. 1). This latter antibiotic has also been identified separately from fermentations of *S. verticillatus*[2] and *S. ardus*[3]. In our search for analogs that may have greater activity than mitomycin C, we attempted to modify the fermentation by the process of directed biosynthesis[4]. The formation of new active metabolites through the addition of suitable precursors to antibiotic fermentations had been demonstrated many years ago. During the early studies on the penicillin fermentation, a large number of precursors were fed to the producing culture to yield a modified antibiotic. This work has been reviewed by Demain[5]. Since that time other antibiotic fermentations have been studied, resulting in the development of numerous analogues by directed biosynthesis, including the novobiocins[6],

Fig. 1. Metabolites of *Streptomyces caespitosus*.



|  | X | R |
|---|---|---|
| Mitomycin A | $OCH_3$ | H |
| Mitomycin C | $NH_2$ | H |
| Porfiromycin | $NH_2$ | $CH_3$ |

Mitomycin B

echinomycins[7~9], pyrrolnitrins[10], polyoxins[11], phleomycins[12], bleomycins[12], actinomycins[13], tyrocidins[14], tallysomycins[15], tetracyclines[16], lincomycins[17], celestosaminides[18], viridogriseins[19] and indolmycins[20].

Japanese workers[21,22] and more recently IYENGAR et al.[23] and SAMI et al.[24] have reported on the development of new mitomycin C and porfiromycin analogs through chemical modification of mitomycin C. A number of the new products showed superiority to mitomycin C when measured by various *in vivo* antitumor and leukopenic tests. The synthetic route required a conversion of mitomycin C to mitomycin A prior to C7 substitution, with overall yields being somewhat low[23]. Our interest in the generation of some new analogs that might be formed by supplementation of a normal fermentation medium was prompted by this report. The present paper describes the results of the modification of the normal fermentation of mitomycin C by a strain of *S. caespitosus* through the addition to the medium of low levels of a number of amines.

In certain limited cases, the analog of mitomycin C was produced that corresponded to the amine added. However, in addition, a new derivative containing the added amine in the C7 position, but having a nucleus related to mitomycin B, was also formed.

To date, the products from the addition of methylamine, ethylamine, propylamine, propargylamine and 2-methylallylamine have been isolated from the fermentation mixtures. In addition, a number of other amines have been shown to lead to the formation of new mitomycins, but have not yet been isolated and characterized. Addition of secondary amines, diamines, and $\beta$-thioethylamines gave no incorporation. These studies are continuing.

## Materials and Methods

### Mitomycin C Fermentations

*S. caespitosus* ATCC 27422 was used for the fermentations, the culture being maintained in the Bristol-Myers culture collection as a lyophile preparation. For day to day use the culture was held on slants of BENNETT's agar stored at 4°C. The growth was scraped from a slant and used to inoculate

Table 1. *In vitro* and *in vivo* activities of mitomycin analogs.

| Compound | TLC Rf[a] | Level amine addition (% w/v) | Time harvest (days) | Prophage induction activity[b] (T/C at 0.5 $\mu$g/ml) | L1210 activity[c] T/C | L1210 activity[c] mg/kg/day |
|---|---|---|---|---|---|---|
| Mitomycin C | 0.30 | | 10~14 | 22 | 160 | 6 |
| Mitomycin A | 0.56 | | | 34.6 | | |
| Porfiromycin | 0.48 | | | 8.9 | | |
| Methylamine I | 0.48 | 0.5 | 10 | 5.9 | 124 | 24 |
| 〃 II | 0.37 | | | 0.9 | 100 | 24 |
| Ethylamine I | 0.53 | 1.0 | 10 | 8.7 | 142 | 24 |
| 〃 II | 0.50 | | | 1.3 | 95 | 24 |
| Propylamine I | 0.51 | 0.5 | 10 | 8.1 | 166 | 6.25 |
| 〃 II | 0.50 | | | 1.0 | 100 | 24 |
| Propargylamine I | 0.54 | 0.25 | 10 | 11.6 | 186 | 24 |
| 〃 II | 0.52 | | | 1.7 | 100 | 24 |
| 2-Methylallylamine I | 0.51 | 0.5 | 10 | 2.5 | 126 | 24 |
| 〃 II | 0.49 | | | 1.4 | 100 | 24 |

a   Thin-layer chromatography, Analtech silica gel GF, CHCl$_3$ - MeOH (9: 1).

b   Procedure of PRICE, K. E.; R. E. BUCK & J. LEIN: Appl. Microbiol. 12: 428~435, 1964.

c   Therapeutics Program Division of the National Cancer Institute, Washington, D.C.

100 ml of the following medium in a 500-ml Erlenmeyer flask and incubated for 33 hours at 27°C at 250 rpm: Cerelose 1%, Staclipse J starch 3%, Mellasoy 2%, washed brewer's yeast 0.5%, $K_2HPO_4$ 0.25% and $CaCO_3$ 1%. This vegetative growth was then transferred (8% inoculum) to 100 ml of medium in a 500-ml Erlenmeyer of the following composition: Cerelose 4%, Staclipse J starch 3%, Mellasoy 2%, Pharmamedia 0.5%, safflower meal 1%, $(NH_4)_2SO_4$ 0.1%, NaCl 0.5%, $K_2HPO_4$ 0.5%, $CaCO_3$ 1.5% and vitamin $B_{12}$ 5 μg/ml final concentration, then incubated for varying periods of time under the same conditions. This incubation time varied with the amine employed.

The amines used were either the hydrochloride salts or the free bases neutralized with concentrated hydrochloric acid, sterilized separately by filtration, then added to the medium at final concentrations varying from 0.25% to 1.0% (w/v) at the time of inoculation. The level added and the time of harvest for each of the amines reported is given in Table 1.

### Detection of Mitomycins

EtOAc extracts from samples of fermentation broths were spotted on Analtech silica gel GF thin-layer plates and developed in a $CHCl_3$ - MeOH (9:1) mixture. The normal pattern of mitomycins plus the new metabolites could readily be seen by examination under UV light at 254 nm or by overlaying the thin-layer plates with BBL Streptomycin Assay Agar containing yeast extract seeded with *Bacillus subtilis* ATCC 6633. The new biosynthetic mitomycins move in the region between mitomycins C and A in this system. Despite having identical UV spectra in methanol they can readily be distinguished on silica under visible light by the characteristic purple hue for the mitomycin C related analogs (Type I) and greenish-blue for the mitomycin B (Type II) related compounds. The Rf values for the compounds in the two thin-layer systems are given in Table 1.

### Isolation

Preliminary studies were employed to reveal the optimal level of the various amines added to the fermentation and the time of maximal appearance of the new mitomycins. These varied according to the precursor. From a 10-liter batch, the cells were removed by centrifugation and the supernatant was extracted three times with an equal volume of EtOAc. The solvent was concentrated under vacuum to remove the volatiles, then an equal volume of toluene was added, followed by a second concentration *in vacuo*. This latter step removed $H_2O$ by azeotropic distillation. The oily residue was then dried in a desiccator under vacuum. The crude mixtures obtained at this stage were purified by a sequence of five column chromatographies (Fig. 2).

The first was a $2.5 \times 50$ cm column containing 115 g Woelm silica gel for dry column chromatography (activity III) packed as a slurry in $CHCl_3$. The oily residue was applied to the top of the column followed by a rinse of 1 liter of $CHCl_3$ to remove the non-polar materials. The column was eluted sequentially with 100-ml portions of $CHCl_3$ containing 1, 2, 3 and 4% MeOH, then developed with 5% MeOH in $CHCl_3$ to elute successively the Type I followed by the Type II analogs, then mitomycin C. The column fractions were monitored by thin-layer chromatography.

The crude mitomycins I and II fractions were then purified separately, each on two successive columns. A $1.5 \times 90$ cm column containing the same Woelm silica gel (76 g) was developed with
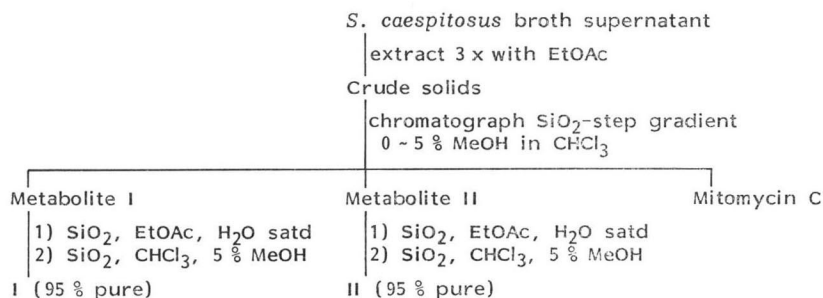
Fig. 2. Isolation of Type I and II mitomycin metabolites.

Table 2. NMR spectra of mitomycin analogs.

| Compound | Solvent | H10′ | H10 | H3 | H9 | H3′ | H1 | H2 | OCH₃ | NCH₃ | C6-CH₃ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Mitomycin C | $C_5D_5N$ | 5.40 | 5.11 | 4.57 | 4.04 | 3.63 | 3.14 | 2.76 | 3.27 | — | 2.07 |
| Type I* analogs | $C_5D_5N$ | 5.40 | 5.13 | 4.57 | 4.01 | 3.63 | 3.12 | 2.75 | 3.25 | — | 2.14~ 2.29 |
| Porfiromycin | $C_5D_5N$ | 5.39 | 4.83 | 4.51 | 4.01 | 3.54 | 2.56 | 2.15 | 3.21 | 2.25 | 2.04 |
| | $CDCl_3$ | 4.69 | 4.39 | 4.24 | 3.69 | 3.44 | 2.27 | 2.27 | 3.20 | 2.23 | 1.80 |
| $\varDelta\delta$ | | 0.70 | 0.44 | 0.27 | 0.32 | 0.10 | 0.29 | −0.12 | 0.01 | 0.02 | 0.24 |
| Type II* analogs | $C_5D_5N$ | 5.48 | 5.22 | 4.46 | 4.22 | 3.67 | 2.47 | 2.23 | — | 2.10 | — |
| | $CDCl_3$ | 4.72 | 4.72 | 4.16 | 3.70 | 3.54 | 2.24 | 2.24 | — | 1.96 | — |
| $\varDelta\delta$ | | 0.76 | 0.50 | 0.30 | 0.52 | 0.13 | 0.23 | −0.01 | — | 0.14 | — |

* Average values for five analogs.

$H_2O$ - satd EtOAc. The 20 ml cuts collected were monitored by thin-layer chromatography, pooled and subjected to a second identical column developed with $CHCl_3$ - MeOH (95: 5). The cuts containing the desired analogs were pooled and dried. At this point, the metabolites were obtained at 95% purity.

Yields varied somewhat but were generally in the order of $10 \sim 30$ mg of Type I compounds from $18 \sim 20$ g crude oily extract and from 100 to 200 mg of Type II.

### Physical and Chemical Analysis

The 10 mitomycin derivatives isolated were examined by UV, IR and $^1H$ NMR spectra. $^{13}C$ NMR spectra were run on only a few of the compounds. The UV spectra in MeOH were essentially all the same as that for mitomycin C with characteristically strong adsorption bands at $220 \sim 222$ and $355 \sim 372$ nm. The IR spectra of all in KBr pellets showed only minor variations from mitomycin C.

The $^1H$ NMR spectra of the Type I analogs were all very similar to that of mitomycin C except for protons of the C7 side chain and the methyl group at C6. On the other hand, there were many significant differences in the $^1H$ NMR spectra of the Type II analogs compared to those of both mitomycin C and the Type I compounds. These shifts and peak assignments are summarized in Table 2.

### *In Vitro* and *In Vivo* Analyses

Prophage Induction: The prophage induction assay employed is that of PRICE et al.[25]. The end point is taken as the amount giving at least a three-fold increase in plaque count over a spontaneous control.

*In Vivo* Antitumor Test: The *in vivo* antitumor tests against L1210 leukemia were performed by standard procedures[26], and the results obtained through the courtesy of the National Cancer Institute, Washington, D.C. Tumor cells obtained from DBA/2 passage mice were inoculated ip to sets of $CDF_1$ or $BDF_1$ hybrid mice ($10^6$ cells/mouse) for therapeutic experiments. Treatment was always started 24 hours after tumor implantation and continued once daily for 9 days by the ip route, with a constant volume of 0.5 ml/injection. Evaluation was on the basis of median survival time (MST). The effect T/C=MST treated/MST control $\times 100$ was calculated. A T/C of 125 or over is considered to indicate significant tumor inhibition.

Paper and Thin-layer Chromatography: Strips ($12 \times 450$ cm) of Schleicher and Schuell 589 blue ribbon filter paper were developed descendingly in the top phase of a benzene - MeOH - $H_2O$ (1: 1: 2) system. After air drying, the strips were placed on the surface of agar trays seeded with *B. subtilis* ATCC 6633, followed by incubation at 28°C for an additional 18 hours. For thin-layer chromatography, Analtech silica gel GF ($20 \times 20$ cm) plates were developed either in $H_2O$ - satd EtOAc or $CHCl_3$ - MeOH (9: 1). Bioactive zones were revealed by overlaying the plates with agar seeded with *B. subtilis*, followed by incubation at 28°C for 18 hours.

## Results and Discussion

Initial experiments with the addition of propargylamine to the normal fermentation of mitomycin C employing the *S. caespitosus* culture revealed the presence of a new antibacterially active zone when the broth extracts were chromatographed in the paper chromatographic system followed by bioautography on agar plates seeded with *B. subtilis*. When these same extracts were chromatographed in both of the thin-layer systems, it was shown that not one, but two, new UV absorbing zones, not seen with the unsupplemented fermentation, were present. Both of these two zones had *B. subtilis* activity. The supplemented fermentation was scaled-up to enable the new products to be isolated. It was shown that one metabolite was a normal mitomycin C analogue substituted at C7 with the propargyl - amine (analog Type I). The second metabolite (analog Type II) was also substituted at C7 with the propargylamine but had the substitutions at C9a and on the aziridine N similar to that of mito-

Table 3.   Amines tested in mitomycin fermentation.

| Amines bioincorporated | Amines not bioincorporated |
| --- | --- |
| Methylamine | sec-Butylamine |
| Ethylamine | tert-Butylamine |
| Propylamine | Diethylamine |
| Propargylamine | Benzylmethylamine |
| Allylamine | Di-(2-chloroethyl)amine |
| 2-Methylallylamine | 2-Aminoethanethiol |
| 2-Chloroethylamine | Cystamine |
| 3-Chloropropylamine | 1,6-Hexamethylene diamine |
| Benzylamine | |
| Aniline | |

mycin B (Fig. 1).

These observations prompted us to test other amines as additives to the fermentation. Those found to be incorporated, in addition to those tested that failed to incorporate, are listed in Table 3. All of the amines tested and shown to lead to new mitomycin analogs were primary amines. No secondary amines were incorporated.

In every case examined so far, the analogs in the Type I series are more active than the analogs of Type II with respect to prophage induction and to activity against L1210 lymphatic leukemia in mice (Table 1). The results on the Type I analogs are in agreement with those obtained by other workers who have synthesized some of these compounds chemically[22,23].

From the NMR data, it was clear that both the Type I and Type II analogs were C7-N substituted mitomycin C derivatives. The Type II compounds had significant structural differences however. The methyl group on the C9a oxygen was lacking and a new three proton singlet at 2.05 ~ 2.17 ppm was observed. This was assigned to the methyl group on the aziridine ring nitrogen by comparison with the spectrum of porfiromycin.

The only naturally occurring mitomycins with N-methylated aziridine rings are mitomycin B and porfiromycin (Fig. 1). Porfiromycin has the same configuration at C9 as mitomycin C. The question, therefore, posed itself as to which of these configurations the Type II compounds actually had, or if it was different from both.

Direct comparison to both compounds was unfortunately impossible since mitomycin B was not available to us nor were we able to find any useful ¹H NMR data on it in the literature. A nuclear Overhauser effect experiment showed significant enhancement only between the C9 and C10 protons as would be expected. Other effects were not strong enough to be definitive.

An alternative approach presented itself from the observation that ¹H NMR spectra of mitomycins are almost always first order when run in pyridine as opposed to other solvents. In addition, aromatic solvent induced shifts (ASIS) are observed for key signals. These ring current shifts arise from shielding, or in some cases de-shielding, effects from solvent molecules aligned with the quinone ring of the mitomycin as shown in Fig. 3 for mitomycin B and porfiromycin.

The ¹H NMR spectra of Type II mitomycins and porfiromycin in $C_5D_5N$ and $CDCl_3$ are compared in Table 2. From these data, it appears that the Type II analogs have the configuration of mitomycin B. Several features of the data point to this conclusion and eliminate other possibilities:

1) In $CDCl_3$ the C10 protons for Type II mitomycins are equivalent, whereas they are not for porfiromycin. Given the configuration of the latter and the steric restrictions imposed by the folds at the fused ring system, this would be expected. The opposite configuration should provide an essentially unrestricted situation. In $C_5D_5N$ the signals for the C10 protons become first order for both porfiromycin and the Type II mitomycin analogs but the differences for the C10 protons of porfiromycin are greater due to the relatively restricted rotation about the C9–C10 bond.

2) The ASIS for the C9 proton is 0.10 ppm greater for Type II analogs than for porfiromycin,

Fig. 3.   Aromatic solvent induced shifts (ASIS) for porfiromycin and Type II analogs.
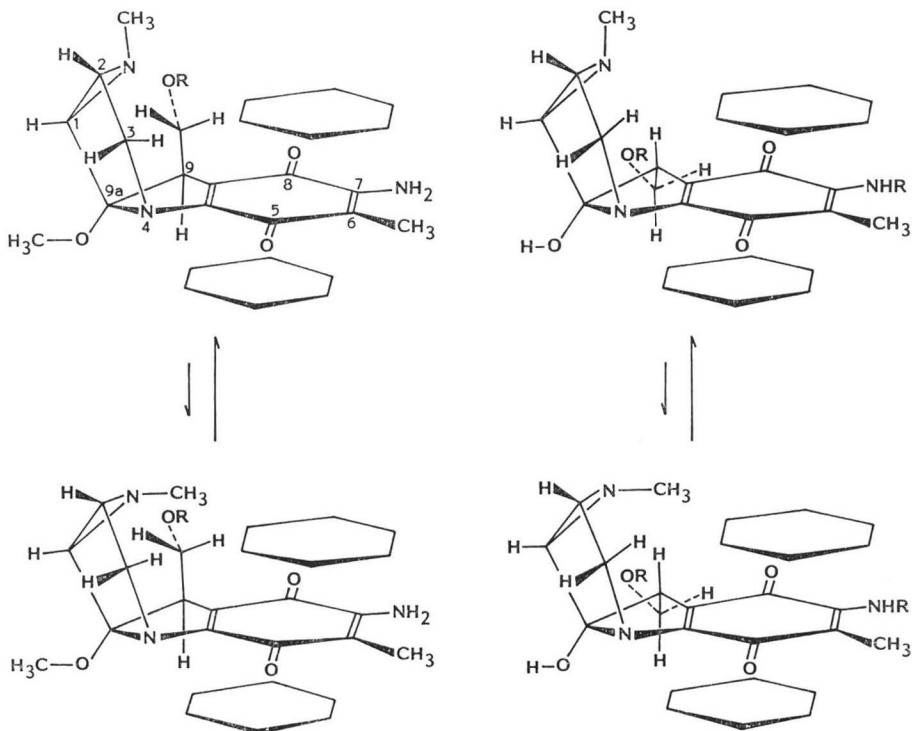


indicating a different environment for it in each of the compounds.

3)   The ASIS for the N-methyl group of porfiromycin was very small compared to that of the Type II analogs (0.02 vs. 0.14 ppm).   If the Type II analogs are in the mitomycin B configuration, we would expect a significant contribution from the aziridine invertomer in which the N-methyl function is brought close to the aromatic solvent-quinone complex.

4)   The above also supports the ring fusion at C9a in Type II analogs being in the configuration shown since the effect of the aziridine nitrogen on C9–C10 bond rotation would be eliminated by inversion at this center.   Also one would expect much weaker shifts for the C1 and C2 protons as well in such a case.   The evidence then points to the Type II analogs being 7-N-alkyl substituted mitomycin B derivatives, presumably arising during fermentation by the same mechanism as mitomycin B does with subsequent amination.

The incorporation of these amines into the mitomycin derivatives has obvious implications for the biosynthesis of this class of antitumor agents.   HORNEMANN[27] has proposed the scheme outlined in Fig. 4 in which pyruvate and erythrose are believed to be the likely precursors of the $mCC_8N$ unit of mitomycin C, possible via a 3-dehydroquinic acid (DHQ) intermediate.   However, direct feeding experiments with 3-[7-$^{14}$C]DHQ did not reveal any incorporation suggesting that it may not be a true intermediate or that its uptake was inefficient at the time of feeding.   The intermediate may be a derivative of an early member in the shikimic acid pathway, 3-deoxy-D-arabinoheptulosonic acid-7-phosphate (DAHP).   ANDERSON et al.[28] have reported on the incorporation of the unusual amino acid, 3-amino-5-hydroxybenzoic acid into the mitomycins, thus the $mCC_8N$ unit may be this compound rather than an unknown postulated intermediate as suggested by HORNEMANN[27].

Fig. 4.   Mitomycin biosynthesis.[27)]
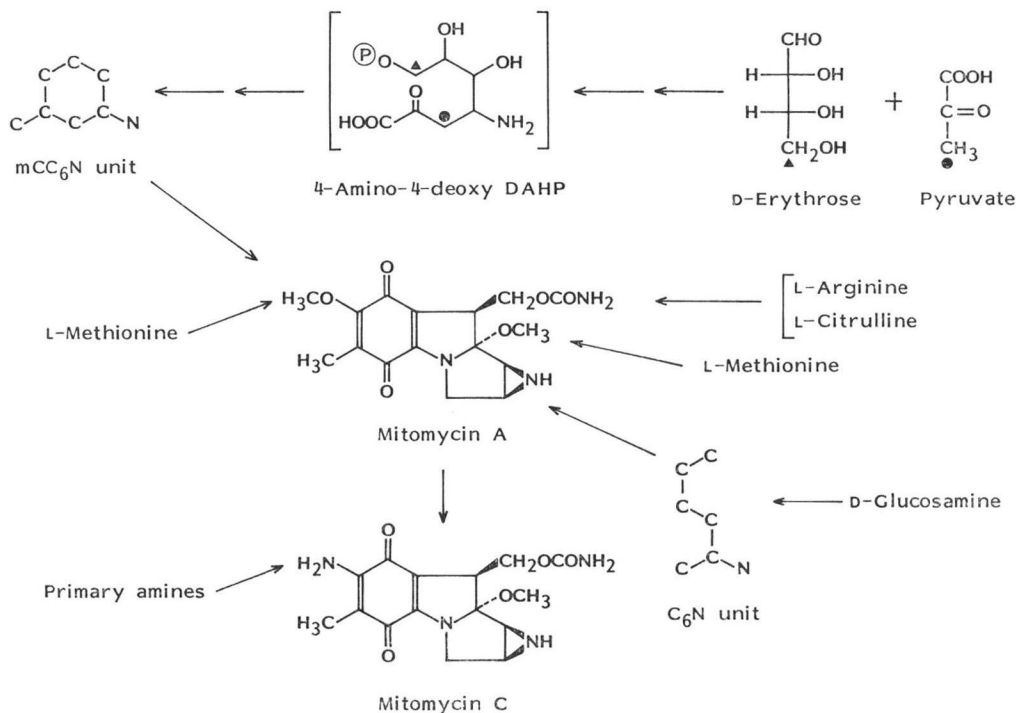


Our observations on the incorporation of the added primary amines at C7 of mitomycin C could be accounted for in the scheme of mitomycin C biosynthesis as outlined by HORNEMANN[27)] (Fig. 4), but the incorporation of these amines at the same position in a mitomycin B analog could also suggest either a separate biogenic scheme for mitomycin B that branches earlier from the mCC$_6$N unit than mitomycin A formation or that a conversion to mitomycin B from mitomycin A involves an invertase to alter the configuration at C9. Such an enzyme is suggestive in the recent study of HORNEMANN and HEINS[29)] which shows that mitomycins A, B and C and porfiromycin all have the same absolute configuration at C1 and C2, with mitomycin B only differing at C9. These authors claim that all the naturally occurring mitomycins thus are the product of one main biosynthetic pathway, differing only in the late stages for individual members. Our results would not contradict this interpretation with the invertase converting mitomycin B to mitomycin A or *vice versa*, mitomycin A to mitomycin B. The presence of such an enzyme has not been sought in *S. caespitosus*.

Recently, TOMOHIRO *et al.*[30)] have reported on a mutant of *S. caespitosus* ATCC 27422 which produces no mitomycin C yet continues to accumulate mitomycins A and B, thus lending credence to HORNEMANN's pathway of mitomycin A being a precursor of mitomycin C. However, mitomycin B could still be produced by a separate pathway in this mutant from an earlier precursor or could branch from mitomycin A or even be in the pathway before mitomycin A. More definitive studies employing isolated enzymes need to be performed to answer some of these questions.

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