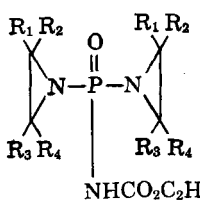
Synthesis of Potential Dual Antagonists IV

Effect of Ring Substituents on the Alkylating and Hydrolytic Properties of Bis-(1-aziridinyl)phosphinyl Carbamates

By T. J. BARDOS, Z. F. CHMIELEWICZ, and C. K. NAVADA

Ethyl [bis-(1-aziridinyl)phosphinyl]carbamate and several of its aziridine-C-methyl derivatives were studied with respect to their comparative alkylating activities, rates of hydrolysis and hydrolytic rearrangements as followed by NMR spectroscopy. It was confirmed that the geminally substituted derivatives radically differ from the parent compound in their chemical reaction patterns; this may explain the important pharmacologic differences found, in particular, between the two experimental antineoplastic agents, AB-100 (I) and AB-132 (III). The synthesis of the fully substituted 2,2,3,3-tetramethyl derivative is reported in this paper.

IN A PRECEDING paper (1), the synthesis of a new series of antitumor agents based on the dual antagonism concept (2) was reported, in which alkyl-substituted aziridine rings were linked, through P—N bonds, to urethan. Preliminary studies in experimental animals indicated that the geminally substituted members of this series (III, IV, and V of the formula below) have pharmacologic properties that are qualitatively (as well as quantitatively) different from those of their unsubstituted (I) or mono-methyl substituted (II) analogs. While the latter compounds behaved like typical alkylating agents, the former showed a surprising lack of hematologic toxicity, and their dosage-limiting side effects appeared to be related rather to central nervous system toxicity (3, 4).



I, $R_1 = R_2 = R_3 = R_4 = H$ (AB-100)
II, $R_1 = CH_3$; $R_2 = R_3 = R_4 = H$ (AB-143)
III, $R_1 = R_2 = CH_3$; $R_3 = R_4 = H$ (AB-132)
IV, $R_1 = R_2 = R_3 = CH_3$; $R_4 = H$ (AB-140)
V, $R_1 = R_2 = R_3 = R_4 = CH_3$ (AB-148)

Subsequent clinical studies (5–8) further demonstrated the strikingly different pharmacologic characteristics of III (AB-132), in comparison to I (AB-100). Particularly, (*a*) the relatively low hematologic toxicity of III, (*b*) its radiation-potentiating effect (9, 10), and (*c*) its interesting cholinesterase-inhibitory activity (apparently due to a metabolic intermediate of III) (11, 12) strongly suggested that the biological action of this compound may be related to chemical reaction mechanisms that are different from those of I or other alkylating agents (13, 14). As part of an effort to determine the chemical and biological mechanisms involved in the action of these and related antineoplastic agents, the authors investigated the comparative alkylating activities and rates of hydrolysis of compounds I–V. In addition, we followed the changes occurring in their NMR spectra during hydrolysis under various conditions to gain some information regarding the possible hydrolytic intermediates of these compounds.

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DISCUSSION

Comparative Alkylating Activities.—A method was developed recently (15) for the comparison of the chemical reactivities of various alkylating agents with respect to nucleophiles resembling the heterocyclic nitrogens of nucleic acid purines and pyrimidines (which are supposedly the biologically significant site of the *in vivo* action of alkylating agents). Epstein's reagent (16) γ-(4-nitrobenzyl)-pyridine (NBP) was chosen as the model nucleophile, and the rates of colored product formation in the reaction of this compound with various types of alkylating agents were measured at standard initial concentrations of the reactants. It was found that throughout an initial time period (usually 30–60 min. from the beginning of the reaction), the increase of absorbance at 600 mμ (E^{600}) *versus* time follows a linear plot. It was shown that, under the limitations employed, the slopes of these linear plots are (in first approximation) proportional to the second-order rate constants for the nucleophilic displacement reactions between the alkylating agents and NBP. Thus, comparative alkylating activity was defined as $k' = [E^{600}/t(\text{min.})]$ at standard initial concentrations of the alkylating agent (0.2 μM) and NBP (240 μM).

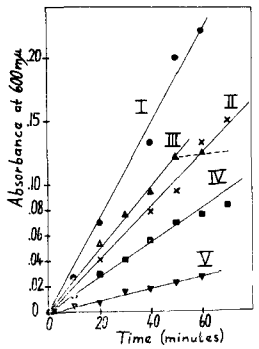


Fig. 1.—Comparative reaction rates of ethyl [bis-(1-aziridinyl)-phosphinyl]carbamate and its ring-substituted derivatives with γ-(4-nitrobenzyl)-pyridine at 80°. Standard initial concentrations: compound, 0.2 μmole/ml.; reagent, 240 μmole/ml.

TABLE I.—COMPARATIVE S_N2 AND S_N1 REACTIVITIES OF ETHYL [BIS-(1-AZIRIDINYL)PHOSPHINYL]-CARBAMATE (I) AND ITS AZIRIDINE-C-METHYL HOMOLOGS

Compd.[a]	Alkylating Activities[b] $k'_{80} \times 10^3$ min.$^{-1}$	Hydrolysis Rate Constants[c] $k_w \times 10^3$ min.$^{-1}$	$k_{ac} \times 10^3$ min.$^{-1}$
I	3.3	12.0	...
II	2.0	7.6	...
III	2.4	274.0	57.6
IV	1.3	...	...
V	0.4	...	11.0

[a] For identification, see text. [b] E^{600}/t(min.) at 80° (15). [c] First-order rate constants for hydrolysis at 37°; k_w determined in ion-free water, k_{ac} in acetone–water, 1:1.

Experimental details as well as the k' values for various types of alkylating agents at 50° and 80° temperatures (k'_{50} and k'_{80}, respectively) were reported (15). In the present investigation, we determined, in the same manner, the k'_{80} values of compounds I–V, as shown in Fig. 1 and Table I.

The results indicate that the unsubstituted compound, I, has the highest and the fully substituted compound, V, the lowest alkylating activity. This agrees with the expected greater stability of the substituted aziridine ring against nucleophilic attack. Compounds II and III would participate in this reaction presumably with their unsubstituted ring carbons, and IV, with its mono-methyl substituted ring carbon, which are sterically the least hindered. However, II shows a somewhat anomalous behavior as it appears to be less reactive than III. A possible explanation for this might be found in the observed tendency (1) of II to lose a carbethoxy group; should this happen under the conditions of the alkylation reaction, it would affect the reactivity of the aziridine rings.

Rates of Hydrolysis.—Figure 2 shows the rates of hydrolysis at 37° of the aziridine rings of I, II, III, and V as measured by the titrimetric method of Allen and Seaman (17). Compounds I, II, and III were hydrolyzed in ion-free water; V, having low solubility in water, was hydrolyzed in 50% aqueous acetone, and, for comparison, a second hydrolysis of III was carried out in this solvent mixture.[1]

This reaction proceeds by first-order kinetics as shown by the linear plots for log-concentration *versus* time. The first-order rate constants are given in Table I.

Here the difference between III and I (or II) is more striking than in the alkylation reaction. Compound III is capable of forming a tertiary carbonium ion by ring opening at the disubstituted carbon and, just as in other well-studied solvolysis reactions, the greater stability of a tertiary carbonium ion (*i.e.*, lower transition-state energy) greatly increases the reaction rate. Compound V, having two disubstituted carbons in each of its aziridine rings, might be expected to hydrolyze at an even faster rate than III; however, the rate of hydrolysis of V in acetone–water is considerably slower than that of III in the same solvent mixture. This seems to indicate that the large number of methyl groups in V make this molecule hydrophobic and suppress its contact with the water molecules. The only unexplained anomaly in the relative hydrolysis rates of these compounds is presented again by II which would be expected to hydrolyze at a somewhat faster rate than I (though slower than III); instead, II shows the lowest hydrolysis rate in the series.

Changes in NMR Spectra.—To correlate the disappearance of titratable ethylenimine groups with the structural transformations of these compounds during hydrolysis, we followed the changes occurring in their NMR spectra at 37° in D_2O, D_2O–D_3CCOCD_3 (1:1), and/or deuterated acetate buffer (2 *M*, pH 5.0). All chemical shifts were expressed in δ(p.p.m.) units (18) with reference to tetramethylsilane (δ = 0), by using the chemical shift of the methylene protons of the carbamate group (δ = 4.20 p.p.m. in $CDCl_3$) in conjunction with the water-peak (σ = 4.7, in D_2O, 4.44 in D_2O–D_3CCOCD_3, and 4.9 p.p.m. in 2 *M* buffer) as internal standards.

The unsubstituted ethylenimine compound, AB-100 (I), in D_2O shows a very slow decrease of its two prominent absorption peaks, *i.e.*, the doublet (δ = 2.38 p.p.m., J_{PH} = 14) corresponding to the eight ring protons (split by the β-phosphorus). Integration of the spectra reveals that in 24 hrs. only about 25% of the ring protons have moved from the doublet and are found in a broad band between

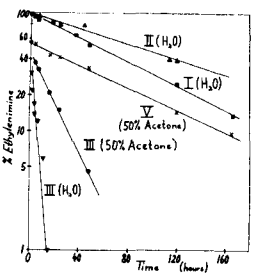


Fig. 2.—Rates of hydrolysis of ethyl [bis - (1 - aziridinyl) - phosphinyl]carbamate and its ring-substituted derivatives at 37°, in water and/or in 50% aqueous acetone. Ordinate: titratable ethylenimine content (17) (expressed as % of calculated total ethylenimine content corresponding to formula).

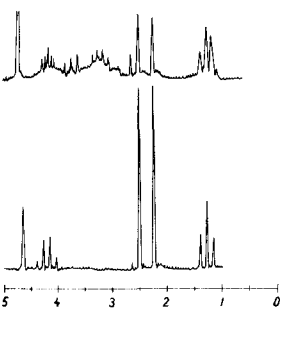
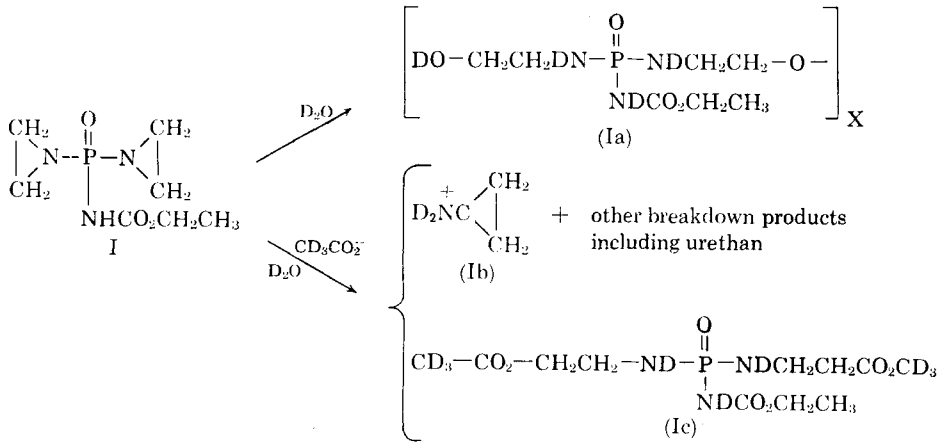


Fig. 3.—NMR absorption spectra of ethyl [bis-(1-aziridinyl) - phosphinyl]carbamate (I) in D_2O. Bottom: at zero-hour. Top: same, after 120 hr. hydrolysis at 37°.

[1] Compound IV could not be included in this study because of a lack of sufficient material.



2.8 and 3.8 p.p.m. After 54 hr., the doublet contains about four of the original ring protons (unchanged) while the remaining four appear now in two multiplets centering at 3.10 and 3.65 p.p.m., respectively.[2] At 120 hr., about 75% of the original ring protons have moved to the latter positions. This rate of change seen in the NMR spectra parallels almost exactly the rate of disappearance of ethylenimine content as determined by the titrimetric method. The new proton absorption bands (multiplets) at 3.10 and 3.65 p.p.m. are compatible with the expected chemical shifts of an aliphatic —CH_2—N—P(O)— and —CH_2—O— group, respectively. The complexities of these bands (as well as of those of the urethan protons at 4.20 p.p.m.) are due to the extensive polymerization exhibited by this compound under hydrolytic conditions. [The final hydrolysis product is a glassy, polymeric material which contains only 19% of the primary alcohol group in free hydroxyl form (19).] (See Fig. 3.)

If I is hydrolyzed in the presence of deuterated acetate buffer, 2 *M* (*i.e.*, four times the molar concentration of the compound), at pH 5.0, the NMR spectrum shows a very rapid change. Within a few hours, the original doublet almost completely disappears giving rise to a sharp singlet at 2.67 p.p.m. (Ib) which, however, accounts only for 10% of the total ring-protons as determined by integration. The major part of the original ring-protons (80%) is found about equally divided in two multiplets, centering at 3.35 and 4.20 p.p.m., respectively, the latter overlapping the carboxyl-linked CH_2-protons of the urethan group. This indicates that, instead of hydrolysis, nucleophilic displacement by the CD_3COO^- ions has occurred.[3] (See Fig. 4.)

Hydrolysis of the mono-methyl derivative (II) in D_2O appears to proceed in a similar manner to that of I; however, the complex spectra of the three different ring protons (*cis*, *trans*, and adjacent to the methyl) make quantitative observation and in-

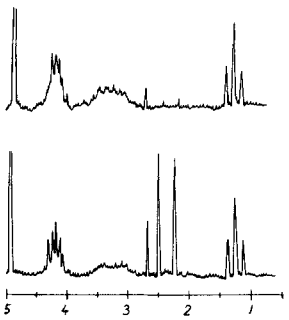


Fig. 4.—NMR absorption spectra of ethyl [bis-(1-aziridinyl) - phosphinyl]-carbamate (I) in deuterated acetate buffer (2 *M*, pH 5.0). Bottom: after 2 hr. hydrolysis at 37°. Top: same, after 23 hr.

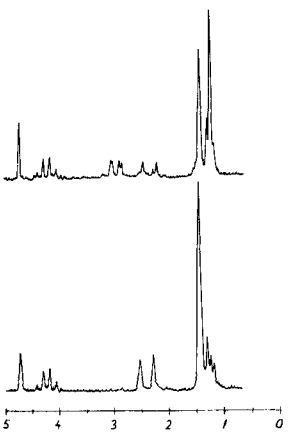


Fig. 5.—NMR absorption spectra of ethyl [bis-(2,2-dimethyl - 1 - aziridinyl)phosphinyl]-carbamate (III) in D_2O. Bottom: at zero-hour. Top: same, after 2 hr. hydrolysis at 37°.

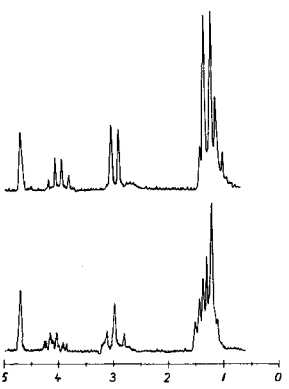


Fig. 6.—Same as Fig. 5. Bottom: after 11 hr. Top after 40 hr.

[2] In addition, there appears a small, sharp peak at 2.67 p.p.m. which is probably due to free ethylenimine (20) and indicates that about 2–5% of the P—N bonds split during the hydrolysis.

[3] Beroza and Borkovec (20) recently reported NMR studies of the hydrolysis of *tris*-(1-aziridinyl)phosphine oxide (TEPA). They used saturated phosphate buffers and did not observe a similar reaction between the phosphate ions and the aziridine groups.

terpretation of the occurring spectral changes more difficult.

In contrast to the first two compounds, the spectral changes of the geminally substituted dimethyl derivative, AB-132 (III), in D_2O occur very rapidly and are indicative of the formation of different types of products. The singlet at 1.44 p.p.m., corresponding to the 12 protons of the gem-dimethyl groups, and the doublet at 2.38 p.p.m., corresponding to the ring protons, decrease by more than 50% (as determined by integration) within the first 2 hr. and almost disappear after 8 hr. at 37°; thus, the rate of change in the NMR spectrum is in good agreement with the rate of disappearance of the titratable ethylenimine groups (Fig. 2). During the same time, another singlet rises at 1.21 p.p.m., and another doublet (or, rather, two multiplets) around 2.93 (2.83 and 3.02) p.p.m. However, before this change is completed, 6–8 hr. after beginning the reaction, the quartet at 4.20 p.p.m. corresponding to the methylene protons of the carbamate group begins to split up and to shift upfield (in the course of the next 8 hr.) to a new position at 4.05 p.p.m. Simultaneously, the absorption peaks corresponding to the ring protons and methyl-group protons undergo several new changes until the former stabilize at 2.96 and 3.08 p.p.m., and the latter at 1.38 and 1.24 p.p.m. in the course of about 30 hr.; at this time, the urethan peaks appear at 1.16 p.p.m. (triplet) for the methyl, and at 4.03 p.p.m. (quartet) for the methylene protons. (See Figs. 5 and 6.)

These changes in the NMR spectrum are compatible with the results of previous studies (19, 21) which indicated that the hydrolysis of AB-132 in CO_2-free water proceeds with the breaking of all P—N bonds (13) and leads to a variety of products, including urethan as well as phosphate salts and esters of 1-amino-2-methyl-2-propanol. The final positions of the proton signals at 2.96 and 3.08 p.p.m. may correspond to free $-CH_2ND_2$ and $-CH_2\overset{+}{N}D_3$ groups, and the shift of the urethan peaks may indicate the breaking of the P—N bond linking urethan. (It is interesting that this seemed to occur only after both aziridine rings were opened.) It has been postulated (14) that the mechanism of this reaction involves a covalent bond formation between the tertiary carbonium ion and the P → O oxygen, to give rise to an unstable cyclic intermediate; such mechanism would be analogous to the rearrangements of *N*-acyl-aziridines to oxazolines (22) and would explain the potent cholinesterase-inhibitory properties (11, 12) of the intermediary hydrolysis products of III. However, the present NMR study does not seem to offer unequivocal evidence for the proposed mechanism.

Hydrolysis of III in 2 *M* deuterated acetate buffer (pH 5.0) proceeds even faster than in D_2O, but the sequence of spectral changes appears to be similar. An observable difference is the transient appearance of two sharp lines at 1.16 and 2.65 p.p.m.; these are probably due to 2,2-dimethylaziridine being split off the molecule. There is no evidence for a ring-opening S_N2 reaction with the acetate ions (as was observed in the case of I), as only the two methylene protons of urethan continue to be found in the area downfield of 4.0 p.p.m.; this again indicates that in the reactions of III the S_N1 mechanism prevails over second-order nucleophilic displacements.

The trimethyl-substituted compound, IV, has low solubility in water and therefore, was hydrolyzed in D_2O–D_3CCOCD_3 (1:1). The broad, somewhat blurred signal around 2.80 p.p.m. corresponding to the lone methine-proton of the aziridine rings, migrates on hydrolysis to a new position at 3.52 p.p.m., and simultaneously, the peaks of the methyl protons (1.55, 1.32, 1.28, and 1.17 p.p.m.) undergo some changes in positions and complexities. The quartet corresponding to the methylene protons of the urethan group (at 4.20 p.p.m.) shows similar changes and shift (to 4.02 p.p.m.) as were seen in the case of III. The rate of these changes appears to be similar to the rate of comparable changes in the NMR spectrum of III when the latter compound was hydrolyzed in the same solvent mixture.

The tetramethyl-substituted compound, V, was hydrolyzed in D_2O–D_3CCOCD_3. The first visible change in its large singlet corresponding to the methyl protons (at 1.48 p.p.m.) occurs after 50 hr. Eventually this peak breaks up into several signals, but in the absence of ring-hydrogens, it is difficult to interpret the chemical significance of these spectral changes. The changes involving the urethan signal (at 4.20 p.p.m.), however, appear to be similar to those observed in the case of III and IV.

Conclusions.—These studies confirm that the important pharmacologic differences between I and some of its ring-substituted analogs are paralleled by characteristic differences in their chemical reaction patterns. It is possible that the unusual properties of III, which were demonstrated in the clinical studies of this compound as an experimental antineoplastic agent, may be the result of a fortuitous combination of alkylating activity and hydrolyzability (due to its ability to react with one of its ring carbons by an S_N1, or with the other by an S_N2 mechanism) as well as to the rapid hydrolytic breakdown of its molecule to various biologically active intermediates. Further clarification of these questions may lead to conclusions that will be helpful in the design of better agents.

EXPERIMENTAL

Methods used in this work for the determination of comparative alkylating activities (15) and rates of hydrolysis (13) were described previously. A Varian Associates model A-60 spectrometer was used for the NMR studies; solvents, etc. as indicated in the individual cases (see above).

The preparation of compounds I–IV was described previously (1, 23).

Ethyl [Bis(2,2,3,3-tetramethyl-1-aziridinyl)phosphinyl]carbamate (V).—Compound V was prepared according to the method described previously (1) by reacting 2,2,3,3-tetramethylaziridine (24) with ethyl dichlorophosphinylcarbamate in the presence of triethylamine. After filtration of the triethylamine hydrochloride (96% theoretical yield), and concentration of the filtrate to dryness, the residue was dissolved in *n*-hexane and crystallized at −27°. After recrystallization from *n*-hexane, the pure product was obtained in a yield of 20%; m.p. 103–104° (uncorrected). Titratable ethylenimine (17) 56% of the total ethylenimine content.

Anal.[4]—Calcd. for $C_{15}H_{30}N_3O_3P$: C, 54.38; H, 9.06; N, 12.69. Found: C, 54.23; H, 9.19; N, 12.85.

Infrared absorption bands $\lambda_{max.}^{CHCl_3}$ (μ): 2.93 (w) (N—H); 3.35 (s) (C—H); 5.78 (s) (C=O); 6.95 (s) (sh at 6.85) (C—CH_3; C—CH_2); 7.28 (s) (C—CH_3); 7.65 (m) (P=O); 8.1–8.55 (s) (C—O; C—N); 8.84 (s) [C(CH_3)$_2$]; 9.35 (s); 10.38 (s) 10.55 (w); 11.10 (w).

The NMR spectrum (in $CDCl_3$, with tetramethylsilane as the internal standard) shows, at δ = 1.35 p.p.m., a singlet for the 24 methyl protons of the aziridine groups. In comparison, the urethan signals (δ = 4.10 and 1.30 p.p.m.) are very small and blurred.

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[4] Microanalysis by Dr. S. Nagy, Massachusetts Institute of Technology, Cambridge, Mass.

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Effects of Amo-1618, Maleic Hydrazide, and Gibberellin Seed Treatment on the First and Second Generation of *Datura tatula*

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Treatment of *Datura tatula* with Amo-1618 increased the fresh and dry weights of the first-year plants, but decreased the alkaloid content. Maleic hydrazide (MH) treatment induced an increase in plant weight and total alkaloid content but a decrease in plant height. Second generation studies indicated a reduction of the fresh and dry weights of the Amo-1618 group, a considerable decrease in the alkaloid concentration of the MH group, and a decrease in the total alkaloid content of both groups. The chlorophyll concentration was not affected. A selective solvent extraction was performed on the leaf-tops. Gibberellin treatment of seeds obtained from plants previously treated with the chemicals generally reversed any inhibitory effect demonstrated by the chemicals on growth, alkaloid concentration and content, and the concentration of various selective-solvent fractions. Gibberellin seed treatment of the controls caused decreases in fresh weight, total alkaloid content, and the alcohol-soluble fractions of the plant.

AMO-1618 (4-hydroxyl-5-isopropyl-2-methylphenyl trimethyl ammonium chloride, 1-piperidine carboxylate) in appropriate concentrations inhibits stem elongation of plants (1–6). It is classified as a growth retardant, *i.e.*, a chemical that slows cell division and cell elongation in shoot tissues and regulates plant height physiologically without formative effects (1). Wirwillie and Mitchell (7) found that Amo-1618 was translocated into the seeds of treated black valentine snap beans, and growth retardation was noted in the following generation of plants. Mutual antagonism has been found between gibberellin and Amo-1618, or similar acting growth retardants, in altering plant growth. This antagonism has been reported for the stem growth of bean (8, 9) and potato (10) and in the cell division of chrysanthemum (11). Lockhart (9) indicates that several growth retardants interact competitively with gibberellin on stem growth, and they act to retard stem elongation by partially blocking the system which provides active gibberellin to the growth mechanism. Zeevaart and Lang (12) have shown that gibberellin completely overcomes the inhibition of flower formation induced by Amo-1618 in *Bryophyllum daigremontianum*. Kende *et al.* (13) found that gibberellic acid (GA) biosynthesis, but not growth, was inhibited

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