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Atrazine Metabolism by Nocardia: Elucidation of Initial Pathway and Synthesis of Potential Metabolites

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The metabolism of atrazine herbicide by *Nocardia*, a soil bacterium previously shown to degrade the herbicide to 4-amino-2-chloro-1,3,5-triazine, has been studied. The primary route of the metabolism appeared to be *N*-dealkylation and was similar to that found in several fungi. The data suggested that the isolated dealkylated metabolites were precursors of 4-amino-2-chloro-1,3,5-triazine. The supposed metabolites 4-alkylamino-2-chloro-1,3,5-triazines were synthesized but there was not direct proof of their involvement in the degradation process.

Numerous workers have shown that triazine herbicides can be degraded by pure cultures of micro-organisms and that in many cases these compounds can act as nutrient sources for the growth of microbial species.

In a previous work we have demonstrated that a *Nocardia* strain, isolated from a soil treated with atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) (**I**), is able to degrade the herbicide to 4-amino-2-chloro-1,3,5-triazine (**IIa**). This metabolite, obtained on the 6th day of bacterial growth, is the first example of a new class of herbicide metabolites in the micro-organisms and demonstrates the existence of a new pathway in atrazine metabolism.[1)]

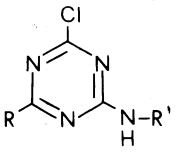
As an extension of the work previously reported, we have investigated the degradation pathway leading to 4-amino-2-chloro-1,3,5-triazine (**IIa**). The formation of **IIa** is possible either through the dealkylated compounds **IIIa, b, c** or through the compounds **IIb, c** (Fig. 1). Examples of dealkylation of atrazine are known; compounds **III** are frequently recovered in different biological materials[2～6)] but there is no available information regarding their recovery by soil bacteria. On the contrary the compounds **IIb, c** are not reported in literature and therefore their synthesis reported in this paper is the first step to ascertain their presence in bacterial culture during the atrazine biodegradation process. In view of the easy displacement of the chlorine atom, compounds of the type **II** should also be useful as intermediates for the synthesis of a variety of 4-alkylamino-1,3,5-triazines substituted in C_2. These compounds are important because they have been found as products of the photochemical degradation of triazine herbicides.[7～10)]

MATERIALS AND METHODS

The *Nocardia* strain used in this study was isolated from soil by enrichment culture techniques.[1)]

Thin-layer and preparative chromatography was carried out on silica gel plates Merck F_{254}, in thicknesses 0.25 and 1 mm activated by heating at 110°C for 30 min. High performance thin-layer chromatography (HPTLC) was carried out on RP-8 plates Merck F_{254}. The following eluents were used: A=chloroform–acetone (9:1); B=benzene–acetonitrile (1:1); C=benzene–ethyl acetate (7:3); D=benzene–chloroform–ethyl acetate (8:6:3); E=acetonitrile–benzene (3:2); F=benzene–ethyl acetate–chloroform (4:3:2); G=water–acetonitrile (1:1); H=chloroform–methanol–formic acid–water (80 : 15 : 4 : 2). Detection was carried out using a UV lamp (254 nm) or, when necessary, Koudela's method; after chlorination the triazines appeared as blue spots.[11)]

Melting points are uncorrected. UV spectra were re-



	R	R′
(IIa)	H	H
(IIb)	H	C_2H_5
(IIc)	H	i-C_3H_7
(IIIa)	NH_2	H
(IIIb)	NH_2	C_2H_5
(IIIc)	NH_2	i-C_3H_7

FIG. 1.

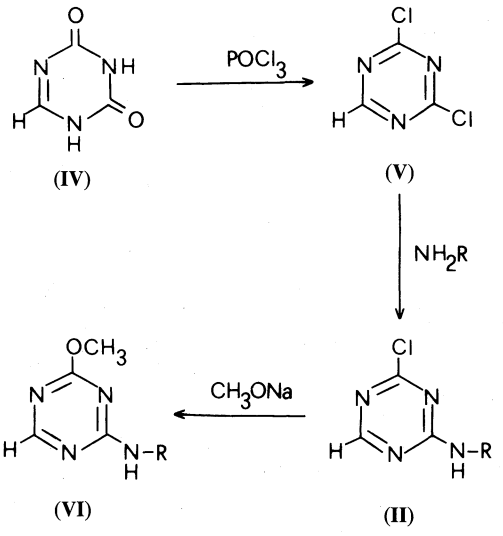


FIG. 2.

corded in chloroform on a Perkin-Elmer spectrometer 124. IR spectra were recorded in chloroform on a Perkin-Elmer 257.

GLC analyses were performed with a Perkin-Elmer 900 gas chromatograph equipped with a flame ionization detector. Two columns were used: 2 m × 2 mm gas column packed with 3% Reoplex 400, 80 ~ 100 mesh Chromosorb W HMDS (column J) or 3% Carbowax C 20 M, 80 ~ 100 mesh Chromosorb W HP DMCS (column K). Operating temperatures were: detector 250°C, injector 220°C, columns 200°C (T_1) and 170°C (T_2). The nitrogen carrier gas flow was 50 ml/min. The ratio of the peak-areas **IIIc**/**IIIb** = 8.6 was calculated and expressed relative to the areas with standard solutions.

GLC/MS analyses were carried out on a Micromass VG 7070 F equipped with a Dani (Dani, Monza, Italy) gas chromatograph Model 3900. Column K was used. The column temperature was programmed in isothermal runs for 5 min, rate 5°C/min up to 200°C. Helium was used as the carrier gas and the flow rate was 30 ml/min. Electron impact spectra were made at the ionizing voltage of both 70 eV and 20 eV and the source temperature was 200°C. The same apparatus and conditions were used for direct insertion probe analyses.

The high-pressure chromatograph used was a Perkin-Elmer Model 3B equipped with a Waters Associates Model U 6K injection valve and a Perkin-Elmer LC-75 variable wavelength spectrophotometric detector. The detector was set at 260 nm. Separation was performed using a 25 cm × 4.5 mm column packed with 10 μ C-8 reversed-phase packing. The mobile phase was water–acetonitrile (1 : 1) and the flow rate was 0.7 ml/min. The pressure in the column corresponded to 360 psi.

All solvents were pesticide grade, the chloroform (Fluka solvent for HPLC) was not stabilized with ethanol, which reacts with products **II**.[12]

The hydrolysis reactions were conducted at 30 ± 2°C in the dark. Solutions of 6.10^{-2} M samples in distilled water or in buffer pH 7 (phosphate) were injected into the LC in no more than 10 μl. The disappearance of **IIa, b, c** in aqueous media was followed by noting the decrease in peak areas of the compounds at different time intervals.

SYNTHESIS AND ANALYTICAL STANDARDS

Compounds **II** were obtained starting from 2,4-dichloro-1,3,5-triazine (**V**) by selective displacement of the chlorine at low temperature with the appropriate amine (Fig. 2). Synthesis of **V** has been reported by drastic reaction between hydrogen cyanide and cyanogen chloride[13]: because of the dangerous reaction involved in this conventional synthesis, we preferred to get **V** by an alternative route although in poor yield, starting from 2,4-dioxo-1,2,3,4-tetrahydro-1,3,5-triazine (**IV**) in the presence of $POCl_3$ and diisopropylethylamine. This new route has also been used to obtain **IIa**, which was compared with the sample prepared according to Huffman.[14] Chemical analyses, mass spectra and IR[15,16] are in agreement with the expected structure of **II**. Further proof of authenticity was obtained by synthesizing the methoxy derivatives **VI** and by spectrometric characterization of the resulting products.

2,4-Dioxo-1,2,3,4-tetrahydro-1,3,5-triazine (**IV**). This compound was prepared according to Piskala.[17]

2,4-Dichloro-1,3,5-triazine (**V**). To a mixture of $POCl_3$ (11 ml), (H_3PO_4 85% 1 ml) and diisopropylethylamine (15 ml) was added **IV** (4 g) with stirring at room temperature; stirring was continued until a vivid reaction took place. Then the mixture was heated to 50°C for 1 hr. The excess $POCl_3$ was removed at reduced pressure and the reaction product extracted with petroleum benzine 40°C; this extract on sublimation at 40°C and 0.1 mmHg produced the compound **V** (28% yield), mp 54 ~ 55°C.[19]

The compound **V** was identified by its conversion with diiospropylamine (1 : 4 mol) into 2,4-diisopropylamino-1,3,5-triazine (**VII**) in refluxing ether (1 hr). The product was chromatographed using eluent D, mp 215~217°C, MS *m/z*: 195 (M^+), 180 (M^+-CH_3), 153 ($M^+-CH_3CH=CH_2$), 138 (153−CH_3), 111 (153−$CH_3CH=CH_2$).[7,9]

4-Amino-2-chloro-1,3,5-triazine (**IIa**). The compound **V** (1 g) was dissolved in anhydrous ether (60 ml), the solution chilled in a dry ice-bath to −20°C and anhydrous ammonia passed into the suspension at a rapid rate. There was a tendency for the temperature to rise and the rate of ammonia addition was regulated so that the temperature did not exceed −15°C. The reaction product was extracted with ether **II**. The filtrate was evaporated and the obtained residue was purified by preparative TLC using eluent E. **IIa** could not be recrystallized (35% yield). **IIa** was identical to the product prepared according to Huffman (UV, TLC, GLC and MS), mp >350°C.[1,14]

4-Alkylamino-2-chloro-1,3,5-triazine (**IIb, c**). The appropriate amine (0.02 mol) in 20 ml of anhydrous ether, was added dropwise to a stirred solution of **V** (0.01 mol) in 50 ml of anhydrous ether at −20°C. After 30 min the cooling bath was removed, the white precipitate was filtered and washed with ether. The filtrate was evaporated and the residue chromatographed on a silica gel column using eluent F (70~80% yields).

2-Chloro-4-ethylamino-1,3,5-triazine (**IIb**). Crystallization solvent: petroleum benzine 80~100°C, mp 119~120°C. UV $\lambda_{max}^{CHCl_3}$: 240 nm. IR cm^{-1}: 3420–3250 (NH), 2990 (CH), 1580, 1515 (C=N). MS *m/z*: 158 (M^+), 160 ($M^+ +2$), 143 (M^+-CH_3), 130 ($M^+-CH_2=CH_2$), 95 (130−Cl). *Anal.* Found: C, 37.85; H, 4.45; N, 35.34. Calcd. for $C_5H_7N_4Cl$: C, 37.87; H, 4.45; N, 35.33%.

2-Chloro-4-isopropylamino-1,3,5-triazine (**IIc**). Crystallization solvent: petroleum benzine 40°C, mp 104~105°C. UV $\lambda_{max}^{CHCl_3}$: 240 nm. IR cm^{-1}: 3420–3250 (NH), 2990 (CH), 1580, 1515 (C=N), 1387, 1370 (CH_3). MS *m/z*: 172 (M^+), 174 ($M^+ +2$), 157 (M^+-CH_3), 130 ($M^+-CH_3CH=CH_2$), 69 (130−ClCN). *Anal.* Found: C, 41.81; H, 5.22; N, 32.47. Calcd. for $C_6H_9N_4Cl$: C, 41.75; H, 5.25; N, 32.46%.

4-Alkylamino-2-methoxy-1,3,5-triazine (**VIb, c**). These compounds were prepared from the reaction of 4-alkylamino-2-chloro-1,3,5-triazine with sodium methoxide (1 : 1 mol) in methanol; the mixture was left at 25°C for 20 min, then the solvent was removed and the residue chromatographed with eluent B.

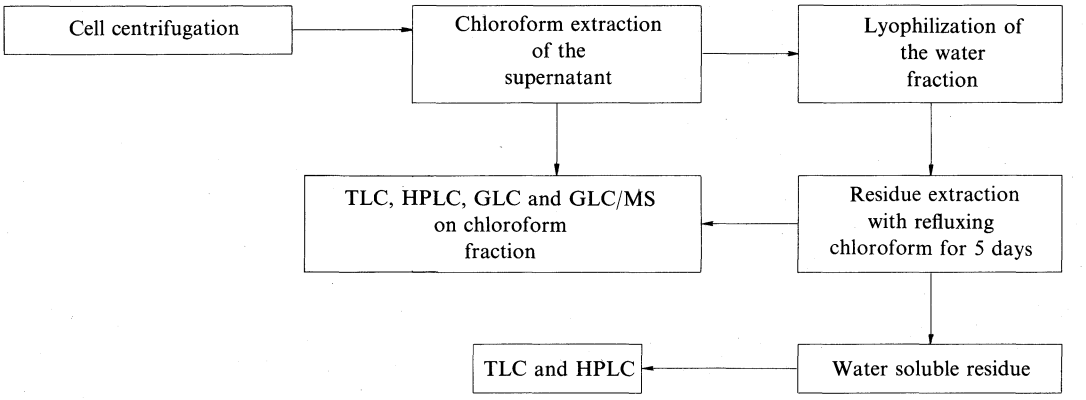
4-Ethylamino-2-methoxy-1,3,5-triazine (**VIb**). mp 82~83°C. UV $\lambda_{max}^{CHCl_3}$: 240 nm. IR cm^{-1}: 3420–3140 (NH), 2990 (CH), 1580, 1530 (C=N), 1310 (OCH_3). MS *m/z*: 154 (M^+), 139 (M^+-CH_3), 126 ($M^+-CH_2=CH_2$), 96 (126−CH_2O).

4-Isopropylamino-2-methoxy-1,3,5-triazine (**VIc**). mp 66~68°C. UV $\lambda_{max}^{CHCl_3}$: 240 nm. IR cm^{-1}: 3420–3140 (NH), 2990 (CH), 1580, 1530 (C=N), 1310 (OCH_3). MS *m/z*: 168 (M^+), 153 (M^+-CH_3), 126 ($M^+-CH_3CH=CH_2$), 96 (126−CH_2O).

Atrazine (**I**), *2-chloro-4-amino-6-ethylamino-1,3,5-triazine* (**IIIb**), *2-chloro-4-amino-6-isopropylamino-1,3,5-triazine* (**IIIc**) and *2-chloro-4,6-diamino-1,3,5-triazine* (**IIIa**) were gifts from Ciba-Geigy (Basle). **I** and **IIIb, c** were recrystallized from benzene; **IIIa** was recrystallized from water.

RESULTS

In order to identify the precursors of **IIa**, a *Nocardia* strain was incubated in the presence of atrazine (30 ppm) according to the techniques described in our previous work.[1] There was no attempt to measure the rate and the extent of the transformations. The isolation and identification of the metabolites were performed from 15 liters of culture at different days of incubation (3rd, 4th and 6th day). The cells were separated by centrifugation at 8000 rpm for 40 min. No chemical degradation of the atrazine occurred on incubating the solution of the herbicide in the absence of bacterium. We carried out experiments, in the absence of bacteria, in the liquid medium fortified with the synthesized standards in order to choose the best extraction and clean-up procedure. We observed that lyophilization caused a total loss of the added triazines **IIb, c** because of their volatility. Besides, comparative trials between the extraction with Soxhlet for 5 days after lyophilization[1] and mechanical shaking of the aqueous medium with chloroform, showed that the former procedure is convenient for the recovery of **IIIa** and **IIa**, while the latter represents the sole possibility for simultaneous recovery of **IIb, c** and **IIIb, c**. Therefore the extraction of **II** and **III** was assured using both methods (Scheme 1). The mixtures obtained according to Scheme 1 were examined by chromatographic techniques. Table I summarizes the chromatographic be-



SCHEME 1.

TABLE I. CHROMATOGRAPHIC PROPERTIES OF STANDARDS

Compounds	TLC* *Rf* values eluent systems		GLC* retention times (min)				HPLC* retention vol. (ml)
			J		K		
	A	B	T_1	T_2	T_1	T_2	
I	0.81	0.85	4.0	10.0	5.0	14.2	8.0
IIa	0.45	0.56	2.8	7.0	3.0	8.0	3.8
IIb	0.76	0.76	1.4	2.0	1.3	2.3	4.9
IIc	0.81	0.81	1.1	1.8	0.8	1.5	5.9
IIIa	0.08	0.28	20.0	50.0	38.0	—	3.5
IIIb	0.41	0.62	9.7	30.0	19.0	50.0	4.2
IIIc	0.57	0.73	6.0	20.0	14.0	33.0	4.9

* See MATERIALS AND METHODS section for composition of TLC eluents and for GLC and HPLC conditions.

haviour of standard **II** and **III**.

On the 3rd and 4th day of bacterial incubation, we could detect the appearance of dealkylated compounds **IIIb** and **IIIc**. The high pressure LC retention volumes, TLC *Rf* and gas LC retention times of two metabolites matched those of authentic samples of **IIIb** and **IIIc**. Certain proof of their authenticity was obtained by combined GLC/MS. The mass spectra of metabolites **IIIb** and **IIIc** are depicted in Fig. 3.

Using the above techniques we observed, on the 6th day of bacterial incubation, the disappearance of metabolites **IIIb** and **IIIc** and the formation of metabolite **IIa**. These results are summarized in Table II.

Many peaks were present at different days of incubation in the GLC/MS chromatograms which were not identified by mass spectrometry because the mass spectral fragmentation patterns could not easily be solved. The polar metabolites soluble in water were revealed by Koudela's method in TLC (eluent H) and HPTLC (eluent G). These substances could not be identified by GLC/MS because of their high polarity and low vapor pressure. However, it should be pointed out that these data indicate the involvement of some other degradation reactions which are not clarified in the present study.

The detection of dealkylated compounds demonstrates that bacteria are able to dealkylate the triazine herbicides as other biological systems do. The ratio between **IIIc**

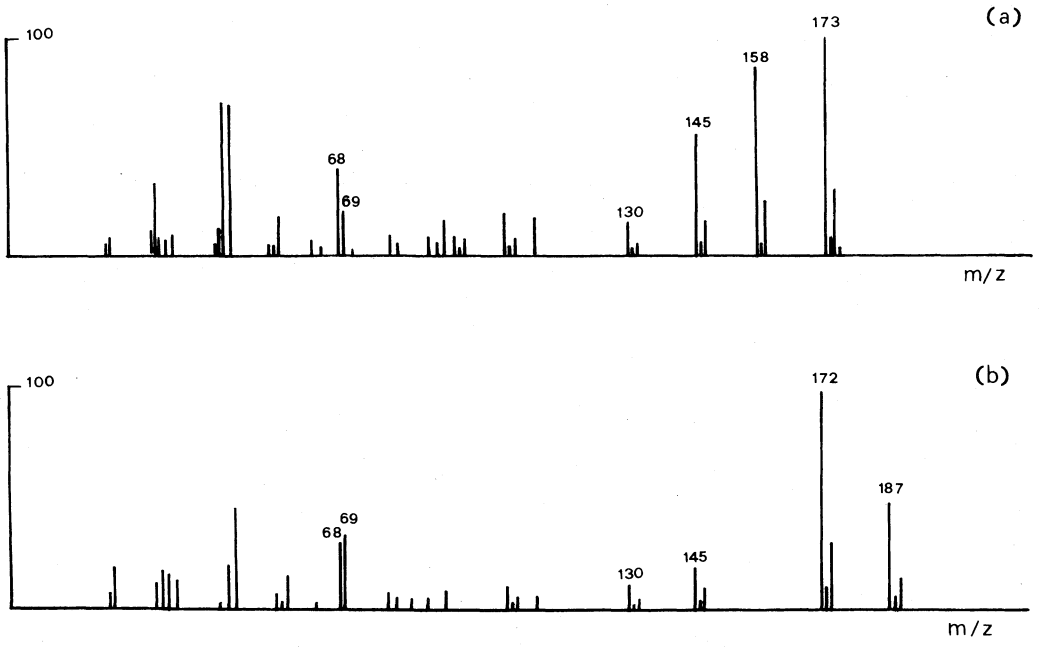


FIG. 3. (a): Mass Spectrum of 4-Amino-2-chloro-6-ethylamino-1,3,5-triazine (**IIIb**).
(b): Mass Spectrum of 4-Amino-2-chloro-6-isopropylamino-1,3,5-triazine (**IIIc**).

TABLE II. APPEARANCE OF ATRAZINE METABOLITES AT DIFFERENT DAYS OF INCUBATION

Days of incubation	I	IIa	IIb	IIc	IIIa	IIIb	IIIc
3rd	+	−	−	−	−	+	+
4th	+	−	−	−	−	+	+
6th	+	+	−	−	−	−	−

+, present; −, absent.

and **IIIb** on the 3rd day of incubation shows that the attack on the ethyl group occurs to a large degree while the isopropyl group is attacked to a lesser extent. The latter observation is in accordance with the observation made with some soil fungi: *Aspergillus fumigatus* removed the ethyl side chain in preference to the isopropyl group as other fungi do.[18] *N*-Dealkylation is of considerable importance as the first stage in the metabolism of atrazine and seems to be a general reaction occurring in homologous molecules (atrazine, simazine and propazine)[19,20] and in analogous molecules (hydroxysimazine and prometryne).[21~23]

From the results expressed in Table II we suggest that *N*-dealkylation should be the starting point for a further degradative process because we did not observe the accumulation of dealkylated products with time. Both these compounds **IIIb, IIIc** may then undergo side chain modification and deamination. The subsequent dealkylation of **IIIb, c** would be expected to result in the formation of **IIIa** while the deamination of **IIIb, c** would be expected to result in the formation of **IIb, c**. Therefore, indirect evidence strongly suggests the existence of 4-alkylamino-2-chloro-1,3,5-triazine (**IIb, c**) and/or 2-chloro-4,6-diamino-1,3,5-triazine (**IIIa**) intermediates, but our attempts to obtain direct proof of their involvement, as appears from the results reported in this paper,

were unsuccessful. Since **IIa** was found, however, both decomposition routes through **IIb, c** and **IIIa** might be probable. If so, the absence of the supposed metabolites **IIIa** and **IIb, c** in the bacterial medium could be ascribed to different reasons. Concerning the difficulty of detecting **IIIa**, Wolf *et al.* have mentioned the rapidity of degradation and association with fungal cells and fungal products in a pure culture.[24] In addition, preliminary data show that the reactivity of alkylamino-2-chloro-1,3,5-triazine and 4-amino-2-chloro-1,3,5-triazine is such that they would be rapidly lost from the culture medium by hydrolytic reaction. Figure 4 shows the breakdown of **IIb** in distilled water at 30°C. It can be seen that **IIb** rapidly disappears and after 2 days 50% of the original amount is lost. Similar behaviour was observed for the hydrolysis of **IIa, c**. The chemical degradation of **IIa, b, c** is considerably faster in distilled water than in a buffered medium at pH 7; in fact after 10 days in a buffered medium at pH 7, about 50% of the original amounts of **IIa, b, c** were still present.

This experimental evidence indicates that most metabolites have a high turnover rate; their variable behaviour and the small amounts obtained can preclude their identification. It can be seen that the deamination process is masked by other chemical and microbiological transformations. In this connection, the detection of **IIa** was made possible since it was present in the culture medium at a high level.[1] We could not rule out nonbiological alterations of metabolite **IIa** and supposed metabolites **IIb, c** which could occur in the bacterial medium or during extraction and analysis.

Studies are in progress in order to identify the products of further chemical degradation of **IIa, b, c** and to ascertain their presence in the

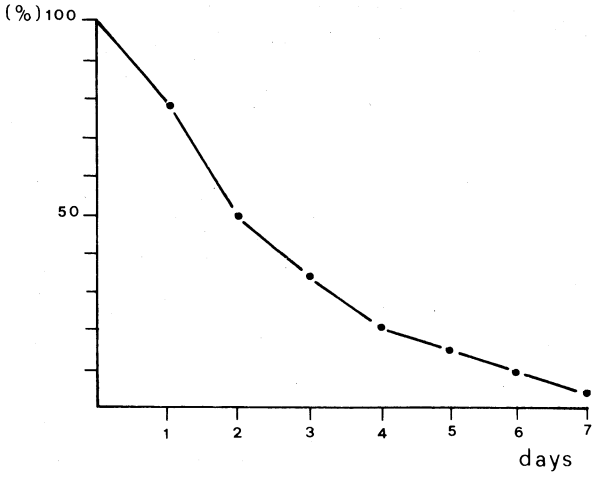


FIG. 4. 2-Chloro-4-ethylamino-1,3,5-triazine (**IIb**) Degradation Rate in Distilled Water at 30°C.

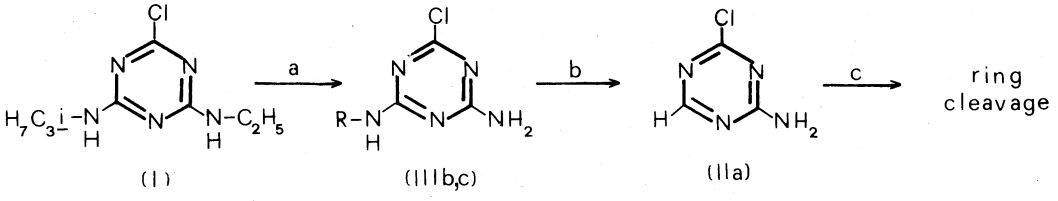


FIG. 5. Degradative Pathway Proposed for the Transformation of Atrazine by a Culture of *Nocardia*. a, b, biological processes; c, chemical process.

bacterial medium. Some observations made by chromatographic techniques, UV and mass spectra show that these substances are mainly polar and not chromophoric, indicating that cleavage of the triazine ring occurs (unpublished data).

Figure 5 shows a proposed pathway for the transformation of atrazine by the culture of *Nocardia* based on the metabolites and experimental evidence reported in this study.

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