# BIOSYNTHESIS OF 4-AMINOBENZOIC ACID IN *AEROBACTER AEROGENES**

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Received 26 May 1971

## 1. Introduction

4-Aminobenzoic acid is formed together with other compounds from chorismic acid [1], the branching intermediate of aromatic amino acid biosynthesis. Mutants of both *Neurospora crassa* [2] and *Escherichia coli* [3] requiring 4-aminobenzoic acid for growth have been divided into two classes by genetic mapping. Hendler and Srinivasan [4] reported cross-feeding between the mutants of *N. crassa*, but no cross-feeding was observed by Huang and Pittard [3] with the *E. coli* auxotrophs. In contrast we found cross-feeding with mutants of *E. coli* [5], which require 4-aminobenzoic acid for growth. Although Huang and Gibson [6] were able to show that two enzymes are involved in the biosynthesis of 4-aminobenzoic acid from chorismic acid in *E. coli*, no intermediate compound could be detected. In earlier reports [7, 8] we described the isolation of a mutant 62-1 AC of *Aerobacter aerogenes* producing compound A, a labile intermediate of the 4-aminobenzoic acid biosynthesis. The present paper reports some spectrometric properties of compound A. In addition the isolation of further mutants of *A. aerogenes* are described, which require 4-aminobenzoic acid for growth.

## 2. Materials and methods

4-Aminobenzoic acid auxotrophs, 62-1 P 4 and 62-1 P 6 were isolated after treatment of *A. aerogenes* 62-1 with *N*-methyl *N'*-nitro-*N*-nitrosoguanidin as described previously [8]. *A. aerogenes* 62-1 AC was used for the isolation of compound A. Cultivation and accumulation conditions were essentially as described by Gibson [9]. Regarding the purine requirement of mutant 62-1 AC, guanine (20 mg/l) was added to the media. Compound A could be isolated as follows: The culture medium was centrifuged and the supernatant was passed through a column of charcoal. The column was washed with deion. water and subsequently eluted with ethanol–2 N ammonia (1:3, v/v). The eluate was evaporated to a small volume *in vacuo* and placed on a column of Sephadex G-10. The column was developed with deionised water and fractions containing compound A were combined and evaporated to dryness under reduced pressure. The resulting material was dissolved in 0.1 M tris-HCl buffer (pH 8) and the solution was applied to a column of DEAE-Sephadex A-25. The column was eluted with tris-HCl buffer and fractions containing compound A were pooled and evaporated to a small volume under reduced pressure. This solution was desalted on Sephadex G-10 and Dowex 50 W X 8

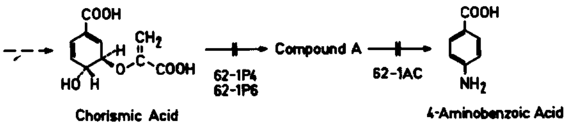

Fig. 1. Additional genetic blocks of *A. aerogenes* mutants in the pathway of 4-aminobenzoic acid synthesis.

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* Part III. Part II = [8].

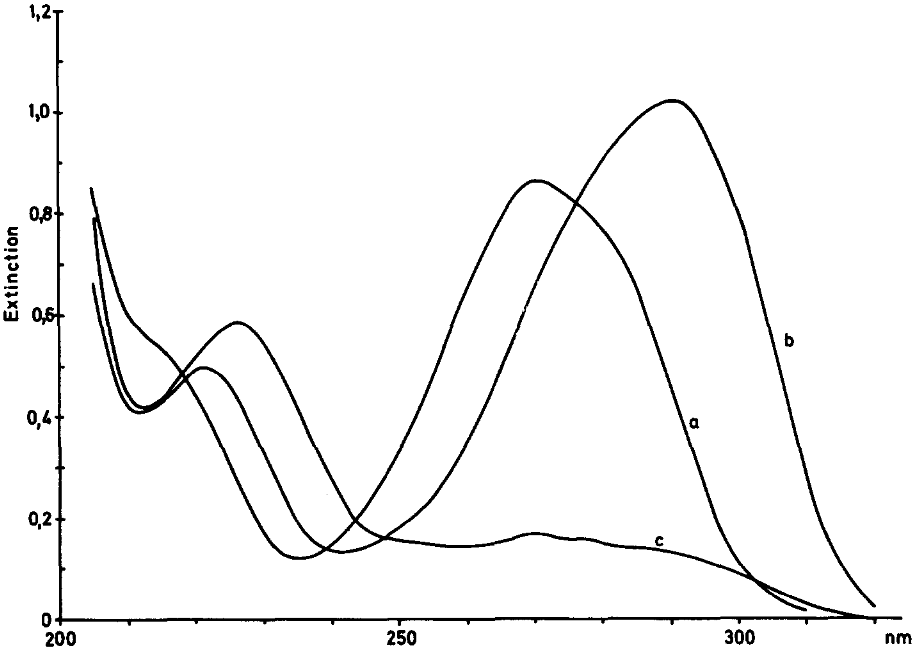

Fig. 2. UV-spectra of compound A; (a) aqueous solution, (b) 0.1 N HCl, (c) after about three hours in 0.1 N HCl.

(97% $NH_4^+$-form mixed with 3% $H^+$-form). Finally, compound A was further purified by chromatography on Sephadex LH-20 (50% and 90% ethanol) and on Sephadex G-10 (deionised water).

## 3. Results

We have found previously [7] that the mutant 62-1 AC of *A. aerogenes* produces an intermediate (compound A) of 4-aminobenzoic acid biosynthesis. This was demonstrated by cross-feeding tests using mutants of *E. coli* (K 5151 and K 430), which are deficient in 4-aminobenzoic acid synthesis. To strengthen these cross-feeding results, further mutants of *A. aerogenes* requiring 4-aminobenzoic acid for growth have been isolated.

The polyauxotrophic strain 62-1 of *A. aerogenes* which produces about 0.4 g chorismic acid/l under suitable conditions [9], was used as a parent strain. After treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidin we obtained two new mutants, 62-1 P 4 and 62-1 P 6, exhibiting an additional block in the pathway of 4-aminobenzoic acid. Cross-feeding tests were set up to determine the additional genetic block. No cross-feeding was detected between mutants 62-1 P 4 and 62-1 P 6, but mutant 62-1 AC fed both of these. Hence it follows that mutant 62-1 P 4 and mutant 62-1 P 6 show an additional block prior to compound A (fig. 1).

The isolation of compound A is seriously hampered by the fact that it becomes converted to 4-aminobenzoic acid under acid conditions. Therefore preferentially, neutral and basis chromatographic systems had to be used. In some cases these procedures are time consuming and connected with loss of compound A.

Pure compound A exhibits a UV-maximum centered at 271 nm ($\epsilon$ about about 15,000) and a shoulder at 216 nm in aqueous solution (fig. 2). When measured in 0.1 N HCl the absorption maximum is shifted to 290 nm ($\epsilon$ increases) and a further maximum with a lower extinction coefficient at 221 nm appears. Under these acid conditions the extinction at 290 nm slowly decreases and after about three hours compound A is completely converted to 4-aminobenzoic acid.

No fluorescence was detected in an aqueous solu-

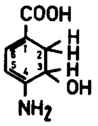

Fig. 3. Proposed structure of compound A.

tion of compound A. After addition of 0.1 N HCl to the aqueous solution of compound A fluorescence slowly arises after a few minutes. The identity of the 4-aminobenzoic acid formed was confirmed by excitation and fluorescence spectra (excitation 290 nm, fluorescence 345 nm) [1].

Compound A was found to be optically active by optical rotatory dispersion measurements, exhibiting a pH-dependent negative cotton effect. The spectrum contains a trough at 296 ± 2 nm and a crossover point at 272 ± 2 nm in aqueous solution. At pH about 3.0 the trough was shifted to 302 ± 2 nm and the crossover point to 288 ± 2 nm.

Because chorismic acid is a precursor of 4-aminobenzoic acid, it was assumed that the enolpyruvyl group of chorismic acid is still present in compound A. After conversion of compound A to 4-aminobenzoic acid no pyruvate could be detected using lactic dehydrogenase.

The NMR spectrum of compound A in hexadeuterodimethyl sulphoxide (DMSO) or NaOD, at 90 MHz, can be explained in part. The spectrum exhibits a pair of doublets near $\tau = 2.36$ and 3.44 ppm, $J = 8.6$ cycles/sec (DMSO). The pair of doublets, characteristic of an enolpyruvyl sidechain [10] (i.e. chorismic acid, $\tau = 5.10$, 465 ppm) is not present.

## 4. Discussion

From the formation of compound A in a 4-aminobenzoic acid auxotroph of *A. aerogenes* and from the conversion of compound A to 4-aminobenzoic acid under acid conditions, we concluded that compound A is a labile intermediate of 4-aminobenzoic acid biosynthesis. The position of compound A, between chorismic acid and 4-aminobenzoic acid, in the biosynthetic pathway and the formation of 4-aminobenzoic acid as a degradation product suggest that compound A is a derivative of benzoic acid with amino function on C-4 and oxygen function on C-3. The extended conjugation between the carboxyl group, the diene system and the amino group is suggested by the high extinction coefficient ($\epsilon$ about 15,000) of the ultraviolet absorption maximum. A system with cross conjugation (i.e. chorismic acid, $\epsilon = 2630$) would yield a much lower extinction coefficient. The chemical shift of the two doublets in the NMR spectrum suggests that the two protons (H-5 and H-6) are olefinic. Of these H-6 has a chemical shift characteristic of a proton in $\beta$-position to a carboxyl group in an $\alpha,\beta$-unsaturated acid [11]. The absence of the pair of doublets, characteristic of the enolpyruvyl sidechain is in agreement with the fact, that after conversion of compound A to 4-aminobenzoic acid no pyruvate could be detected. Therefore, we assume that a hydroxyl group is attached to C-3. In agreement with all these results the structure of compound A is proposed as shown in fig. 3.

Studies to confirm this structure are presently undertaken.

## Acknowledgements

*A. aerogenes* 62-1 was kindly supplied by Prof. F. Gibson, Canberra, Australia. The technical assistance of Miss M. Herrmann is acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the Stiftung Volkswagenwerk. The NMR measurements were kindly performed by Dr. R. Price and V. Formacek, Bruker-Physik AG, Karlsruhe-Forchheim, Germany, and Dr. H. Rüterjans, Grafschaft, Germany. We want to thank Dr. H. Rau, Universität Tübingen, Germany, for the fluorescence measurements.

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