Phytochemistry, 1968, Vol. 7, pp. 1861 to 1866. Pergamon Press. Printed in England

THE ISOLATION AND IDENTIFICATION OF NICOTIANINE: A NEW AMINO ACID FROM TOBACCO LEAVES

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(*Received* 1 *May* 1968)

Abstract—A hitherto unknown amino acid, nicotianine, was isolated from the leaves of tobacco plants. By the use of i.r., u.v. and NMR spectrophotometry, colour reactions and synthesis the new amino acid was identified as L(+)-*N*-(3-amino-3-carboxypropyl)-β-carboxypyridinium betaine.

INTRODUCTION

IN THE course of our studies on nitrogen metabolism in tobacco plants, we have observed several ninhydrin-positive compounds whose behaviours on paper and ion-exchange chromatography are not coincident with any of known naturally occurring amino acids. Among them, an amino acid was conspicuous as it could also be detectable on paper chromatograms by Pauly's reaction[1] and by u.v. absorption. The compound, nicotianine, has an elution volume similar to that of cystathionine, being eluted before methionine at 30–50° run with an amino acid analyser.

Nicotianine is consistently found in all organs of tobacco plants (*Nicotiana tabacum*) and much more was present in younger leaves than older ones.

In order to clarify the chemical structure and physiological role of nicotianine, the isolation and identification of the compound was undertaken and is described in this paper. A preliminary account on this work has been reported.[2]

RESULTS

A 70% aqueous methanolic extract of green tobacco leaves (*Nicotiana tabacum*, "Bright Yellow") was treated with Dowex 50 × 4 resin (H^+ form) and the separated cationic constituents were subjected to displacement chromatography according to the method of Partridge and Brimley.[3] The crude nicotianine thus obtained was further purified by cellulose column chromatography. Final purification was achieved by preparative paper chromatography and recrystallization from aqueous methanol yielding colourless needles decomposing at 241–243°, $[\alpha]_D^{24}$ +24·0 in water, with an empirical formula of $C_{10}H_{14}O_5N_2$.

The substance gave a blue to violet colour on paper chromatograms with ninhydrin and tests for sulphur,[4] secondary amine,[5] aromatic amine,[6] benzene ring,[7] carbonyl group,[8]

[1] T. MANN and E. LEONE, *Biochem. J.* **53**, 140 (1953).
[2] M. NOGUCHI, H. SAKUMA and E. TAMAKI, *Arch. Biochem. Biophys.*, **125**, 1017 (1968).
[3] S. M. PARTRIDGE and R. C. BRIMLEY, *Biochem. J.* **51**, 628 (1952).
[4] F. L. HOHN, *Ind. Engng Chem. Anal. Ed.* **17**, 199 (1954).
[5] F. FEIGL and V. ANGER, *Mikrochim. Acta* **1**, 138 (1937).
[6] M. ISHIDATE and T. SAKAGUCHI, *J. Pharm. Soc. Japan* **70**, 398 (1950).
[7] A. L. LE ROSEN, R. T. MORAVEK and J. K. CARLTON, *Anal. Chem.* **24**, 1335 (1952).
[8] O. L. BRADY, *J. Chem. Soc.* **756** (1931).

hydroxyl group[9] and tertiary pyridine group[10] were all negative. Successive treatments of nicotianine on a paper chromatogram with cupric nitrate and ninhydrin proved it to be α-monoamino acid.[11] It also becomes visible on a paper chromatogram by Pauly's reaction giving an orange colour, although the sensitivity is relatively low. The u.v. spectra of nicotianine and nicotinic acid in water are shown in Fig. 1. As seen in the figure, the spectrum of nicotianine (λ_{max}, 265 nm; shoulders, 258 and 271 nm) is closely similar to that of nicotinic acid (λ_{max}, 262 nm; shoulders, 256 and 268 nm). In addition nicotianine, on reduction with sodium borohydride, has two absorption bands at 260 and 350 nm; the spectrum is coincident with those of 1,6-dihydro compounds of *N*-substituted nictotinic acid derivatives.[12] Furthermore, nicotianine, after being heated up to 230° in a sealed tube, gives a positive test for tertiary pyridine compounds.[10]

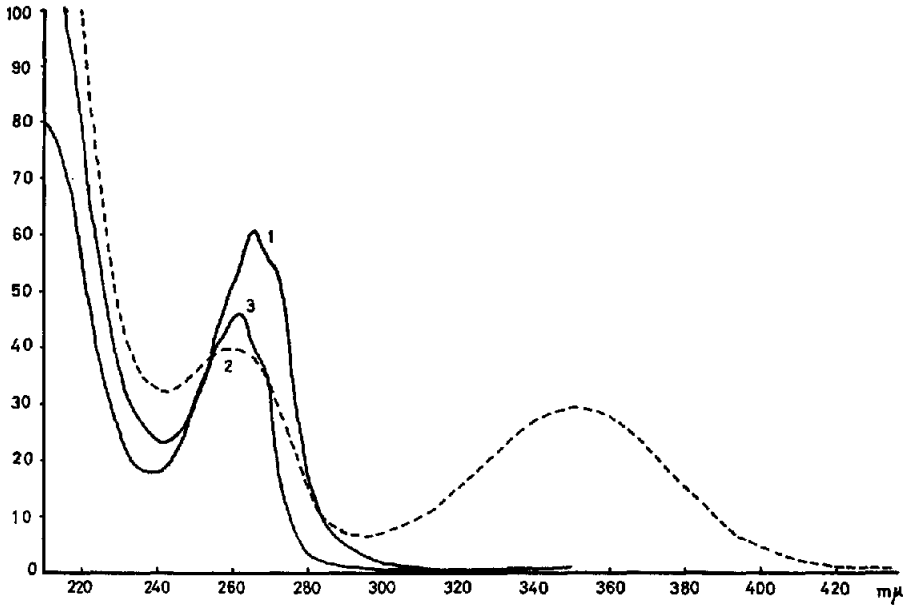


FIG. 1. THE U.V. SPECTRA OF NICOTIANINE (1), NICOTINIC ACID (2) AND BOROHYDRIDE REDUCED NICOTIANINE (3).

These results suggest that in nicotianine, a pyridine ring is present in which the nitrogen atom is substituted by some group resulting in a pyridinium compound.

The NMR spectrum of nicotianine in deuterium oxide shows six bands (τ in ppm); a quartet (7·35), four triplets (6·21, 5·16, 1·80 and 1·00 respectively) and a singlet (0·65) (Fig. 2). The band at 5·16τ is partially overlapped by a strong signal due to HDO, but from its shape and intensity it seems most reasonable to assume the band is a triplet whose area is similar to that of the 1·00τ band. Thus, the relative areas of the six bands are approximately 2:1:2:1:2:1. This indicates that of the fourteen protons in the molecule, nine are not exchanged and five are easily exchangeable with deuterium. The three bands in low-field region are

[9] F. FEIGL, *Spot Tests*, Vol. II, p. 129, Elsevier, New York (1954).
[10] E. KODICEK and K. K. REDDI, *Nature* **168**, 475 (1951).
[11] P. O. LARSEN and A. KJAER, *Biochim. Biophys. Acta* **38**, 148 (1960).
[12] K. WALLENFELS and H. SCHÜLY, *Ann.* **621**, 106 (1959).

interpreted as due to four protons attached to pyridine ring. The other three bands in relatively high-field region seem to be due to five protons attached to aliphatic carbon atoms.

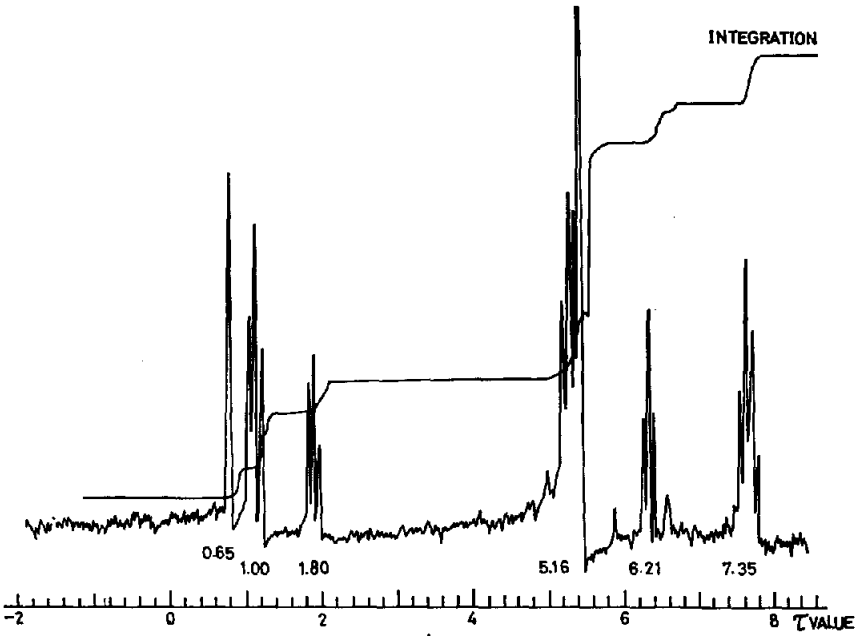
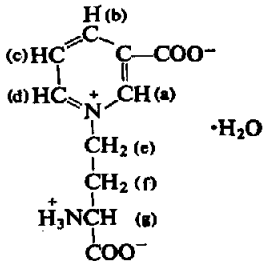


FIG. 2. THE NMR SPECTRUM OF NICOTIANINE IN DEUTERIUM OXIDE.

The above results indicate that the possible structure of nicotianine to be *N*-(3-amino-3-carboxypropyl)-*β*-carboxypyridinium betaine (monohydrate) (I).



(I)

The NMR spectrum of nicotianine is well consistent with the structure. The signal at 7·35τ is attributable to the two H(f) protons which are coupled with H(g) proton and H(e) protons thus giving a quartet formed by partial overlapping of two triplets. The triplet at 6·21τ is attributable to H(g) proton coupled with the two H(f) protons and the triplet at 5·16τ is understood to correspond to the H(e) protons whose signals are strongly shifted downfield by the deshielding effect of the pyridine ring. The triplet at 1·80τ is attributable to H(c) proton attached to the *beta* carbon of the pyridine ring. Two doublets caused by the

couplings between H(c) and H(b), and between H(c) and H(d) would form the triplet by partial overlapping. The 1·00τ triplet seems to correspond to H(b) and H(d) protons. The chemical shift of H(b) proton seems to become near that of H(d) proton owing to the deshielding effect of the adjacent carboxyl group. The singlet at 0·65τ is interpreted as due to H(a) proton whose signal is shifted downfield as compared to that of H(d) proton by the adjacent carboxyl group.

As shown in Fig. 3, the i.r. spectrum of nicotianine does not have the absorption corresponding to C=O stretching vibration of non-ionized —COOH (generally falls in the range 1730 to 1680 cm^{-1}) but has a broad absorption near 1630 cm^{-1} which seems to be ascribed to —COO^-. When nicotianine was treated with excess hydrochloric acid, only its monohydrochloride ($C_{10}H_{13}O_4N_2Cl$) was obtained. The i.r. spectrum of the hydrochloride still has a strong absorption at 1630 cm^{-1}, which seemed to correspond to the —COO^- of the

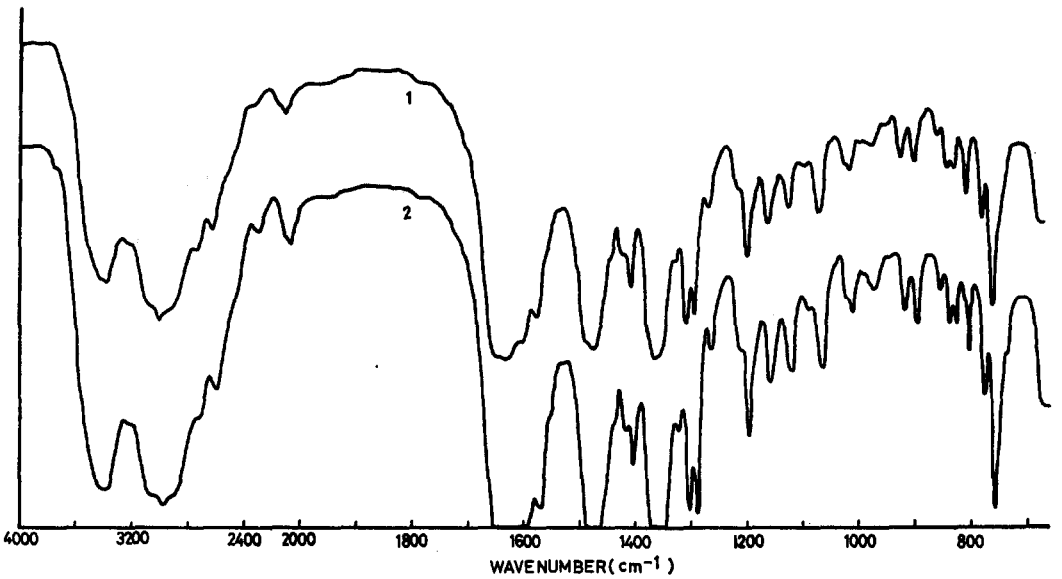


FIG. 3. THE i.r. SPECTRA OF NICOTIANINE (1) AND L-*N*-(3-AMINO-3-CARBOXYPROPYL)-β-CARBOXYPYRIDINIUM BETAINE MONOHYDRATE (2) IN KBr DISKS.

nicotinic acid moiety. In addition, a new absorption band appeared at 1700 cm^{-1} which is attributable to the —COOH of the α-aminobutyryl moiety of the hydrochloride. Thus the betaine structure with 1 mole of water of crystallization seems to be most probable for the crystalline nicotianine.

The final confirmation of the structure was attained by comparing nicotianine with synthetic L(+)-*N*-(3-amino-3-carboxypropyl)-β-carboxypyridinium betaine.

According to the method of Frankel and Knobler,[13] methyl L-α-amino-γ-iodobutyrate hydrochloride was prepared from L-homoserine, which was then heated with ethyl nicotinate to yield the ester of the pyridinium salt. The ester was hydrolysed with 3 N hydrochloric acid and the hydrolysate was then freed from chloride ion by successive treatment with silver oxide and hydrogen sulphide. After recrystallization from aqueous methanol, L-*N*-(3-amino-3-carboxypropyl)-β-carboxypyridinium betaine monohydrate was obtained in colourless fine

[13] M. FRANKEL and Y. KNOBLER, *J. Am. Chem. Soc.* **80**, 3147 (1958).

needles; $[\alpha]_D^{24}$ +28·4 in water (c=5·0); λ_{max} 265 nm (ϵ=4480). It begins to char above 200° and decomposes at 243° as in the case of nicotianine.

As seen in Fig. 3, the i.r. spectra of nicotianine and the synthetic compound in KBr disks are perfectly identical. Their u.v. and NMR spectra were also identical. The R_f values of the synthetic compound in paper chromatography with various solvent systems and its elution volume in the ion-exchange chromatography with an amino acid analyser were in good agreement with those of nicotianine. Thus the chemical structure of nicotianine was demonstrated to be L(+)-*N*-(3-amino-3-carboxypropyl)-β-carboxypyridinium betaine.

DISCUSSION

Nicotianine appears to be the first example of a naturally occurring pyridinium containing amino acid. Although its physiological role in tobacco plants is still unknown, the presence of nicotinic acid moiety in its molecule is of great interest in considering the metabolisms of nicotine or nicotinamide compounds in plants. The experiments on the biosynthesis and conversion of nicotianine in tobacco plants are now being undertaken.

EXPERIMENTAL

Isolation of Nicotianine from Tobacco Leaves

Green tobacco leaves (*Nicotiana tabacum*, "Bright Yellow"), 30 kg, were homogenized with 100 l. of 70% aqueous methanol. The homogenate was filtered through three layers of nylon cloth and the filtrate was concentrated *in vacuo* to about 20 l. The water-insoluble matters precipitated were removed by filtration through a Buchner funnel with the aid of celite. The filtrate was passed through a column of Dowex 50×4 resin (H^+ form, 50–100 mesh, 4·7 l.). The column was washed with deionized water and the cations on the column were then eluted with 15 l. of 2 N aqueous NH_3. The eluate was concentrated *in vacuo* to a syrupy mass, which was then taken up into 5 l. of deionized water. Activated charcoal, 300 g, was added to the solution and the mixture was stirred for 1 hr at room temperature. The mixture was filtered through a Buchner funnel and the charcoal on the funnel was washed with deionized water. The filtrate and washing were combined. According to the method of Partridge and Brimley,[3] the decolorized solution was subjected to displacement chromatography using five columns of Dowex 50×4 resin connected in a series of units of diminishing dia. (5·2×54, 3·9×27, 2·5×28, 1·6×22, 0·9×23 cm). The cations on the columns were displaced with 0·2 N aqueous NH_3 until the effluent from the second column became positive to ninhydrin, and thereafter 0·14 N aqueous NH_3 was employed as the developer. The effluent from the last column was collected in 20-ml fractions. Nicotianine was eluted with leucine, isoleucine, γ-aminobutyric acid and minor amounts of other amino acids.

The fractions containing nicotianine were combined and the combined solution was evaporated *in vacuo* to dryness. The residue (1·7 g) was dissolved in a small amount of distilled water and the solution was transferred onto the top of a cellulose column (2·4×90 cm) which was previously equilibrated with a mixture of *n*-butanol, acetic acid and water (6:3:1). Elution was carried out with the same solvent as used for the equilibration and the effluent was collected in 20-ml fractions. The fractions containing nicotianine were combined and the combined effluent was concentrated to dryness *in vacuo*. The residue (870 mg) was dissolved in a small volume of water-saturated phenol and the solution was absorbed on the top of a cellulose column (2·4×90 cm) which was previously equilibrated with the same solvent. Elution was carried out using water-saturated phenol and the effluent was collected in 10-ml fractions. The fractions containing nicotianine were combined and, after the addition of water, excess phenol was extracted with $CHCl_3$. The aqueous phase was evaporated *in vacuo* to dryness, giving 250 mg of amorphous substance. The final purification was undertaken by preparative paper chromatography. The amorphous substance was dissolved in a small volume of water and the solution was spotted in line on purified sheets of Whatman No. 1 filter paper. Chromatography was carried out with solvent system of *n*-butanol:acetic acid:water (4:1:5).

Nicotianine was detected on the chromatograms under u.v. light. The bands of nicotianine on the chromatograms were cut out and extracted with distilled water. The extract was concentrated *in vacuo* to a small volume and treated with activated charcoal. After removal of the charcoal, the solution was evaporated to dryness, giving 160 mg of white substance. The substance was dissolved in a small amount of water, to the solution was added methanol until the solution became slightly turbid and the mixture was kept in a refrigerator overnight. The separated crystals were further recrystallized three times similarly as above to yield 54 mg of fine colourless needles of nicotianine; decomp. 241–243°; $[\alpha]_D^{24}$ +24 (c=2·0 in water). (Found: C, 49·4; H, 5·9; N, 11·3. $C_{10}H_{14}O_5N_2$ required: C, 49·6; H, 5·8; N, 11·6 per cent.)

Preparation of Methyl L-*α-Amino-γ-Iodobutyrate Hydrochloride*

According to the method of Frankel and Knobler for the DL-compounds,[13] 2·4 g of L-homoserine ($[\alpha]_D^{26}$ −8·8 in water (obtained from Kyowa Hakko Kogyo Co. Ltd., Tokyo)) was refluxed with 50 g of constant boiling HI and 50 ml of toluene for 6 hr. After removal of HI and toluene by azeotropic distillation, the residue was extracted successively with $CHCl_3$ and ether until a yellowish granular product (L-α-amino-γ-iodobutyric acid hydroiodide) was obtained; m.p. 143°; yield 6·4 g. Further purification by dissolving the substance in acetone and precipitating with excess of ether gave a pale yellow crystalline substance; m.p. 143°; $[\alpha]_D^{28}$ −23·2 (c=5·0 in ethanol).

Methyl L-α-amino-γ-iodobutyrate hydrochloride was prepared by esterification of L-α-amino-γ-iodobutyric acid hydroiodide (5·4 g) in dry methanol saturated with HCl (yield, 4·2 g). The crude product was dissolved in ethanol and precipitated with excess of ether, giving colourless needles; m.p. 102–103°; $[\alpha]_D^{28}$ +25·2 (c=5·0 in ethanol).

Preparation of L-*N-(3-Amino-3-Carboxypropyl)-β-Carboxypyridinium Betaine*

Methyl L-α-amino-γ-iodobutyrate hydrochloride (1·4 g, 5 mmoles) was dissolved in 7·5 g (50 mmoles) of hot ethyl nicotinate and the mixture was kept at 80–85° for 12 hr. A greasy material which separated out during the reaction was removed from the solution, washed with ether and then dissolved in 5 ml of ethanol. To the solution was added 100 ml of ether under vigorous stirring and the precipitate was hydrolysed with 20 ml of 3 N HCl at 100° for 3 hr. The solution was evaporated to dryness and the residue was dissolved in 10 ml of distilled water. The solution was passed through a column of Dowex 50 × 4 (H form, 100–200 mesh, 1·5 × 19 cm). After washing the column with distilled water, the cations on the column were eluted with NHCl and the eluate was collected in 20-ml fractions. Each fraction was examined by paper chromatography and the fractions containing only the reaction product were combined. The combined solution was evaporated to dryness, the white residue was dissolved in a small volume of water, to the solution was added excess of alcohol and the precipitate was collected (yield 0·6 g). Further recrystallizations by the similar method gave the pure betaine monohydrochloride (decomp. 237–238°). (Found: C, 46·2; H, 5·2 N, 10·5; Cl, 13·3. $C_{10}H_{13}O_4N_2Cl$ required: C, 46·1; H, 5·0; N, 10·8; Cl, 13·6 per cent.)

The hydrochloride (500 mg) was dissolved in 5 ml of water, to the solution was added 0·5 g of Ag_2O and, after removal of the insoluble precipitates by filtration, the filtrate was treated with H_2S. The precipitated Ag_2S was removed and the solution was evaporated to dryness. The residue was once recrystallized from aqueous methanol giving colourless fine needles of *N*-(3-amino-3-carboxypropyl)-β-carboxypyridinium betaine monohydrate; yield 340 mg; decomp. 243°; $[\alpha]_D^{24}$ +28·4 (c=5·0 in water). (Found: C, 50·1; H, 6·0; N, 11·6. Calc. for $C_{10}H_{14}O_5N_2$: C, 49·6; H, 5·8; N, 11·6 per cent.)

Acknowledgements—We express our thanks to the staff of the Utsunomiya Tobacco Experiment Station for supplying the tobacco leaves. Thanks are also due to Professor Onda, Kitazato University, and to Professor Kobayashi, Tokyo Metropolitan University, for their helpful discussions.