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THE OCCURRENCE OF 2-(2-PROPENYL)-Δ^1-PIPERIDEINE IN THE LEAVES OF POMEGRANATE (*PUNICA GRANATUM* L.)

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Abstract—The press juice of leaves of young plants of Pomegranate contains an alkaloid having a structure similar to that of γ-coniceine (2-*n*-propyl-Δ^1-piperideine), one of the major alkaloids of Hemlock (*Conium maculatum* L.). This alkaloid which was isolated as the hydrochloride proved to be very unstable and on characterization was shown to have the structure of 2-(2-propenyl)-Δ^1-piperideine.

INTRODUCTION

PRELIMINARY experiments on young actively growing plants of Pomegranate carried out by Cromwell and Rothwell[1] showed that the saturated alkaloids isopelletierine, *N*-Methyl isopelletierine and pseudo-pelletierine which normally occur in the bark and wood of mature plants[2] were absent from the leaves of young plants. However, chromatography of extracts of both fresh and dried leaves of young plants revealed the presence of unsaturated alkaloids which gave a positive nitroprusside reaction[3] for Δ^1-piperideine. Reference to the occurrence of these alkaloids in young tissues of Pomegranate has been made in previous publications.[4,5] In this communication the results of further work on the structure of the unsaturated alkaloids are presented.

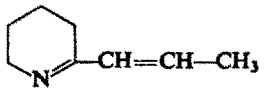
RESULTS AND DISCUSSION

In earlier experiments[1] the alkaloids were isolated from the tissues by steam distillation into dilute hydrochloric acid and by extraction with ethanol. Chromatography of the steam distillate showed three distinct spots having R_f values of 0·2, 0·3 and 0·45 and ethanolic extracts showed two spots of R_f values 0·3 and 0·45. These results suggested that steam distillation from a solution made alkaline with magnesium oxide resulted in the breakdown of one or more of the alkaloids to give the compound of R_f value 0·2. The experiment was repeated using the method of band chromatography to obtain larger amounts of the alkaloids. Elution of the band of R_f value 0·2 with distilled water gave a solution which reacted strongly with the nitroprusside reagent thus confirming the presence of a compound having the structure of a Δ^1-piperideine. Sufficient material was obtained by elution of the band of R_f value 0·2 to enable further investigation of the compound to be made. On hydrogenation over Adam's catalyst a compound was isolated which showed the same R_f value (0·44) as 2-methyl-piperidine. The reduction product was further characterized by preparation of the hydrochloride and picrate which melted at 209° and 116° respectively (m.p. of authentic

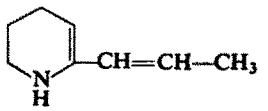
[1] B. T. CROMWELL and K. ROTHWELL, Unpublished results.
[2] L. MARION, In *The Alkaloids* (Edited by R. H. F. MANSKE), Vol. 6, p. 125. Academic Press, New York (1960).
[3] B. T. CROMWELL, *Biochem. J.* 64, 259 (1956).
[4] B. T. CROMWELL and M. F. ROBERTS, *Phytochem.* 3, 369 (1964).
[5] B. T. CROMWELL, In *Biosynthetic Pathways in Higher Plants* (Edited by J. B. PRIDHAM and T. SWAIN), p. 147. Academic Press, New York (1965).

2-methyl-piperidine hydrochloride 210°; m.p. of authentic 2-methyl-piperidine picrate 116–117°). These results indicated that the breakdown product of R_f value 0·2 was 2-methyl-Δ^1-piperideine and confirmation was obtained by chromatography of synthetic 2-methyl-Δ^1-piperideine.

Further examination of eluates of bands of R_f value 0·30 and 0·45 showed that the compound of R_f value 0·30 was responsible for the production of 2-methyl-Δ^1-piperideine on alkaline hydrolysis and it was assumed that this compound is unstable in the presence of air and moisture. This assumption was strengthened by the fact that an eluate of the band on exposure to air and subjected to further chromatography showed a variable number of spots which reacted positively with the nitroprusside reagent. Recent work using thin-layer chromatography has shown that rapid extraction of the plant material with ethanol at low temperature resulted in the appearance of a single spot which gave a red colour with the nitroprusside reagent. In order to ensure rapid extraction of the alkaloid at low temperature the plant material was treated with liquid nitrogen, allowed to thaw out and the sap pressed out in a hydraulic press. During the course of the operation the temperature of the material was not allowed to rise above 5°. The alkaloid was isolated as the hydrochloride, a white crystalline substance (m.p. 112–114°) which was very unstable in the presence of air and moisture. Alkaline hydrolysis of the hydrochloride with magnesium hydroxide yielded 2-methyl-Δ^1-piperideine together with other products which reacted positively with iodine but negatively with the nitroprusside reagent when subjected to paper chromatography. Hydrogenation of the hydrochloride over Adam's catalyst yielded a product having an i.r. spectrum identical with that of coniine. The hydrochloride of the reduction product melted at 212° (m.p. of authentic coniine hydrochloride 212°). These findings suggest that the alkaloid has the structure of 2-(2-propenyl)-Δ^1-piperideine (I) or 2-(2-propenyl)-Δ^2-piperideine (II).



(I)



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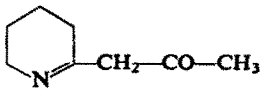

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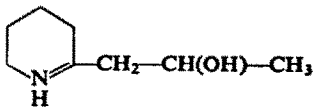

(IV)

Further study of the structure of the alkaloid was carried out by examination of the i.r. and NMR spectra and by determination of "active hydrogen" by the method of Zerewitinoff. The i.r. spectra of the hydrochloride and the free base (Fig. 1) showed a band at the frequency 1660 cm^{-1} which is within the expected range for the absorption of the C=N and C=C stretching vibrations. Both these vibrations would be expected in structures (I) and (II). The spectrum of the free base shows two bands in this region, i.e. at 1660 cm^{-1} and 1620 cm^{-1}. Experience suggests that the former is due to a C=N stretch and the latter to a C=C stretch. Beyermann *et al.*[6] state that the C=N vibration of γ-coniceine occurs at 1663 cm^{-1} and it

[6] H. C. BEYERMANN, M. VAN LEEUWEN, J. SMIDT and A. VAN VEEN, *Rec. Trav. Chim.* **80**, 513 (1961).

would be expected that the C=N vibration of (I) would be similar. The spectrum also shows bands at 680 cm^{-1} and 980 cm^{-1} which would indicate a C=C structure having both *cis* and *trans* configurations. Examination of the frequency range in the region of 3300 cm^{-1} gives no indication of an N–H vibration due to active hydrogen on the nitrogen atom. Evidence from i.r. spectra therefore suggests that the alkaloid has structure (I) rather than structure (II). The small band in the 3300 cm^{-1} frequency range which was probably due to the presence of moisture in the sample would be unlikely to mask any vibration due to N–H particularly when it is compared with the band obtained in this region with piperidine and 2-methyl-piperidine. Infrared spectra of the impure alkaloid showed vibrations indicating the presence of —OH (other than moisture) and C=O groups suggesting that structures (III) and (IV) were present. However, there can be little doubt that these compounds are

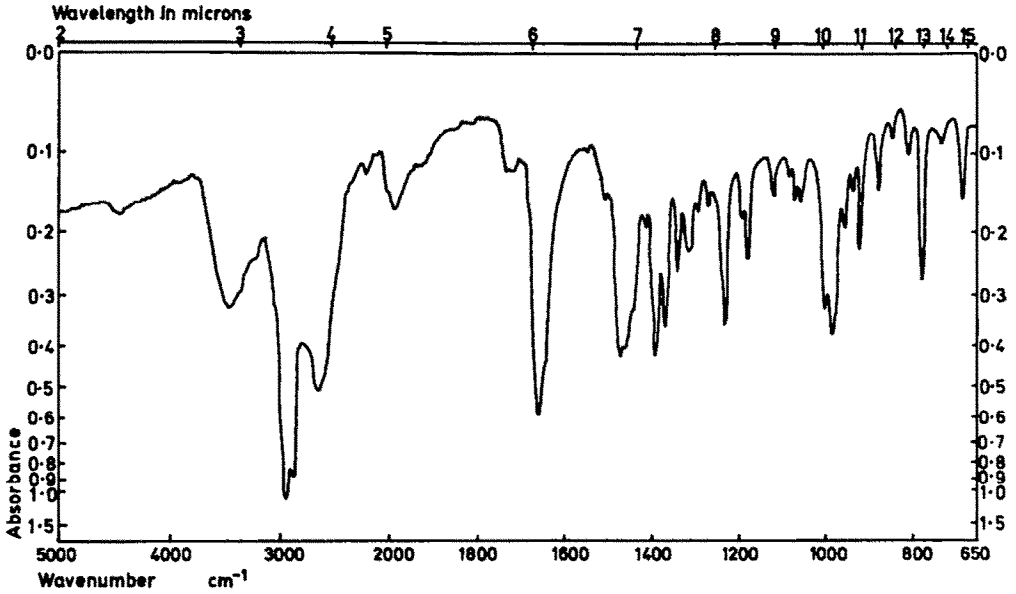

FIG. 1. I.R. SPECTRUM OF HYDROCHLORIDE OF UNSATURATED ALKALOID ISOLATED FROM POMEGRANATE.

artefacts possibly resulting from addition of water across the double bond of the side-chain of (I) followed by oxidation. When the alkaloid was extracted under strictly controlled conditions and a pure product obtained there was no evidence for the presence of —OH and C=O groups.

The NMR spectrum of the alkaloid (Fig. 2) shows four resonance peaks at 2·9τ (d), 6·2τ (a), 7·0τ (b) and 7·9τ and 8·0τ (c and e). The spectrum of the hydrochloride of the alkaloid was better resolved than that of the free base. In general the NMR spectra of Δ^1-piperideines are not well resolved due to coupling. The peak (d) is found in the expected position for the protons of the group N=C—CH=CH. The peak (a) due to two protons on the 6-carbon atom of the heterocyclic ring is in the expected position. The slight shift downfield from the position of these protons in piperidine which are at 6·8τ, is due to the adjacent double bond. Beyermann *et al.*[6] obtained a peak at 6·1τ for these protons in the molecule of γ-coniceine and this similarity of 2-(2-propenyl)-Δ^1-piperideine to γ-coniceine is understandable since one would not expect these protons to be affected by the variation

in the structure of the side-chain. The peak (b) given by the two protons on the 3-carbon atom of the ring also shows a slight shift downfield from 7·8τ for γ-coniceine, due to the effect of unsaturation in the side-chain. The peaks (c) and (e) represent an overlapping of the peak due to the protons of the 4- and 5-carbon atoms of the ring and a peak due to the protons in the methyl group of the side-chain.

It is apparent that peaks (a) and (b) are of equal area, hence the ring is a Δ^1-piperideine. Had the ring structure been that of a Δ^2-piperideine the peak (b) would be absent, and the peak due to the hydrogen on the 3-carbon atom would have occurred in the range 3–6τ. The spectrum would also have contained a peak due to the proton on the nitrogen of the ring.

The NMR spectrum confirms that structure (I) which has been assigned to the alkaloid is correct. If this is so, the areas under the peaks should be in the ratio d:a:b:c and d as 2:2:2:7. Our spectrum gave a ratio of 1·7:2:2·6:6·8 which is in fair agreement in view of

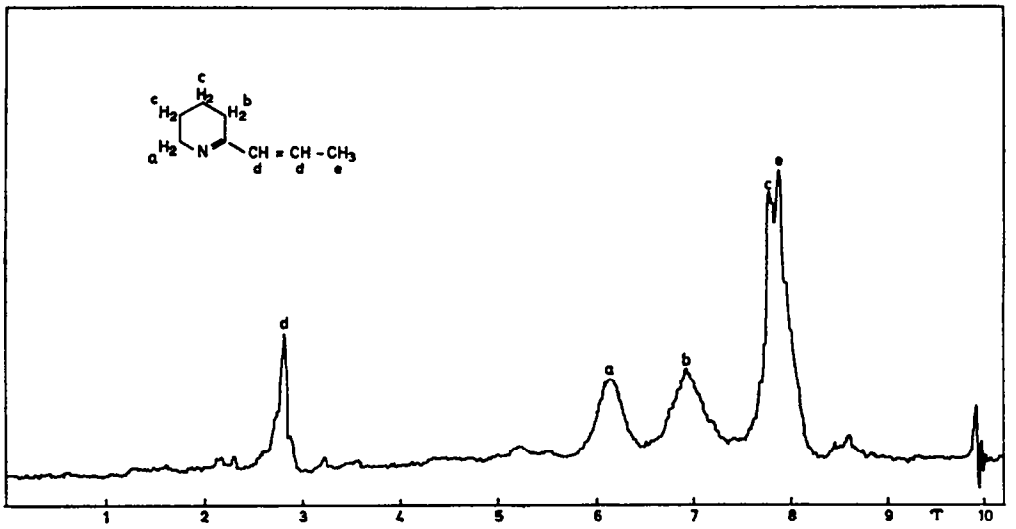

FIG. 2. NMR SPECTRUM OF HYDROCHLORIDE OF UNSATURATED ALKALOID ISOLATED FROM POMEGRANATE.

the poor resolution of the spectrum and the tendency of the alkaloid to undergo oxidation yielding a carbonyl group which would give a reduction in (d) and a slight increase in peak (b).

Further confirmation of structure (I) was obtained by determination of the "active hydrogen" in samples of the alkaloid. Theoretically if structure (I) is correct no evolution of gas should take place when the alkaloid reacts with lithium aluminium hydride under the specified conditions. Piperidine, which contains "active hydrogen", was used as a check in the experiment. The results of the experiment are summarized in Table 1. The small evolution of gas obtained could be interpreted as due to the presence of a small amount of the Δ^2-piperideine (II).

The occurrence in Pomegranate of 2-(2-propenyl)-Δ^1-piperideine is of interest from the point of view of the biogenesis of the piperidine alkaloids. This unstable compound occurs in the cotyledons of the young seedling and in the leaves and actively growing tissues of both young and older plants and appears to be the only detectable alkaloid present in the press juice of fresh tissues which have been frozen with liquid nitrogen. Conventional methods

of extraction of the tissues (especially oven-dried material) yield extracts which, when subjected to chromatography, reveal the presence of at least two and sometimes three alkaloids. The present work has shown that in the presence of moisture and air 2-(2-propenyl)-Δ^1-piperideine gives rise to compounds in which the side-chain becomes saturated as the result of hydration and possibly subsequent oxidation. The need for rapid extraction of alkaloids from fresh tissues at low temperature in studies of intermediates of alkaloid biogenesis is thus emphasized.

TABLE 1. DETERMINATION OF "ACTIVE HYDROGEN" IN PIPERIDINE AND THE ALKALOID ISOLATED FROM POMEGRANATE

(1) Piperidine
59 mg yielded 15·6 ml of hydrogen at 20°/760 mm.
Found: 1·20% "active hydrogen".
Calc.: $C_5H_{11}N$ (mol. wt. 85·146) 1·18% "active hydrogen".

(2) Alkaloid isolated from Pomegranate, 2-(2-propenyl)-Δ^1-piperideine.
55 mg yielded 1·4 ml of hydrogen at 20°/760 mm.
Found: 0·114% "active hydrogen".
Calc.: $C_8H_{13}N$ (mol. wt. 123) 0·813% "active hydrogen".
282 mg yielded 5·05 ml of hydrogen at 20°/760 mm.
Found: 0·08% "active hydrogen".

Previous work[3] on Hemlock (*Conium maculatum* L.) has shown that in seedlings and actively growing vegetative tissues γ-coniceine (2-propyl-Δ^1-piperideine) is the major alkaloid which may be a precursor of the saturated alkaloids. Similarly 2-(2-propenyl)-Δ^1-piperideine may be an important intermediate compound in the formation of the saturated alkaloids of Pomegranate accumulating only in young tissues which are metabolically very active and subsequently undergoing translocation to mature tissues in which hydration and oxidation in the side-chain and reduction in the ring take place. However, it is possible that 2-(2-propenyl)-Δ^1-piperideine may be a product of metabolic activity in a particular phase of growth of young tissues and that the important intermediates in the biogenesis of the saturated alkaloids which are normally found in the bark of the root system are enzyme bound and not detectable by chromatographic means.

EXPERIMENTAL

Plant Material

Seeds from Pomegranate fruits obtained commercially were sown in pans in J.I. compost. The seedlings were pricked out and grown on in borders in a cool greenhouse. Leaves and young shoots were harvested when required.

Extraction of Alkaloids

In earlier experiments both fresh and dried leaf material were used. Leaves were dried at 60° in an oven with forced draught, finely powdered and stored in a desiccator until required for use. Steam distillation of the alkaloids was carried out according to the method described by Cromwell[3] for the isolation of Hemlock alkaloids. MgO was used to free the bases prior to steam distillation. Fresh material was extracted with ethanol in a Waring Blendor, the cell debris removed by filtration and the ethanolic solution of the alkaloids evaporated to dryness on a water bath at 40°. The residue was taken up with distilled water,

the solution made alkaline with 10% w/v Na_2CO_3 and the alkaloids shaken out with $CHCl_3$ in small portions. The alkaloids were removed from the combined $CHCl_3$ solutions by shaking with 10% HCl. The acid solution was evaporated to dryness on a water bath at 55° and a solution of the residue in ethanol was used for chromatography.

In recent experiments young leaves of Pomegranate in batches of 300 g were placed in the perforated cylinder of a hydraulic press (S. H. Johnson Ltd., London, E.15). The leaves were frozen with liquid nitrogen and allowed to thaw out. The temperature was kept below 5°. The material was carefully pressed at 4500 lb/in^2 and the sap collected in tubes kept at −10° in NaCl/ice mixture. The sap was made alkaline with 10% Na_2CO_3 and filtered at 2–3° to remove the precipitate which formed. The filtrate of pale straw colour was rapidly extracted at 2° with six portions of $CHCl_3$ ($6 \times \frac{1}{8}$ vol. of sap). The extracts were combined and dried (Na_2SO_4), saturated with dry HCl and the solvent removed using a Rotary Film Evaporator with a cold trap. The residue formed white crystals which melted at 112–114° and remained stable without discoloration provided that moisture and air were rigorously excluded. This preparation was used for chromatography, i.r. spectra, NMR spectra and the determination of "active" hydrogen.

Paper Chromatography

Acid-washed Whatman No. 3 MM papers were used and the solvent system described by Cromwell[3] was employed for the separation of the alkaloids. Chromatograms were run for 18 hr at a temperature of 25°.

Thin-layer Chromatography[7]

Plates were prepared with Silica Gel G.254 (Merck). The Silica Gel was made to a suitable consistency with 5% KOH. The plates were activated by drying in an oven at 150° for 30 min and stored in a desiccator until required for use. The solvent system used was chloroform–ethanol 9:1.

The following reagents were used for detection of the alkaloids on chromatograms.

(1) Iodine (0·2% w/v) in light petroleum, b.p 40–60°.
(2) Modified Dragendorff's Reagent.[8]
(3) Nitroprusside reagent.[3]

The nitroprusside reagent was modified by substitution of tetraethyl ammonium hydroxide for sodium carbonate. Better colour development and definition with less tendency for spreading of spots was thus achieved.

Determination of I.R. Spectrum

A Unicam S.P. 200 Spectrometer with sodium chloride prisms was used throughout the course of this work.

Determination of NMR Spectrum

The spectrum of the hydrochloride of the alkaloid in deuterated chloroform was recorded using a Perkin-Elmer 40 m/c spectrometer.

[7] D. WALDI, In *Thin-Layer Chromatography* (Edited by E. STAHL), p. 291. Academic Press, New York (1965).
[8] B. T. CROMWELL and S. D. RENNIE, *Biochem. J.* **58**, 318 (1954).

Determination of "Active Hydrogen" by the General Method of Zerewitinoff[9]

$LiAlH_4$ was used following the procedure described by Linstead, Elvidge and Whalley.[10] The solvent (*n*-butyl ether) was redistilled and dried by passage through a column of Woelm Alumina (neutral-activity I). All measurements were taken at 20°/760 mm. The maximum temperature of the reaction was 60°. Solvent blanks were determined with each estimation and, as a check, the "active hydrogen" in pure piperidine freshly distilled from potassium hydroxide was determined.

Hydrolysis of the Alkaloid with Magnesium Oxide

An aqueous solution of the hydrochloride of the alkaloid was refluxed for 4 hr after addition of excess MgO. The solution was distilled in steam for 20 min and the distillate shaken with small portions of $CHCl_3$. The combined extracts were dried (Na_2 SO_4) and saturated with dry HCl. The solvent was evaporated off and the residue examined by paper and thin-film chromatography. The residue consisted principally of 2-methyl-Δ^1-piperideine together with small amounts of the original compound and traces of compounds which reacted positively with iodine but negatively with the nitroprusside and Dragendorff reagents.

Preparation of 2-Methyl-Δ^1-Piperideine

The method of Lipp[11] was used for the preparation of 2-methyl-Δ^1-piperideine from 6-bromohexan-2-one prepared by the method of Anderson, Crawford and Sherrill.[12] The final product was purified by steam distillation with MgO and the distillate shaken with $CHCl_3$. The hydrochloride was prepared by saturating the dry solution with dry HCl and evaporating off the solvent.

Acknowledgements—We thank Mr. G. Collier for assistance in the interpretation of i.r. spectra, Mrs. J. Hampshire for technical assistance and Professor N. F. Robertson for his interest and encouragement throughout the course of this work.

[9] T. Zerewitinoff, *Ber. Deut. Chem. Ges.* **40**, 2023 (1907).
[10] R. P. Linstead, J. A. Elvidge and M. Whalley, *A Course in Modern Techniques of Organic Chemistry*. Butterworth, London (1955).
[11] A. Lipp, *Ber. Deut. Chem. Ges.* **25**, 2190 (1892).
[12] E. P. Anderson, J. V. Crawford and M. L. Sherrill, *J. Am. Chem. Soc.* **68**, 1294 (1946).