ISOLATION AND CHARACTERIZATION OF 1-ALANYL-URACIL (WILLARDIINE) AND 3-ALANYL-URACIL (*ISO*-WILLARDIINE) FROM PISUM SATIVUM

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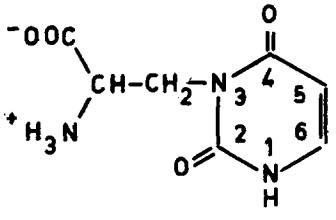
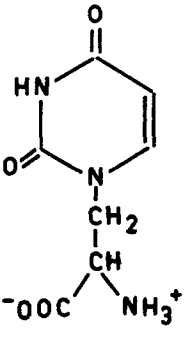
Introduction

During germination of pea seeds (Pisum sativum L. var. Rondo), a number of uracil-containing amino acids appear in increasing amounts. In a previous communication, the structure of the quantitatively most important product was proposed to be 3-alanyl-5-ribosyl-6-amino-uracil (compound I) (Lambein and Van Parijs, 1968). A second compound is now identified as 3-alanyl-uracil (II), the isomer of willardiine (1-alanyl-uracil, IV). Another compound, present in smaller amounts, is identical with willardiine. Compound III is very similar to I, but has not been further characterized.



II (*iso*-willardiine)



IV (willardiine)

Two alanyl-uracil derivatives have previously been isolated from plant material. 1-alanyl-uracil (willardiine) (Gmelin, 1959)

was found in seeds of Acacia Willardiana and a 5-alanyl-uracil derivative, described by Brown and Silver (1966), was isolated from Pisum sativum.

While the structure of willardiine has been unequivocally demonstrated by chemical synthesis (Kjaer, et al., 1961 ; Dewar and Shaw, 1962 ; Shvachkin, et al., 1964) no comparison has been made between the product isolated by Brown and Silver (1966) and the 5-alanyl-uracil synthesized by Springer and coworkers (1965). The latter authors also synthesized 6-alanyl-uracil.

Our products can be easily purified in small amounts by paper chromatography of crude extracts in n-butanol:acetic acid:water (60:15:25 , v/v/v) and ethanol:water (80:20, v/v). Large scale preparation is described below.

Characterization of the compounds.

The identity of the isolated compound IV with willardiine, synthesized by Dewar and Shaw, was demonstrated by chromatography in 8 different solvents, electrophoretic mobility at pH 1.9 and 7.0 ; U.V.-spectra at different pH-values and spectrophotometric pK-values (8.0 and 10.2) ; rate of photolysis under U.V.-light (254 nm), and rate of regeneration of the initial spectrum in ammoniacal medium (Fikus and Shugar, 1966).

The U.V.-spectrum of the other compound (II, Fig. 1) is very similar to the spectrum of 3-substituted uracil.

The bathochromic shift, and the increase of the extinction coefficient in alkaline medium, point clearly to a uracil ring with a substituent at position 3 and no substituent at position 1. Uracil and 5-bromo-uracil were isolated after bromination and acid hydrolysis.

After bromination under mild conditions, and boiling of the addition product in absolute ethanol (30 min at 100°C), a

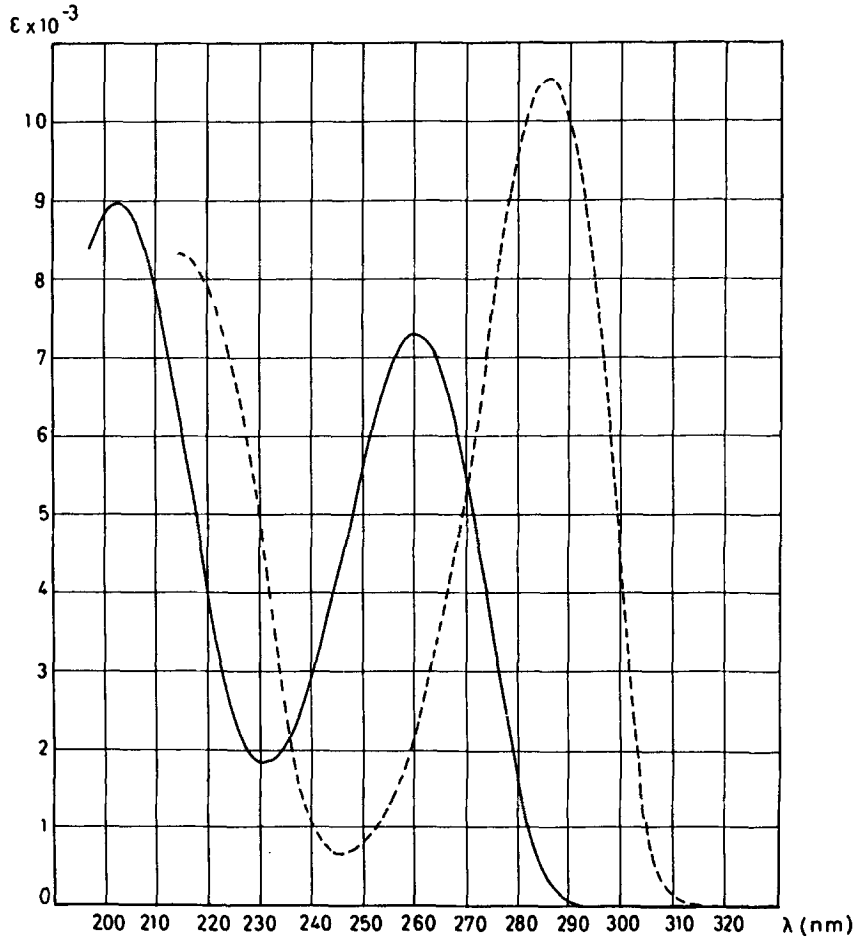


Fig. 1. U.V.-spectra of compound II (*iso*-willardiine) in water

pH_2 ——————— pH12 — — — —

5-bromo-uracil derivative was obtained with a substituent at position 3, which still gave a positive ninhydrin reaction. This result shows that the amino acid chain in compound II is not present at position 5.

The results of elementary analysis and the determination of the molecular weight by potentiometric titration (see experimental part) are completely in agreement with an alanyl-uracil

structure. Therefore the structure of compound II must be 3-alanyl-uracil.

Discussion

With the isolation and identification of 3-alanyl-uracil (II), referred to as *iso*-willardiine by Shvachkin and Azarova (1964), the four possible alanyl-uracil isomers are now known, and two of these (II and IV) are present in pea seedlings. Furthermore, compound I, also present in pea seedlings, is a substituted 3-alanyl-uracil (Lambein and Van Parijs, 1968).

The compounds seem to play an important role in the first stages of embryo growth during seed germination. They are not present in the dry seeds, but fairly high concentrations are found during germination in all organs of the seedling.

Experimental

565 g of 6-days old pea seedlings (cotyledons discarded) are squeezed out with a rotative fruit-press. The juice, containing 69.10^6 optical units (260 nm), is dialyzed against distilled water. Following 48 hours dialysis, 60% of the optical density passes trough the membrane, and this is concentrated in a vacuum evaporator. The solution is then adsorbed on a column of Dowex 50 W (x 4) 200-400 mesh (H^+) (100 cm x 4 cm). The column is washed with distilled water, and eluted with a linear HCl gradient of 10 l, with an end concentration of 2 N HCl. After 5 l of effluent, a large peak is eluted which contains 11.25×10^6 optical units. In this peak, compounds I, II and IV are present, together with free amino acids as impurities. This mixture is further purified by passing through a column of Dowex 1 (x 4) 200-400 mesh ($HCOO^-$) (40 cm x 1.5 cm), on which the three compounds are not retained. On concentration of the effluent, a part of compound I crystallizes out. Further separation is obtained by preparative thin layer chromatography on Al_2O_3, type PF 254 (Merck, Germany). The Al_2O_3 powder is suspended in 0.01 N HCl. Plates of 30 cm length are covered with a layer of Al_2O_3 2 mm thick, and 1500 optical

units are applied to each plate. Compound I is separated from II and IV with the solvent system acetone : water (15 : 85). Compound II and IV are partially separated by chromatography on a column of Dowex 50 W (x 4) 200-400 mesh (H^+) (50 cm x 1.2 cm) ; elution with 0.75 N HCl. After a second passage through the Dowex 50 W column, separation of II and IV is almost complete. A final purification is effected by passage through the Dowex 1 ($HCOO^-$) column.

Compound II, from three subsequent preparations, is crystallized from distilled water, and recrystallized, first from a mixture of ethanol : water, and then from acetone : water. 180 mg fine crystals are obtained.

Properties of compound II.

M.P. 239 (decomposition - non corrected).

U.V.-spectrum : pH 2 : λmax 260 nm εmax 7.4×10^3
λmin 231 nm εmin 1.8×10^3
pH 12: λmax 286 nm εmax 10.6×10^3
λmin 245 nm εmin 0.64×10^3

Elementary analysis, found : C:38.3 H : 5.15 N : 19.1
theor.: C:38.71 H : 5.11 N : 19.35

By spectrophotometric titration, a pK of 10.45 is found. Potentiometric titration shows 2 pK-values, 8.45 and 10.25. In 4% formaldehyde (5% methanol is added as stabiliser) the first pK is lowered to 6.63. A molecular weight of 212.5 is estimated by titration (theor. : 217.2). The compound gives a purple colour with ninhydrin.

The crystallization with one molecule of water is also found for 1-alanyl-uracil (Gmelin, 1959), 5-alanyl-uracil and 6-alanyl-uracil (Springer, et al., 1965).

The small amount of compound IV was not crystallized. Taking into account the known M.W. and εmax, 2 mg of IV were isolated.

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