ISOLATION AND CHARACTERIZATION OF 2-(β,D-GLUCOPYRANOSYL)-4-ALANYL-3-ISOXAZOLIN-5-ONE : A SECOND UV-SENSITIVE HETEROCYCLIC α-AMINO ACID FROM *PISUM SATIVUM L.*

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In addition to 2-alanyl-3-isoxazolin-5-one (I) an other UV-sensitive heterocyclic α-amino acid (III) has been isolated and purified from pea seedlings. Some properties of III are compared with those of synthetic isoxazolinone derivatives and with those of I. The chemical structure was analyzed by chemical methods and by spectroscopy. The structure was found to be : 2-(β,D-glucopyranosyl)-4-alanyl-3-isoxazolin-5-one.

INTRODUCTION

In CCl_3COOH-extracts of seedlings of *Pisum sativum L. (Cv. Rondo)* four heterocyclic α-amino acids were found. These compounds are not present in the dry seeds, but they are rapidly formed during the early stages of germination (1).

Two of these amino acids (compound II and IV) were identified as uracil derivatives (3-alanyl-uracil and 1-alanyl-uracil resp.) (2, 3). Compounds I and III show, in contrast to II and IV, a much higher sensitivity towards UV-irradiation and alkaline pH. Using different methods, such as spectroscopy, identification of degradation products which were formed by alkaline degradation or by UV-irradiation, elementary analysis, comparison of the properties with those of synthetic products, etc., we were able to show that compound I has the following structure : 2-alanyl-3-isoxazolin-5-one (4, 5).

In this paper we examine the structure of compound III. Some properties of compounds I and III are compared in detail.

ISOLATION AND PURIFICATION OF COMPOUND III

Small amounts of III were isolated from a homogenate in cold 5 % CCl_3COOH of 4 to 6 days old seedlings (seedleaves discarded). The homogenate was centrifuged at 2 500 x *g*. The supernatant was extracted four times with ether to eliminate the greatest part of

the CCl_3COOH. III was isolated by subsequent paper chromatography with ethyl alcohol : water (80 : 20, v : v) (Rf = .21), butyl alcohol : acetic acid : water (60 : 15 : 25, v : v : v) (Rf = .19) and isopropyl alcohol : water (70 : 30, v : v) (Rf = .16).

Larger scale preparations were achieved by cation exchange on Dowex 50 W (H^+) as described for the isolation of II and IV (2). The column was eluted with a linear gradient of 10 l, (from 0 to 2 N HCl). The peak containing III, was eluted after 3.3 l of effluent. The fractions, which belonged to this peak, were evaporated under vacuum to dryness, and purified by paper chromatography with ethyl alcohol : water. The band with Rf = .21 was eluted with water and was further purified by cation exchange on a column Dowex 50 W (x 4) 200-400 mesh (H^+) (60 cm x 1.25 cm); this column was eluted with 0.5 N HCl; only one peak was detected by the Uvicord. The fractions were evaporated under vacuum to dryness, dissolved in water, and the solution was passed through a column of Dowex 1 (X 4) 200-400 mesh ($HCOO^-$) (40 cm x 1.4 cm), which does not retain compound III.

It was difficult to crystallyze the purified product. Crystals were obtained from a mixture of methyl alcohol and n-propyl alcohol by slow evaporation of the methyl alcohol.

From 500 g fresh seedlings, 175 units of O.D. were obtained.

PROPERTIES OF COMPOUND III

The product is very soluble in water but nearly insoluble in organic solvents. Decomposition point : 182°C.

By potentiometric titration in 10% formaldehyde of 8.1 mg crystalline product, a molecular weight of 355.1 was estimated (theor. : 334.3). Spectrophotometric titration indicates a pK-value of about 8. Due to the lability of III in alkaline medium only a rough estimate of the pK-value was obtained.

After spraying of paperchromatograms with ninhydrin, III gives a normal purple colour, in contrast to I (4).

Elementary analysis :

exp.	:	N : 8.37 %	C : 42.47 %	H : 5.87 %		
theor.	:	N : 8.38 %	C : 43.11 %	H : 5.39 %		
UV-spectra :	pH 6.2	: λmax : 265 nm		εmax : 10 600		
		λmin : 217 nm		εmin : 1 200		
	pH 10	: λmax : 267 nm		εmax : 10 130		
		λmin : 223 nm		εmin : 1 300		

THE CARBOHYDRATE MOIETY.

After heating of a concentrated solution of pure III in 1 N HCl at 100°C during 3 hours, some unchanged III was found together with a product which reduces alkaline $AgNO_3$. This product was identified by paper chromatography in six different solvent systems, and by enzymic oxydation, as D-glucose.

This glucose was quantitatively estimated by using a commercial D-glucose-oxydoreductase preparation (Boehringer, Mannheim, Germany). After acidic hydrolysis, 48% of the weight of the initial material was found as D-glucose. After irradiation of III with UV-light (λ= 254 nm), 40.5% of the weight of the initial material was found as D-glucose. Other degradation products of III inhibited the enzymatic reactions, and the D-glucose formed by degradation of III had to be purified by paperchromatography.

THE α-AMINO ACID MOIETY.

The α-amino acid character of III was demonstrated by the ninhydrin reaction at room temperature and by paper chromatography in the presence of Cu^{++}-ions. After irradiation with UV-light, four ninhydrin-reacting products were found by paper chromatography. One of these products was identified by chromatography in six different solvent systems and by electrophoresis at pH 1.9 and 7. In every case the mobility of the isolated product was identical with that of glutamic acid.

Glutamic acid was also found and identified after hydrolysis of compound III in 6 N HCl at 100°C for 18 hours.

THE HETEROCYCLIC RING.

The heterocyclic ring of compound III was studied by spectroscopy and by comparison of some specific properties of the product with those of synthetic products and those of compound I.

Spectroscopy.

Fig. 1 shows the UV-spectra of III in water at pH 6.2 and 10. Spectrophotometric titration indicated only one dissociation (pK = 8). The bathochromic shift of 2nm is due to the deprotonation of the α-amino group, this phenomenon was also observed in the UV-spectra of I (4). While the UV-spectra of III show a hypochromicity of 4.5% in alkaline medium, the UV-spectra of I showed a hyperchromicity of 7.2% in the same conditions. The UV-

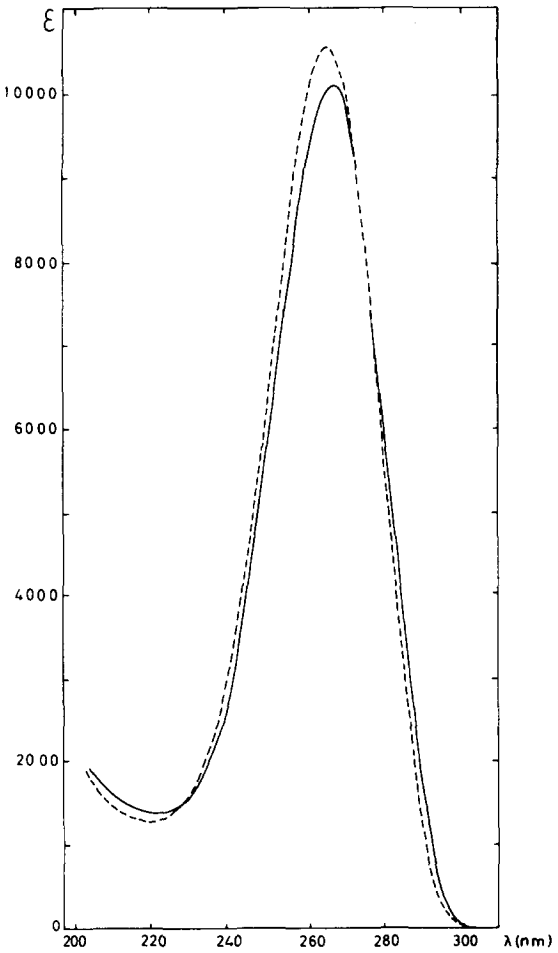


Fig. 1 : UV-spectra of compound III in water at pH = 6.2 (----) and at pH = 10 (——). In ordinate : the molecular extinction coefficient (ε).

spectra of both compounds I and III in neutral or in acidic solutions, are almost the same.

Fig. 2 shows the 60 MHz NMR-spectra of III (a), and of 2,4-dimethyl-3-isoxazolin-5-one (b) in D_2O. In the spectrum of III, doublet peaks which could be due to an olefinic double bond are not present, in contrast to the NMR-spectrum of I (4). The singlet peak at 8.25 ppm is due to one proton, as was demonstrated by integration; the high chemical shift is comparable to a singlet at 8.05 ppm in the spectrum of 2,4-dimethyl-3-isoxazolin-5-one. Both peaks are due to a proton at C-3 of the isoxazolinone ring; in both cases, a substituent at C-4 must be present.

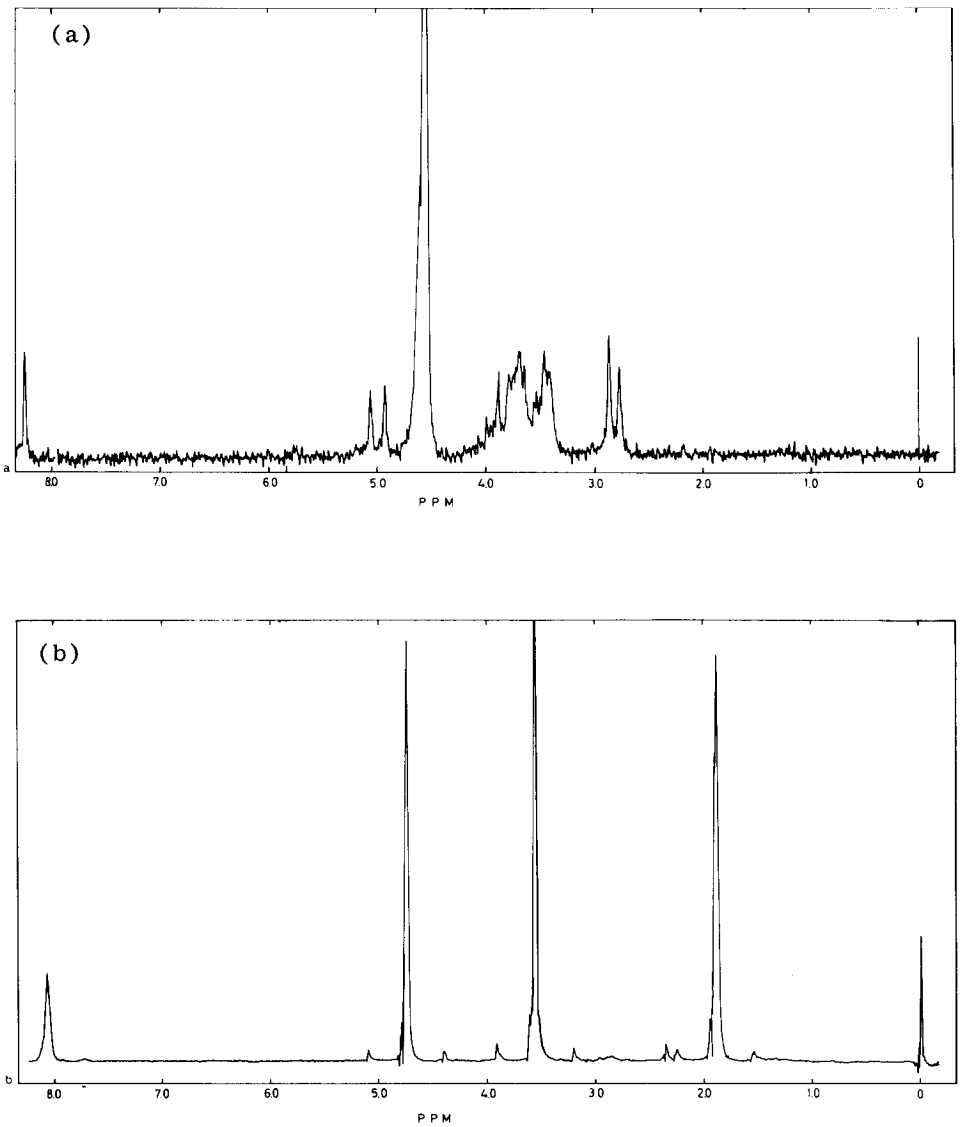


Fig. 2 : 60 MHz NMR-spectra of compound III (a) and of 2,4-dimethyl-3-isoxazolin-5-one (b) in D_2O, at room temperature. TMS was used as an external standard.

The presence of an alanyl-substituent at C-4 is in agreement with a deformed triplet peak at 3.9 ppm, and a doublet peak at 2.8 ppm. In the spectrum of I (4) an identical triplet at 3.9 ppm was due to the proton in α-position of the alanyl-moiety. Double irradiation demonstrated a coupling of this triplet peak with the doublet peak at 2.8 ppm, which is due to the two protons in β-position of the alanyl-moiety. The chemical shift of 2.8 ppm is in agreement with a C-C bond in β-position.

The doublet peak at 4.99 ppm and the peaks at 3.8 to 3.4 ppm are due do the carbon-linked protons of the carbohydrate moiety, in agreement with the spectrum of 1-(β,D-glucopyranosyl)-uracil. In both cases, a doublet peak at 5 ppm is due to the proton at C_1', their coupling constant of 8 cps indicates a diaxial position of H_1' and H_2' in agreement with the β-configuration.

Fig. 3 shows the IR-spectrum of III in a KBr-disc.

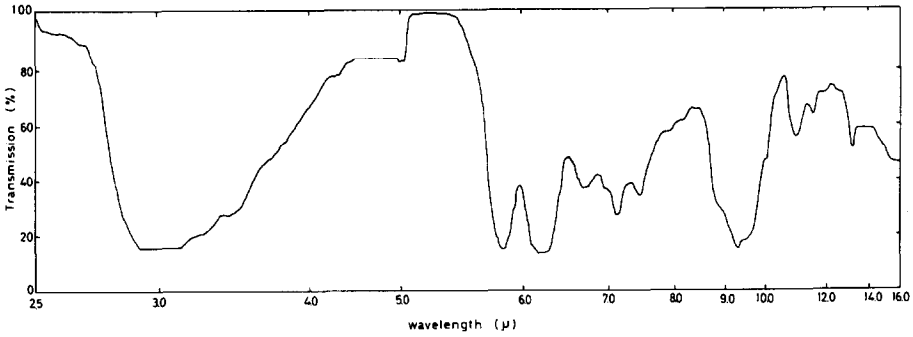


Fig. 3 : IR-spectrum of compound III in a KBr-disc.

Alkaline degradation.

Both compounds I and III undergo irreversible transformations at room temperature at pH 10 and at higher pH. The specific UV-spectra disappear. This behaviour is in agreement with the alkaline ring-opening of N-substituted 3-isoxazolin-5-ones with a proton at position 3 (6).

The rate of transformation of these compounds in alkaline

medium is decreased by addition of 4% formaldehyde.

Degradation by UV-light.

Both compounds I and III, and synthetic 2,4-dimethyl-3-isoxazolin-5-one, show a very similar sensitivity to UV-irradiation (λ = 254 nm). The rate of this photochemical transformation is about twenty times higher than the rate of photolysis of uridine. In D_2O-solutions, the relative quantum yields were lowered to 78% of the initial value for I, to 81% for III, to 72% for 2,4-dimethyl-3-isoxazolin-5-one, and to 47% for uridine.

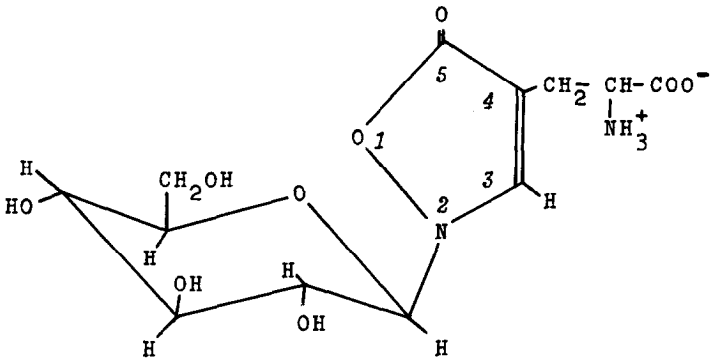
By photolysis of III, glutamic acid and free D-glucose were formed, together with other ninhydrin-reacting products. This is in agreement with the opening of the isoxazolinone ring at different places, as was already observed for compound I (5).

DISCUSSION

The formation of glutamic acid, as a degradation product of compound III, instead of α,β-diaminopropionic acid and derivatives which were liberated by degradation of compound I, points to an other linkage of the α-amino acid moiety to the heterocyclic ring. The NMR-spectrum clearly indicates the presence of a carbon-linked α-alanyl group. At C-3, a proton must be present, in agreement with the lability in alkaline medium (6), and with the NMR-spectrum. Following the observations of Springer *et al.*(7) the presence of the α-alanyl group at C-4 is also in agreement with the formation of a normal violet coloration by ninhydrin.

The only partial liberation of glucose in conditions in which the purine nucleosides are hydrolyzed and the pyrimidine nucleosides are not, points to a N-C bond. This is confirmed by the NMR-spectrum, which also indicates a β-configuration.

Finally, the UV-spectra, the instability of compound III in alkaline medium and under UV-irradiation, and the NMR-spectrum, clearly indicate the presence of the same heterocyclic ring in both compounds I and III. The chemical structure of compound III must be : 2(β,D-glucopyranosyl)-4-alanyl-3-isoxazolin-5-one.



COMPOUND III

The presence in plant extracts of products with a similar sensitivity towards UV-light has not yet been reported. This can be due to the instability of these products. Meanwhile, we also found compounds I and III in other leguminous seedlings (unpublished results). The physiological importance of these compounds is not yet known.

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