NEW ISOXAZOLINONE AMINO ACIDS FROM *LATHYRUS ODORATUS* SEEDLINGS.

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In addition to β-(isoxazolin-5-one-2-yl)-alanine (I) and β-(2-β-D-glucopyranosyl-isoxazolin-5-one-4-yl)-alanine (III), previously found in *Pisum sativum* seedlings, two new amino acids and an amine containing the same heterocyclic ring have been isolated from *Lathyrus odoratus* seedlings. Their structures were determined as α-amino-γ-(isoxazolin-5-one-2-yl)-butyric acid (VI), 2-aminoethyl-isoxazolin-5-one (VII) and the γ-glutamyl derivative of the latter (V).

INTRODUCTION

Seedlings of sweet peas (*Lathyrus odoratus L.*) appear to be a very rich source of heterocyclic compounds containing an isoxazolinone ring. Up to now eight such compounds were isolated and characterized. These products are present in high concentrations during germination.

Two isoxazolinone amino acids (I and III) have been found in pea seedlings (*Pisum sativum L.*) (4,6). These compounds are also present in sweet pea seedlings at concentrations similar to those found in pea seedlings.

Extracts of sweet pea seedlings have an absorption maximum at 265 nm. This is mainly due to the presence of isoxazolinone derivatives. From the molar extinction coefficient and the molecular weight of the most abundant compounds it was calculated that isoxazolinone derivatives account for about 10 % of the dry matter of 10-day-old seedlings.

Three isoxazolinone derivatives isolated from sweet pea seedlings have been reported recently including a nitrile (VIII) (7), a glucoside (IX) and a carboxylic acid (X) (11). While the structure of compound I has been confirmed by enzymic studies (8), the structures of compounds VIII and IX have been confirmed by chemical synthesis (10).

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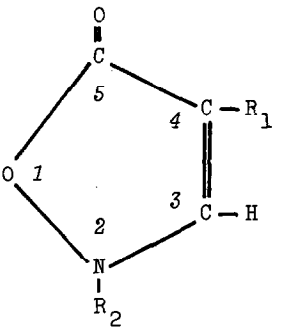

I	: R_1 = H	R_2 = $-CH_2-CH(NH_2)-COOH$
III	: R_1 = $-CH_2-CH(NH_2)-COOH$	R_2 = -β-D-glucopyranosyl
V	: R_1 = H	R_2 = $-CH_2-CH_2-NH-CO-CH_2-CH_2-CH(NH_2)-COOH$
VI	: R_1 = H	R_2 = $-CH_2-CH_2-CH(NH_2)-COOH$
VII	: R_1 = H	R_2 = $-CH_2-CH_2-NH_2$
VIII	: R_1 = H	R_2 = $-CH_2-CH_2-CN$
IX	: R_1 = H	R_2 = -β-D-glucopyranosyl
X	: R_1 = H	R_2 = $-CH_2-COOH$

Table 1 : Structures of natural occuring isoxazolinones.

The isolation and characterization of three ninhydrine reacting isoxazolinone derivatives is described below.

DETECTION AND ISOLATION OF ISOXAZOLINONE DERIVATIVES

After imbibition for 24 hrs, the seeds were germinated on wet filter paper in the dark at 25°C for up to 10 days. The cotelydons were discarded and the seedlings were homogenized in the cold, the extract was centrifuged and the supernatant concentrated under vacuum. From 100 g of fresh material, 55 000 units of optical density (at 265 nm) or about 5 mmoles were extracted.

A suitable amount was applied on paper strips Whatmann 3MM. After chromatography with ethyl alcohol : water (80 : 20) the UV-absorbing bands were located with a UV-lamp in the dark room. The bands were cut out and eluted with water. The eluate was

Table 2 : Concentration of isoxazolinone-derivatives in 10 day old seedlings of *Lathyrus odoratus* as % of dry weight, and Rf-values in solvent
1 : ethyl alcohol : water (80 : 20) ;
2 : butyl alcohol : acetic acid : water (60 : 15 : 20)
3 : butyl alcohol : pyridine : water (50 : 50 : 50)
4 : isopropyl alcohol : water (70 : 30).

Compound	Concentration	Solvent 1	2	3	4
I	1.9 %	47	20	28	36
III	0.1 %	43	10	31	27
V	1.2 %	45	28	43	44
VI	3.5 %	45	31	45	47
VII	0.02 %	58	52	60	54
VIII	0.8 %	79	75	90	75
IX	0.4 %	70	34	82	68
X	0.3 %	50	60	47	52

rechromatographed with butyl alcohol : acetic acid : water (60 : 15 : 25). The purity of each band was tested by paper chromatography in a variety of solvents and by paper electrophoresis.

The purification of the compounds on a preparative scale was achieved mainly by io exchange chromatography as described previously (11). From a preparative Dowex 50W (H^+)-column, compounds V and VI were eluted with a HCl-gradient at about 1.5 N HCl as a double peak, The front part containing mainly V. Complete separation was obtained by repeated ion exchange chromatography and crystallization of VI or by paper chromatography in butyl alcohol : acetic acid : water (60 : 15 : 25) during 36 hrs.

Compound VII is eluted from the Dowex 50W column at a concentration of 2 N HCl, together with some other UV-absorbing material. Further purification was done by paper chromatography.

The N-substituted isoxazolin-5-one derivatives have UV-spectra with a low and broad minimum around 230 nm and a maximum around 265 nm. In alkaline solutions the UV-absorption disappears irreversibly with a half-life time of about 2 hrs at pH = 10 and about 6 min at pH = 12.

Upon irradiation with UV-light (λ = 254 nm) the UV-absorption disappears irreversibly with a quantum yield of about 0.5 moles/einstein in water (compared to uridine as a standard).

Conclusive evidence for the presence of an isoxazolin-5-one was derived from NMR-spectroscopy, the ring-protons yielding highly specific signals.

Synthetic isoxazolinones, especially the N-substituted-3,4-unsubstituted isoxazolin-5ones (10) demonstrate the same spectroscopic properties and the same sensitivities towards UV-irradiation and alkaline solutions.

COMPOUND VI : α-amino-γ-(isoxazolin-5one-2-yl)-butyric acid

Photolysis of VI with UV-light (λ = 254 nm) yields two ninhydrin reacting photoproducts with Rf-values of 14 and 37 in butyl alcohol : acetic acid : water (60 : 15 : 25).

Paper chromatography in six solvents, paper electrophoresis at pH = 7 and automatic amino acid analysis identified these photoproducts at α,γ-diaminobutyric acid (Rf = 14) and α-amino-γ-acetylamino butyric acid. The color obtaines after ninhydrin spray as well as the Rf-values in all solvent systems used were identical with those of the standard product.

Since after UV-photolysis of compound I α,β-diamino propionic acid and α-amino-β-acetylamino propionic acid were identified (4), the photolysis of VI, together with the UV-spectrum and the quantum yield, suggest the homology of compounds I and VI. This was confirmed by the NMR-spectrum of VI. The 60 MHz NMR-spectrum of VI is very similar to the spectrum of I (4) and clearly shows the presence of an additional CH_2 in the amino acid side chain (fig. 1).

Elementary analysis : N : 14.87 ; C : 45.07 ; H : 5.50.
Theoretical values for $C_7H_{10}N_2O_4$: N : 15.05 ; C : 45.16 ; H : 5.41

UV-spectrum in water : λ max : 265 nm ε max : 12 400
λ min : 225 nm ε min : 745

COMPOUND VII : 2-aminoethyl-isoxazolin-5-one.

VII bears a positive charge. From a Dowex 50 W (H^+) column it is eluted with 2 N HCl. From an automatic amino acid analyzer, using the buffers described by Spackman et al. (9), VII was eluted out 13 min later than arginine.

After spraying of paper chromatograms with a ninhydrine solution and heating, VII like compound I, produces a bright oran-

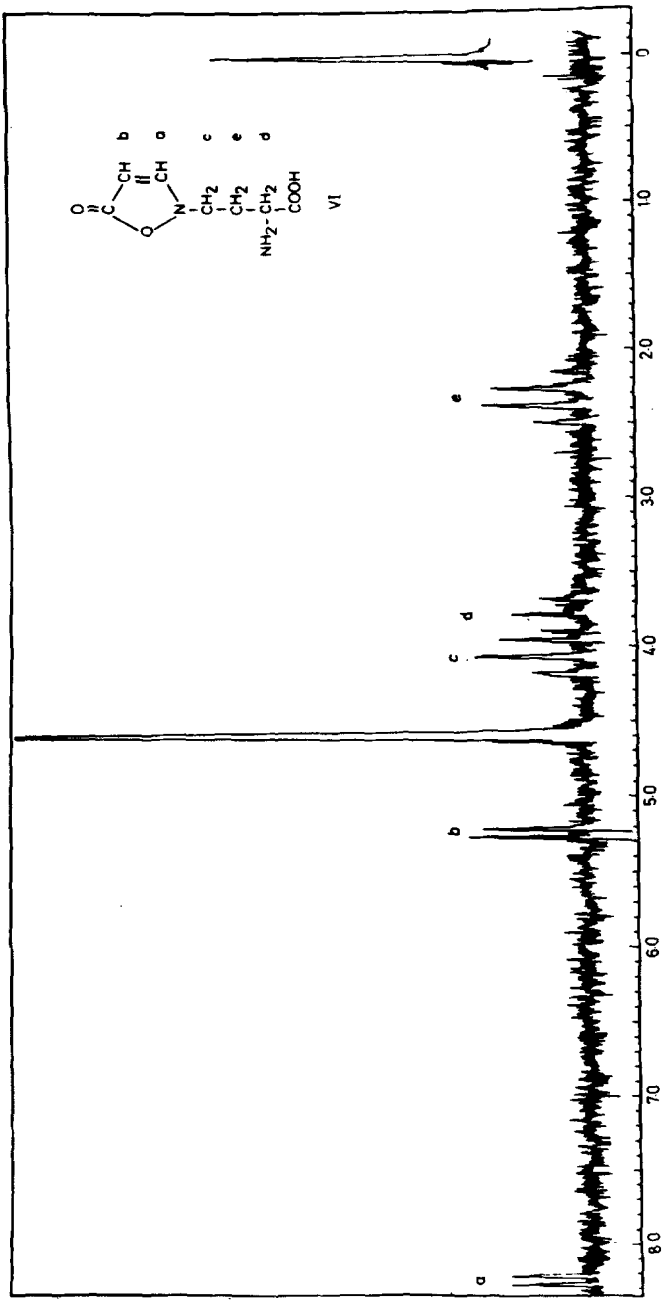

Fig. 1 : NMR-spectrum of compound VI at 60 MHz in D_2O, at room temperature. TMS was used as an external standard.

ge red spot which gradually turns into a normal purple color. After UV-irradiation of VII, ethylene diamine and monoacetyl ethyle diamine were identified by an amino acid analyzer. The mono acetyl compound was eluted 51 min before VII, while for the elution of the diamine a 1 N buffer at pH = 6.18 was used.

The 60 MHz NMR-spectrum of VII clearly demonstrates the presence of a N-substituted isoxazolin-5-one (doublets at 5.32 and 8.25 ppm, 3.5 cps apart). Two triplet peaks at 3.43 ppm and at 4.66 ppm are due to the amino ethylene side chain(fig. 2)

COMPOUND V : 2-γ-glutamylamino ethyl-isoxazolin-5-one.

The amino acid V is much less stable than I or VI. After photolysis, two ninhydrin reacting photoproducts were found. Paper electrophoresis at pH = 7 showed that one photoproduct had a positive charge, and the second photoproduct was neutral. These photoproducts could not be identified by paper chromatography.

In the 60 MHz NMR-spectrum of V, two doublet peaks are present at 5.17 ppm (one proton) and at 8.17 ppm (one proton) and two clusters of peaks at 2 to 2.4 ppm (four protons) and at 3.4 to 4.1 ppm (five protons). The doublet peaks can be assigned to the ring protons. Double irradiation experiments indicated coupling of a triplet peak at 4.04 ppm with a triplet peak at 3.47 ppm, similar to the NMR-spectrum of VII. Asmaler triplet at 3.73 ppm is assigned to the proton in α-position of the α-amino acid moiety. Double irradiation demonstrated coupling of this triplet with a quadruplet around 2.25 ppm which is overlapped by a triplet at 2.3 ppm.

These data are in agreement with the presence of glutamic acid that is bound to the free aminogroup of compound VII by an amide bond. The presence of glutamic acid was confirmed by hydrolysis of V in 1 N HCl at 100°C during 45 min. Glutamic acid and compound VII were identified by paper chromatography in six solvent systems and by paper electrophoresis at pH = 7. According to Kasai and Sakamura (2) the position of the α-CH in the NMR-spectrum agrees with a γ-glutamyl amide derivative.

DISCUSSION.

The presence of both glucosides III and IX in the same spe-

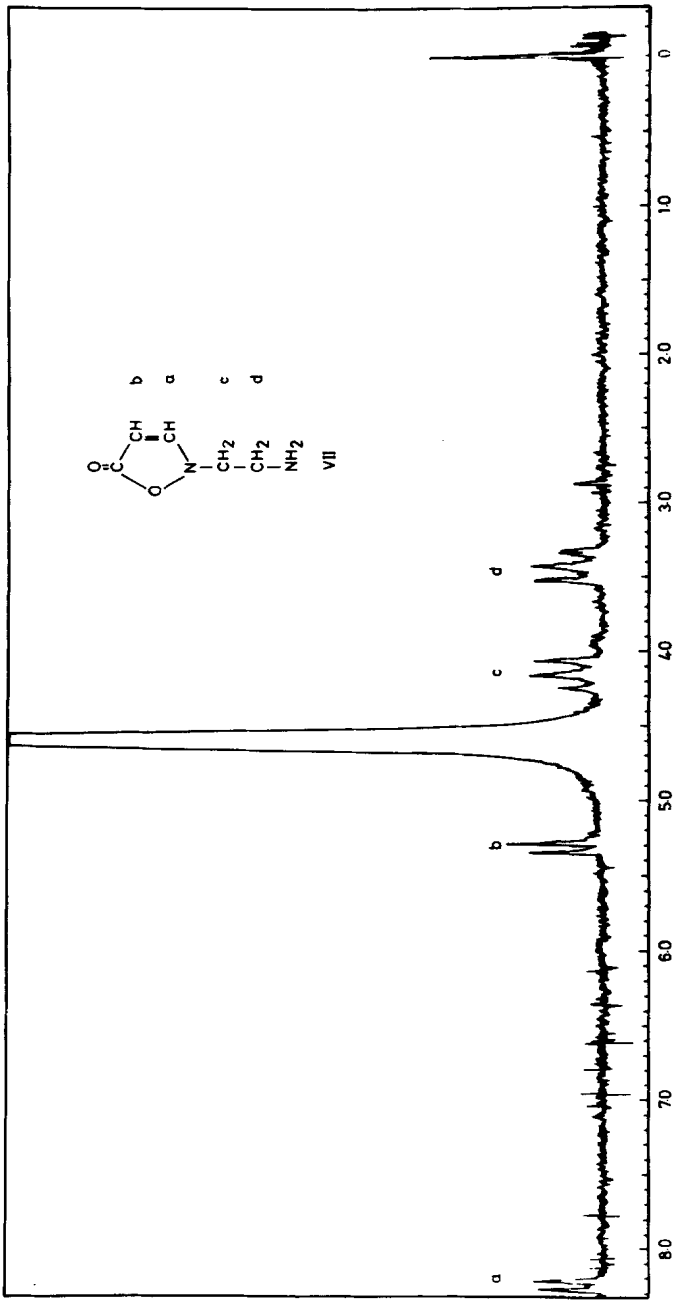

Fig. 2 : NMR-spectrum of compound VII at 60 MHz in D_2O, at room temperature. Tetradeutero-3-trimethylsilyl-propionate was used as standard.

cies strongly suggests that IX may be the precursor for III, alanylation of heterocyclic rings being a frequently occuring reaction in plants (3). As has been reported for compounds I (8) and II (3-alanyl-uracil)(1, 5), the alanylation also occurs in pea seedlings.

For the biosynthesis of both compounds I and II, O-acetyl serine seems to be the best donor for the alanyl side chain (1, 8). Then O-acetyl homoserine may well be a donor for the side chain of compound VI. The presence of both homologues I and VI in sweet pea seedlings, while O-acetyl homoserine is present in pea seedlings (12) but VI is not, offers an interesting case of enzyme specificity.

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