UDC 547.992:581.134.5

GIBBERELLINS AND RELATED SUBSTANCES

IV. Gibberellins and Gibberellin-like Substances from the Leaves of Nicotiana tabacum

N. Ya. Grigor'eva, V. F. Kucherov, V. N. Lozhnikova, and M. Kh. Chailakhyan

Khimiya Prirodnykh Soedinenii, Vol. 5, No. 4, pp. 296-304, 1969

In 1958, M. Kh. Chailakhyan et al. [1] showed that an alcoholic extract obtained from the leaves of the common tobacco plant Nicotiana tabacum possesses a gibberellin-like broad-spectrum physiological activity. It accelerates the growth of the dwarf pea Pioneer and promotes the passage through vernalization of winter wheat and barley and also the stratification of the seedlings of perennial fruit plants. One of the most interesting properties of this fraction is its capacity for affecting the photoperiodic reaction of plants in the same way as gibberellic acid. Thus, Nicotiana sylvestris treated with an extract from common tobacco N. tabacum flowers normally and bears fruit under short-day conditions A similar treatment permits the long-day plant Rudbeckia bicolor to pass from the rosette stage and give a flowering stem with short-day illumination.

We have attempted to elucidate the nature of the physiological activity of an ethanolic extract of common tobacco N. tabacum. The initial material for the investigation was 77 kg of the leaves of this tobacco grown in 1964 on open ground with long-day illumination. The physiologically active substances were purified by the scheme on page 252

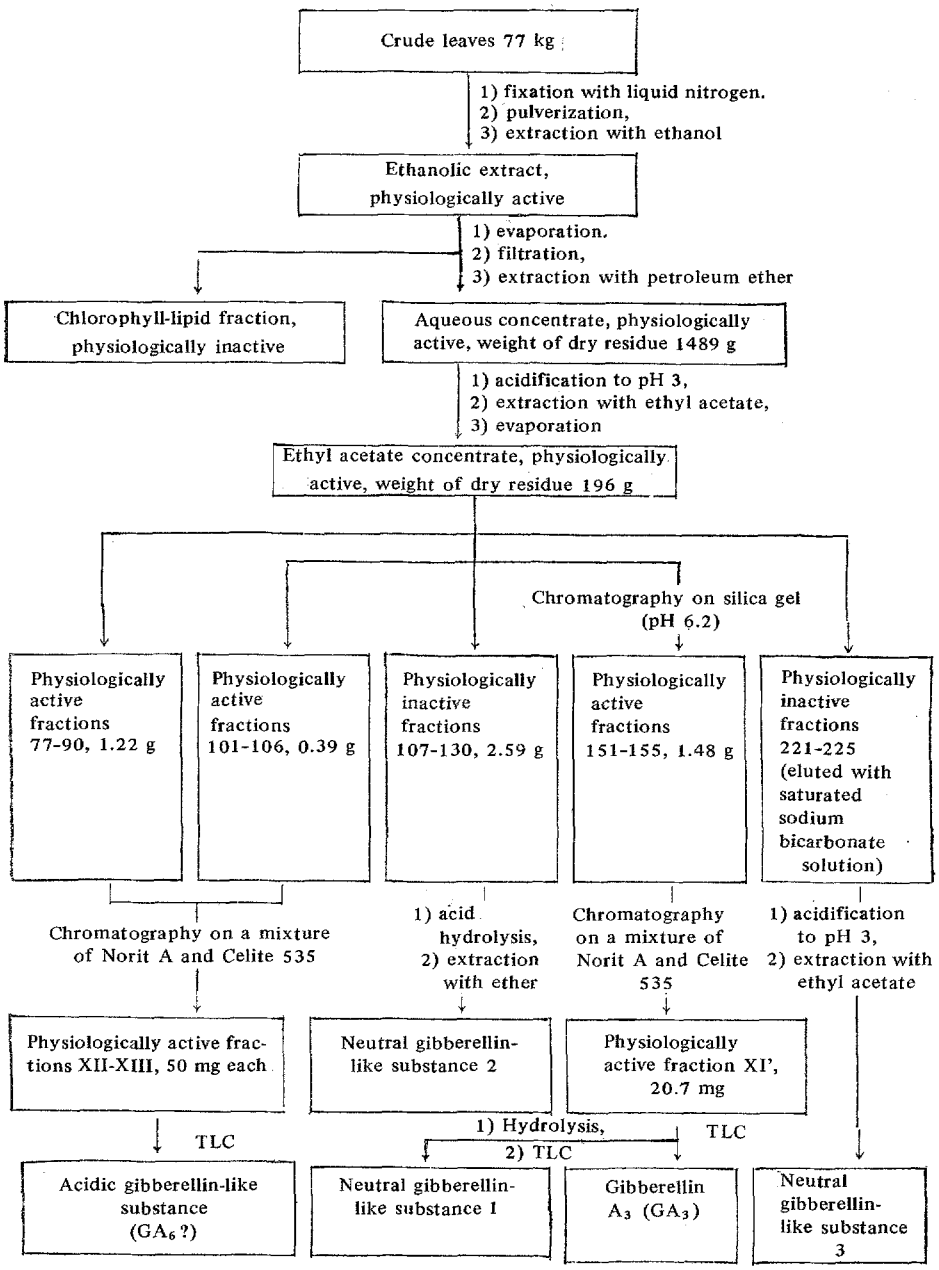
As can be seen from the scheme, the physiologically active substances are extracted by ethyl acetate from the acidified aqueous concentrate remaining after the evaporation of an ethanolic extract of the leaves of common tobacco N. tabacum and the separation of the fraction containing the chlorophyll and lipids. An analysis of the ethyl acetate extract by thin-layer chromatography (TLC) on a nonfixed layer of KSK silica gel in a neutral system (12% of methanol by volume in chloroform) and the biological development of the chromatograms showed that the main part of the active substances was concentrated in a zone with R_f 0.25–0.5, which is characteristic for gibberellin A_3 (GA_3). In addition, active substances were present in the zone adjacent to the start and in the zone with R_f 0.6–0.85. When the ethyl acetate extract was chromatographed on paper in an alkaline system [butan-1-ol–5 N ammonia (1:1)] and the chromatogram was developed biologically, the active substances were again found in the zone characteristic for GA_3 (R_f 0.11–0.33) and in a zone with R_f values close to 1. At this stage of purification, the chemical behavior of the substances gives information only of the composition of the accompanying inert materials. The positions of the physiologically active zones did not coincide with the positions of the spots revealed after the treatment of the chromatograms with chemical reagents. Spots characteristic of the gibberellins, fluorescing after treatment of the plates with sulfuric acid and heating, could not be found because of the contamination of the active zones of the chromatograms with inert substances, probably of polyphenolic nature.

The purification of the ethyl acetate extract on columns of silica gel impregnated with a pH 6.2 phosphate buffer [2] led to the isolation of three groups of physiologically-active fractions (77–90, 101–106, and 151–155). When the latter were chromatographed on columns containing a mixture of Norit A carbon and Celite 535 (1:2) we got rid of a considerable part of the inert compounds and concentrated the active substances into two narrow fractions (XI' and XII–XIII).

The analysis of these fractions by the TLC method on a nonfixed layer of KSK silica gel (150–200 mesh) and the chemical treatment of the chromatograms again gave only information on the inert substances. Analysis on Merck silica gel G likewise showed no spots characteristic for the gibberellins. The search for a more sensitive and effective method of analysis of the mixture studied led us to the use of plates with a fixed layer of KSK silica gel having grain dimensions of 3–6 μ.

Identification of GA_3. The analysis of the active fraction XI' on such plates showed the presence in it of a substance giving the spot characteristic of the gibberellins with a green-blue fluorescence after treatment with sulfuric acid and heating at 80° C for 1 min [3–5]. On chromatography in neutral, acid, and alkaline solvent systems, this spot had the same R_g values as GA_3.

When fraction XI' was chromatographed on a preparative plate with a fixed layer of finely disperse KSK silica gel in system 1 [chloroform–ethyl acetate–acetic acid (12:8:1)], it was found that the spot fluorescing after treatment with sulfuric acid and heating was present in the zone with R_g 0.28–0.5, which had a high gibberellin-like activity. The weight of dry substance in this zone was ~2 mg. A repeat analysis of the eluate from this zone by the TLC method on a fixed layer of finely disperse KSK silica gel showed that this contained, besides GA_3, a small amount of an impurity having in system 1 a R_f value close to that of GA_3 but migrating in system 2[butan-1-ol–3 N ammonia (5:1)] with the solvent front (Fig. 1).



Consequently, for the final purification of the active substance we used the method of two-dimensional chromatography on preparative plates with a fixed layer of finely disperse KSK silica gel. In direction I we used system 1 and in direction II system 2.* The treatment of the plates with sulfuric acid and subsequent heating gave a spot with a green-blue fluorescence which was intersected by straight lines drawn parallel to the starting line through the spots of the GA_3 label chromatographed together with the sample under investigation in both directions. The fluorescence spectrum of the eluate of this spot (recorded by V. I. Ivanov, Chemical Faculty of Moscow State University) showed that it was identical with the fluorescence spectrum of GA_4 [3] (Fig. 2).

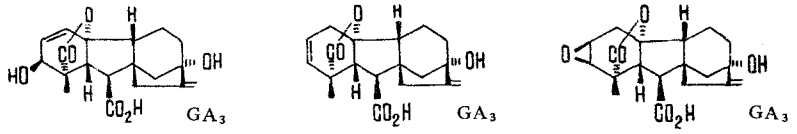
The facts given permit the conclusion that the active substance of fractions 151–155 obtained from the silica gel column and of fraction XI' from a Celite-carbon column is gibberellin A_3 (GA_3).

To evaluate the amount of GA_3 in the plant material, we used two independent methods:

*Two-dimensional chromatography under the conditions recommended by McMillan et al. [3] proved to be inapplicable in working with KSK silica gel, since, on chromatography in ethyl acetate, the gibberellins have fairly high R_f values on it.

1) A comparison of the physiological activity of pure GA_3 in the range of concentrations from 10^{-4} to 10^{-8} g/l with the activity of a chromatographically pure fraction from the leaves of common tobacco N. tabacum containing GA_3.

2) A comparison of the fluorescence yield of a solution of pure GA_3 of known concentration and a solution of the active fraction containing GA_3 after heating the corresponding sample with an ethanolic solution of sulfuric acid [3].



The concentration of GA_3 in the leaves determined by method 1 was 0.2–2 μg/kg, and by method 2 it was 1–1.5 μg/kg. Thus, the two methods give results agreeing satisfactorily.

Our evaluation on the content of gibberellin from the leaves of tobacco N. tabacum agrees with the results appearing in recent years on the concentration of gibberellines in the vegetative organs of plants. Thus, from 44 tonnes of bamboo shoots Tamura et al. isolated 14 mg of a substance which they called bamboo gibberellin [6], and from 11 tons of potato peel Hayashi and Rappaport isolated 4.5 mg of a substance which they called potato factor II [7]. The content of gibberellins in the five types of herbs that they analyzed was estimated at about the same magnitude (0.2–2.9 μg/kg) by Jones, McMillan, and Radley [3].

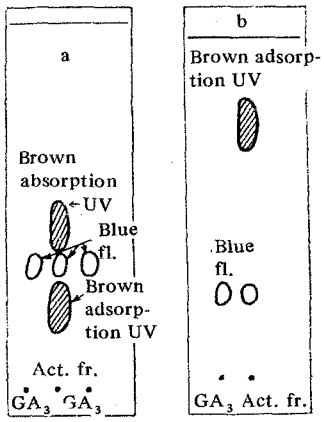


Fig. 1. TLC analysis of the active zone containing GA_3 on a fixed layer of finely-disperse KSK silica gel in systems 1 (a) and 2 (b).

Acidic gibberellin-like substances. When the active fractions XII and XIII from the Celite-carbon column were chromatographed on analytical plates with finely-disperse KSK silica gel in various solvent systems, we obtained no spots fluorescing fairly clearly after treatment with sulfuric acid and heating. The chromatography of these fractions on a preparative plate with finely-disperse silica gel in system 1 and the biotesting of the chromatograms permitted the detection of active substances in the zone with R_f 0.5–0.74, which corresponds to the R_f values of gibberellins A_5 (GA_5) and GA_6 under the conditions used. On re-analysis of the eluates of the active zones by the TLC method with system 1, after the treatment of the plates with sulfuric acid and heating a spot fluorescing extremely weakly was observed with an R_f value close to the values for GA_5 and GA_6, and also a considerable amount of inert substances in the region with R_f values close to those of GA_3–GA_7. In system 2, the inert substances had values approximating to 1; however, the spot fluorescing at the level of GA_5 and GA_6 remained very weak even when considerable amounts of the active fractions were deposited on the plate. No reliable chemical development of this spot in the physiologically active zones from fractions XII and XIII could be achieved even when two-dimensional chromatography was used.

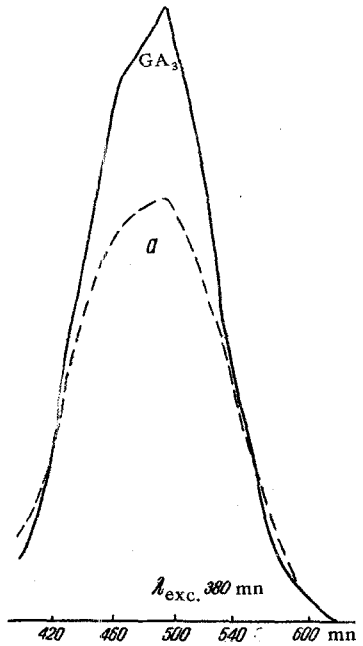


Fig. 2. Fluorescence spectra of GA_3 and a chromatographically pure physiologically-active fraction from the leaves of tobacco N. tabacum (a) containing GA_3.

In view of the fact that the chemical identification of the active fraction considered was impossible, we attempted its biological identification. For this purpose we used the biotest with the dwarf corn mutants d-1 and d-5. It is known that GA_5 is extremely active on d-5 and is comparatively inactive on the mutant d-1, while GA_6 has an activity comparable with that of GA-5 on the mutant d-1 and an even weaker activity on the mutant d-5 [8]. A study of the biological activity of the fraction under consideration showed* that it possessed a weak activity on mutant d-1 and a weaker activity on mutant d-5. These results show that the fraction under investigation does not contain gibberellin A_5 but it does, probably, contain gibberellin C_6. However, we cannot exclude an an alternative possibility—the presence in the active fraction of an unknown gibberellin with a structure similar to that of GA_5 and GA_6.

The difficulties connected with the chemical detection of this gibberellin under the conditions of TLC can apparently be explained by the fact that GA_5 GA_6 are revealed by sulfuric acid and subsequent heating considerably more poorly than GA_3. Thus, to show the presence of GA_3 on silica gel G it is sufficient to heat the plate treated with sulfuric acid at 80° C for 10–20 min [3].

*The experiment was carried out by Dr. Zembdner (Institute for the Study of Crop Plants, AS GDR, Hatersleben).

Consequently, at such low concentrations of gibberellins as are present in tobacco leaves, the sensitivity of the method of detection makes it possible to identify GA_3 but not reliably to establish the presence of GA_6.

Neutral gibberellin-like substances. When fraction XI' containing GA_3 (see above) was chromatographed in the acid solvent system 1 on a fixed layer of KSK silica gel with grain dimensions of 3–6 μ, followed by the biological examination of the chromatograms in the zones with $R_f > 0.6$, substances were found which showed a gibberellin-like activity at high dilutions (the activity of 0.1 part of the eluate of this zone on the dwarf pea Pioneer was 175% relative to a control, and on ten-fold dilution the activity rose to 300%). When this fraction was chromatographed in a neutral system 3 [methanol–chloroform (4 : 96)], physiological activity was found only at the level of GA_3, and when it was chromatographed in an alkaline system [butan-1-ol–5 N ammonia (1 : 1)] on paper it was found only in the frontal zone of the chromatogram, we assumed that fraction XI' contained, in addition to GA_3, a gibberellin-like substance in the combined form [9]. Under the conditions of slow chromatography on finely disperse silica gel or on paper, hydrolysis of the bound form took place with the liberation of a neutral substance more polar than the known gibberellins. This assumption was confirmed by the isolation of a physiologically-active substance of low polarity in the acid hydrolysis of the zones remaining at the start isolated in the chromatography of fraction XI'. We called this substance neutral gibberellin-like substance 1 of tobacco (GLS 2).

The search for similar substances in the other fractions obtained from the silica gel and Celite-carbon columns showed that fractions 107–130 also contained a bound form of a substance of low polarity possessing a high gibberellin-like activity which was liberated on acid hydrolysis. This substance was called neutral gibberellin-like substance 2 of tobacco (GLS2). A gibberellin-like substance of low polarity was also found in the fraction isolated from the silica gel columns with a saturated solution of sodium bicarbonate (see Experimental). It had a high R_f value (>0.8) on chromatography in neutral, acid, and alkaline solvent systems and is probably formed by the alkaline hydrolysis of a bound form remaining in the column after elution with methanol. This substance we called neutral gibberellin-like substance 3 of tobacco (GLS 3). The low content of GLSs 1, 2, and 3 in the plant extract has not so far permitted a more detailed characterization of these compounds.

Experimental

The work was carried out with KSK silica gel purified in the following way. A mixture of equal volumes of ground silica gel and concentrated hydrochloric acid was boiled with stirring for 12 hr. The acid was decanted off and the operation was repeated with a fresh portion of acid until the reaction for Fe^{3+} ion in a fresh acid extract was negative. Then the silica gel was washed with water to a strictly neutral reaction, dried in the air, fractionated by means of a sieve, and, finally, the fractions were dried at 120° C for 48 hr.

To prepare the finely-disperse silica gel, a fraction with particle dimensions <250 mesh was slurried with such an amount of water that the height of the column above the 5-centimeter layer of silica gel was 50 cm. Fractions were collected according to the rate of settling of the particles as described by Pitra and Sterbr [10]. The fractions settling after 12 hr but not settling in 8 hr had particle dimensions of 3–6 μ (the dimensions of the particles of Merck silica gel G are 30–40 μ.

In order to prepare analytical plates with a fixed layer of finely disperse silica gel, we used a suspension of silica gel and gypsum in chloroform as described by Peifer [11]. The preparative plates were obtained from a suspension of silica gel and gypsum in water by means of a Stahl apparatus and were dried in air at room temperature for 2 hr. The layer thickness was 0.5 mm.

All the biological tests were carried out on the dwarf pea Pioneer by the method described previously [12] (the activity is shown in percentages of an aqueous control taken as 100%).

The fluorescent spectra were taken on a Jobin and Ivon spectrofluorimeter. The fluorescence was excited with a source of light with λ 380 mμ.

First extraction of the physiologically active substances of the leaves of common tobacco N. tabacum. Freshly-cut leaves in portions of 15–17 kg were fixed with liquid nitrogen, ground to a powder, and then covered with two volumes of ethanol and left for a day. The ethanolic extract was filtered off and the residual green mass was extracted with one and a half volumes of ethanol with stirring in an enamelled reactor for a day. The combined ethanolic extracts were evaporated in vacuum at 40° C to an aqueous residue. The evaporation was accompanied by the formation of an oily deposit containing the bulk of the chlorophyll and lipids of the leaves, which was readily eliminated by filtration. For final removal of the chloroform-lipid fraction, the aqueous solution was extracted with petroleum ether. The volume of the total aqueous concentrate was 12 *l* and the weight of the dry matter in it was 1489 g.

Biological tests showed that the elimination of the chlorophyll-lipid fraction had practically no effect on the physiological activity of the aqueous concentrate. Evaporation of the solution in vacuum at ~40° C and its storage for a short time at pH 3 also had no effect on its activity.

Further extractive purification of the physiologically active substances. Ethyl acetate concentrate. A study of the biological activity of the chloroform, ether, ethyl acetate, and isobutanol extracts from a native and an acidified aqueous concentrate showed that the active substances are readily extracted by ethyl acetate from an acidified aqueous concentrate.

The bulk of the active substances was extracted by shaking the aqueous concentrate acidified to pH 3 with ethyl acetate (4:3) at room temperature for 2 hr. The operation was repeated 15 times. Evaporation of the dried ethyl acetate extract gave a concentrate containing 196 g of dry matter. It was established by paper chromatography that the concentrate contained only traces of amino acids and carbohydrates.

Chromatography of the ethyl acetate concentrate on columns of buffered silica gel. The ethyl acetate concentrate was separated into three equal portions, each of which was mixed with 200 ml of KSK silica gel (60–100 mesh), evaporated, and transferred in the form of a suspension in chloroform to a column containing 2 kg (4200 ml) of KSK silica gel (60–100 mesh) impregnated with pH 6.2 phosphate buffer [2]. The columns were eluted with chloroform containing increasing amounts of ethyl acetate. The composition of the eluting mixture was changed after each 40 *l*, the concentration of ethyl acetate being increased by 10%. Two-liter fractions were collected. The analogous fractions from the three column were combined, evaporated, and analyzed by means of biotests and TLC on silica gel. Three groups of physiologically active fractions were detected (Table 1).

After elution with pure ethyl acetate, the columns were eluted with methanol (36 *l*) and then with a saturated solution of $NaHCO_3$ (60 *l*, amount necessary for the complete decoloration of the silica gel).

The bicarbonate eluate was evaporated at ~40° C in vacuum to a volume of 4.5 *l*, and this was kept at 0° C for 12 hr, filtered, and extracted with ethyl acetate. The aqueous solution was acidified to pH 3 and extracted with ethyl acetate (1 *l* × 5). Evaporation yielded a concentrate containing 0.3 g of dry substance and possessing a considerable physiological activity (activity of a 0.01 fraction—163%).

Chromatography of the active fractions on Celite-carbon columns. A) Fractions 77–90 and 101–106 were combined and mixed with 15 ml of silica gel. The mixture was evaporated, suspended in water, and transferred to a column 2.6 cm in diameter filled with a mixture of 20 g of Norit A carbon and 40 g of Celite 535 (the height of the column of adsorbent was 31.5 cm). The column was eluted with water containing increasing amounts of acetone, 400-ml fractions being collected. The composition of the eluting mixture was changed after each 400 ml, the content of acetone being increased by 5%.

Biological tests permitted the detection of active fractions XII and XIII (Table 2).

B) When fractions 151–155 were chromatographed on a mixture of Norit A carbon and Celite 535 (1:2), the physiologically active fraction XI' was obtained similarly (see Table 2).

The neutral gibberellin-like substances of common tobacco N. tabacum. An eluate of the zones with R_f 0.0–0.28 obtained in the chromatography of fraction XI' on preparative plates of finely-disperse KSK silica gel was evaporated, mixed with a solution of 2 ml of acetic acid in 20 ml of acetone, and left at 20° C for 5 days. The solution was evaporated and the residue was dissolved in 10 ml of water and extracted with ether. The biotest showed the presence in the extract of a substance with gibberellin-like activity. When the extract was chromatographed by the TLC method in system 3 with the biological development of the chromatogram, the active substance had $R_f > 0.8$.

A similar substance was found by the same method when fractions 107–130 from the silica gel columns were hydrolyzed.

Conclusions

1. A study of the gibberellin-like substances of the leaves of tobacco N. tabacum has shown that the latter contains gibberellin A_3 and possibly A_6 in the free state.

The amount of GA_3 in the leaves of this plant is approximately 0.2-2 μg/kg.

Table 1

Fraction No.	Weight of dry residue	Eluting mixture	Activity of a 0.01 fraction (by volume), %
77—90	1.22	Chloroform-ethyl acetate (70:30)	210
101—106	0.39	Chloroform-ethyl acetate (60:40)	215
151—155	1.48	Chloroform-ethyl acetate (30:70)	235

Table 2

Fraction No.	Weight of dry residue, mg	Eluting mixture	Activity of a 0.03 (by volume) fraction, %
XII	50	55% acetone	196
XIII	52	60% acetone	160
XI′	20.7	50% acetone	300

2. In addition, tobacco leaves contain neutral gibberellin-like substances in the bound form which are liberated on hydrolysis.

REFERENCES

1. M. Kh. Chailakhyan, T. V. Nekrasova, L. P. Khlopenkova, and V. N. Lozhnikova, Fiziologiya rastenii, 10, 465, 1963.
2. V. F. Kucherov, I. A. Gurvich, A. V. Simolin, and I. M Mil'shtein, DAN SSSR, **163**, 765, 1965.
3. D. F. Jones, J. McMillan, and M. Radley, Phytochem., **2**, 307, 1963.
4. D. F. Jones, Nature, **202**, 1309, 1964.
5. G. Sembdner and K. Schreiber, Phytochem., **4**, 49, 1965.
6. S. Tamura, N. Takahashi, N. Murofushi, and S. Griuchijima, Tetrah. Let., 2465, 1966.
7. F. Hayashi and L. Rappaport, Plant Physiol., **41**, 53, 1966.
8. P. W. Brian, H. G. Hemming, and D. Lowe, Ann. Bot., **111**, 369, 1964.
9. G. Sembdner, G. Schneider, J. Wieland, and K. Schreiber, Exper., **20**, 89, 1964.
10. J. Pitra and I. Sterbr, Chem. Listy, **56**, 544, 1962.
11. J. Peifer, Mikrochem. Acta, 529, 1962.
12. V. N. Lozhnikova, L. P. Khlopenkova, and M. Kh. Chailakhyan, Agrokhimiya, 132, 1967.

11 August 1967

Institute of Organic Chemistry AS USSR
Institute of Plant Physiology AS USSR